Ph.D. THESIS

STUDY OF SELENIUM INDUCED STRESS RESPONSES IN ARABIDOPSIS THALIANA L. AND PISUM SATIVUM L. PLANTS, THE POSSIBILITY OF BIOFORTIFICATION

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INTRODUCTION

The actuality of the present topic is based on the fact that research on selenium started just a few decades ago. Researchers pointed out whilst selenium is not essential for plants, it is a necessary microelement for animals and humans, furthermore, there is more and more research on this topic in order to study the possible role of selenium.

Selenium exposure, like other environmental stress factors, can induce stress-induced morphogenetic responses (SIMR) within the plant body, since plants control their organs' growth and development adjusting to the current conditions of the environment (e.g. actual water- and mineral conditions, presence of organic and inorganic contamination). The stress-induced morphogenic responses can appear both in the root- and shoot systems. Not only the environmental conditions but the endogen hormonal status of the root (e.g. auxin, cytokinin and ethylene) can control the morphological responses, which means that changing the metabolism and transport of plant hormones plays a role in the formation of SIMR. The active signal transduction between the exogen, environmental and endogen, hormonal regulatory elements *via* signal molecules, ensures the phasing of the growth and developmental signals.

A new group within the signal molecules, is the nitric oxide (NO) and its derivatives, so called reactive nitrogen species (RNS). The NO as a signal transductor, plays an important role in the developmental processses, and more recent studies confirm that NO and its derivatives are not specific, rather they can be considered as general and multifunctional signal molecules. However, during stress responses not only the RNS play signal transductive role but the reactive oxygen species (ROS) contribute to the morphogenetical changes. There is an active signal transduction between RNS and ROS, and researchers think today that we can speak about nitro-oxidative stress, during ROS and RNS formation, induced by biotic or abiotic stress, and they induce the changes together, of which targets are macromolecules.

OBJECTIVES

During my work, I aimed at focusing on the study of the selenium treatment induced hormonal and signal transductive processes and how these responses indicate the morphogenetic response in the *Arabidopsis* plants, furthermore the background mechanisms behind these phenomenons. In addition to the model plant *Arabidopsis* (*Arabidopsis thaliana* L.), I also wanted to use a well-known, common foodplant, the pea plant (*Pisum sativum* L.) in order to study selenium biofortification, since the quality famine causes serious health and ecological problems among Se-deficient areas worldwide.

During my experiments I aimed at finding answers to the following questions:

1. How do the applied selenium concentrations affect the development of the model plant *Arabidopsis*, and is there a morphogenetic response during selenium stress?

2. What kind of changes are induced by selenium in the hormonal system?
3. What kind of changes are induced by selenium exposure in the level of growth-regulating signal molecules (nitric oxide and hydrogen peroxyde) within the root system of the *Arabidopsis* plants?
4. What kind of interactions exist between the development-regulating hormonal and signalling systems during selenium stress?
5. How does selenium affect the development and ripening of the crop of the pea plants during long-term treatment?
6. Would it be possible for me to use the applied method as a selenium biofortification method?

MATERIALS AND METHODS

Experiments on *Arabidopsis thaliana* L. plants

I used 2-4-7- and 14-day-old (DAG2/DAG4/DAG7/DAG14; days after germination) *Arabidopsis thaliana* L. plants to carry out my experiments. Beside the wild-type (*Col-0*), the *nia1nia2* double mutant, the *gsnor1-3* mutant, furthermore the β-glucuronidase (GUS) transgenic lines were used in order to examine the hormonal status (*DR5::GUS*, *ARR5::GUS* and the *ACS8::GUS/GFP*). During my experiments I also worked with three different *AtCKX::GUS* (*AtCKX4*, *AtCKX5*, *AtCKX6*) lines. I studied the *ipt-161* and *35S:CKX2 Arabidopsis* lines, as well. In addition, during my experiments the *aux1-7*, *hookless* (*hls1-1*) and *etr1-1 Arabidopsis* plants were also used. From the modified ascorbic acid (Asa) containing lines, the *vtc2-1* and the *miox4* were used. I chose the *CYCB1;1::GFP Arabidopsis* line in order to study the cell proliferation. I applied sodium selenite (Na_2SeO_3) as main treatment at 10, 20 and 40 μM concentrations, added directly into the agar media, thus the plants germinated and grew on the Se-containing agar. As control, plants without Se treatment were used. I also applied the following treatments together with Se: S-nitroso-N-acetyl-penicillamine (SNAP) as NO donor at 10 μM concentration, and 6-benzylaminopurine (BA) as exogen CK at 0.1 μM concentration.

The selenium and sulphur contents were measured by inductively coupled plasma mass spectrometry (ICP-MS) in 4-day-old wild-type *Arabidopsis* plants root and shoot systems.

On the sample collecting days the following morphological parameters were recorded: cotyledon area, hypocotyl length, primary root length. The measurements were carried out on digital images, using Fiji and Zeiss Axiovision Rel. 4.8 softwares. Taking the digital images, Zeiss Axioskope 200-C stereomicroscope and Zeiss Axiovert 200M invert microscope were used. Based on the morphological parameters, selenium tolerance index was calculated.

In those transgenic *Arabidopsis* lines, which carry β-glucuronidase (GUS) activity, 5-bormo-4-chloro-3-indolil glucuronid (X-Gluc) staining was used. The samples

were detected by a Zeiss Axiovert 200M invert microscope. The X-Gluc staining of the *DR5::GUS* plants made it possible for me to localize and count the lateral roots, furthermore I could specify their developmental stage.

Fluorescently stained samples were *in situ* and in *vivo* measured by a Zeiss Axiovert 200M type invert microscope. The pixel intensity was determined both in the root and shoot. The level of NO was visualized by 4-amino-5-methylamino-2'7'-difluofluorescein diacetate (DAF-FM DA), while the level of hydrogen peroxyde of the plants by Amplifu™ (or 10-acetyl-3,7-dihydroxyphenoxazine or Amplex Red). In order to study the viability of root tips and cotyledons, I used the fluorescein diacetate (FDA) dye.

The GFP expression of the *CYCB1;1::GFP* plants was measured using Zeiss LSM 700 Axio Observer.Z1 and Olympus LSM 700 laser scanning confocal microscopes. I used propidium iodide (PI) staining on the 4-day-old plants in order to make the cell walls visible. The intensity and localization of the GFP signal was analyzed on digital images using Zeiss Zen2010, Olympos Fluoview FV100, and Fiji softwares. The distance between the quiescent centre (QC) and the beginning of the transition zone (TZ), where the elongation of the cells is powerful, was determined. The size of the root meristem was measured on digital images using the Fiji software.

Experiments on *Pisum sativum* L. plants

During my experiments in the biofortification topic, I used *Pisum sativum* L. Petit provencal pea plants. The seedlings were transferred to perlite-filled 5-litre pots (4 plants/pot and 6 pots/treatment) and grew under greenhouse conditions. Watering of the plants was carried out applying Hoagland solution. The pea plants were grown under control conditions for 35 days, then they got 10, 50 and 100 μM Na_2SeO_3 treatment, added into the Hoagland solution, for 50 (50 and 100 μM Se) or 56 days (10 μM Se). During the experimental period, seeds were collected three times, using two-week breaks.

The following morphological parameters were measured manually: shoot length, shoot fresh weight, leaf length, primary root length and root fresh weight. The

crop-related parameters were the following: number of the pods/plant, fresh and dry weight of the seed containing pod, fresh weight of the seeds, number of the seeds/pod.

Beside selenium, other micro- (Zn, Mn, Fe, Co, Cu, Mo) and macro elements (K, Mg, Ca) were determined in the shoot, root and crop of the plants by ICP-MS method.

RESULTS AND DISCUSSION

During my Ph.D. studies, I investigated the developmental, hormonal and signalling responses of the model organism *Arabidopsis thaliana* L. with the help of genetical and biochemical methods under different concentrations of selenite treatment. Furthermore, I studied in our experimental system whether selenium accumulates in the seeds of pea plants during the biofortification experiments and how this long-term Se exposure affects the development of *Pisum sativum* L. plants. With the use of these experimental systems, it became possible to widen our understanding not only in the field of plant physiology but also add useful information to the opportunity of biofortification.

Based on my results, I can summarise:

1. The higher Se concentrations (20 and 40 µM) caused the **growth inhibition of the shoot and primary root** however, long-term (14 days), mild Se exposure (10 µM) resulted in stress induced morphogenetic response. These developmental responses are considered **elements of the adaptation process** since the re-orientation of means from development for protection mechanisms ensures better survival. Besides the growth inhibition, the death of cells is also possible, which may happen *via* incorporation of Se into proteins (as selenocystein and selenomethionne).
2. I pointed out that Se exposure can cause significant **changes in hormone homeostasis**: Se decreases the auxin response (DR5-dependent GUS activity), increases the biosynthesis of ethylene (ACS8::GUS expression). The activity and spatial distribution of the cytokinin response promoter (ARR5)-dependent

GUS change under selenite stress as well, presumably *via* the inhibition of root-to-shoot translocation and the regulation of AtCKX4 and AtCKX5.

3. During the early development of the seedlings, selenium excess decreased the amount of **nitric oxide** (which happens independently from NR), and increases the **hydrogen peroxide** level in the root, which implicates the antagonistic relationship between these two molecules. Furthermore, using biochemical (NO donor treatment) and genetical (*gsnor1-3* and *nia1nia2* mutants) methods, I proved, that **high NO levels contribute to the induction of selenium tolerance, while the optimal level of H_2O_2 is necessary for the Se endurance.**
4. It can be hypothesized, that the **Se induced H_2O_2 decreases the auxin-dependent genexpression during early development, whereas in older roots NO inhibits the transport of auxin, resulting in the reduction of total auxin level and growth inhibition of the root**. The selenium-induced intensified **ethylene** biosynthesis (ACS8::GUS activity) takes part in cell death induction, thus in the inhibition of growth and the **H_2O_2 is a downstream component of the signalling**. Furthermore, my results show, that in selenium treated roots there is **no regulatory relationship between NO and ethylene**. Under control conditions, there is a **mutual negative relationship between CK and NO** in *Arabidopsis* roots. **In case of Se exposure, CK influences the metabolism of NO, and the decrease of NO level proved to be necessary for the activation of ARR5 promoter. This result shows a negative CK-NO cross talk under selenium stress.**
5. Selenium affects **negatively the long-term development of pea** plants, and in the reproductive phase plants survival strategy focuses on the producing and ripening of the seeds.
6. The development of seeds was also limited by selenium treatment, however, the **biofortification proved to be successful because selenium accumulated in the new seeds**.

As a result, it is clear, that the elements of the hormonal (auxin, cytokinin and ethylene) and signalling (NO and H_2O_2) networks act together and are in a relationship, they regulate the Se exposure induced developmental changes. Our research group reported the changes in NO metabolism caused by selenium for the first time, and the role of NO in selenium tolerance. Our new and important result is the investigation of the relationship between NO and cytokinin, and its nature under an abiotic (selenium) stress condition.

In our opinion, with this work, I could contribute to the understanding of the role and interactions of nitric oxide in plants.

PUBLICATION LIST

Topic-related publications:

1. **Lehotai N**, Pető A, Erdei L, Kolbert Zs (2011) The effect of selenium (Se) on development and nitric oxide levels in *Arabidopsis thaliana* seedlings. Acta Biologica Szegediensis 55: 105-107.
2. **Lehotai N**, Pető A, Bajkán Sz, Erdei L, Kolbert Zs (2011) *In vivo* and *in situ* visualization of early physiological events induced by heavy metals in pea root meristem. Acta Physiologiae Plantarum 33: 2199-2207. (IF: 1,639)
3. **Lehotai N**, Kolbert Zs, Pető A, Feigl G, Ördög A, Kumar D, Tari I, Erdei L (2012) Selenite-induced hormonal and signalling mechanisms during root growth of *Arabidopsis thaliana* L.. Journal of Experimental Botany 63: 5677-5687. (IF: 5,364)

Other publications:

1. Pető A, **Lehotai N**, Lozano-Juste J, León J, Tari I, Erdei L, Kolbert Zs (2011) Involvement of nitric oxide and auxin in signal transduction of copper-induced morphological responses in *Arabidopsis* seedlings. Annals of Botany 108: 449-457. (IF: 4,030)
2. **Lehotai N**, Pető A, Weisz M, Erdei L, Kolbert Zs (2011) Generation of reactive oxygen and nitrogen species in pea cultivars under copper excess. Acta Biologica Szegediensis 55: 273-278.
3. Kolbert Zs, Pető A, **Lehotai N**, Feigl G, Ördög A, Erdei L (2012) *In vivo* and *in vitro* studies on fluorophore-specificity. Acta Biologica Szegediensis. 56: 37-41.
4. Kolbert Zs, Pető A, **Lehotai N**, Feigl G, Erdei L (2012) Long-term copper (Cu^{2+}) exposure impacts on auxin, nitric oxide (NO) metabolism and morphology of *Arabidopsis thaliana* L.. Plant Growth Regulation. 68:151-159. (IF: 2,859)
5. Feigl G, Kumar D, **Lehotai N**, Tugyi N, Molnár Á, Ördög A, Szepesi Á, Gémes K, Laskay G, Erdei L, Kolbert Zs (2013) Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern) and rapeseed (*Brassica napus* L.) to copper stress. Ecotoxicology and Environmental Safety, 94: 179-189. (IF: 2,482)
6. Pető A, **Lehotai N**, Feigl G, Tugyi N, Ördög A, Gémes K, Tari I, Erdei L, Kolbert Zs (2013) Nitric oxide contributes to copper tolerance by influencing ROS metabolism in *Arabidopsis.* Plant Cell Reports, 32: 1913-1923. (IF: 2,936)
7. Elfogadva: Feigl G, Kumar D, **Lehotai N**, Pető A, Molnár Á, Rácz É, Ördög A, Erdei L, Kolbert Zs, Laskay G: Comparing the effects of excess copper in the leaves of *Brassica juncea* (L. Czern) and *Brassica napus* (L.) seedlings: growth inhibition, oxidative stress and photosynthetic damage. Acta Biologica Hungarica. (IF: 0,563)

Total impact factor: 19.873

Scientific report:

1. **Lehotai N** (2012) The possibilities and enzymatic background of selenium and zinc biofortification of pea plants. Report for Short-Term Scientific Mission (STSM) in the framework of COST Action FA 0905 (Reference code COST-STSM-ECOST-STSM-FA0905-010212-013321).

Scientific lectures:

1. Pető A, **Lehotai N**, Kolbert Zs, Erdei L (2010) The effect of heavy metal-induced reactive oxygen- (ROS) and nitrogen species (RNS) generations on cell viability of pea roots. p27, L18. 3rd Plant NO Club International Meeting, July 15-16. 2010, Olmütz, Czech Republic.
2. Kolbert Zs, Pető A**, Lehotai N**, Erdei L (2011) A nitrogén-monoxid (NO), mint a nehézfém-indukált növekedési válaszok regulátora. S4-02. 10th Congress of the Socieatas Biologiae Plantarum Hungarica, August 31- September 2. 2011, Szeged, Hungary.
3. Feigl G, Kumar D, Pető A, **Lehotai N**, Szepesi Á, Erdei L, Kolbert Zs (2012) Studying the effect of copper in *Brassica juncea* and *Brassica napus* root tips: metabolism of reactive oxygen and nitrogen species and morphological adaptation. p51. 7th PhD Student Conference, Scandinavian Plant Physiology Society (SPPS), September 12-15. 2012, Laulasmaa, Estonia.
4. Feigl G, Kumar D, Pető A, **Lehotai N**, Ördög A, Molnár Á, KolbertZs, Erdei L (2012) The effect of zinc on the microelement homeostasis and the metabolism of reactive signal molecules in *Brassica juncea* and *Brassica napus*. Third Annual Workshop of COST Action FA 0905 – Mineral improved crop production for healthy food and feed. October 23-26. 2012, Lisbon, Portugal.
5. **Lehotai N**, Lyubenova L, Drews N, Ördög A, Feigl G, Kolbert Zs, Erdei L, Schröder P (2012) The possibilities and enzymatic background of Se and Zn biofortification of pea plants. Third Annual Workshop of COST ACTION FA 0905 – Mineral improved crop production for healthy food and feed. October 23-26. 2012, Lisbon, Portugal.
6. Horváth E, Kolbert Zs, **Lehotai N**, Feigl G, Tari I, Erdei L (2012) Role of reactive oxygen- and nitrogen species in poplar plants during zinc, copper and polyethylene glycol treatments. Characterization and oxidative stress tolerance in plants: from models to trees (OXIT) HUSRB/1002/214/036. Interim Conference, November 20. 2012, Szeged, Hungary.
7. **Lehotai N**, Feigl G, Koós Á, Erdei L, Kolbert Zs (2014) Cytokinin-nitric oxide relationship in selenium-stressed *Arabidopsis*. Socieatas Biologiae Plantarum Hungarica, Conference of Young Biologists, January 30. 2014, Budapest, Hungary.
8. Feigl G, **Lehotai N**, Molnár Á, Erdei L, Rodríguez-Ruiz M, Palma JM, Corpas FJ, Kolbert Zs (2014) Zinc induced nitro-oxidative stress in *Brassica* species. L23. 5th Plant NO Club Meeting, July 24-25. 2014, Munich, Germany.
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1. Kolbert Zs, Vashegyi Á, Ördög A, **Lehotai N**, Méri Á, Erdei L (2009) Short time effect of copper ion (Cu^{2+}) on nitric oxide (NO) production in *Sorghum sudanense* L. roots. p58., P19. COST 859 Workshop of WG1 and WG2 on Uptake, Sequestration and Detoxification - an Integrated Approach, April 16-17. 2009, Szeged, Hungary.
2. Pető A, **Lehotai N**, Erdei L, Kolbert Zs (2010) Metal content and nitric oxide (NO) production in the roots of heavy metal-treated pea plants. SB-23. ISIRR 11th International Symposium Interdisciplinary Regional Research, October 13-15. 2010, Szeged, Hungary.
3. **Lehotai N**, Pető A, Weisz M, Kolbert Zs, Erdei L (2011) The effect of long-term copper (Cu^{2+}) exposure on reactive nitrogen- and oxygen species generation in two pea cultivars. P110, p174. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants, July 5-8. 2011, Budapest, Hungary.
4. Kolbert Zs, Pető A, **Lehotai N**, Erdei L (2011) Nitric oxide as negative regulator of auxin during copper induced morphological responses. P141, p220. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants, July 5-8. 2011, Budapest, Hungary.
5. Kolbert Zs, Pető A, **Lehotai N**, Tari I, Erdei L (2011) Endogenous reactive oxygen species (ROS) status and cell death in nitric oxide (NO) mutants under copper excess. XXIV Scandinavian Plant Physiology Society (SPPS) Congress, August 21-25. 2011, Stavanger, Norway.
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7. **Lehotai N**, Pető A, Feigl G, Kumar D, Erdei L, Kolbert Zs (2011) Early responses in root meristem of *Pisum sativum* and *Arabidopsis thaliana* induced by copper and selenium. Second Annual Conference and MC Meeting COST Action FA 0905, Mineral Improved Crop Production for Healthy Food and Feed, November 23-26. 2011, Venice, Italy.
8. Kolbert Zs, **Lehotai N**, Pető A, Feigl G, Kumar D, Erdei L (2012) Selenium-induced growth responses and their hormonal background. Plant growth, Nutrition & Environment Interactions, February 18-21. 2012, Vienna, Austria.
9. **Lehotai N**, Pető A, Feigl G, Kumar D, Erdei L, Kolbert Zs (2012) Study of selenite-induced hormonal and signalling mechanisms during root growth of *Arabidopsis thaliana* L. by light- and fluorescence microscopy. p54. 7th PhD Student Conference, Scandinavian Plant Physiology Society (SPPS), September 12-15. 2012, Laulasmaa, Estonia.
10. Kolbert Zs, Pető A, **Lehotai N**, Feigl G, Tugyi N, Ördög A, Erdei L (2013) Relationship between nitric oxide (NO) and reactive oxygen species (ROS) in copper-treated *Arabidopsis* roots. p189. Society for Experimental Biology (SEB) Annual Main Meeting, July 3-6. 2013, Valencia, Spain.
11. Kolbert Zs, **Lehotai N**, Pető A , Feigl G, Tugyi N, Erdei L (2013) Cytokinin overproducing *ipt6-1 Arabidopsis* shows altered NO generation and insensitivity to selenite. 11th International POG Conference, July 17-19. 2013, Varsaw, Poland.

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13. Feigl G, Pető A**, Lehotai N**, Molnár Á, Erdei L, Kolbert Zs (2013) Comparison of the effect of copper and zinc in *Brassica juncea* and *Brassica napus* roots: microelement homeostasis, metabolism of reactive signal molecules and morphological adaptation. p44. Biomedica Minikonferencia, December 13. 2013, Szeged, Hungary.
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15. Feigl G, **Lehotai N**, Molnár Á, Erdei L, Kolbert Zs (2014) Zinc excess affects root architecture and reactive oxygen- and nitrogen species metabolism in *Brassica juncea* and *Brassica napus*. C7.39. Society for Experimental Biology (SEB) Annual Main Meeting, July 1-4. 2014, Manchester, England.
16. Feigl G, **Lehotai N**, Molnár Á, Erdei L, Kolbert Zs (2014) Detection of protein tyrosine nitration in zinc-treated *Brassica* plants. S1-P03. Socieatas Biologiae Plantarum Hungarica, 11th Congress, August 27-29. 2014, Szeged, Hungary.
17. **Lehotai N**, Feigl G, Koós Á, Pető A, Erdei L, Kolbert Zs (2014) The role of nitric oxide under selenium tolerance. S1-P09. Socieatas Biologiae Plantarum Hungarica, 11th Congress, August 27-29. 2014, Szeged, Hungary.
18. Molnár Á, Feigl G, **Lehotai N**, Erdei L, Kolbert Zs (2014) Microscopic study of zinc localization in *Brassica* roots. S1-P14. Socieatas Biologiae Plantarum Hungarica, 11th Congress, August 27-29. 2014, Szeged, Hungary.