Project closing report (K 120383, 2016-2020)

Introduction, the aim of the project

Heavy metals (HMs), as external signals can cause the stress-induced morphogenic response (SIMR) root phenotype, which results in the formation of a less penetrating, rather horizontally situated root architecture. The main goals of the project were to study the effect of heterogeneous HM distribution on root SIMR and on its nitric oxide (NO)-related background signalling in crops grown in rhizotrons, to observe the connection between the capability for shaping of SIMR and for HM tolerance and accumulation. Additionally, the project intends to reveal the involvement of NO-dependent macromolecule modifications in HM tolerance or sensitivity. A part of the planned research focuses on the signal interactions of NO with novel phytohormones (ethylene, strigolactones) in heavy metal-induced root growth.

Results

In the first two years of the project, we set up a **rhizotron system** in which we analysed the changes in the root system structure of seedlings in the presence of zinc (Zn) or nickel (Ni).

In case of **Zn** we applied growth inducing (10 ppm zinc sulphate) and growth inhibiting (500 ppm) concentrations for rapeseed (*Brassica napus).* The growth inhibiting Zn treatment resulted in elevated protein tyrosine nitration due to the imbalance in reactive oxygen species (ROS) and reactive nitrogen species (RNS) homeostasis without the change of the nitration pattern. Callose and pectin formation and viability loss was observed as the effect of high Zn supply, while as the effect of beneficial Zn dosage the reorganisation of protein nitration pattern was observed which was accompanied by unmodified ROS and RNS balance and root viability. These results revealed that different root growth responses are accompanied by distinct changes in the pattern and strength of tyrosine nitration proposing that nitrosative signalling have a relevant role in root growth responses triggered by Zn supply (FEIGL ET AL. 2019a, NITRIC OXIDE). Beyond the homogenous Zn distribution, several heterogeneous layouts were prepared (top/bottom half or top/middle/bottom third, respectively): control/10 ppm Zn; 10 ppm Zn/control; control/10 ppm Zn/control; control/500 ppm Zn; 500 ppm Zn/control; control/500 ppm Zn/control; 500 ppm Zn/10 ppm Zn/control. In these cases, diverse root structures developed and local growth responses were evolved by local Zn amounts.

In another system, nitro-oxidative signalling was compared in sunflower (*Helianthus annuus*) and rapeseed grown in the presence of **combined HM treatments**. The model-sewage contained cadmium, chromium, copper, mercury, lead and Zn at their highest concentrations legally allowed. Sunflower was relatively sensitive while rapeseed showed better metal translocation capability and root growth which was accompanied by elevated NO production and changes in tyrosine nitration pattern. These suggest that increased NO content and reorganisation of protein nitration pattern may contribute to tolerance against combined HM treatment (FEIGL ET AL. 2020a, PLANTS).

Seeds collected from nickel hyperaccumulator *Odontarrhena lesbiaca* (previously *Alyssum lesbiacum*) from two different locations (Ampeliko and Loutra, Lesbos, Greece) were germinated in soil-filled rhizotrons in the presence of 3000 mg/kg **Ni**. Plants from the Ampeliko site accumulated NO, showed reduced tyrosine nitration and improved tolerance in case of Ni exposure compared to plants derived from the Loutra site. The results suggest that Ni hypertolerance and hyperaccumulation might be connected to the plants' capability of maintaining their RNS balance and to reduce the sensitivity of the proteome to nitration. Moreover, the fact that the descendants of the two ecotypes have different sensitivity to excess Ni, raises the possibility that such properties of a species' might be influenced by their living space and they can be genetically inherited (FEIGL ET AL. 2020b, ANTIOXIDANTS).

Collectively, the applied rhizotron system proved to be suitable for studying root structure, as numerous parameters could be determined in seedlings (e.g. primary root [PR] length, lateral root [LR] number, lateral root length, angle of lateral root growth). However, the rhizotron cultivation system couldn't be used for all elements (e.g. Se did not cause a visible effect on root development). The results clearly show that the effect of excess element on root structure and nitrosative signalling is concentration-dependent (Zn), species-dependent (combined HM treatment), and also depends on the hyperaccumulation capacity of the plant (Ni). Plant tolerance against excess elements is associated with the decrease in the rate of protein nitration, which may be due to activated protein degradation mechanisms.

We studied the effect of **Se** treatments on non-accumulator, accumulator and hyperaccumulator plant species cultivated in nutrient solution or in agar-solidified medium. In the first system, Indian mustard (*Brassica juncea)* as a selenium accumulator was grown hydroponically in the presence of 0, 20, 50 or 100 μM selenite or selenate. Selenite proved to be more toxic than selenate, and more intense protein nitration as well as alterations in nitration pattern was observed suggesting a correlation between the degree of Se forms-induced toxicities and nitroproteome size, composition in *Brassica* organs (MOLNÁR ET AL. 2018a ECOTOX ENVIRON SAF). In the second system, the selenite-induced responses of Indian mustard were compared with those of thale cress (*Arabidopsis thaliana).* In case of *Arabidopsis*, relative Se sensitivity was accompanied by decreased stomatal density and induced stomatal opening, callose accumulation, pronounced oxidative stress and moderate nitrosative modifications. These suggest that selenite tolerance or sensitivity is rather associated with oxidative processes than secondary nitrosative modifications in these plants (MOLNÁR ET AL. 2018b ECOTOX ENVIRON SAF). Using *Astragalus bisulcatus* (Se hyperaccumulator and hypertolerant) and *Astragalus membranaceus* (non-accumulator of Se), we first revealed that Se sensitivity of *A. membranaceus* coincides with Se-induced disturbances in superoxide ($O_2^{\cdot-}$) metabolism leading to its accumulation. Se increased the production or disturbed the metabolism of RNS (NO; peroxynitrite, $ONOO^-$; S-nitrosoglutathione, GSNO) consequently resulting in intensified protein tyrosine nitration in sensitive *Astragalus* species. In contrast, Se caused no significant alterations in ROS/RNS levels and tyrosine nitration in the hypertolerant *A. bisulcatus* suggesting that this species is able to prevent Se-induced nitro-oxidative stress (KOLBERT ET AL. 2018, PLANT CELL PHYSIOL).

These results confirm our previous observations that excess Se causes intensified protein nitration leading to nitrosative stress. Additionally, the results point out that the degree of nitrosative signalling depends on the Se form, on Se concentration and also on the sensitivity or hyperaccumulation capacity of the plant species.

We are conducting further experiments using *Astragalus bisulcatus* (selenium hyperaccumulator) and *Astragalus cicer* (non-accumulator of selenium) grown on nutrient agar media supplemented with 0, 5, 10, 25, 50 or 75 μM sodium selenate. We are currently examining oxidative parameters in this comparative system, in order to be able to evaluate the involvement of oxidative stress and antioxidant defence in Se tolerance of hyperaccumulators. Additionally, in the frame of a collaboration, organic Se-compounds have been determined in *Astragalus* plants and the results show that methylated-Se-compounds are abundant in *A. bisulcatus*, but not in *A. cicer*. We will prepare and submit a manuscript from these results within the next three months.

The modulating effect of **Se on root development** has been investigated in *A. thaliana* model plant and found that as the effect of 15 μM selenite, the number of visible (emerged) lateral roots increased, which led to a more branched root system. This was accompanied by shortening of the primary root which can be considered as a symptom of SIMR. The results collectively indicate that in the Se-stressed root system, increased ethylene (ET) levels inhibit

NO generation and NO negatively influences ET levels. It was shown for the first time that the antagonistic interplay between the two gasotransmitters in turn regulates the emergence of LRs in *Arabidopsis* under Se stress (FEIGL ET AL. 2019b, J PLANT GROWTH REGUL).

Related to this topic, we published review articles regarding ET-NO interplay (KOLBERT ET AL. 2019a ANTIOXIDANTS) and Se effects on plant proteome (KOLBERT ET AL. 2019b, J PLANT PHYS). We published a book chapter on Se (KOLBERT ET AL. 2019c, IN: PLANT TOLERANCE TO ENVIRONMENTAL STRESS, CRC PRESS). Árpád Molnár wrote his PhD dissertation on this topic which he will publicly defend in November 2020.

We set an experimental system in which we grew and treated *Arabidopsis thaliana* (Col-0, non-accumulator) and *Brassica juncea* (L. Czern cv. Negro Caballo, accumulator) with Ni (0, 25, 50, 75, 100 µM $NiCl_2$). Studying GUS-tagged *Arabidopsis* lines (*ARR5::GUS*, *ACS8::GUS* and *DR5::GUS*) revealed that **Ni**-increased LR emergence and concomitantly reduced LR initiation were accompanied by elevated levels of auxin (AUX), cytokinin (CK) and ethylene in the LRs or in upper root parts, whereas Ni-induced PR shortening is related to decreased AUX and increased CK and ET levels in the root tip. These suggest the **Ni-induced disturbance of hormonal balance in the root system**. Results of the comparative study showed that weaker Ni tolerance of *Arabidopsis* was coupled with a Ni-induced increase in RNS, ROS and hydrogen sulphide levels, as well as with an increase in redox signalling and consequent increment of protein nitration. However, in relative Ni tolerant *Brassica juncea,* redox signalling (except for peroxynitrite) was not modified and Ni-induced intensification of protein tyrosine nitration was less pronounced suggesting that **Ni tolerance is associated with the capability of preventing protein nitration** (KOLBERT ET AL. 2020, ECOTOX ENVIRON SAFETY).

Comparing the **Zn** tolerance of the wild-type (WT), GSNO reductase (GSNOR) overexpressor *35S::FLAG-GSNOR1* and GSNOR-deficient *gsnor1-3*, we observed relative Zn tolerance of *gsnor1-3* which was not accompanied by altered Zn accumulation capacity. Moreover, in *gsnor1-3* plants Zn did not induce NO/*S*-nitrosothiol (SNO) signalling possibly due to the enhanced activity of NADPH-dependent thioredoxin reductase. In WT and *35S::FLAG-GSNOR1*, GSNOR was inactivated by Zn and Zn-induced H_2O_2 is directly involved in GSNOR activity loss. In WT seedlings, Zn resulted in slight intensification of protein nitration detected by western blot and protein *S*-nitrosation observed by resin assisted capture of SNO proteins (RSNO-RAC). LC-MS/MS analyses indicate that Zn induces the *S*-nitrosation of ascorbate peroxidase 1. Our data collectively show that **Zn-induced H_2O_2 may influence its own level which involves GSNOR inactivation-triggered SNO signalling.** These data provide new evidence for the interplay between H_2O_2 and SNO signalling in *Arabidopsis* plants affected by metal stress (KOLBERT ET AL. 2019d, PLANT CELL PHYSIOL).

Beyond the classical phytohormones that regulate root system development, we examined the possible **relationship of strigolactones (SL) with NO** in *Arabidopsis thaliana* seedlings. Deficiency of SL synthesis or signalling (*max1-1* and *max2-1*) resulted in elevated NO and SNO levels due to decreased GSNOR protein abundance and activity indicating that there is a signal interaction between SLs and GSNOR-regulated levels of NO/SNO. This was further supported by the down-regulation of SL biosynthetic genes (*CCD7, CCD8* and *MAX1*) in GSNOR-deficient *gsnor1-3*. Based on the more pronounced sensitivity of *gsnor1-3* to exogenous SL (*rac*-GR24, 2 µM), we suspected that functional GSNOR is needed to control NO/SNO levels during SL-induced primary root (PR) elongation. Additionally, SLs may be involved in GSNO-regulated PR shortening as suggested by the relative insensitivity of *max1-1* and *max2-1* mutants to exogenous GSNO (250 µM). **Collectively, our results indicate a connection between SL and GSNOR-regulated NO/SNO signals in roots of *A. thaliana***

grown in stress-free environment (OLAH ET AL. 2020a, FRONT PLANT SCI). As this work used *max2-1* mutant and *rac*-GR24 exerting unspecific effects to both SL and karrikin signalling, it cannot be ruled out that karrikins are partly responsible for the observed effects. This issue is currently being investigating.

Related to this topic, we discussed literature regarding SL-NO interplay in a minireview article (KOLBERT 2019e, PHYSIOL PLANT). An addendum article (to Oláh et al. 2020, Front Plant Sci) has recently been submitted to the journal Plant Signaling & Behavior.

In the frame of the project, we synthesized the **nitro-oleic acid** (NO_2-OA) standard and detected its amount in *Brassica napus* seeds and differently developed seedlings. In all samples, NO_2-OA was detectable at nanomolar concentrations. The seeds showed the highest NO_2-OA content, which decreased during germination. In contrast, NO levels increased in the early stages of germination and seedling growth. Exogenous NO_2-OA treatment (100 µM, 24h) of *Brassica* seeds resulted in significantly increased NO level and induced germination capacity compared to untreated seeds. The results of *in vitro* approaches (DAF-FM fluorescence, NO-sensitive electrode) supported the NO liberating capacity of NO_2-OA. **We observed for the first time that *Brassica* seeds and seedlings contain free NO_2-OA which may be involved in germination as a NO donor** as suggested both by the results of exogenous NO_2-OA treatment of seeds and *in vitro* approaches. Due to their high NO_2-OA content, *Brassica* sprouts can be considered as good source of dietary NO_2-OA intake (VOLLÁR ET AL. 2020, PLANTS).

We prepared further publications related to the topic of the project. **Three book chapters** have been published (KOLBERT AND FEIGL 2018 IN: SINGH V ET AL. (EDS.) REACTIVE OXYGEN SPECIES IN PLANTS BON OR BANE REVISITING THE ROLE OF ROS, WILEY; FEIGL ET AL. 2020c IN: IQBAL M ET AL. (EDS.) IMPROVING ABIOTIC STRESS TOLERANCE IN PLANTS, CRC; OLÁH ET AL. 2020b IN: POÓR P ET AL. (EDS.) OXIDATÍV STRESSZ ÉS ANTIOXIDÁNS VÉDEKEZÉS A NÖVÉNYVILÁGTÓL A KLINIKUMIG, MAGYAR SZABADGYÖK-KUTATÓ TÁRSASÁG). **Five additional review papers** have been published related to the research topic (KOLBERT ET AL. 2017, PLANT PHYSIOL BIOCHEM; KOLBERT ET AL. 2019f, NITRIC OXIDE; GUPTA ET AL. 2020ab NEW PHYTOL; LOPES-OLIVEIRA ET AL. 2020, J EXP BOT accepted).

Summary

The common features of the observed elements (non-essential Se, non-redox active, essential Zn, redox-active essential Ni) is that in sensitive plant species (*Arabidopsis thaliana*, *Astragalus membranaceus*, *Helianthus annuus*) they cause disruption of ROS and RNS metabolism thereby triggering protein tyrosine nitration which is a marker of nitro-oxidative stress. This means that the increase in protein nitration occurs regardless of the essentiality and redox nature of the element. Excess Zn supply has also been shown to enhance S-nitrosation in *Arabidopsis*. In tolerant species (*Brassica juncea*, *Astragalus bisulcatus*, *Odontarrhena lesbiaca*), Se, Zn, and Ni caused slight or no changes in RNS metabolism and did not cause significant protein tyrosine nitration thus nitro-oxidative stress. All of these suggest a strong correlation between the degree of nitro-oxidative stress and plant tolerance to excess elements, thus secondary nitro-oxidative stress occurs in plant cells in a non-element-specific manner (Figure 1). Among the elements studied, Se and Ni triggers changes in phytohormone levels in *Arabidopsis* roots and these alterations explain the changes in root system architecture (SIMR). The root growth of *Arabidopsis* is regulated by the interplay between SL and NO in a stress-free environment (Fig 2). Additionally, *Brassica napus* seeds and seedlings were shown to contain a considerable amount of NO_2-OA, which promotes their germination as an endogenous NO donor.

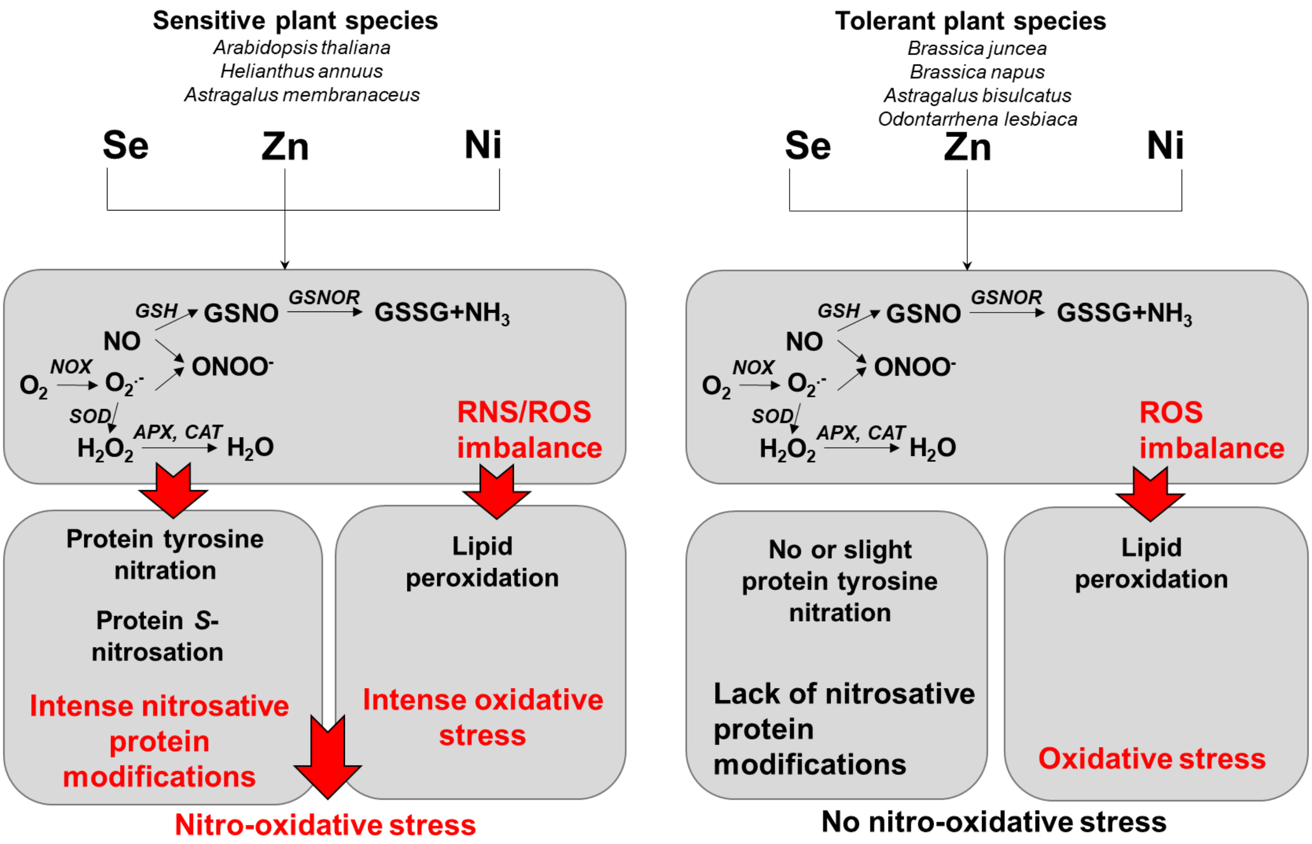

Fig 1. Summary of the examined parameters and results of the project. In sensitive plant species, Se, Zn, Ni-induced ROS and RNS imbalance leads to nitro-oxidative stress, while in tolerant species RNS metabolism is not or only slightly affected and no nitro-oxidative stress is observable. Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; NH_3, ammonia; NOX, NADPH-oxidase; SOD, superoxide dismutase; APX, ascorbate peroxidase; CAT, catalase.

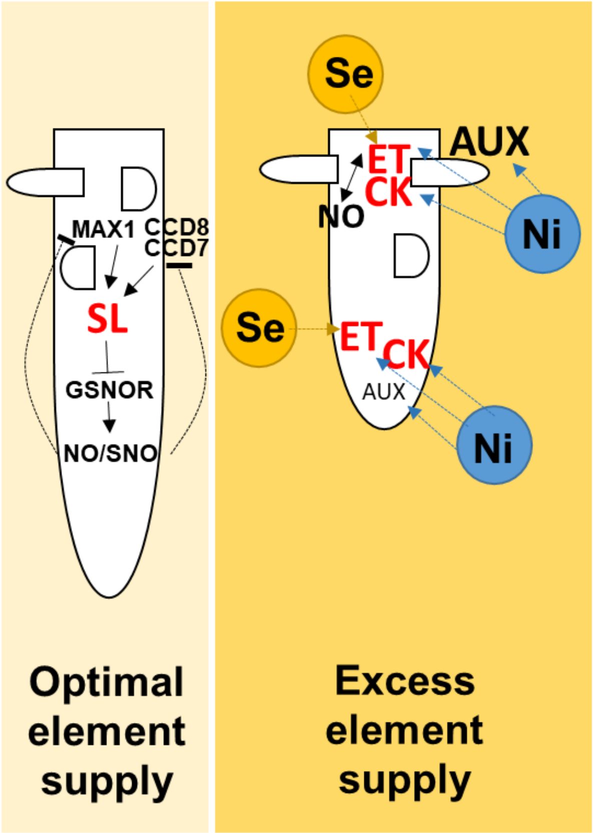

Fig 2. Summary of the examined parameters and results of the project. In case of optimal supply of elements, SL inhibits GSNOR enzyme allowing NO and SNO accumulation. In turn, high NO/SNO levels down-regulate SL biosynthetic genes (*MAX1*, *CCD7*, *CCD8*) further supporting the mutually negative interplay between SL and NO in root growth regulation. Excess Se induces ethylene (ET) accumulation which antagonizes with NO signalling, while excess Ni induces ET and cytokinin (CK) increase in root tips and also in upper root zones and modifies auxin (AUX) distribution within the root. These hormonal alterations may be responsible for the appearance of SIMR root phenotype.

The results of this project are primarily relevant for basic science and form the basis of our further research. We would like to thank the National Research, Development and Innovation Office for the support.

Own publications related to the project

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