Final report of the 120962 project

From morphological adaptation to growth inhibition: role of heavy metal-induced nitro-oxidative stress in plant root development

The project was carried out over three years (01. 10. 2016 – 31. 09. 2019), in three equal annual periods.

1. Theoretical background

The early development of the root system is crucial in terms of the life of a plant; besides ensuring physical stability for the whole plant, it is responsible for water and nutrient uptake and also might be practically (phytoremediation) relevant. It has a noteworthy plasticity: due to different stress conditions its architecture can change to favour seedling vigour and yield stability.

The delicate balance of the endogenous signal system responsible for the development of the root can be affected by various environmental stimuli, such as the excess of essential or non-essential HMs. Heavy metal contamination of soils and water is an existing and growing problem. This has partly originated from agricultural processes, such as excessive use of fertilisers or application of sewage (Tóth *et al*. 2016).

HMs at low concentrations are able to induce the morphological and physiological adaptation of the root system called stress-induced morphogenic response. SIMR is a special combination 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 (Potters *et al*. 2007; Kolbert 2016], however this conjecture needs further investigations. On the other hand, HMs at high concentration lead to growth inhibition due to their phytotoxic effect by altering the most important plant physiological and metabolic processes (Kalaivanan and Ganeshamurthy, 2016).

In addition to reactive oxygen species (ROS) like superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) (Morina *et al.* 2010; Jain *et al.* 2010), 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 ($ONOO^-$) and S-nitrosoglutathione (GSNO), (Wang *et al.* 2013). 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 (Corpas *et al.* 2007, 2011).

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 (Corpas and Barrosso, 2013). A typical example of ROS-RNS crosstalk is the reaction of $O_2^{\cdot -}$ and NO resulting in the formation of $ONOO^-$, which is accountable for post-translational modification protein tyrosine nitration (PTN), the covalent modification on specific tyrosines in proteins forming 3-nitrotyrosine (Corpas *et al.* 2013). The addition of the nitro group to one of the ortho carbons in the aromatic ring of tyrosine residues (Gow *et al.* 2004) 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 (van der Vliet *et al.* 1999). PTN 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 (Greenacre and Ischiropoulos, 2001; Radi, 2004, Corpas *et al.* 2013). Moreover, PTN is furthermore able to disturb signal transduction pathways by the inhibition of tyrosine phosphorylation (Galetsky *et al.* 2011).

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 (Jordan, 1992), trees (Pagés, 1992), Arabidopsis (Devienne-Barret *et al.* 2005) or Brassica napus under phosphorus deficiency (Yuan *et al.* 2016). 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.

2. Introduction – setting up the experimental system and optimisation

2.1. Zinc and nickel

As the first step, the **rhizotron plant growing system** was set up, according to the growth rate of the species involved in the study.

Custom-made plexi panels were ordered and 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 is 1 cm.

The rhizotrons are 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_2O_5/l) mixed with 20% sand; the initial water content is set to 70%. Homogenous rhizotrons are just simply filled with the mixture (approximately 250 grams/piece), while in heterogeneous setups different metal-containing soils are carefully layered.

The seeds are pre-germinated for 24 hours on 26°C and then transferred to the soil surface of the pre-filled rhizotrons. In the first 48 hours after the seeding, the young seedlings are covered with transparent plastic foil to provide optimal humidity, then the growing plants are supplemented with 10 ml distilled water in every two days. Rapeseed, wheat and corn seedling are grown for 10 days, while sunflower seedlings are grown only for 6 days since their faster development compared to the other species.

The development of the root system is monitored throughout the entire growth period, digital images (with using a flatbed scanner) are taken regularly for manual digital analysis (ImageJ).

After the optimisation of the rhizotron system, a **wide range of zinc (Zn) and nickel (Ni) concentrations were tested**, to determine the stress-induced morphological response (SIMR) and 50% growth inhibition-causing concentrations. Besides homogenous application of the heavy metals several heterogeneous setups were tested.

In case of **zinc**, 10 ppm Zn supplementation proved to cause a modified, SIMR-like reaction and the addition of 500 ppm Zn triggered 50% growth inhibition in **oilseed rape**.

In case of **nickel** – despite the screening the effect of 10, 20, 40, 80, 120, 160 and 200 ppm on **rapeseed** – the emergence of SIMR could not be detected, while 180 ppm nickel caused 50% growth inhibition. Upon the repetition of the pilot experiments, I encountered a phenomenon, during which the appearance of the 50% growth inhibition was not reproducible with the same amount of Ni supplementation. Repeated experiments revealed that depending on the actual plant generation, in some cases lower and in other cases higher Ni concentrations inhibited plant growth effectively, however I could not find any explanation or background process which could explain of its occurrence.

In case of **sunflower** neither the emergence of SIMR nor the 50% growth inhibition could not be detected in the tested Zn or Ni range. Interestingly, neither **wheat** nor **corn** plants showed no response to the presence of either Zn or Ni in the soil, since neither the low nor the high Zn concentration altered root architecture significantly in the pilot experiments.

Based on these, **oilseed rape** was chosen for further, detailed experiments in **rhizotron system supplemented with Zn**, since it produced reproducible and reliable results unlike the other setups. The selection of one experimental setup allowed me to perform experiments promising high quality results.

Besides homogenous application of the heavy metals several heterogeneous setups were tested, and according to the pilot experiments and the experiences mentioned above **the effect of spatial distribution of Zn on oilseed rape root growth** was examined.

2.2. Combined treatment

Pilot experiments were conducted with three experimental designs: besides control, the effect of model-sewage in two different layouts (control soil watered with model-sewage during seedling growth and soil pre-equilibrated with model-sewage) was simulated in rhizotron system by the use of the highest HM concentrations (Cd, Cr, Cu, Hg, Ni, Pb, Zn) legally allowed. Dicotyledonous species (oilseed rape and sunflower) responded to the treatment, while (similar to the previous, one-metal treatment) monocotyledonous species are not showing clear morphological alterations.

3. Applied methods

Characterisation of the root system was carried out on the scanned images by using ImageJ software. Changes in the concentrations of microelements were measured by inductively-coupled plasma mass spectrometry (ICP-MS). A number of microscopic staining was performed on the root tips, to determine the changes in Zn distribution, root cell wall composition, lipid peroxidation, apical meristem viability, DNA replication capability, and reactive oxygen- and nitrogen species content. S-nitrosoglutathione and 3-nitrotyrosine content of the root tips was also detected by using immunofluorescent microscopy. Tyrosine nitration and S-nitrosoglutathione reductase protein abundance were detected by western blot analysis. The biotin switch method was also adapted for the detection of S-nitrosylation. Superoxide dismutase, NADPH-oxidase and S-nitrosoglutathione reductase activity was detected and measured by using native polyacrylamide gel electrophoresis method. In addition, activity of catalase in soil was measured by a titrimetric method.

4. Results

4.1. Examination the effect of Zn on oilseed rape in a homogeneous rhizotron system

The results of this part of the project is published in the following scientific article: G. Feigl *et al.* (2019) Zinc-induced root architectural changes of rhizotron-grown *B. napus* correlate with a differential nitro-oxidative response. Nitric Oxide 90 (2019) 55–65. (IF 2018: 3.371)

Highlights:

- Different levels of Zn induce distinct alterations in the root growth of rapeseed
- Low Zn supplementation changes protein nitration pattern and stimulates root growth
- High Zn treatment increases nitrosative stress and nitration, inhibiting root growth
- Nitrosative processes have an important role in Zn-induced root growth responses

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 (summarised in Fig. 1) were studied in a soil-filled rhizotron system.

The growth-inhibiting Zn treatment resulted in elevated protein tyrosine nitration (PTN) due to the increased ROS and RNS production, 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 PTN pattern reorganisation was observed; there were no substantial changes in ROS and RNS levels 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 PTN, proposing that nitrosative processes have an important role in the stress-induced root growth responses.

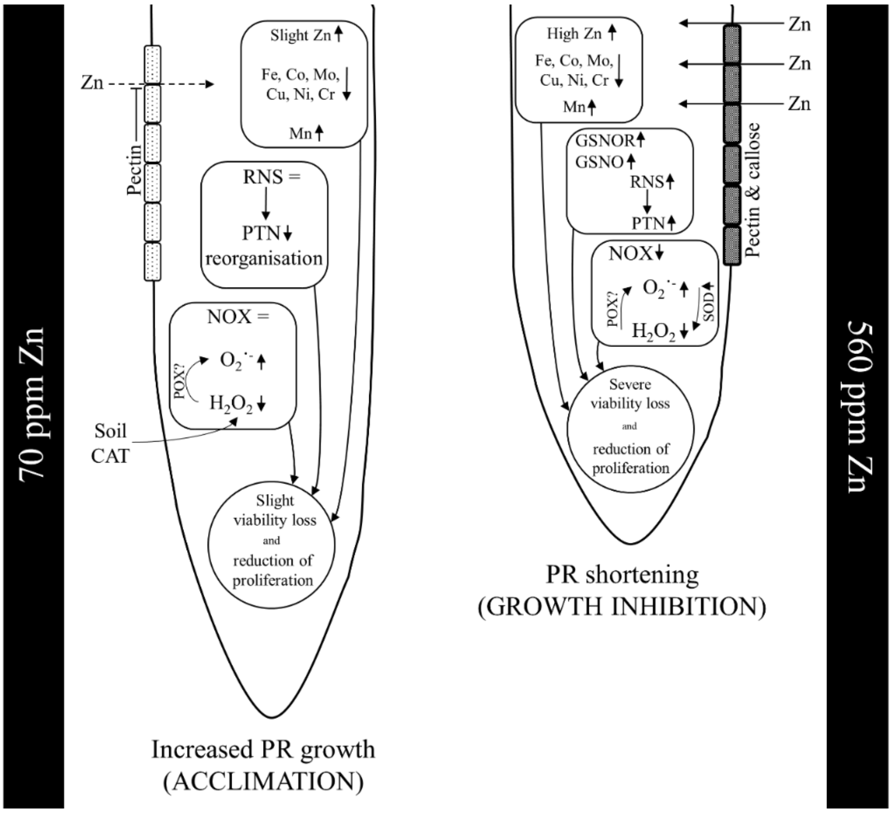

Fig. 1. Schematic model summarising the results presented in this study. 10 ppm Zn supplementation (resulting 70 ppm total Zn) caused a positive growth response with slight Zn uptake and PTN reorganisation in the background, while no oxidative or nitrosative stress was detectable. 500 ppm Zn treatment (resulting 560 ppm total Zn) inhibited root growth, and this stress response was accompanied by high Zn uptake and indicated by increased cell wall modifications, PTN and ROS/RNS levels. (An

upward arrow indicates increase while a downward arrow shows decrease; = means no significant change.)

4.2. Examination the effect of Zn on oilseed rape in a heterogeneous rhizotron system

The results of this part of the project is published in G. Feigl *et al.* (2018) The effect of different vertical zinc distribution on the early root development and zinc accumulation of *Brassica napus*. Proceedings of the 24th International Symposium on Analytical and Environmental Problems, 286-289.

In these experiments, to be able to determine and characterise the exact effect of excess zinc (Zn) on the root growth of *Brassica napus* L., several different layouts were used in soil-filled rhizotrons. This setup allowed me to examine the root growth of rapeseed in a practically 2D system, submitted to heterogeneously layered Zn-contaminated soil. According to preliminary experiments 10 and 500 ppm Zn supplementation as treatment were chosen as SIMR-inducing and growth-inhibiting concentrations, respectively. Several heterogeneous setups were assembled (top/bottom half or top/middle/bottom third, respectively): control/10 ppm Zn (layout 1); 10 ppm Zn/control (layout 2); control/10 ppm Zn/control (layout 3); control/500 ppm Zn (layout 4); 500 ppm Zn/control (layout 5); control/500 ppm Zn/control (layout 6); 500 ppm Zn/10 ppm Zn/control (layout 7) (Fig. 2.)

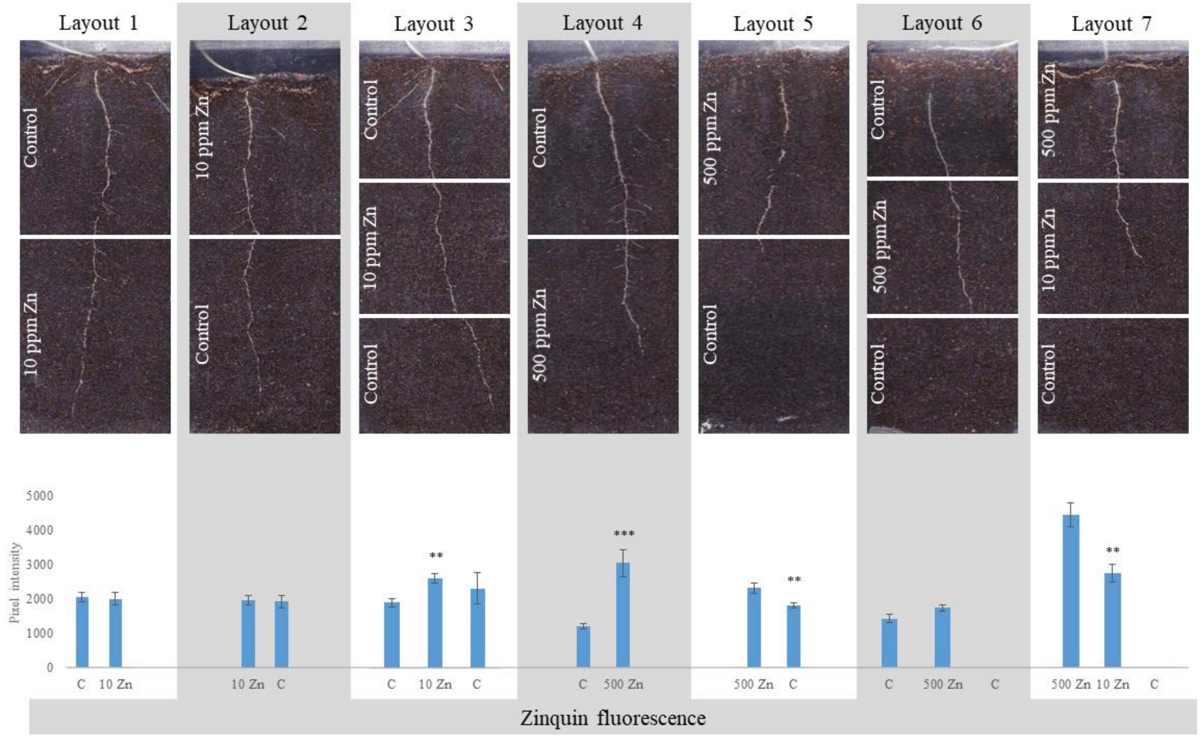

Fig. 2. Representative images of the root growth in the different heterogeneous rhizotron system layouts and the Zn content-dependent Zinquin-fluorescence values in the root tips of the corresponding soil zone. Significant differences according to Student's t-test (n = 10, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$) between each layouts' control and Zn-containing zones are indicated.

Highlights:

- Roots grown in layout 1 and 2 showed approximately the same growth and Zn-contents, the low Zn-treatment did not alter the morphology significantly.

- In layout 3 the growing root met with the Zn supplement in a younger developmental state, causing higher Zn accumulation of the root tips grown in the 10 ppm Zn zone
- In layout 4 the growing root met with the control soil at its younger age and remained more active metabolically, while in layout 5 the growing root was in a stressed state and tried to exclude Zn
- In layout 6 the growing root was relatively older upon encountering the same layer, resulting in the arrest of root growth and exclusion of Zn from its root tips
- In layout 7 the developing root to grew through the 500 ppm Zn zone, but the high Zn concentration ultimately caused the inhibition of root development in the subsequent, low-Zn-containing soil layer.

Results obtained from the heterogeneous Zn distribution experiments point towards the presumption that the growth inhibiting effect of the 500 ppm Zn treatment depends on the developmental stage of the rapeseed seedlings. When the root encountered the high Zn dose in a younger stage, it did not suffer growth inhibition, while the toxic effect of Zn is more pronounced on older roots. Considering the Zn accumulation, there was no close connection between the applied treatment and the Zn content of the root tips in all applied layouts.

4.3. Examination the effect of combined heavy metal treatment on oilseed rape and sunflower a rhizotron system

The results of this part of the project is published in G. Feigl *et al.* (2019) Combined heavy metal treatment affects nitro-oxidative status of rapeseed and sunflower roots differently. 14th International Conference on Reactive Oxygen and Nitrogen Species in Plants: Conference Abstract Book p. 97 Paper: P 40. Munich, Germany.

Currently, a manuscript is under preparation and is expected to be submitted to an internationally relevant scientific journal in the near future.

The goal of this study was to determine the nitro-oxidative status in the root system of rapeseed (*Brassica napus* L.) and sunflower (*Helianthus annuus* L.) subjected to combined HM treatment. The effect of model-sewage was compared to control conditions (simulating ideal circumstances) in two different layouts: control soil watered with model-sewage during seedling growth (setup #1: simulating irrigation with sewage for the first time) and soil pre-equilibrated with model-sewage (setup #2: simulating a condition, where sewage was used for irrigation for a longer preceding period). The experiments were carried out in the rhizotron system by the use of the highest HM concentrations (Cd, Cr, Cu, Hg, Ni, Pb, Zn) legally allowed.

In sunflower roots

- both treatment setup inhibited root growth, proportional to the severity of the HM treatment
- levels of ROS and RNS decreased
- protein tyrosine nitration increased significantly in setup #1.

Meanwhile in oilseed rape roots

- root growth only decreased in setup #1, while viability of the root tips increased proportional to the severity of the HM treatment
- in setup #1 levels of ROS and RNS decreased, while in setup #2 nitrosative stress could be detected

- protein tyrosine nitration pattern was re-organised.

Brassica napus showed better tolerance to combined HM treatment compared to *Helianthus annuus*: in setup #2, simulating a more severe pollution, longitudinal root growth remained unaffected, with elevated NO content and remodelled tyrosine nitration in the background. Results suggest species-specific molecular responses to combined HM stress.

5. Conclusions

The main result of the performed research is the exploration of the dose-dependent effect of zinc on the root architecture of rapeseed. 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 obtained results suggest that Zn in different amounts triggers different root growth responses accompanied by distinct changes in the pattern and strength of PTN, proposing that nitrosative processes have an important role in the stress-induced root growth responses.

According to experiments using combined heavy metal treatment, rapeseed tolerates the heavy metal content of wastewater in a system modelling a more severe pollution than sunflower because it did not reduce the length of its root, while in the background the increased nitric oxide resulted in protein tyrosine nitration rearrangement. The results suggest the species-specificity of the molecular level responses to combined heavy metal treatment.

A further - less positive - result of the research carried out is the recognition that in the applied soil-filled rhizotron system, the other investigated species did not show growth responses to zinc or nickel that were appropriate to the objectives of the research or were not reproducible properly.

The results obtained in the project were published in several forms and levels from conference posters and talks to scientific articles. Moreover, the experiences gained during the completion of the work has contributed to the publication of some (thematically related) other papers, thesis works and the composition of a new research project proposal. Also, in the near future, a manuscript discussing the results of the combined heavy metal treatment is expected to be submitted to an internationally relevant scientific journal.

6. References

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