SELENITE TREATMENT INDUCES NITRO-OXIDATIVE STRESS AND DECREASES VIABILITY IN INDIAN MUSTARD

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Abstract

Selenium (Se) is an essential microelement for all living organisms, except higher plants, where it has not been proven yet. Like other micronutriens, non-optimal amounts of Se in organisms has negative effects. Some agricultural crops can accumulate large amounts of Se, that decreases the yield and renders it harmful for consumption. In our study, we examined the long-term effects of selenite on secondary stress processes, in order to reveal molecular mechanisms of Se toxicity.

We examined indian mustard (*Brassica juncea* L.), which is a secondary Se accumulator agricultural crop. Plants were precultivated in hydroponic culture for nine days and treated with 0 μM (control), 20 μM, 50 μM or 100 μM sodium selenite for seven days.

Among reactive nitrogen species, the level of nitric oxide remained at control level in case of all treatment concentrations. The two reactive oxygen species, superoxide radical and hydrogen peroxide had elevated concentrations in case of 100 μM sodium selenite treatment. Peroxynitrite, a molecule connected to both families of reactive molecules showed elevated levels as the effect of all selenite concentrations. Selenite induced lipid peroxidation in the root tips of *Brassica*, while, protein tyrosine nitration only slightly intensified compared to control plants. Viability significantly decreased in root tips of 50 and 100 μM sodium selenite-treated plants.

In supraoptimal concentrations selenite disturbs the natural homeostasis of ROS and RNS resulting in secondary nitro-oxidative stress, which is partly responsible for the Se-induced viability loss.

Introduction

Se is naturally present in the environment; although various natural and anthropogenic factors may influence its content. Anthropogenic factors can rapidly increase Se content in soil to toxic levels; therefore Se is widely examined today. The two main bioactive forms of Se are selenate (SeO_4^-) and selenite (SeO_3^-). Supraoptimal Se content can cause crop yield reduction and *via* accumulator plants it can endanger human health [1]. Selenosis, the disease of elevated Se levels has the following effects: defective nails and skin, hair loss, unsteady gait and paralysis.

Reactive oxygen (ROS) and nitrogen species (RNS) are molecule families which are present under natural conditions. If a stress disrupts their homeostasis, they cause further damages through secondary oxidative and nitrosative stress. Since both the molecules and the harmful processes are similar, as well as they control each other's levels, recently the term nitro-oxidative stress is used [2]. The secondary nitro-oxidative stress can cause notable damages in macromolecules such as proteins and membrane lipids.

Based on the above, it is important to examine environmentally hazardous elements and their toxicity mechanisms, especially in agricultural crops being the first organisms that encounter them. The aim of this study was to characterize the processes behind Se induced nitro-oxidative stress in order to be able to alleviate yield reduction and increase food safety in the future.

Materials and methods

In all cases, approx. two cm-long segments were cut from the root tips and were incubated in 2 mL dye/buffer solutions in Petri-dishes. After the staining procedure, the root samples were prepared on microscopic slides in buffer solution. We used different specific fluorophores for each staining, which is shown below (Table 1).

Fluorophores	DAF-FM DA	DHE	DHR	Amplex Red	FDA	Schiff's reagent
Labelled molecule/ process	Nitric oxide	Superoxide radical	Peroxynitrite	Hydrogen peroxide	Viability	Lipid peroxidation

Table 1. Fluorophores and the examined processes

Although these staining methods allow semi-quantitative determinations, they are reliable tools for *in situ* detection of ROS and RNS, since their specificity were proved *in vivo* and *in vitro*. The roots of the plants labeled with different fluorophores were investigated under a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss Jena, Germany) At least 10–15 root tips were measured in each experiment.

Root tissues of *Brassica juncea* were grounded with double volume of extraction buffer (50 mM Tris–HCl buffer pH 7.6–7.8) containing 0.1 mM EDTA, 0.1% TritonX-100 and 10% glycerol. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant was stored at -20 °C. Protein concentration was determined using the Bradford (1976) assay with bovine serum albumin as a standard.

10 μg of root and 25 μg of shoot protein extracts per lane were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % acrylamide gels. For western blot analysis, separated proteins were transferred to PVDF membranes using the wet blotting procedure (30 mA, 16h). After transfer, membranes were used for cross-reactivity assays with rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000 Immunodetection was performed by using affinity isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody in dilution of 1:10000, and bands were visualized by using NBT/BCIP reaction. As a positive control nitrated bovine serum albumin was used.

Results and discussion

Nitric oxide levels in root meristems remained similar to control in case of all treatment concentrations (Figure 1. A.).

Mild selenite stress did not affect superoxide levels, but 50 μM sodium selenite decreased it. Contrary, the highest selenite concentration significantly induced the formation of superoxide (Figure 1. B.)

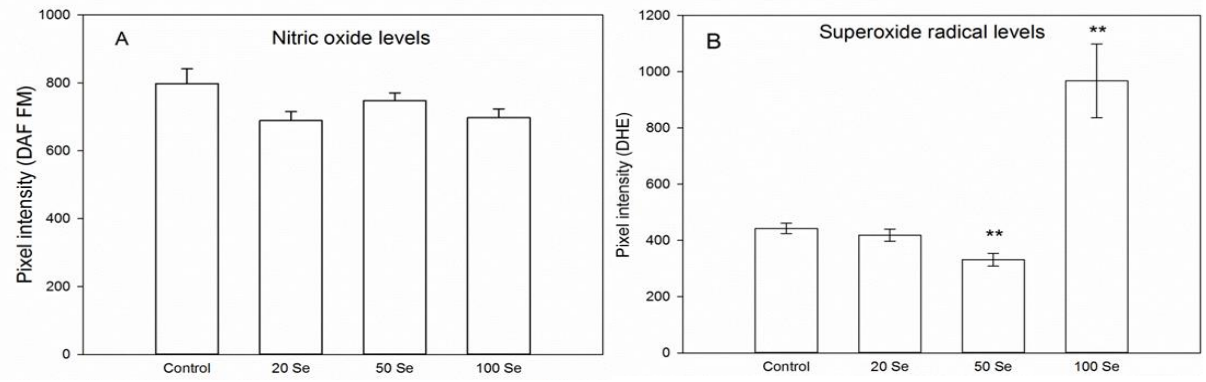

Figure 1. Nitric oxide (A) and superoxide (B) levels in root tips of *B. juncea* treated with 0, 20, 50 or 100 μM selenite for 7 days. Statistically significant differences were determined by Student t -test (**P≤0.01).

Peroxynitrite is formed in the reaction between nitric oxide and superoxide radical. The levels of peroxynitrite significantly increased in 50 and 100 μM selenite-treated root tips, (Figure 2.A.).

Hydrogen peroxide levels increased depending on Se treatment; however only 100 μM sodium selenite resulted in significant difference from control (Figure 2.B.).

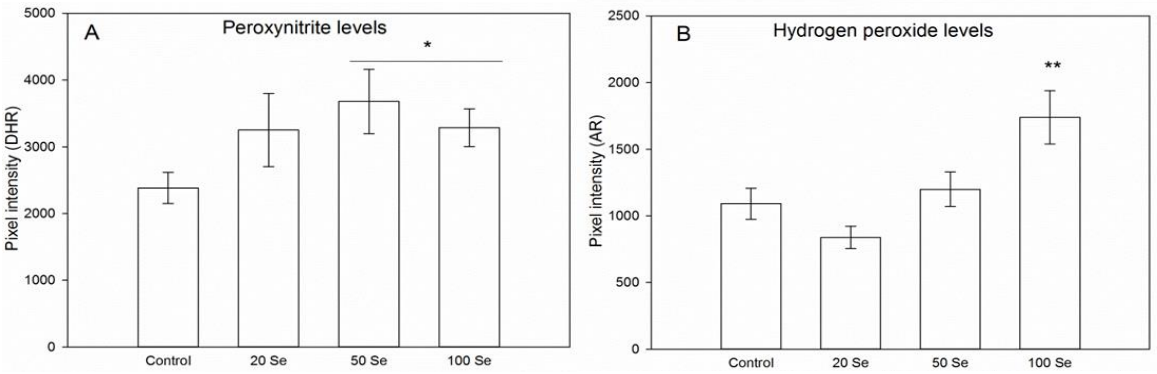

Figure 2. Peroxynitrite (A) and hydrogen peroxide (B) levels in selenite-treated *B. juncea* roots. Statistically significant differences were determined by Student t -test (*P≤0.05, **P≤0.01).

We analyzed two markers of nitro-oxidative stress: lipid peroxidation and protein tyrosine nitration. *Brassica juncea* roots had only slightly increased protein nitration compared to control plants. Newly nitrated protein bands could not be observed in treated samples (Figure 3.A.). In case of lipid peroxidation, selenite had a more obvious effect, since every concentration led to the intensification of membrane damage. (Fig 3B).

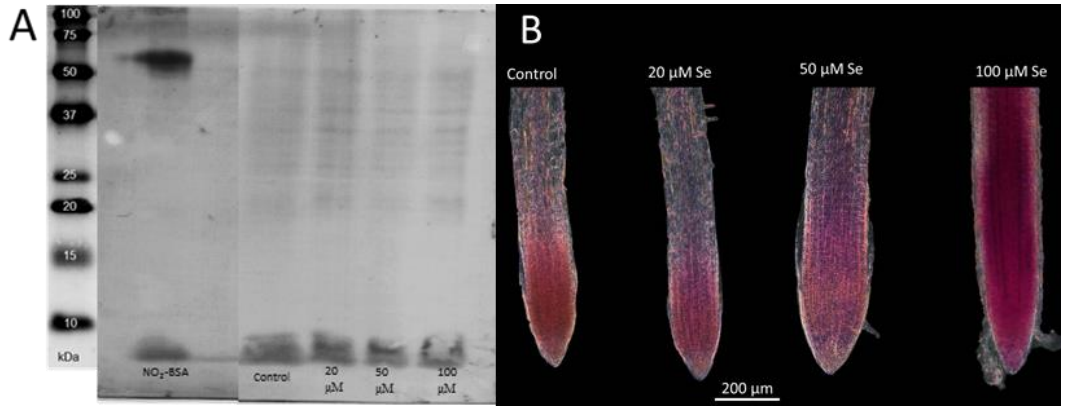

Figure 3. (A)Representative immunoblots showing protein tyrosine nitration in roots of *Brassica juncea* treated with 0, 20, 50 or 100 μM selenite. Commercial nitrated BSA was used as a positive control. (B) Lipid peroxidation in root tips of control and Se-treated *Brassica juncea*. Bar=200 μm

Cell viability in the root meristem is essential to root growth and plant biomass production, thus a decrease in it reflects the overall effect of stress on the organism. In case of sodium selenite, 50 and 100 μM treatment significantly decreased viability compared to control (Figure 4.).

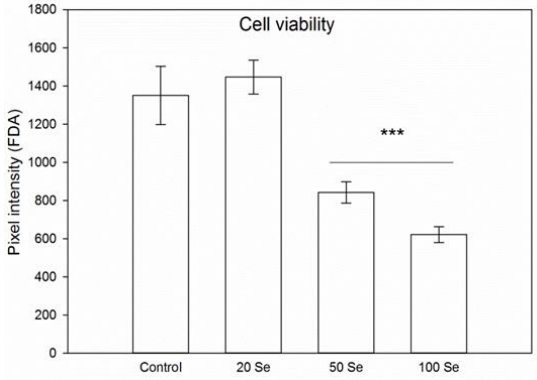

Figure 4. Cell viability in *B. juncea* root meristem. Roots were treated for 7 days with 0, 20, 50 or 100 μM selenite. Statistically significant differences were determined by Student t -test (***P≤0.001).

Conclusion

In case of selenite stress, natural homeostasis of ROS and RNS was disturbed and higher Se concentrations induced nitro-oxidative stress. Nitric oxide levels remained at control level, but the increase in peroxynitrite showed a slight increase. Most likely, newly generated NO molecules immediately reacted with superoxide radical, forming peroxynitrite. The level of superoxide radical increased only as the effect of 100 μM selenite, and 50 μM sodium selenite decreased it significantly. One mechanism behind this could be the peroxynitite generation, or activation of antioxidant enzimes and other metabolic pathways may be involved. Hydrogen peroxide could be generated from superoxide radical by enzimatic activity. The analyzed oxidative and nitrosative stress markers responded to selenium treatment, which indicates that higher concentrations of Se have harmful effects on plant macromolecules. The observed nitro-oxidative stress processes potentially contributed to the viability loss of root meristem cells, leading to cell death. The slight increase in viability as the effect of 20 μM sodium selenite suggests that Se can alleviate stress as an antioxidant.

In this study, for the first time nitration pattern changes where observed in selenium accumulator and tolerant crop plants. The lipid peroxidation proved to be more intense than the protein nitration suggesting that from nitro-oxidative processes, oxidative stress is more severe in selenium accumulator *Brassica* plants.

Acknowledgements

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