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Selenite-induced nitro-oxidative stress processes in *Arabidopsis thaliana* and *Brassica juncea*

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ABSTRACT

Extremes of selenium (Se) exert toxic effects on plants' physiological processes; although plant species tolerate Se differently. This study focuses on the effect of Se (0, 20, 50 or 100 μM sodium selenite) on secondary nitro-oxidative stress processes mainly using *in situ* microscopic methods in non-accumulator *Arabidopsis thaliana* and secondary Se accumulator *Brassica juncea*. Relative Se tolerance or sensitivity of the species was evaluated based on growth parameters (fresh and dry weight, root growth) and cell viability. Besides, selenite-triggered cell wall modifications (pectin, callose) and stomatal regulations were determined for the first time. In case of *Arabidopsis*, relative selenite sensitivity was accompanied by decreased stomatal density and induced stomatal opening, callose accumulation, pronounced oxidative stress and moderate nitrosative modifications. In contrast, the selenite-treated, relatively tolerant *Brassica juncea* showed larger number of more opened stomata, pectin accumulation, moderate oxidative and intense nitrosative stress. These suggest that selenite tolerance or sensitivity is rather associated with oxidative processes than secondary nitrosative modifications in higher plants.

1. Introduction

Selenium (Se) is a naturally occurring non-metal element, which is in many ways special and exists in many interchangeable oxidized and organic, inorganic forms. Elevated Se concentrations are naturally found in soils derived from Cretaceous shale rock (Kabata-Pendias, 1998) and Se may accumulate in the environment as the result of anthropogenic activities (Terry et al., 1992). Se shows chemical similarities with sulphur (S), therefore plants use their S uptake and metabolism system to assimilate Se. Some species in *Brassicaceae* family like *Brassica juncea* are sulphur loving and consequently are capable of accumulating larger amount of Se in their tissues (Pilon-Smits and Quinn, 2010). Additionally, these so-called secondary accumulators show reduced sensitivity to the presence of Se. On the other hand, most plant species, like the model plant *Arabidopsis thaliana* are non-Se-accumulators since they accumulate less than 25 μg Se/g dry weight and they cannot tolerate elevated Se levels in their environment (El-Ramady et al., 2015). Besides the plant species, also the applied Se form and the plants' age determine the rate of Se toxicity. The main consequences of Se excess, which are responsible for its toxicity, are the malformation of non-specific selenoproteins, reactive oxygen species (ROS) production and oxidative stress (Van Hoewyk, 2013). For the pro-oxidant properties of Se forms, the depletion of the major antioxidant, glutathione is principally responsible (Van Hoewyk, 2013). Also the disturbance in reactive nitrogen species (RNS) homeostasis and the consequent nitrosative stress are induced as the effect of Se (Lehotai et al., 2016a; Kolbert et al., 2016). The term RNS is used to describe the family of nitric oxide (NO.) originated molecules like, *inter alia*, peroxynitrite ($ONOO^-$), *S*-nitrosoglutathione (GSNO) or nitrogen dioxide radical ($.NO_2$) (Corpas et al., 2007). The intense production of RNS leads to macromolecule modifications resulting in nitrosative stress (Corpas et al., 2007; Valderrama et al., 2007). Posttranslational modification of proteins caused by tyrosine nitration is becoming a useful marker of nitrosative processes in plant systems (Corpas et al., 2013). Nitration of certain tyrosine amino acids occurs in two steps resulting in the formation of 3-nitrotyrosine which induces alterations in protein structure and function and through the prevention of tyrosine phosphorylation it may influence signal transduction as well (reviewed by Kolbert et al., 2017).

Plants evolved protection mechanisms against damaging effects of excess Se such as the production and emission of volatile compounds like dimethyl(di)selenide (DMDSe) (El-Ramady et al., 2015). The rate of

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Se volatilization varies in plant kingdom which is determined also by the forms of Se (de Souza et al., 2000). In addition to protecting against Se toxicity within the plant tissues, Se volatilization may also have important ecological role like deterring herbivores and affecting neighbouring plants. (Schiavon and Pilon-Smits, 2017).

Several plant species are capable of modifying the chemical composition of their cell walls in order to prevent heavy metals from entering the cytoplasm. Increased formation and deposition of lignin or callose effectively reduces metal absorption thus facilitates plant survival and at the same time can partly be responsible for growth inhibition in metal-exposed environment (Le Gall et al., 2015). Moreover, heavy metal-triggered alterations in contents and methylesterification, acetylation status of pectins greatly determine heavy metal binding and the porosity of the cell wall thus its capability for growth (Le Gall et al., 2015).

Our research was motivated partly by the fact that Se-induced cell wall modifications and their possible correlations with Se tolerance are almost completely unknown. Similarly, our knowledge is incomplete regarding the regulatory effect of Se on stomata; although Se volatilization has been extensively studied in several plant species. Se has shown to induce nitro-oxidative stress in non-accumulator pea (Lehotai et al., 2016a), but the relationship between nitrosative processes and Se tolerance has not been examined so far. Our further aim was to evaluate the possible nitro-oxidative stress-inducing effect of selenite in non-accumulator *Arabidopsis thaliana* and secondary accumulator *Brassica juncea*.

2. Materials and methods

2.1. Plant growth conditions

Experiments were carried out with *Brassica juncea* L. Czern (cv. Negro Caballo) and *Arabidopsis thaliana* L. Heynh (Columbia-0). Seeds of both species were surface sterilised in 5% (v/v) sodium hypochlorite, then placed on perlite (in case of *Brassica* seeds) or on ½ Murashige-Skoog medium (in case of *Arabidopsis*) in Eppendorf tubes floating on Hoagland solution. In case of *Brassica,* the seedlings were pre-cultivated for nine days and then treated with 0 (control), 20, 50 or 100 μM sodium selenite (Na_2SeO_3) for one week meaning that *Brassica* plants were 16-days-old at the time of the sampling. In order to obtain the appropriate amount of biomass, *Arabidopsis* plants were grown in Hoagland solution for three weeks before being treated with the same selenite concentrations like mustard for one week. *Arabidopsis thaliana* plants were 28-days-old at the time of harvesting. Anoxia was prevented with constant aeration of the nutrient solution. Both plant species were grown during controlled conditions (150 μmol m^{-2}/s photon flux density, 12 h/12 h light/dark cycle, relative humidity 55–60% and temperature 25 ± 2 °C). All chemicals used during the experiments were purchased from Sigma-Aldrich unless stated otherwise.

2.2. Se content analysis

Leaf and root materials of *Arabidopsis* and *Brassica* were harvested separately and washed in distilled water then dried at 70 °C for 72 hours. Nitric acid (65% w/v, Reanal, Hungary) and hydrogen peroxide (H_2O_2, 30%, w/v, VWR Chemicals, Hungary) were added to dried plant material. The samples were destructed at 200 °C and 1600 W for 15 min. After appropriate dilutions, Se concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700 Series, Santa Clara, USA). Se concentrations are given in μg/g dry weight (DW).

2.3. Evaluation of growth parameters, root system morphology and root cell viability

Fresh and dry weights of plant materials were measured using a balance and the values are given in mg. Primary root length was measured manually and expressed as centimetre. Also the number of visible lateral roots were counted manually and expressed as pieces/root.

In order to evaluate Se tolerance of the species, cell viability in root apical meristem was determined by using fluorescein diacetate (FDA) fluorophore according to Lehotai et al. (2011). Root tips were incubated in 10 μM FDA solution (prepared in 10/50 mM MES/KCl buffer, pH 6.15) for 30 min in darkness and were washed four times in buffer.

2.4. Microscopic visualization of cell wall modifications in the root system

Callose was detected with aniline blue fluorescent dye according to Cao et al. (2011) with slight modifications. The stain was used in 0.1% (w/v) solution containing 1 M of glycine. Root tips were incubated in dye solution for 5 minutes at room temperature, then washed once with distilled water.

Cell wall pectin content was visualized using 0.05% (w/v) ruthenium red (RR) solution prepared with distilled water. Root samples were incubated in RR solution for 15 minutes and were washed once with distilled water according to Durand et al. (2009).

2.5. Examination of stomatal parameters

Plant leaves were submerged in MES/KCl buffer (10/50 mM, pH 6.15) and the epidermal layers were carefully removed using forceps. In every case, strips were prepared from the same part of the leaf blade, avoiding leaf veins. The epidermal cell layers were put on slides using the previous buffer. Pictures were taken with a microscope (Zeiss Axiovert 200 M) using 10x and 40x object lenses. Image analysis was carried out using Axiovision Rel. 4.8 software. Stomatal density (pieces/mm^2) was analysed by counting all stomata in a 200 μm diameter circle. For the stomatal opening analysis, the widths of the stomatal pores were measured and the data are given as μm.

2.6. In situ detection of ROS, glutathione, cell-wall peroxidase activities, lipid peroxidation and RNS in the root tips

Dihydroethidium (DHE) at 10 μM concentration was applied for the detection of superoxide anion levels. Root segments were incubated in darkness at 37 °C for 30 min, and washed two times with Tris-HCl buffer (10 mM, pH 7.4) (Kolbert et al., 2012).

Hydrogen peroxide levels were examined using 50 μM Amplex Red (10-acetyl-3,7 dihydroxyphenoxazine) dye solution in sodium phosphate buffer (50 mM, pH 7.5), then washed once with the same buffer according to Lehotai et al. (2012).

Cellular glutathione levels were detected with the help of monobromobimane (MBB) fluorophore. Root tips were stained in 100 μM dye solution (prepared in distilled water) for 60 min, and then washed once (Lehotai et al., 2016a).

Cell wall peroxidase (POD) activity was examined using 0.2% (w/v) pyrogallol solution containing 0.03% (v/v) hydrogen peroxide prepared in 10 mM phosphate buffer (pH 7.0). Samples were incubated for 15 minutes in room temperature and washed two times with distilled water (Eleftheriou et al., 2015).

Reactive aldehydes produced during lipid peroxidation were visualized using Schiff's reagent according to Arasimowicz-Jelonek et al. (2009). Root tips were incubated in dye solution for 20 minutes and then the reagent was replaced by 0.5% (w/v) $K_2S_2O_5$ (prepared in 0.05 M HCl) for a further 20 min.

Nitric oxide level of the root tips was monitored with the help of 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF-FM DA) according to Kolbert et al. (2012). Root segments were incubated in 10 μM dye solution for 30 min (darkness, 25 ± 2 °C), and washed twice with Tris-HCl (10 mM, pH 7.4).

Peroxynitrite was visualised with 10 μM dihydrorhodamine 123