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The intensity of tyrosine nitration is associated with selenite and selenate toxicity in *Brassica juncea* L.

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Abstract

Selenium phytotoxicity involves processes like reactive nitrogen species overproduction and nitrosative protein modifications. This study evaluates the toxicity of two selenium forms (selenite and selenate at 0, 20, 50 and 100 μM concentrations) and its correlation with protein tyrosine nitration in the organs of hydroponically grown Indian mustard *(Brassica juncea* L.).

Selenate treatment resulted in large selenium accumulation in both *Brassica* organs, while selenite showed slight root-to-shoot translocation resulting in a much lower selenium accumulation in the shoot. Shoot and root growth inhibition and cell viability loss revealed that *Brassica* tolerates selenate better than selenite. Results also show that relative high amounts of selenium are able to accumulate in *Brassica* leaves without obvious visible symptoms such as chlorosis or necrosis. The more severe phytotoxicity of selenite was accompanied by more intense protein tyrosine nitration as well as alterations in nitration pattern suggesting a correlation between the degree of Se forms-induced toxicities and nitroproteome size, composition in *Brassica* organs. These results imply the possibility of considering protein tyrosine nitration as novel biomarker of selenium phytotoxicity, which could help the evaluation of asymptomatic selenium stress of plants.

Key words: *Brassica juncea*, nitric oxide, protein tyrosine nitration, reactive nitrogen species, selenite, selenate

1. Introduction

Selenium (Se) is an essential micronutrient for all living organisms with the exception of higher plants and fungi, where the capability of utilizing Se as an essential micronutrient has probably been lost (Schiavon and Pilon-Smits 2017). Among naturally occurring oxidized selenium forms, selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are water-soluble and are the most bioavailable for plants (Dungan and Frankenberger 1999), both having large accumulation potential in nature (Kaur et al. 2014). Globally, soil selenium concentrations are within the range of 0.01–2.0 mg kg^{-1} with an overall mean of 0.4 mg kg^{-1}. Higher concentrations up to 1200 mg kg^{-1} are found in soils derived from seleniferous parent materials like shales or sandstones (Fordyce 2005; Johnson et al. 2010).

Despite the fact that selenium is non-essential for higher plants, it is still metabolised by them. Selenium shows chemical similarities with sulphur (S), therefore plants use their S uptake and metabolism system to assimilate selenium. However, selenite, the other selenium form metabolized by plants likely enters cells *via* phosphate transporters (Li et al. 2013). Some species in *Brassicaceae* family like *Brassica juncea* are sulphur-loving and consequently are capable of accumulating large amount of Se in their tissues (Pilon-Smits and Quinn 2010). Additionally, these so-called secondary accumulators show reduced sensitivity to the presence of selenium.

Selenium at low concentrations behaves as an antioxidant; delays senescence and promotes plant growth (Kaur et al. 2014). The 0.5 mg kg^{-1} Se concentration proved to be beneficial for promoting growth and yield of Indian mustard (Singh et al. 1980).

Although, extremes of excess selenium have negative effects on plant growth inducing symptoms like stunting of growth, chlorosis, withering and drying of leaves as well as decreased protein synthesis (El-Ramady et al. 2015). These alterations are caused by the sum of complex molecular processes like non-specific selenoprotein formation, disturbance in hormonal balance, in carbon and macro/microelement homeostasis and the evolution of nitro-oxidative stress (reviewed by Kolbert et al. 2016).

Excess selenium is known to induce the overproduction of reactive oxygen species (ROS) leading to oxidative stress (Lehotai et al. 2012; Van Hoewyk 2013; Dimkovikj and Van Hoewyk 2014). Besides, the metabolism of nitric oxide (NO) and its derivatives, the reactive nitrogen species (RNS) like peroxynitrite is also affected by selenium (Chen et al. 2014; Lehotai et al 2012, 2016 a, b). Consequently, nitrosative stress may develop involving mostly protein S-nitrosylation (Corpas et al. 2007), protein nitration and lipid nitration (Mata-Pérez et al. 2016). The RNS-related protein tyrosine nitration is a two-step process caused by peroxynitrite ($ONOO^-$)-originated agents such as hydroxyl radical ($OH^\cdot$), carbonate radicals ($CO_3^{\cdot -}$) and nitrogen dioxide radical ($NO_2^\cdot$) (Souza et al. 2008). Peroxynitrite itself is formed in a rapid, non-enzymatic reaction ($k = 6.7 \times 10^9$ liter $mol^{-1}s^{-1}$) between NO and superoxide anion radical (Padmaja and Huie 1993).

During nitration, a nitro group is added to aromatic rings on one of the two *ortho* carbons of tyrosine amino acids (Tyr) (Gow et al. 2004). The nitration of Tyr is most likely selective and relatively rare in physiological conditions (Bartesaghi et al. 2007). The rare occurrence of nitrated tyrosine residues and the selectivity suggests that protein tyrosine nitration may be a signalling process (Corpas et al. 2011). As a posttranslational modification, nitration has different effects on protein activity. In plant systems, nitration most generally induces activity loss or triggers no changes in function (reviewed by Kolbert et al. 2017). Moreover, protein tyrosine nitration is considered as an indicator for the intensity of nitrosative stress processes (Corpas et al. 2009). Selenite-induced nitrosative and oxidative stress has recently been observed in the non-accumulator *Pisum sativum* (Lehotai et al. 2016 b), but tyrosine nitration as indicator for secondary nitrosative stress in selenium accumulator plants has not been characterized yet. Moreover, RNS metabolism and protein nitration affected by different selenium forms is still unknown. Therefore, this study compares the effect of selenite and selenate in particular on RNS generation and protein tyrosine nitration as indicators for nitrosative stress contributing to selenium toxicity in secondary selenium accumulator *Brassica juncea*. Interestingly, relatively high amounts of selenium can accumulate in different food plants without causing visible symptoms in aerial plant parts (Hawrylak-Nowak et al. 2015; Lehotai et al. 2016; Jiang et al. 2017) which makes difficult visually identifying selenium-rich plants and at the same time poses risk for human health as well. Therefore, this study intends to answer the further question whether tyrosine nitration can be a biochemical marker for selenium toxicity.

2. Materials and methods

2.1. Plant growth conditions

The surface of *Brassica juncea* L. Czern (cv. Negro Caballo) seeds were sterilised in 5% (v/v) sodium hypochlorite then placed on perlite in Eppendorf tubes floating on Hoagland solution. Anoxia was prevented with constant aeration of the nutrient solution. The solution contained 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 0.01 mM Fe-EDTA,10 µM H_3BO_3, 1 µM $MnSO_4$, 5 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$ and 10 µM $CoCl_2$. Seedlings were pre-cultivated for nine days and then treated with 0 (control), 20, 50 and 100 µM sodium selenite (Na_2SeO_3) or selenate (Na_2SeO_4) for two weeks. Conditions in the greenhouse were the following: 150 µmol m^{-2}/s photon flux density with 12h/12h light/dark cycle, relative humidity 55–60% and temperature 25±2 ºC. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

2.2. Analysis of total selenium concentration

Control and treated plant material were harvested and washed in distilled water then dried at 70 ºC for 72 hours. 6 ml of nitric acid (65% w/v, Reanal, Hungary) was added to 100 mg of dried plant material, and after 2 hours of incubation the samples were supplemented with 2 ml of hydrogen peroxide (30%, w/v, VWR Chemicals, Hungary). The samples were destructed at 200 ºC and 1600 W for 15 min. The selenium concentrations of leaf and root samples were determined by inductively coupled plasma mass spectrometry (Agilent 7700 Series, Santa Clara, USA) and the data are given in µg/g dry weight (DW). These analyses were carried out two times with three samples each (n=3).

2.3. Evaluation of selenium tolerance

Shoot and root fresh weight was determined using an analytical scale and then the plant material was dried at 70 ºC for 72 hours for dry weight measurements. Primary root length was measured manually and the data was used to calculate tolerance index according to the following formula:

$$Tolerance\ index = \frac{treated\ root\ lenght}{mean\ control\ root\ lenght} * 100\%$$

Morphological data was acquired from three separate generations and in each generation 15 plants were examined (n=15).

2.4. Fluorescent microscopic analysis

In all microscopic methods, 2 cm-long root tips were used for staining. The root segments were incubated in dye/buffer solution in Petri-dishes and were washed according to the protocols and prepared on microscopic slides in buffer. Microscopic experiments in case of selenite were carried out on three separate plant generations with 10 root tips examined (n=10). In case of selenate, two separate generations were examined with the same sample number.

For the determination of cell viability, fluorescein diacetate (FDA) fluorophore was used according to Lehotai et al. (2011). Root tips were stained for 30 min in darkness with 10 μM fluorophore solution in MES buffer (10/50 mM MES/KCl, pH 6.15) and were washed four times with the same buffer.

To evaluate NO content of root tips, 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF-FM DA) stain was used. Root segments were incubated for 30 min in darkness at room temperature in 10 μM dye solution, and washed twice with Tris-HCl (10 mM, pH 7.4) buffer (Kolbert et al. 2012).

For the detection of superoxide content in roots, dihydroethidium (DHE) was applied at 10 μM concentration. Roots were incubated in darkness at 37 ºC, and were washed with Tris buffer two times (Kolbert et al. 2012).

Peroxynitrite was visualised with dihydrorhodamine 123 (DHR). DHR was applied in 10 μM concentration in Tris buffer for 30 min in darkness. After incubation, root tips were washed with buffer two times (Sarkar et al. 2014).

Cellular gluthatione levels were examined with monobromobrimane (MBB) fluorophore. Root tips were stained in 100 μM dye solution, and then washed once with distilled water (Lehotai et al. 2016 a).

Microscopic analysis of different stained root tips was accomplished under a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (AxiocamHR, HQ CCD, Carl Zeiss, Jena, Germany). Filter set 10 (exc.: 450–490, em.: 515–565 nm) was used for FDA, DAF-FM and DHR, filter set 9 (exc.:450–490 nm, em.:515–∞ nm) for DHE and filter set 49 (exc.: 365 nm, em.: 445/50 nm) was applied for MBB. Circles of 100 µm radii were applied for measuring pixel intensity on digital photographs, using Axiovision Rel. 4.8 software (Carl Zeiss, Jena, Germany).

2.5. Detection of nitrated proteins

Plant tissues 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 and centrifuged at 12,000 rpm for 20 min at 4 °C. After centrifugation, the protein extract was stored at -20 °C. Protein concentration was determined using the Bradford (1976) assay with bovine serum albumin as a standard.

25 µg of root and shoot protein extracts were denaturated and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % acrylamide gels. The proteins were transferred to PVDF membranes using the wet blotting procedure (30 mA, 16h) for immunoblotting. 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 NBT/BCIP reaction. Nitrated bovine serum albumin served as positive control. Western blot was applied to 3 separate protein extracts from different plant generations, multiple times per extract, meaning a total of 7 acquired blotted membranes (n=3).

2.6. Statistical analysis

All results are shown as mean values of raw data (±SE). For statistical analysis, mostly Duncan's multiple range test (One way ANOVA, $P<0.05$) was used in SigmaPlot 12. For the assumptions of ANOVA we used Hartley's F_{max} test for homogeneity and Shapiro-Wilk normality test.

3. Results

3.1. Selenium forms are differentially accumulated and distributed in *Brassica* organs

Both selenium forms were taken up by *Brassica* plants from the nutrient solution; however, the translocation showed differences. Selenate concentration-dependently accumulated in large quantities in both organs, especially in the leaves exceeding 1000 µg/g DW. In contrast, selenite treatment caused remarkably lower selenium accumulation rate in the same organ. In roots, selenite treatment-induced selenium accumulation exceeded that caused by selenate treatment (Fig 1).

3.2. Selenium forms are differentially tolerated by *Brassica juncea*

Selenium accumulation exerted severe toxic effects on plant growth in both organs (Fig 2). The 20 µM selenate treatment had beneficial effect on shoot growth (Fig 2 a,b,f), while 50 µM selenate decreased shoot fresh weight (Fig 2b) and did not significantly influence dry weight (Fig 2f) compared to control. The highest applied selenate concentration (100 µM) remarkably decreased shoot size and biomass (Fig 2 a,b,f). In case of selenite, both shoot fresh and dry weight decreased as the effect of 20 µM concentration (Fig 2 a,c,g). Further concentrations of selenite significantly inhibited shoot fresh and dry biomass production. Furthermore, in 100 µM selenite-treated *Brassica*, serious growth arrest was accompanied by the accumulation of purple pigments (Fig 2 a,c,g). The tendencies were similar regarding root growth, where 20 µM selenate increased and higher concentrations of selenate or selenite decreased biomass (Fig 2 d,e,h,i).

In order to evaluate the overall endurance of *Brassica juncea* to selenium stress, root tolerance index was calculated based upon root growth inhibition (Fig 3 a). Selenate at all applied concentrations resulted in a decreased tolerance index; however, this reduction was significant only in case of 50 and 100 μM. The highest selenate concentration decreased the tolerance index by 30%. Selenite had a similar effect at lower concentration, but 100 μM selenite proved to be more toxic, since it induced 70% loss in tolerance index. The tolerance of *Brassica* plants to different selenium forms was further evaluated by detecting viability of root meristem cells (Fig 3 b,c). In case of selenate, cell viability only slightly and non-significantly diminished as the effect of all applied concentrations. 20 μM selenite exposure did not influence cell viability, but 50 and 100 μM selenite significantly reduced it compared to control resulting in 40 and 20% viability, respectively.

3.3. Selenium forms differentially influence the levels of reactive intermediates in Se-accumulator *Brassica juncea*

Nitric oxide content of *Brassica* root tips slightly elevated in case of almost all treatment concentrations, but these alterations did not prove to be statistically significant (Figure 4 ab). Superoxide radical is the other component participating in peroxynitrite formation. The 20 μM selenate treatment reduced superoxide radical levels in the root tips, and higher concentrations did not affect them significantly compared to control (Figure 4c). Similarly to selenate, non-significant reduction of superoxide radical level in 20 μM selenite-treated root tips was detected. However, in contrast to selenate, 100 μM selenite significantly enhanced superoxide levels resulting in ~2.5-fold accumulation (Figure 4d). The mildest selenate exposure (20 μM) did not influence peroxynitrite levels in roots; however, in case of higher concentrations, peroxynitrite levels decreased compared to control (Figure 4e). In contrast, selenite (especially 50 and 100 μM) significantly induced the formation of peroxynitrite in *Brassica* root tips (Figure 4f). Both ROS and selenium can deplete antioxidants, especially glutathione (GSH), so experiments were performed to evaluate changes in glutathione levels of the root tips. Both selenium forms decreased glutathione levels, but the effect of selenite (Figure 5b) was concentration-dependent and much more pronounced compared to selenate (Figure 5a).

3.4. Selenium forms exert diverse effects on protein tyrosine nitration in *Brassica juncea* organs

Nitrosative stress was characterized by detecting the nitrated proteome using western blot analysis. Compared to the basal nitration pattern, the leaves of selenate-treated plants showed only mild increase in nitrotyrosination without the appearance of newly nitrated protein bands (Fig 6a). In case of selenite, protein nitration enhanced more intensively compared to selenate (Fig 6b). Moreover, the immunopositivity decreased in two protein bands (marked by white arrows on Fig 6b) as the effect of selenite treatments and a newly nitrated band with approximately 60 KDa molecular weight could be detected in the leaves of 100 μM selenite-treated *Brassica* (marked by black arrow on Fig 6b). In roots, selenate treatment exerted more severe effects on proteome nitration compared to leaf nitration (Figure 6c). The most intense nitration was caused by 50 μM selenate, because nitration increased in four protein bands (marked by blue arrows on Fig 6c). Interestingly, 100 μM selenate caused only a mild elevation in nitration, compared to the nitration pattern of control plants. The 20 μM selenate treatment had no visible effect on physiological nitration. Contrary, the other applied selenium form already at 20 μM concentration increased protein nitration (Figure 6d) and the intensification of selenite-triggered protein nitration proved to be more pronounced compared to the effect of selenate treatment. It is important to note, that similarly to selenate, 50 μM selenite caused the most intense tyrosine nitration of the root proteome.

Discussion

In case of both selenium forms *Brassica juncea* were able to uptake and accumulate large amounts of selenium (Fig 1) in tissues similarly to previous data (Sharma et al. 2010). Selenite showed a poor root-to-shoot translocation in agreement with the results of Hawrylak-Nowak et al. (2015), which can be explained by a rapid formation of organic selenium compounds in the roots (de Souza et al. 1998; Zayed et al. 1998). Selenate on the other hand, had a good shoot accumulation rate, slightly supressing selenium levels in the root system (Ramos et al. 2010).

The applied selenite or selenate concentrations influenced growth parameters of *Brassica juncea*. Selenate at low concentrations (20 µM) proved to be beneficial for organ growth (Fig 2), most likely because of the antioxidant effect of selenium which was reportedly able to alleviate stress and consequently promote plant growth (Djanaguiraman et al. 2010; Garcia-Banuelos et al. 2011; Kaur et al. 2014; Hawrylak-Nowak et al. 2015; Ebrahimi et al. 2015; Hajiboland and Keivanfar 2012). At the same time, in case of higher concentrations the growth stunting effect of selenite was more conspicuous than that of selenate suggesting the more intense toxicity of selenite (Hawrylak-Nowak et al. 2015). Results also show that relative high amounts of selenium are able to accumulate in *Brassica* leaves without obvious visible toxic symptoms such as chlorosis or necrosis. Compared to the shoot system, root growth was more sensitive to selenite or selenate stress (Fig 2 d,e,h,i,) due to the large amount of selenium accumulated in this organ. In agreement with earlier studies (Smith and Watkinson 1984; Jun et al. 2015) our cell viability (Fig 3 b,c), root (Fig 3 a) and shoot growth (Fig 2) data indicate that selenate is better tolerated by *Brassica juncea* than selenite, which can be explained by the faster incorporation of selenite into selenoamino acids (Lyons et al. 2005).

The observed inhibitory effect of selenate or selenite on growth partly originates from the fact that excess selenium can disturb the metabolism of reactive oxygen and nitrogen species leading to oxidative and nitrosative stress (Kolbert et al. 2016). Nitric oxide is the key molecule of inducing nitro-oxidative stress; thus, its levels were examined in most experimental designs. Selenite or selenate treatment can result in NO overproduction as was observed in *Pisum sativum* or *Brassica rapa* (Lehotai et al. 2016b; Chen et al. 2014). However, in *Arabidopsis* roots, selenite caused nitrate reductase-independent NO diminution (Lehotai et al. 2016 a). In the present experiments, neither selenite nor selenate influenced significantly the NO levels of *Brassica juncea* root tips (Fig 4 ab). These results indicate that the effect of selenium forms on NO metabolism may depend on plant species. The level of superoxide radical was shown to be elevated by selenium treatment (Tamaoki et al. 2008; Freeman et al. 2010). Interestingly, in *Brassica*, only high concentration of selenite but not selenate caused superoxide accumulation (Fig 4 cd) indicating prooxidative and consequently toxic effect of this selenium form. In case of mild selenate exposure, the level of superoxide decreased, which supports the antioxidant role of low selenate dose as was shown in earlier works (Xue et al. 2001; Djanaguiraman et al. 2010; Ekanayake et al. 2015; Bachiega et al. 2016). Peroxynitrite is a strong oxidative and nitrosative agent in plant cells (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011), thus its concentration could reflect overall stress severity. In selenite-treated plants, $ONOO^-$ levels decreased (Fig 4 e,f,g) due to the possible activation of scavenging mechanisms. Contrary to selenate, selenite induced peroxynitrite generation in roots of *Brassica*, which implies the more severe prooxidant and pronitrant effect of this Se form. Glutathione, as an antioxidant and peroxynitrite-scavenging molecule has a key role in protection against abiotic stress and in plant development as well (Gill et al. 2013). Selenite exposure resulted in the diminution of GSH content in root tips (Fig 5), while selenate did not significantly affect GSH levels. Selenium-induced GSH depletion is widely reported in plants (Van Hoewyk et al. 2008; Tamaoki et al. 2008; Hugouvieux et al. 2009; Freeman et al. 2010; Dimkovikj and Van Hoewyk 2014). In case of selenite, GSH depletion could be explained by a non-enzymatic reduction of selenite, which generates seleno-glutathione and superoxide radical leading to oxidative stress (Wallenberg et al. 2010).

Protein tyrosine nitration is a basal posttranslational modification in plants, regulating protein activity under control conditions (Corpas et al. 2009; Chaki et al. 2009; Chaki et al. 2015). Furthermore, results indicate that the physiological nitration of the proteome is more intense in roots compared to leaves (Corpas et al. 2009; Lehotai et al. 2016 a) suggesting that proteins in the root may be more sensitive to this modification. Our study using *Brassica juncea* confirms both previous observations. In leaves, the two selenium forms had different effects, since despite its high accumulation rate; selenate only slightly affected the degree of protein nitration and did not influence its pattern. In contrast, selenite treatment - which surprisingly caused slight selenium accumulation in leaves - increased the nitration of proteome more intensely and it changed the composition of the nitrated proteome as well. In roots, the nitration patterns are not affected either by selenate or by selenite, but in case of the more toxic selenite, the intensification of protein nitration is more pronounced. Both excess selenite and selenate proved to be pronitrant in *Brassica juncea*, although the intensity of protein nitration as well as the pattern seemingly depends on the applied Se form. Moreover, the rate and pattern of selenium-induced protein nitration shows organ-dependence in Indian mustard.

This study reveals selenate and selenite-induced protein tyrosine nitration in secondary selenium accumulator *Brassica juncea* for the first time. Based on the results, sensitivity against selenium forms may be related to the intensity of protein tyrosine nitration in both organs of *Brassica juncea.* This research is the first to propose the possibility that protein tyrosine nitration can be a biomarker for selenium-induced phytotoxicity, which could help the identification of asymptomatic selenium stress of plants. However, further experiments are needed to support and clarify this practically relevant possibility.

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Figure legends

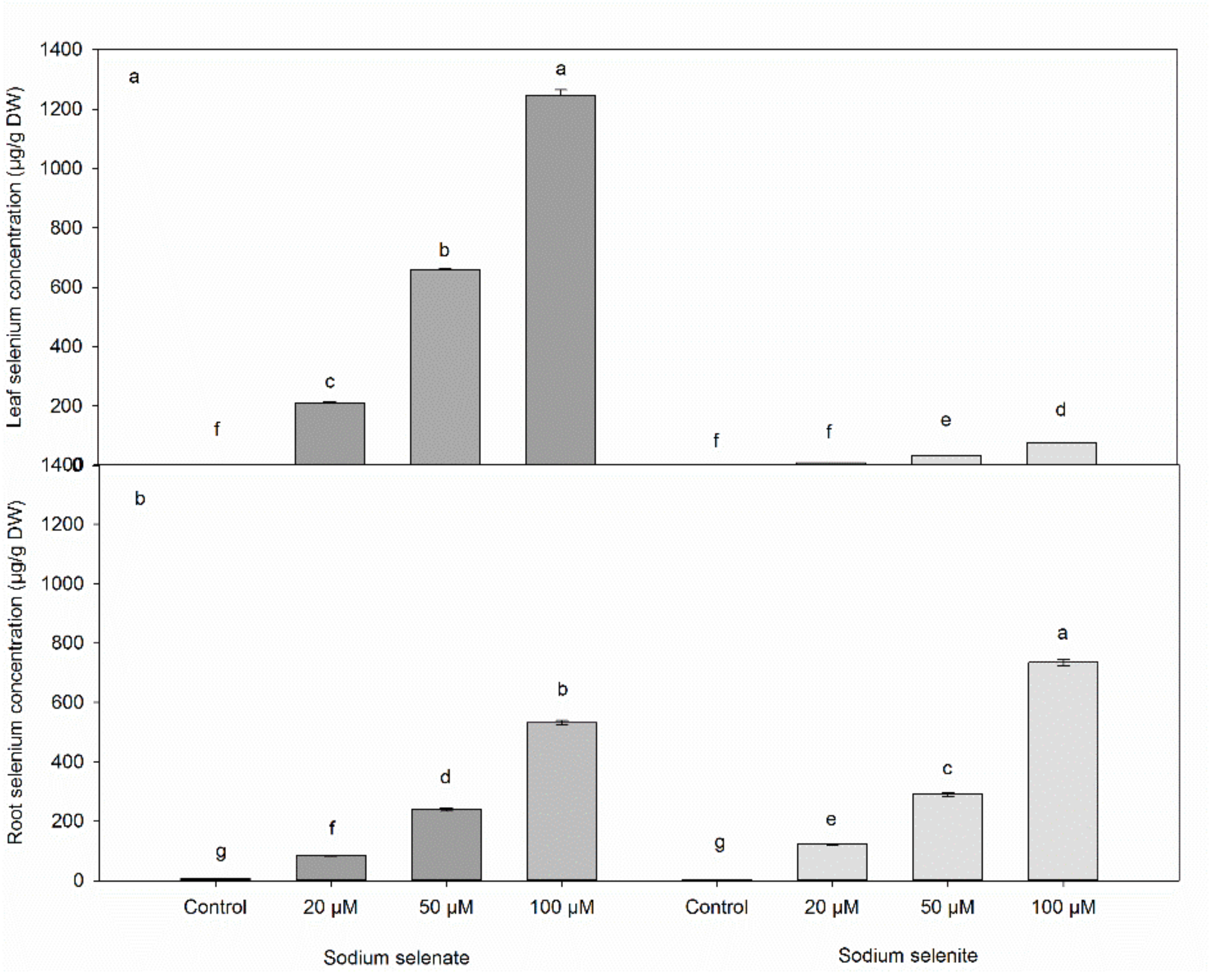

Fig 1 Selenium accumulation in *Brassica* organs

Selenium concentration (µg/g DW) in leaves (a) and roots (b) of *Brassica* treated with different selenium forms. Different letters indicate significant differences according to Duncan’s test (n=3, P≤0.05).

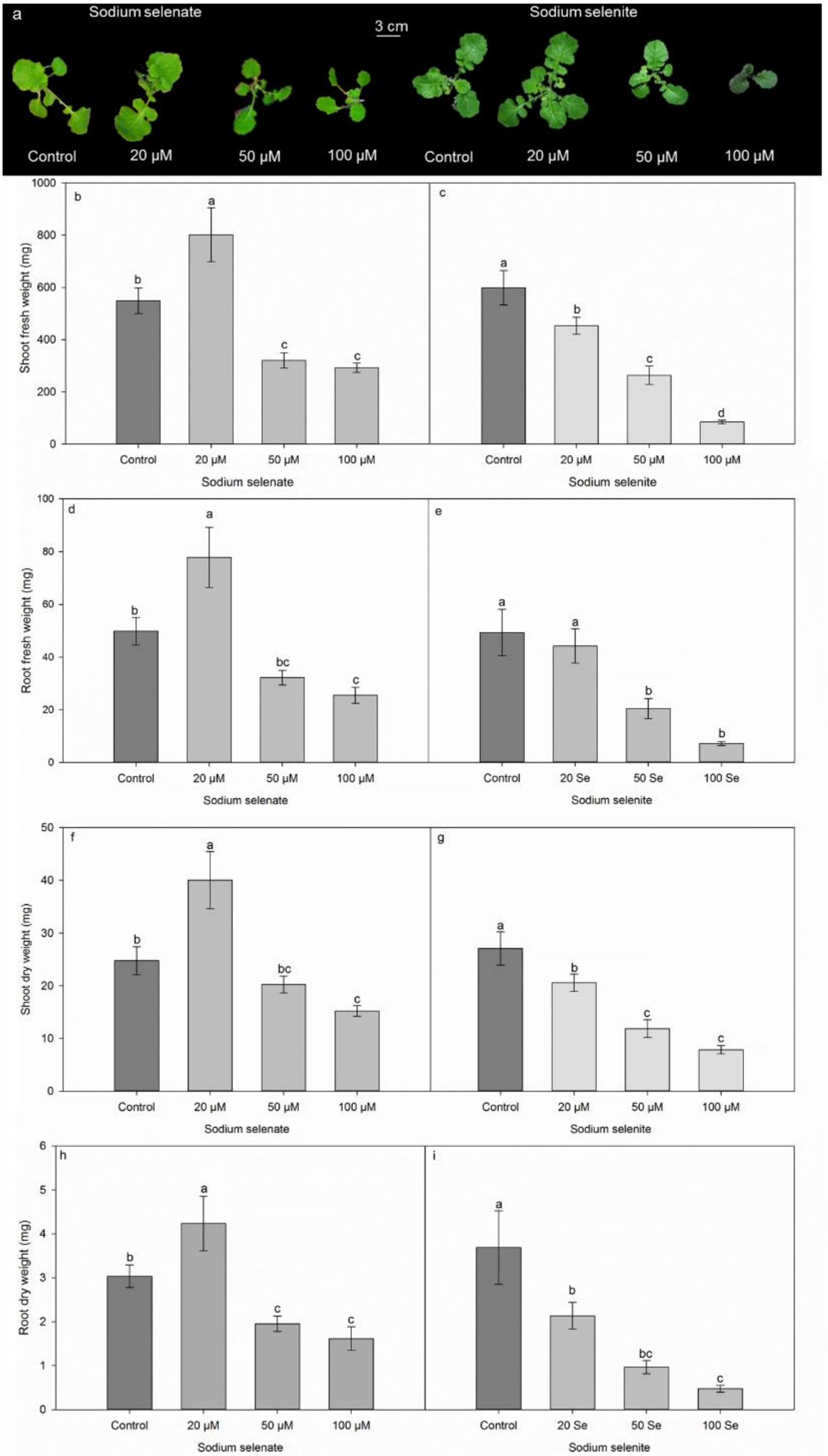
a
Sodium selenate
3 cm
Sodium selenite
Control
20 μM
50 μM
100 μM
Control
20 μM
50 μM
100 μM
b
c
Shoot fresh weight (mg)
0
200
400
600
800
1000
b
a
c
c
a
b
c
d
Control
20 μM
50 μM
100 μM
Sodium selenate
Control
20 μM
50 μM
100 μM
Sodium selenite
d
e
Root fresh weight (mg)
0
20
40
60
80
100
b
a
bc
c
a
a
b
b
Control
20 μM
50 μM
100 μM
Sodium selenate
Control
20 Se
50 Se
100 Se
Sodium selenite
f
g
Shoot dry weight (mg)
0
10
20
30
40
50
b
a
bc
c
a
b
c
c
Control
20 μM
50 μM
100 μM
Sodium selenate
Control
20 μM
50 μM
100 μM
Sodium selenite
h
i
Root dry weight (mg)
0
1
2
3
4
5
6
b
a
c
c
a
b
bc
c
Control
20 μM
50 μM
100 μM
Sodium selenate
Control
20 Se
50 Se
100 Se
Sodium selenite

Fig 2 Selenium forms affect organ development of *Brassica juncea*

Shoot morphology (a), fresh (b,c), dry (f,g) weight and root fresh (d,e) and dry (h,i) weight of 14 day-old *Brassica juncea* plants treated with different selenate or selenite concentrations for 14 days. Bar=3 cm. Different letters indicate significant differences according to Duncan's test (n=15, P≤0.05).

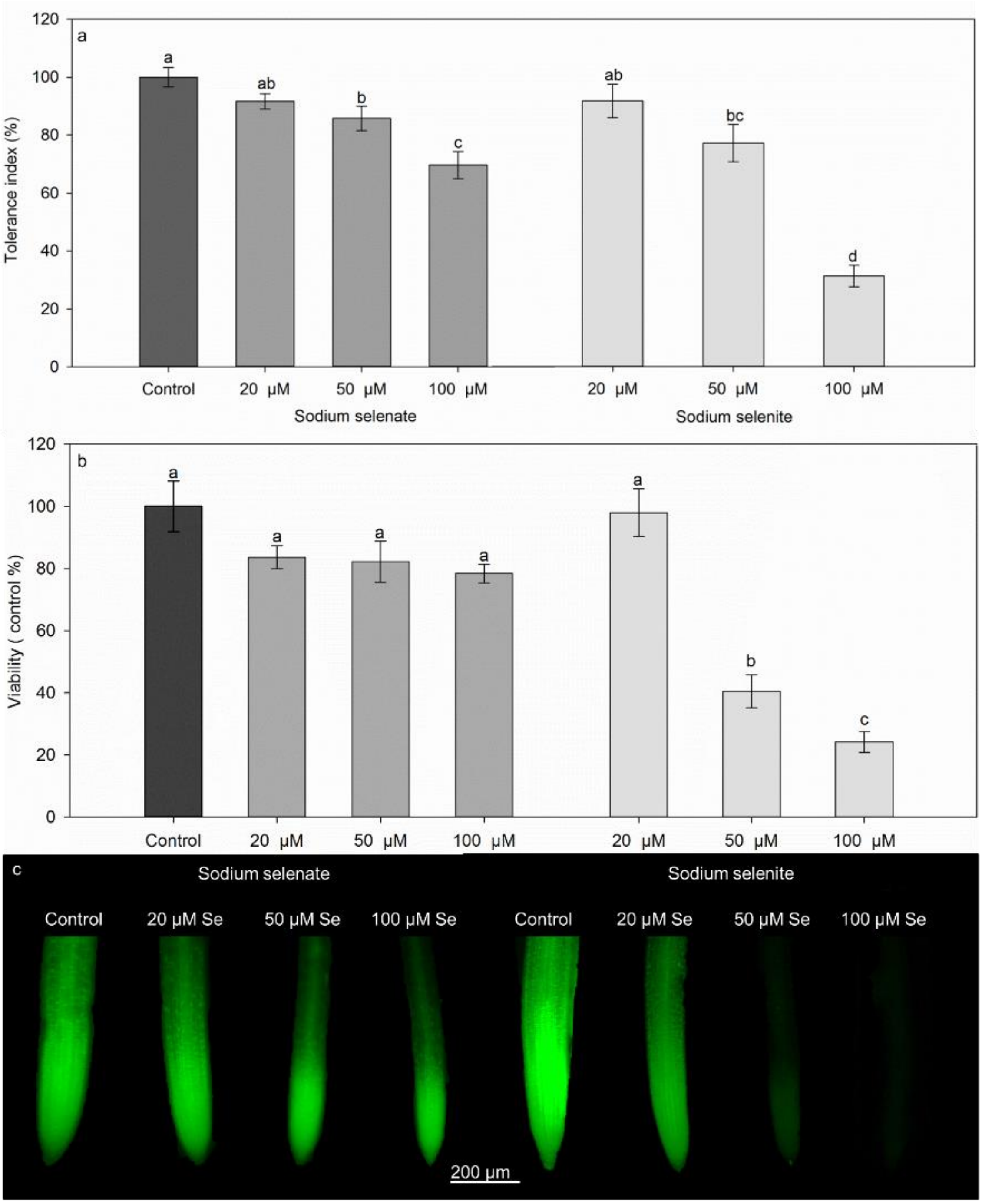

Fig 3 *Brassica* plants differentially tolerates selenite and selenate

Root tolerance index (%) calculated from primary root lengths of selenate- or selenite-treated *Brassica juncea* (a, n=15). Cell viability in meristems of selenate- or selenite-treated *Brassica juncea* roots (b), Different letters indicate significant differences according to Duncan’s test (n=15, $P \leq 0.05$). Representative fluorescent microscopy images showing-FDA stained root tips (c)

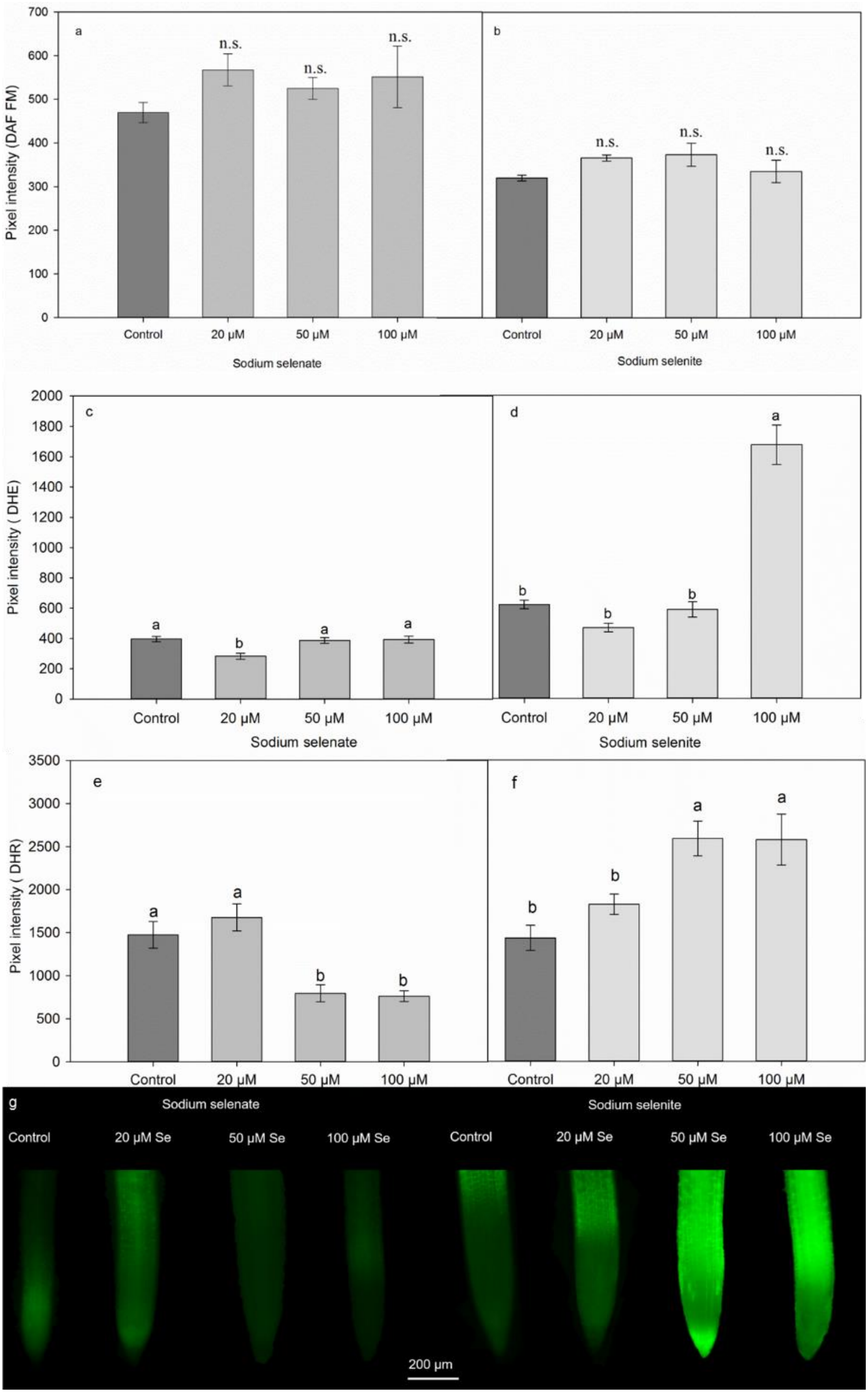
a
b
Pixel intensity (DAF FM)
0
100
200
300
400
500
600
700
n.s.
n.s.
n.s.
n.s.
n.s.
n.s.
Control
20 µM
50 µM
100 µM
Sodium selenate
Sodium selenite
c
d
Pixel intensity (DHE)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
a
b
a
a
b
b
b
a
Control
20 µM
50 µM
100 µM
Sodium selenate
Sodium selenite
e
f
Pixel intensity (DHR)
0
500
1000
1500
2000
2500
3000
3500
a
a
b
b
b
b
a
a
Control
20 µM
50 µM
100 µM
g
Sodium selenate
Sodium selenite
Control
20 µM Se
50 µM Se
100 µM Se
Control
20 µM Se
50 µM Se
100 µM Se
200 µm

Fig 4 Selenium forms disturb RNS homeostasis in *Brassica* roots

Nitric oxide levels in the root meristem of selenate (a)- or selenite (b)-treated *Brassica juncea* visualised by DAF-FM DA. Superoxide radical levels in the roots of selenate (c)- or selenite (d)-treated *Brassica juncea* stained with DHE. Peroxynitrite levels in the roots of selenate (e)- or selenite (f)-treated *Brassica juncea* detected with DHR. N.s. indicates statistically non-significant differences and different letters indicate significant differences according to Duncan's test (n=10, $P \leq 0.05$). Representative fluorescent microscopy images showing DHR-stained root tips (g).

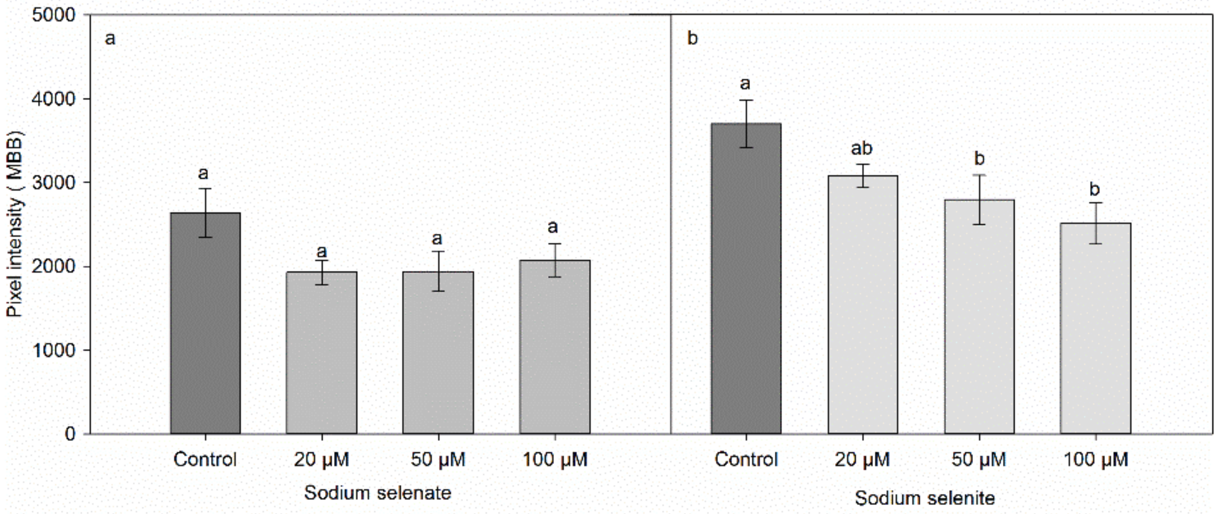

Fig 5 Glutathione depletion induced by selenium forms

Gluthatione levels in selenate (a)- or selenite (b)-treated *Brassica* root meristem detected with MBB fluorophore. Different letters indicate significant differences according to Duncan's test (n=10, P≤0.05).

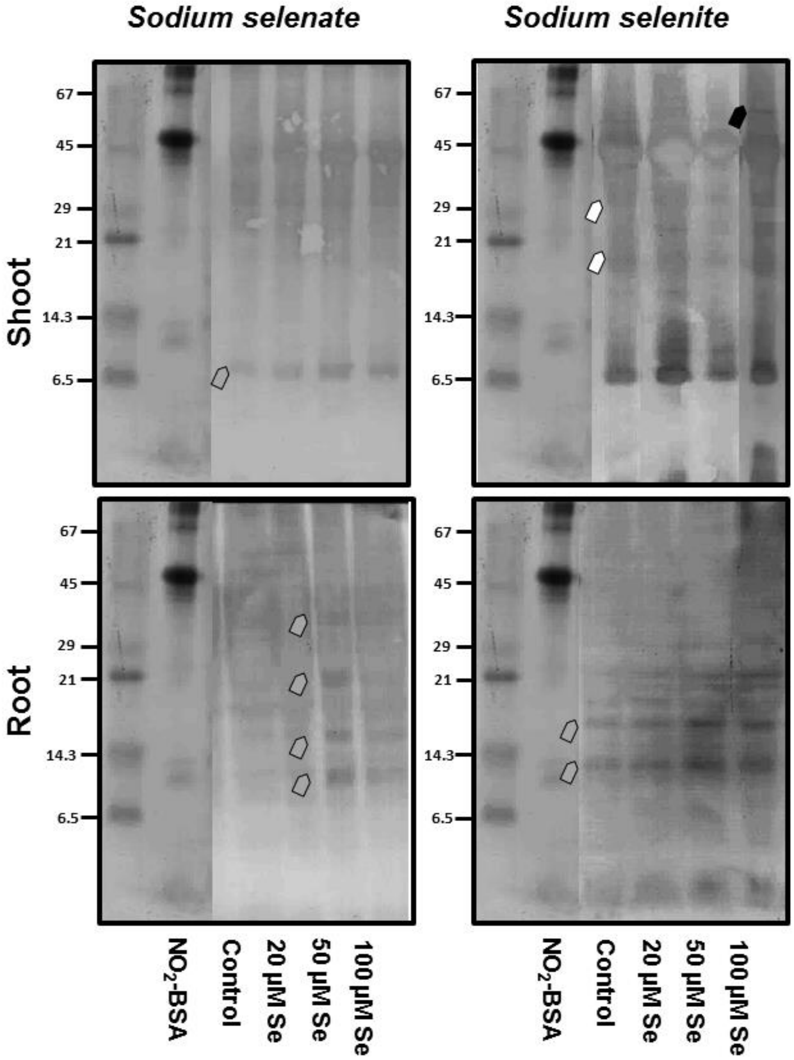

Fig 6 Pronitrant effects of selenium forms in *Brassica* proteome

Representative immunoblots showing protein tyrosine nitration in leaves (a,b) and roots (c,d) of *B. juncea* plants grown under control conditions and treated with selenate (a,c) or selenite (b,d). Commercial nitrated BSA (NO_2-BSA) was used as positive control and molecule marker (MM) is shown as a protein weight indicator. White arrows show decrease in nitration, grey arrows show intensified nitration. Black arrow indicates a newly appeared nitrated protein band.

Reference list

Arasimowicz-Jelonek M, Floryszak-Wieczorek J (2011) Understanding the fate of peroxynitrite in plant cells – from physiology to pathophysiology. Phytochem 72:681–688.

Doi: 10.1016/j.phytochem.2011.02.025

Bachiega P, Salgado J M, de Carvalho J E, Ruiz A L T, Schwarz K, Tezotto T, Morzelle MC (2016) Antioxidant and antiproliferative activities in different maturation stages of broccoli (*Brassica oleracea* Italica) biofortified with selenium. Food Chem 190: 771-776.

Doi: 10.1016/j.foodchem.2015.06.024

Bartesaghi S, Ferrer-Sueta G, Peluffo G, Valez V, Zhang H, Kalyanaraman B, et al. (2007) Protein tyrosine nitration in hydrophilic and hydrophobic environments. Amino Acids 32:501–515.

Doi:10.1007/s00726-006- 0425-8

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248–255.

Doi: 10.1016/0003-2697(76)90527-3

Chaki M, Valderrama R, Fernández-Ocana AM, et al. (2009) Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. J Exp Bot 60:4221–4234.

Doi: 10.1093/jxb/erp263

Chaki M, de Morales PÁ, Ruiz C, Begara-Morales JC, Barroso JB, Corpas FJ, Palma JM (2015) Ripening of pepper (*Capsicum annuum*) fruit is characterized by an enhancement of protein tyrosine nitration. Ann Bot 116(4):637-47.

Doi:10.1093/aob/mcv016

Chen Y, Mo H-Z, Zheng M-Y, Xian M, Qi Z-Q, Li Y-Q et al. (2014) Selenium Inhibits Root Elongation by Repressing the Generation of Endogenous Hydrogen Sulfide in *Brassica rapa*. PLoS ONE 9(10): e110904. Doi:10.1371/journal.pone.0110904

Corpas FJ, Carreras A, Valderrama R, Chaki M, Palma JM, del Río LA, Barroso JB (2007) Reactive nitrogen species and nitrosative stress in plants. Plant Stress 1:37-41.

Corpas FJ, Chaki M, Leterrier M, Barroso JB (2009) Protein tyrosine nitration: a new challenge in plants. Plant Sign Behav 4:920–923.

Corpas FJ, Leterrier M, Valderrama R, Airaki M, Chaki M, Palma JM, Barroso JB (2011) Nitric oxide imbalance provokes a nitrosative response in plants under abiotic stress. Plant Sci 181:604–611. Doi: 10.1016/j.plantsci.2011.04.005

Dimkovikj A, Van Hoewyk D (2014) Selenite activates the alternative oxidase pathway and alters primary metabolism in Brassica napus roots: evidence of a mitochondrial stress response. BMC Plant Biol 14:259. Doi:10.1186/s12870-014-0259-6

Djanaguiraman M, Prasad PV, Seppanen M (2010) Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. Plant Physiol Biochem 48:999–1007. Doi:10.1016/j.plaphy.2010.09.009

Dungan RS, Frankenberger JWT (1999) Microbial transformations of selenium and the bioremediation of seleniferous environments. Bioremediat J 3:171–188. Doi:10.1080/10889869991219299

Ebrahimi N, Hartikainen H, Simojoki A, Hajiboland R, Seppänen M (2015) Dynamics of dry matter and selenium accumulation in oilseed rape (*Brassica napus* L.) in response to organic and inorganic selenium treatments. Agri Food Sci 24(2):104-117.

Ekanayake L J, Vial E, Schatz B, McGee R, Thavarajah P (2015) Selenium fertilization on lentil (*Lens culinaris* Medikus) grain yield, seed selenium concentration, and antioxidant activity. Field Crops Res 177: 9-14. Doi: 10.1016/j.fcr.2015.03.002

El-Ramady H, Abdalla N, Alshaal T, et al. (2015) Selenium and its role in higher plants. In: E. Lichtfouse et al. (eds.), Pollutants in Buildings, Water and Living Organisms, Environmental Chemistry for a Sustainable World 7, Springer, pp 238-285. Doi 10.1007/978-319-19276-5_6

Fordyce F (2005) Selenium deficiency and toxicity in the environment. In: Selinus O, Alloway B, Centeno J, Finkelman R, Fuge R, Lindh U, Smedley P (eds). Essentials of Medical Geology, Elsevier, London, pp 373–415.

Freeman JL, Tamaoki M, Stushnoff C et al. (2010) Molecular mechanisms of selenium tolerance and hyperaccumulation in *Stanleya pinnata*. Plant Phys 153:1630–1652. Doi: 10.1104/pp.110.156570

Garcia-Banuelos ML, Hermosillo-Cereceres A, Sanchez EM (2011) The Importance of Selenium Biofortification in Food Crops. Curr Nutr Food Sci 7:181–190. Doi: 10.2174/157340111797264796#sthash.qSryv1uw.dpuf

Gill SS, Anjum NA, Hasanuzzaman M, Gill R, Kumar DT, Ahmad I, Pereira E, Tuteja N (2013) Glutathione and glutathione reductase: A boon in disguise for plant abiotic stress defense operations. Plant Phys Biochem 70:204–212. Doi: 10.1016/j.plaphy.2013.05.032

Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H (2004) Biological significance of nitric oxide-mediated protein modifications. Am J Physiol Lung Cell Mol Physiol 287:L262–L268.
Doi: 10.1152/ajplung.00295.2003

Jiang C, Zu C, Lu D, Zheng Q, Shen J, Wang H, Li D (2017) Effect of exogenous selenium supply on photosynthesis, Na+ accumulation and antioxidative capacity of maize (*Zea mays* L.) under salinity stress. Sci Rep 7: 42039
Doi: doi:10.1038/srep42039

Hajiboland R, Keivanafar N (2012) Selenium supplementation stimulates vegetative and reproductive growth in canola (*Brassica napus* L.) plants. Acta Agric Slov 99-1:13-19.
Doi: 10,2478/v10014-012-0002-7

Hanson B, Lindblom SD, Loeffler ML, Pilon-Smits EAH (2004) Selenium protects plants from phloem feeding aphids due to both deterrence and toxicity. New Phytol. 162:655–662. doi:10.1111/j.1469-8137.2004.01067.x

Hawrylak-Nowak B, Matraszek R, Pogorzelec M (2015) The dual effects of two inorganic selenium forms on the growth, selected physiological parameters and macronutrients accumulation in cucumber plants. Acta Physiol Plant 37:41.
Doi:10. 1007/s11738-015-1788-9

Hugouvieux V, Dutilleul C, Jourdain A, Reynaud F, Lopez V, Bourguignon J (2009) *Arabidopsis* putative selenium-binding protein1 expression is tightly linked to cellular sulfur demand and can reduce sensitivity to stresses requiring glutathione for tolerance. Plant Phys 151:768–781.
Doi: 10.1104/pp.109.144808.

Johnson CC, Fordyce FM, Rayman MP (2010) Symposium on ‘Geographical and geological influences on nutrition’ factors controlling the distribution of selenium in the environment and their impact on health and nutrition. Proc Nutr Soc 69:119–132.
Doi: 10.1017/ S0029665109991807

Jun Li, Dongli Liang, Siyue Qin, Puyang Feng, Xiongping Wu (2015) Effects of selenite and selenate application on growth and shoot selenium accumulation of pak choi (*Brassica chinensis* L.) during successive planting conditions. Environ Sci Pollut Res 22(14): 11076–11086.
Doi: 10.1007/s11356-015-4344-7

Kaur N, Sharma S, Kaur S, Nayyar H (2014) Selenium in agriculture: a nutrient or contaminant for crops? Arch Agron Soil Sci 60:1593-1624.
Doi: 10.1080/03650340.2014.918258

Kolbert Zs, Feigl G, Bordé Á, Molnár Á, Erdei L (2017) Protein tyrosine nitration in plants: Present knowledge, computational prediction and future perspectives. Plant Physiol Biochem 113:56–63.
Doi: 10.1016/j.plaphy.2017.01.028

Kolbert Zs, Lehotai N, Molnár Á, Feigl G (2016) "The roots" of selenium toxicity: a new concept. Plant Signal Behav 11(10): e1241935
Doi:10.1080/15592324.2016.1241935

Kolbert Zs, Pető A, Lehotai N, Feigl G, Ördög A, Erdei L (2012) In vivo and in vitro studies on fluorophore-specificity. Acta Biol Szeged 56:37–41.

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 Biol Szeged 55:105–107

Lehotai N, Kolbert Zs, Pető A, Feigl G, Ördög A, Kumar D, Tari I, Erdei L (2012) Selenite-induced hormonal and signaling mechanisms during root growth of *Arabidopsis thaliana* L. J Exp Bot 63:5677–5687. Doi: 10.1093/jxb/ers222

Lehotai N, Feigl G, Koós Á, Molnár Á, Ördög A, Pető A, Erdei L, Kolbert Zs (2016a) Nitric oxide-cytokinin interplay influences selenite sensitivity in *Arabidopsis*. Plant Cell Rep Doi:10.1007/s00299-016-2028-5

Lehotai N, Lyubenova L, Schröder P, Feigl G, Ördög A, Szilágyi K, Erdei L, Kolbert Zs (2016b) Nitro-oxidative stress contributes to selenite toxicity in pea (*Pisum sativum* L.). Plant Soil 400:107-122 Doi: 10.1007/s11104-015-2716-x

Li HF, McGrath SP, Zhao FJ (2008) Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. New Phytol 178:92–102. Doi:10.1111/j.1469-8137.2007.02343.x

Lindermayr C, Saalbach G, Durner J (2005) Proteomic identification of S-nitrosylated proteins in *Arabidopsis*. Plant Physiol 137:921–930. Doi: 10.1104/pp.104.058719

Lyons GH, Stangoulis JCR, Graham RD (2005) Tolerance of wheat (*Triticum aestivum* L.) to high soil and solution selenium levels. Plant Soil 270:179–188. Doi: 10.1007/s11104-004-1390-1

Mata-Pérez C, Sánchez-Calvo B, Padilla MN, Begara-Morales JC et al. (2016) Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in *Arabidopsis*. Plant Phys 170(2):686-701. Doi: 10.1104/pp.15.01671

Padmaja S, Huie RE (1993) The reaction of nitric oxide with organic peroxyl radicals. Biochem Biophys Res Commun 195: 539–544.

Doi:10.1006/bbrc.1993.2079

Pilon-Smits EAH, Quinn CF (2010) Selenium metabolism in plants. Cell Biol Metals Nutr 17: 225-241

Doi: 10.1007/978-3-642-10613-2_10

Ramos SJ, Faquin V, Guilherme LRG, Castro EM, Ávila FW, Carvalho GS, Bastos CEA, Oliveira C (2010) Selenium biofortification and antioxidant activity in lettuce plants fed with selenate and selenite. Plant Soil Environ 56(12): 584–588.

Sarkar TS, Biswas P, Ghosh, KS, Ghosh S (2014) Nitric oxide production by necrotrophic pathogen *Macrophomina phaseolina* and the host plant in charcoal rot disease of jute: complexity of the interplay between necrotroph-host plant interactions. Doi:10.0371/journal-pone.0107348

Schiavon M and Pilon-Smits EAH (2017) The fascinating facets of plant selenium accumulation – biochemistry, physiology, evolution and ecology. New Phytol 213:1582–1596.

Doi: 10.1111/nph.14378

Sharma S, Bansal A, Dhillon SK et al. (2010) Comparative effects of selenate and selenite on growth and biochemical composition of rapeseed (*Brassica napus* L.). Plant Soil 329-339.

Doi:10.1007/s11104-009-0162-3

Singh M, Singh N, Bhandari DK (1980) Interaction of selenium and sulfur on the growth and chemical composition of raya. Soil Sci 129:238–244.

Doi:10.1097/00010694-198004000-00007

Smith GS, Watkinson JH (1984) Selenium toxicity in perennial ryegrass and white clover. New Phytol 97(4): 557–564.
Doi: 10.1111/j.1469-8137.1984.tb03619.x

Souza JM, Peluffo G, Radi R (2008) Protein tyrosine nitration. Functional alteration or just a biomarker? Free Rad Biol Med 45:357–366.
Doi: 10.1016/j.freeradbiomed.2008.04.010.

de Souza MP, Pilon-Smits EAH, Lytle CM, Hwang S, Tai J, Honma TSU, Yeh L, Terry N (1998) Rate-limiting steps in selenium assimilation and volatilization by Indian mustard. Plant Phys 117:1487–1494.
Doi: 10.1104/pp.117.4.1487

Tamaoki M, Freeman JL, Pilon-Smits EAH (2008) Cooperative ethylene and jasmonic acid signaling regulates selenate resistance in *Arabidopsis*. Plant Phys 146:1219–1230.
Doi: 10.1104/pp.107.110742

Van Hoewyk D, Takahashi H, Inoue E, Hess A, Tamaoki M, Pilon-Smits EAH (2008) Transcriptome analyses give insights into selenium-stress responses and selenium tolerance mechanisms in *Arabidopsis*. Phys Plant 132:236–253.
Doi: 10.1111/j.1399-3054.2007.01002.x

Van Hoewyk D (2013) A tale of two toxicities: malformed selenoproteins and oxidative stress both contribute to selenium stress in plants. Ann Bot 112:965–972.
Doi: 10.1093/aob/mct163

Wallenberg M, Olm E, Hebert C, Björnstedt M, Fernandes AP (2010) Selenium compounds are substrates for glutaredoxins: a novel pathway for selenium metabolism and a potential mechanism for selenium-mediated cytotoxicity. Biochem J 429:85–93.
Doi: 10.1042/BJ20100368

Xue T, Hartikainen H, Piironen V (2001) Antioxidative and growth-promoting effect of selenium on senescing lettuce. Plant Soil 237(1): 55-61. Doi: 10.1023/A:1013369804867

Zayed A, Lytle CM, Terry N (1998) Accumulation and volatilization of different chemical species of selenium by plants. Planta 206: 284–292. Doi: 10.1007/s004250050402