Title: Nitro-oxidative stress contributes to selenite toxicity in pea (*Pisum sativum* L.)

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Abstract

Background and aims Selenium (Se) phytotoxicity at the cellular level disturbs the synthesis and functions of proteins, together with the generation of an oxidative stress condition. This study reveals the nitro-oxidative stress events, supplemented by a broad spectrumed characterisation of the Se-induced symptoms.

Methods Applying several, carefully selected methods, we investigated the selenite treatment-induced changes in the Se and sulphur contents, pigment composition, hydrogen peroxide level, activity of the most important antioxidative enzymes, glutathione, nitric oxide and peroxynitrite, lipid peroxidation and protein tyrosine nitration.

Results The Se content increased intensively and concentration-dependently in the organs of the treated plants, which led to altered vegetative and reproductive development. The level of the investigated reactive oxygen species and antioxidants supported the presence of the Se-induced oxidative stress, but also pointed out nitrosative changes, in parallel.

Conclusions The presented results aim to map the altered vegetative and reproductive development of Se-treated pea plants. Mild dose of Se has supportive effect, while high concentrations inhibit growth. Behind Se toxicity, we discovered both oxidative and nitrosative stress-induced modifications. Moreover, the presented data first reveals selenite-induced concentration- and organ-dependent tyrosine nitration in pea.

Keywords Nitrosative stress, oxidative stress, *Pisum sativum* L., selenite

Introduction

Selenium (Se) is a non-metal microelement essential for some prokaryotes including archaea, bacteria and protozoa, certain green algae and mammals. However, the essentiality of Se in higher plants has not been proved so far (Van Hoewyk 2013). It has been described that the chemical similarity of Se to sulphur (S) makes possible its uptake and metabolism *via* S pathways in plants. Plants predominantly take up selenium by sulphate or even phosphate transporters in the form of selenate (SeO_4^-) (Terry et al. 2000). Generally, as a naturally occurring element, Se ranges from 0.01 to 2 mg kg^{-1} with an overall mean of 0.4 mg kg^{-1} in soils. Elevated Se levels can also be found naturally, in soils derived from Cretaceous shale rock or as a result of anthropogenic activities, such as mining, agriculture, household or oil production (Dhillon and Dhillon 2003; Dhillon et al. 2008). Selenium levels higher than 5 mg kg^{-1} in the tissues are toxic for most plant species (Reilly 1996). At the whole plant level, the characteristic symptoms of Se toxicity are, necrosis, withering and drying of leaves chlorosis (Mengel and Kirkby 1987), reduced photosynthetic activity and premature death (Tripathi and Misra 1974); however the toxic levels of Se for plants vary between species (Kaur et al. 2014). Moreover, excess of selenium reduces shoot biomass by decreasing fresh weight, hypocotyl length and cotyledon diameter of *Arabidopsis* (Grant et al. 2011; Ohno et al. 2012; Lehotai et al. 2011) and also affects the root system through the inhibition of primary root elongation (Grant et al. 2011; Lehotai et al. 2012). At cellular level, toxicity is partly caused by the alteration of protein synthesis, structure and function, as a result of the incorporation of Se in the amino acids, cysteine and methionine. Other principle mechanism of Se

phytotoxicity is the disruption of the redox balance and the subsequently generated oxidative stress (Van Hoewyk et al. 2013). In the latter process, glutathione (GSH) could have a fundamental role. Its dose- and time-dependent depletion under the influence of selenium, can be the reason for growth inhibition, reactive oxygen species (ROS) production and oxidative stress, and its level has been shown to be associated with Se tolerance (Hugouvieux et al. 2009; Grant et al. 2011).

Besides ROS, reactive nitrogen species (RNS) as the family of nitric oxide (NO)-related molecules, are also produced during diverse stress responses (Procházková et al. 2014). RNS includes non-radical molecules, such as peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), *S*-nitrosoglutathione (GSNO), nitrosonium cation (NO^+) and nitroxyl anion (NO^-) and also radicals such as nitrogen dioxide radical ($NO_2^{\cdot}$) (Wang et al. 2013). The overproduction of RNS in cells results in secondary nitrosative stress (Corpas et al 2007; 2011). Since there is an active interplay between ROS and RNS and their signalling overlaps (Lindermayr and Durner 2015), the secondary stress triggered by them can be considered as nitro-oxidative stress (Corpas and Barroso 2013). An example for the ROS-RNS crosstalk is the *in vivo* formation of peroxynitrite from the reaction between superoxide anion ($O_2^{\cdot -}$) and NO, which is responsible for the protein tyrosine nitration, a reliable biomarker of nitrosative stress in plants (Corpas et al. 2007; 2013). Tyrosine nitration is a two-step posttranslational modification process leading to a nitro group ($-NO_2$) addition to the tyrosine radical in a radical-radical termination reaction (Souza et al. 2008). It causes steric and electronic perturbations, which modifies the tyrosine's capability to function in electron-transfer reactions or to keep the proper protein conformation (van der Vliet et al. 1999). Tyrosine nitration can modify the protein functions in several ways; however the general outcome of nitration is a decreased protein activity (Corpas et al. 2013). Furthermore, tyrosine nitration can indirectly influence other signal transduction pathways e.g. by preventing the phosphorylation of tyrosine residues (Galetskiy et al. 2011).

Despite the importance of green pea (*Pisum sativum* L.) as a traditional edible crop cultivated in large areas and in large quantities worldwide (Santalla et al. 2001), data available on selenium accumulation and toxicity mechanisms are scarce. Moreover, considering the fact that reactive nitrogen species are multifunctional plant signals, it is attractive to hypothesize that they might be involved in selenium phytotoxicity. Therefore, the goal of this study was to characterize the accumulation and phytotoxicity of selenium in green pea, in particular the RNS-associated nitrosative processes and their crosstalk with Se-induced oxidative stress.

Materials and methods

Plant material and growth conditions

Seeds of *Pisum sativum* L. cv. Petit Provençal were surface sterilized by immersion in 5% (v/v) sodium hypochlorite for 10 min, followed by washing with running water for 2 h. Germination took place in Petri dishes between moist filter papers at 26°C for 4 days. Seedlings were placed into perlite-filled plastic pots (4 seedlings/pot) and watered with full-strength Hoagland solution, resulting in semi-hydroponic conditions. Plants were precultivated for seven days and then treated with 0 (control), 10, 50 or 100 μM sodium selenite (Na_2SeO_3) added into the Hoagland solution for fourteen days. Plants were grown in greenhouse at a photon flux density of 150 μmol m^{-2} s^{-1} (12/12 h light/dark cycle) at a relative humidity of 55–60% and at 25 ± 2°C.

All chemicals used during the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Element content analysis

The element analysis was carried out with an inductively coupled plasma mass spectrometer (ICP-MS, Thermo Scientific XSeriesII, Asheville, USA). Roots and leaves of 0, 10, 50 and 100 μM Se-treated pea plants were harvested separately and rinsed with distilled water. After 72 hours of drying at 70°C, nitric acid (65%, w/v) and hydrogen peroxide (H_2O_2, 30%, w/v) (both from Reanal, Budapest, Hungary) were added to the samples, which were subjected to microwave-assisted digestion (MarsXpress CEM, Matthews, USA) at 200°C and 1600 W for 15 min. Values of Se and S concentrations are given as μg g^{-1} dry weight (DW).

Morphological measurements

Fresh weight (g) of the shoot and root material was measured on the 14th day of the treatment. The length (cm) of the shoot and the primary root was also measured manually using a scale. The measurements were performed by the same person to avoid human technical mistakes.

Measurement of chlorophyll and carotenoid contents

Total pigment content was determined according to Lichtenthaler (1987). Leaf material was homogenized in liquid nitrogen and 0.5 g of each sample was centrifuged with 80% acetone for 20 min at 7000 rpm. The supernatants were collected in Falcon tubes and the pellets were subjected to a second and third repeat of the first step. The optical density (OD) was measured using a Spektral photometer (Beckman Coulter 740) at 663, 646 and 470 nm. The amount of pigments was calculated according to the equations: Chl *a* = 12.25 OD_{663} - 2.79 OD_{646}, Chl *b* = 21.5 OD_{646} - 5.1 OD_{663}, Chl *a* + *b* = 7.15 OD_{663} + 18.71 OD_{646}, and carotenoids = (1000 OD_{470} – 1.82 Chl *a* – 85.02 Chl *b*)/198 (Lichtenthaler 1987).

Spectrophotometric determination of hydrogen peroxide

The quantitative determination of H_2O_2 was carried out according to the method of Velikova et al. (2000). Fresh root and leaf materials were homogenized in ice bath with 0.1% (w/v) trichloroacetic acid (TCA). After a 20 min centrifugation at 7000 rpm at 4°C, supernatants were collected and 10 mM phosphate (pH 7.0) and 1 M potassium iodide buffers were added to the samples. The absorbance was determined 10 min after the mixing step, at 390 nm, using phosphate buffer as blank.

Enzyme extraction

The extraction of glutathione S-transferases (GSTs) and antioxidative enzymes was performed by the method of Schröder et al. (2005) with some modifications. Leaves and roots were homogenized in liquid nitrogen with a mortar and pestle to a fine powder and extracted at 4°C in ten-fold volumes (w/v) of 0.1 M Tris/HCl buffer (pH 7.8) containing 1% soluble PVP K90, 5 mM 1,4-dithioerythritol (DTE), 1% Nonidet P40 and 5 mM EDTA. The crude extract was centrifuged at 12 000 rpm and 4°C for 30 min. Proteins in the supernatant were precipitated by stepwise addition of solid ammonium sulfate first to 40% and then to 80% saturation. After each step, the extracts were centrifuged at 5000 rpm and 4°C for 30 min. After the second centrifugation, pellets were resuspended in 2 mL of 25 mM Tris/HCl buffer (pH 7.8), then the extracts were desalted and further purified by passing them through PD10 desalting columns (Pharmacia, Freiburg, Germany). The samples were aliquoted and stored at -80°C. Concentration of proteins in the crude extract was determined according to the method

of Bradford (1976) using bovine serum albumin (BSA) as reference. Absorption was measured at 595 nm at room temperature.

Spectrophotometric assays for antioxidative enzymes and GST determination

Glutathione S-transferase (GST, EC 2.5.1.18) activity was assayed in standard spectrophotometric tests using different model substrates, which cover the enzyme activities of different enzyme isoforms. Aliquots of the enzyme extract were incubated with 0.1 M potassium phosphate buffer (pH 7.8), 1 mM GSH with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, ε_{340} ($mM^{-1}cm^{-1}$)=9.6), with *p*-nitrobenzyl-chloroide (*p*-NBC, ε_{310} ($mM^{-1}cm^{-1}$)=1.8), *p*-nitrophenylacetate (*p*-Npa, ε_{400} ($mM^{-1}cm^{-1}$)=8.79), and with the diphenylether herbicide, fluorodifen (ε_{400} ($mM^{-1}cm^{-1}$)=3.1).

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed following the method of Zhang et al. (1996). Reaction mixture contained 1 mM oxidized glutathione (GSSG) and 2 mM NADPH in 100 mM Tris/HCl buffer (pH 7.5) with 0.1 mM EDTA. After adding the enzyme to the mixture, the decrease of NADPH concentration through reduction of GSSG to GSH by GR (ε_{340} ($mM^{-1}cm^{-1}$)=6.22) was determined.

Ascorbate peroxidase (APX, EC 1.11.1.11) was measured following the method of Vanacker et al. (1998). The reaction mixture contained 1 mM H_2O_2 and 250 µM ascorbic acid in 55.56 mM KH_2PO_4/K_2HPO_4 (pH 7.0). The reaction was started by mixing the reaction mixture and the enzyme extract and the decrease of ascorbic acid concentration was recorded (ε_{290} ($mM^{-1}cm^{-1}$)=2.8).

Catalase (CAT, EC 1.11.1.6) was assayed by measuring the decrease of H_2O_2 concentration at 240 nm by the method of Verma and Dubey (2003). The reaction mixture contained 53 mM H_2O_2 in 100 mM KH_2PO_4/K_2HPO_4 (pH 7.0). The buffer was mixed with the enzyme extract and the decrease of H_2O_2 concentration was recorded at 240 nm (ε_{240} ($mM^{-1}cm^{-1}$)=0.036).

The enzyme activity assays were carried out using a 96-well plate reader SPECTRAMax PLUS 384 spectrophotometer (Molecular Devices, Ismaning) with the data analyzing software SOFTmax PRO 4.6. The 96-well plates from Nunc (Brand, Wertheim) were applied for measuring in the visible light spectrum (390-750 nm); for assays in the UV spectrum range specific plates from Greiner (Greiner, Frickenhausen) were used. In the standard kinetic tests, absorption changes were determined in 15 sec intervals for 5 min at room temperature. The samples were measured using three technical replicates. Reaction

mixtures without enzyme extract were used as blanks; and enzyme activities are expressed as μkat mg protein^{-1}. One kat represents the enzymatic formation of 1 mol end product per second in the extract.

Quantification of total glutathione

The measurement of total glutathione content was carried out after Griffith (1980), with some modifications. This method is based on an enzymatic recyclization through the glutathione reductase. During the reaction, the formation rate of 5-thio-2-nitrobenzoate is directly proportional to the rate of recyclization of the reaction, which is directly proportional to the GSH content. The absorbance was determined at 405 nm, using a KONTRON Uvikon Double-Beam spectrophotometer. Changes in absorbance during 1 min correspond to the concentration of GSH, using GSSG as standard.

Microscopic detection of reactive oxygen and nitrogen species and glutathione

In situ detection of H_2O_2 in the pea leaves was carried out by using 3,3'-diaminobenzidine (DAB) staining (Guan et al. 2009). Whole leaves were incubated for 1 h in DAB solution (2 mg L^{-1}) on a rotary shaker (40 rpm) in the dark at room temperature. Samples were washed once with 2-N-morpholine-ethansulphonic acid/potassium chloride (MES/KCl) buffer (10/50 mM, pH 6.15).

The levels of nitric oxide in leaf discs and root tips were detected by 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF-FM DA) (Kolbert et al. 2012). Samples were incubated for 30 min in the dark at room temperature in 10 μM dye solution, and were washed twice with Tris/HCl buffer (10 mM, pH 7.4).

The fluorophore, 3′-(p-aminophenyl) fluorescein (APF) was applied for the visualization of peroxynitrite level in the root tips and leaf discs of pea (Kolbert et al. 2012). Samples were incubated in the dark in 10 μM dye solution for 1 hour and were washed twice with 10 mM Tris/HCl buffer.

Cellular glutathione levels were visualized *in situ* in the root tips with the help of monobromobimane (MBB) fluorescent staining. The root tips were incubated for 1 hour at

room temperature in 100 μM dye solution (prepared in distilled water), then washed once with distilled water. For control, root tips were pre-incubated in distilled water, while as positive control, root tips were kept in 1 mM GSH solution for 20 minutes before staining. As a negative control, samples were pre-treated with 10 mM CDNB for 10 minutes.

Microscopic investigation of pea samples dyed with different fluorophores was performed under a Zeiss Axiovert 200M 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 DAF-FM and APF and filter set 49 (exc.: 365 nm, em.: 445/50 nm) was applied for MBB. Fluorescence intensity (pixel intensity) was measured on digital images using Axiovision Rel. 4.8 software, within circles of 100 μm radii within the root tip, and of 500 μm radii in leaf discs. Whole leaves stained with the non-fluorescent DAB were examined using Zeiss Axioscope 2000-C stereomicroscope (Carl Zeiss, Jena, Germany).

Determination of lipid peroxidation

The level of membrane lipid peroxidation in the root and leaf tissues was quantified by measuring thiobarbituric acid reactive substances (TBARS) concentration according to the method of Heath and Packer (1968). Freshly grounded shoot and root tissues of pea were centrifuged at 10 000 rpm for 5 min in 0.1% tri-chloro acetic acid (TCA). The supernatant was removed and incubated at 95°C for 30 min in 0.5% 2-thiobarbituric acid (TBA) dissolved in 20% TCA. After cooling the samples on ice, a second centrifigation was applied at 5 000 rpm for 5 min. The absorbance of the supernatant was determined at 440 nm and 532 nm, and corrected for unspecific turbidity after substraction from the value obtained at 600 nm. The level of lipid peroxidation is expressed as μmol TBARS per gramm fresh weight, using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Immuno-detection of nitrotyrosine

Crude protein extracts from plant material were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels. For Western blot analysis, proteins were transferred to PVDF membranes using the wet blotting procedure (Bio-Rad, Hercules, CA, USA). After the transfer, membranes were blocked for 1 h with 5% non-fat milk in TBS-Tween (50 mM Tris-HCl; pH 7.4, 150 mM NaCl and 0.1% Tween-20),

prior used for cross-reactivity assays with rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000 (Corpas et al. 2008). Immuno-detection was performed by using affinity isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody in dilution of 1:10 000, and bands were visualised by using NBT/BCIP reaction. As a positive control nitrated bovine serum albumin was used.

Statistical analysis

The results are expressed as mean±SE. Multiple comparison analyses were performed with SigmaStat 12 software using analysis of variance (ANOVA, $P \leq 0.05$) and Duncan's test. In some cases, Microsoft Excel 2010 and Student's t-test were used (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). All experiments were carried out at least two times. In each treatment at least 5 samples were measured.

RESULTS

Selenium accumulation and translocation in green pea

As an effect of the increasing external selenite concentrations, the selenium content of the root system increased dramatically and in a concentration-dependent manner (Table 1). Insomuch, 100 μM sodium selenite resulted in ~1500-fold increase in Se content of the root system, while in the leaves ~100-fold enhancement was measured. Selenium distribution, expressed as leaf:root ratios, notably decreased as the effect of increasing treatment doses. Sulphur concentrations were significantly increased by all selenium treatments in both organs, compared to the controls (Table 1). However, the effect of selenite on S contents did not prove to be concentration-dependent.

Table 1 Total selenium (Se) and sulphur (S) concentrations (μg/g dry weight) in the leaves and roots of pea plants treated with 0, 10, 50 or 100 μM selenite. Leaf:root ratios of Se concentrations in control and selenite-treated pea plants. Different letters indicate significant differences according to Duncan's test (n=6, $P \leq 0.05$)

	Se (μg/g dry weight)				S (μg/g dry weight)	
Na_2SeO_3 (μM)	leaf	root	leaf:root ratio		leaf	root
0	0.97 ± 0.06 ^{e}	0.92 ± 0.10 ^{e}	1.05		59516.66 ± 7044.72 ^{b}	50400.00 ± 5134.93 ^{b}

10	74.66 ± 4.41 [de]	303.70 ± 23.03 [c]	0.24	79426.66 ± 9636.44 [a]	76343.33 ± 12069.20 [a]
50	104.60 ± 3.65 [d]	1112.00 ± 113.92 [b]	0.09	68060.00 ± 9331.08 [ab]	78370.00 ± 11387.27 [a]
100	123.36 ± 8.35 [d]	1480.00 ± 113.92 [a]	0.08	75236.66 ± 8821.39 [a]	78586.66 ± 17827.59 [a]

Selenite altered vegetative and reproductive development of pea

The high amount of selenium accumulated from the external medium caused alterations in both growth and morphology of pea plants (Fig. 1). As the effect of 10 µM selenite, the length and the fresh weight of the shoot system significantly increased. Regarding the roots, the elongation of the primary root slightly decreased, while the fresh weight of the whole root system increased under the lowest selenite concentration. The more severe Se doses (50 and 100 µM selenite) resulted in the reduction of the shoot, root size and fresh weight. Furthermore, these concentrations of selenite induced the premature development of flowers (see arrows in Fig. 1D).

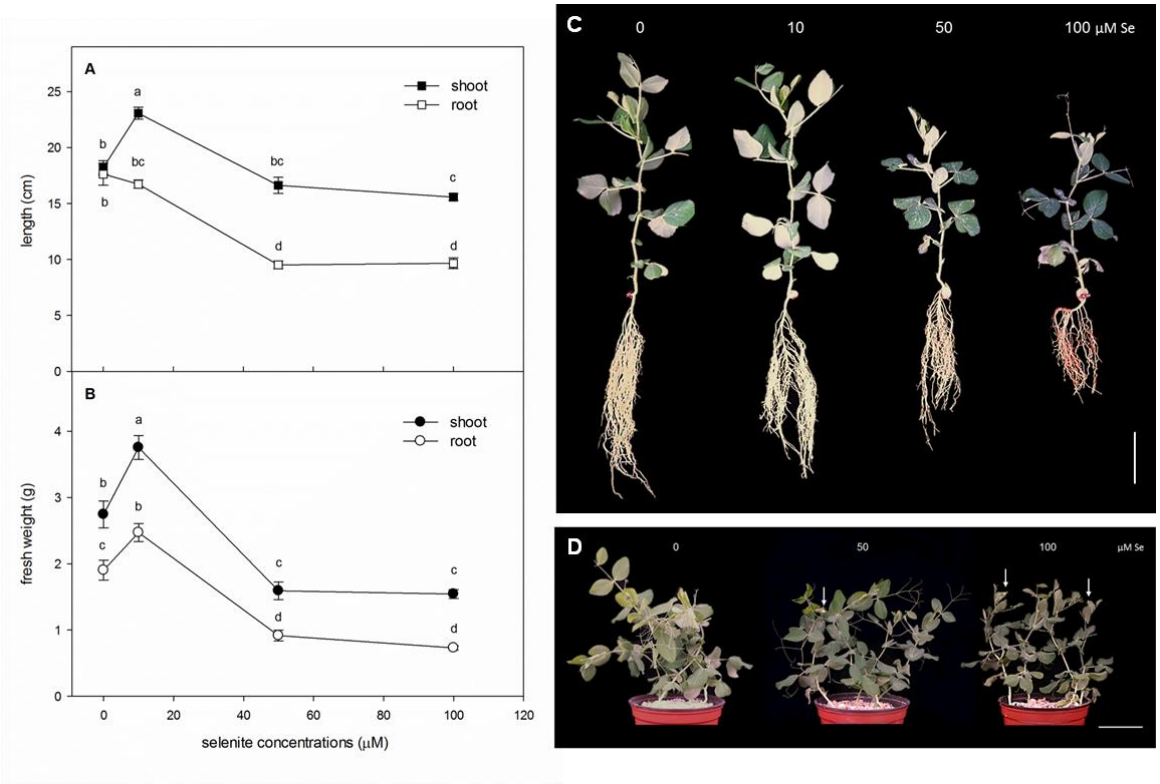


Fig. 1 The length (cm, A) and the fresh weight (g, B) of the shoot and root system of pea plants treated with 0, 10, 50, 100 µM selenite. Different letters indicate significant differences according to Duncan's test (n=6,

P≤0.05). (C) Representative photographs showing the shoot and root system of control (0 μM Se) and Se-treated pea plants. Bar=5 cm. (D) Representative photographs showing the shoot system of control (0 μM Se), 50 or 100 μM Se-exposed pea. White arrows indicate flowers appeared as the effect of the treatments. Bar=10 cm

As a reliable marker for stress endurance, the photosynthetic pigment composition of selenite-exposed pea leaves was also analysed. Excess selenium moderately decreased chlorophyll (chl) *a* and carotenoid contents, while chl *b* concentrations showed slighter diminution. However, in case of chlorophylls, the negative effect proved to be independent from the applied selenite concentrations (Table 2). In general, selenium induced only slight changes in the contents of photosynthetic pigments.

Table 2 Concentrations of photosynthetic pigments (μg/g fresh weight) and the chlorophyll *a*/*b* ratios in the leaves of control and selenite-treated pea plants. Different letters indicate significant differences according to Duncan's test (n=6, P≤0.05)

Na_2SeO_3 (μM)	Chl a	Chl b	Chl a/b	Total chlorophyll	Total carotenoids
0	13.37 ± 0.0115 ^{a}	3.83 ± 0.0027 ^{b}	3.48 ± 0.0041 ^{a}	17.21 ± 0.0136 ^{a}	3.44 ± 0.0030 ^{a}
10	11.82 ± 0.0052 ^{c}	3.50 ± 0.0071 ^{c}	3.37 ± 0.0112 ^{b}	15.32 ± 0.0120 ^{c}	3.28 ± 0.0026 ^{b}
50	12.28 ± 0.0049 ^{b}	4.00 ± 0.0107 ^{a}	3.06 ± 0.0140 ^{c}	16.29 ± 0.0155 ^{b}	3.23 ± 0.0062 ^{c}
100	11.36 ± 0.0048 ^{d}	3.84 ± 0.0050 ^{b}	2.95 ± 0.0081 ^{d}	15.21 ± 0.0070 ^{d}	2.95 ± 0.0018 ^{d}

Selenite induced oxidative stress in a concentration-dependent manner

Selenite-induced oxidative stress was characterized by measuring the H_2O_2 content, the activity of antioxidant enzymes (ascorbate peroxidase, catalase) and the accumulation of TBARS reflecting to lipid peroxidation, which is a steady indicator for oxidative damage (Corpas et al. 2013). Moreover, glutathione and related enzymes were also examined in the selenite-exposed pea.

In both organs, the concentration of H_2O_2 was increased by 50 and 100 μM selenite, but it did not show changes in case of 10 μM Se treatment (Fig. 2A). In the leaves, the 50 and 100 μM selenite-induced H_2O_2 accumulation was less pronounced, however it was confirmed by the intensification of brown colorization during histochemical DAB staining (Fig. 2a). The specific activity of APX slightly decreased under lower selenite doses in both organs; although 100 μM Se caused an induction of the enzyme within the root system (Fig. 2B). As

the effect of 50 and 100 µM selenite, the CAT activities showed enhancement in the leaves but reduction in the roots however, this did not prove to be significant, while 10 µM Se did not cause alterations in the activity of this enzyme (Fig. 2C). According to the TBARS content, remarkable increase was observed in the leaves and minor in the roots of pea treated with higher selenite doses (50 and 100 µM, Fig. 2D).

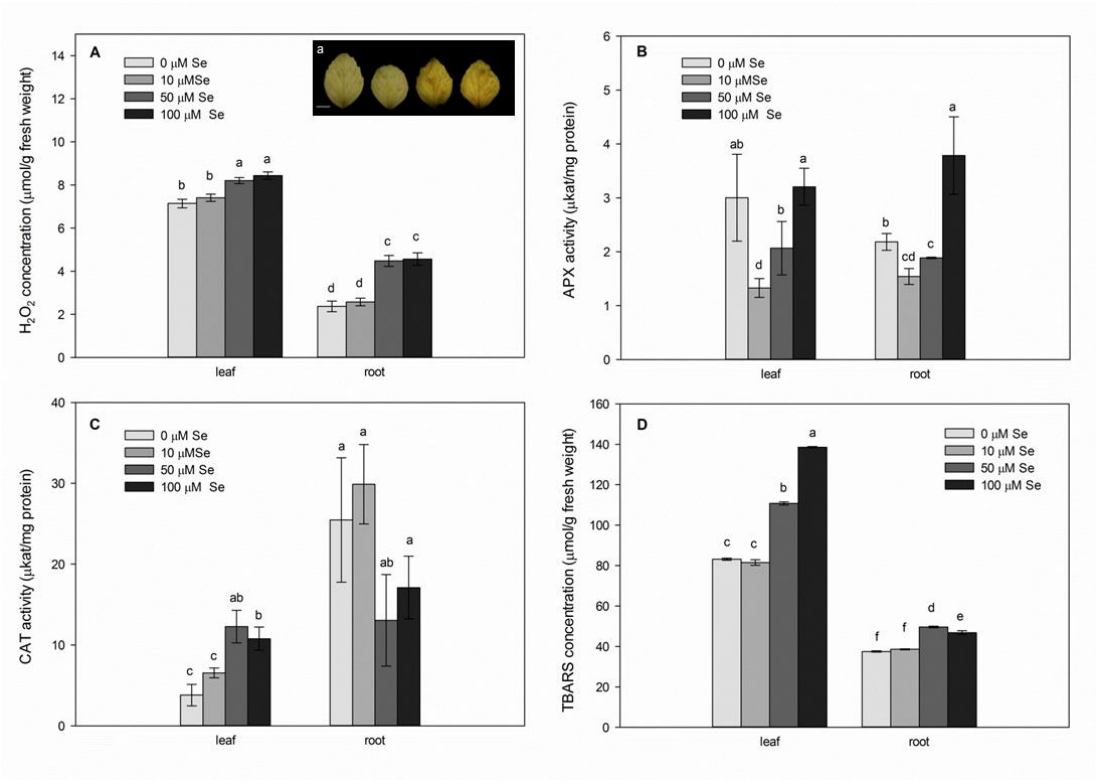


Fig. 2 Hydrogen peroxide concentration in pea leaves and roots, measured spectrophotometrically (A) and detected by DAB staining (a) in the leaves of pea (from left: control, 10, 50 and 100 µM Se, Bar=1 cm). Activity (µkat/mg protein) of ascorbate peroxidase (B) and catalase (C) in the roots and leaves of pea. (D) The concentration of TBARS in the leaf and root of pea plants treated with 0, 10, 50, 100 µM selenite. Different letters indicate significant differences according to Duncan's test (n=6, $P \leq 0.05$)

The glutathione concentration in the leaves was decreased after treatment with 10 and 50 µM selenite compared to the untreated samples, while it was in the range of the control in case of the highest Se dose. It has to be mentioned, that the changes of leaf glutathione contents did not prove to be statistically significant (Fig. 3A). In the whole root system, the total GSH concentration was not affected by milder selenite treatments; however it exceptionally elevated as the effect of severe Se exposure. In contrast, within the root tips, the intensity of the GSH-associated MBB fluorescence decreased depending on the elevating Se

concentrations (Fig. 3B). The GSH-dependence of the MBB fluorophore was verified by exogenous GSH and CDNB pre-treatments (Fig. 3b).

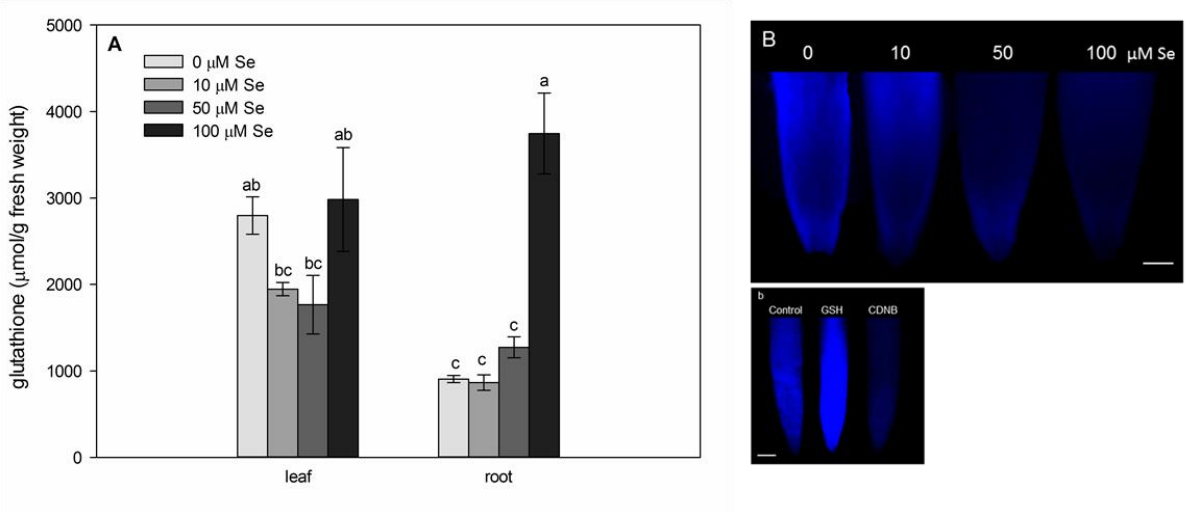
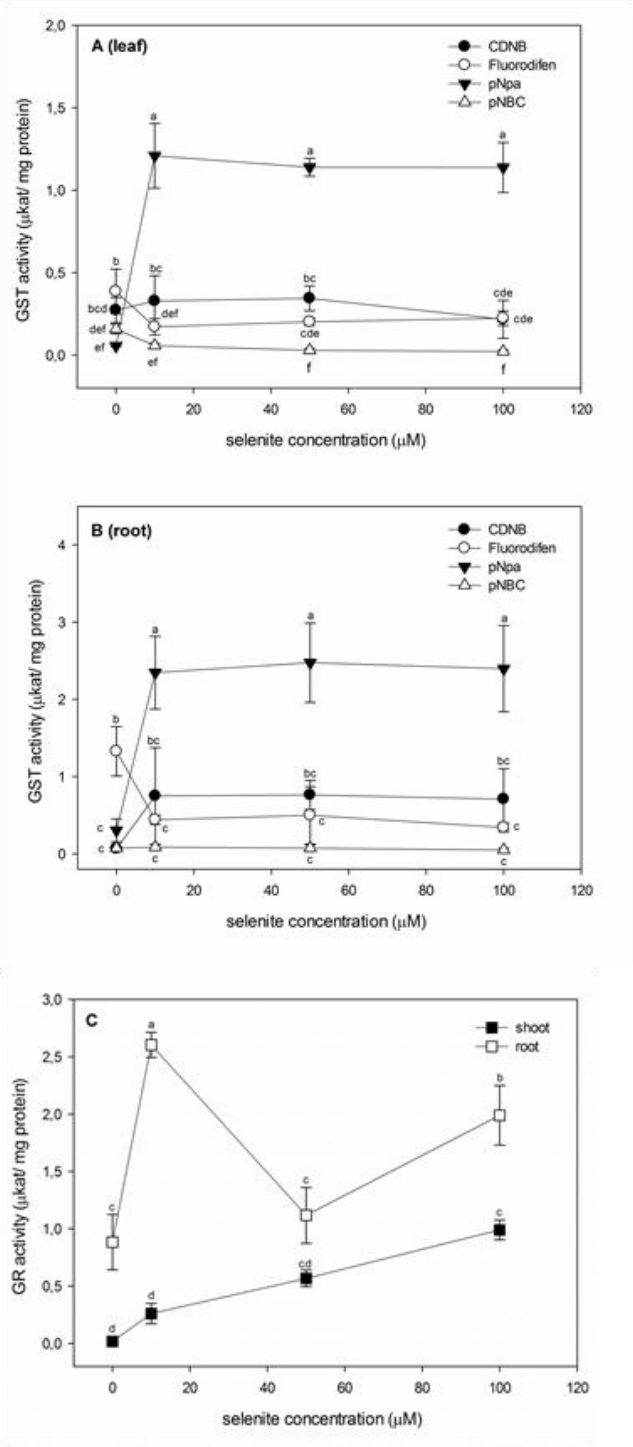


Fig. 3 (A) Concentration of total glutathione (µmol/g fresh weight) in the leaves and roots of control and selenite-exposed pea. Different letters indicate significant differences according to Duncan's test (n=6, P≤0.05). (B) Representative microscopic images of MBB-stained root tips of control (0 µM Se) and 10, 50, 100 µM selenite-treated pea. Bar=100 µm. (b) Representative microscopic images of MBB-stained root tips treated with water (Control), 1 mM GSH or 10 mM CDNB. Bar=100 µm

Selenite exposure affected the activity of GSH-associated enzymes as well. In extracts from both roots and leaves, GST activity was assayed by using model substrates CDNB, pNBC, fluorodifen and pNpa (Fig. 4AB). In general, the root extracts showed higher GST activity compared to the leaf extracts and in both organs, the model substrate pNpa was conjugated at high rates, irrespective of the Se-concentration applied. All treatments caused a significant induction of the pNpa-GST activity independently from the concentration of applied Se. In contrast, GST activity for the model substrate Fluorodifen notably decreased in the leaves and roots of selenite-exposed pea. Also, glutathione reductase activity was higher in the root system than in the leaf (Fig. 4C). In the root, selenium at low dose caused the largest induction of the GR activity, but 50 µM selenite did not affect it. Moreover, the 100 µM Se concentration resulted in a moderate elevation of GR activity. In the leaves, selenite concentration-dependently increased the GR activity; however the effect proved to be statistically significant only in the 100 µM selenite-treated sample.



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389 **Fig. 4** Specific activity (µkat/mg protein) of glutathione-S-transferase in control and 10, 50 or 100 µM selenite-
390 treated pea leaves (A) and root (B) determined by using the model substrates CDNB, fluorodifen, pNpa and
391 NBC. (C) Specific activity (µkat/mg protein) of glutathione reductase in the leaves and roots of pea treated with
392 0, 10, 50 or 100 µM Se. Different letters indicate significant differences according to Duncan's test (n=5,
393 P≤0.05)

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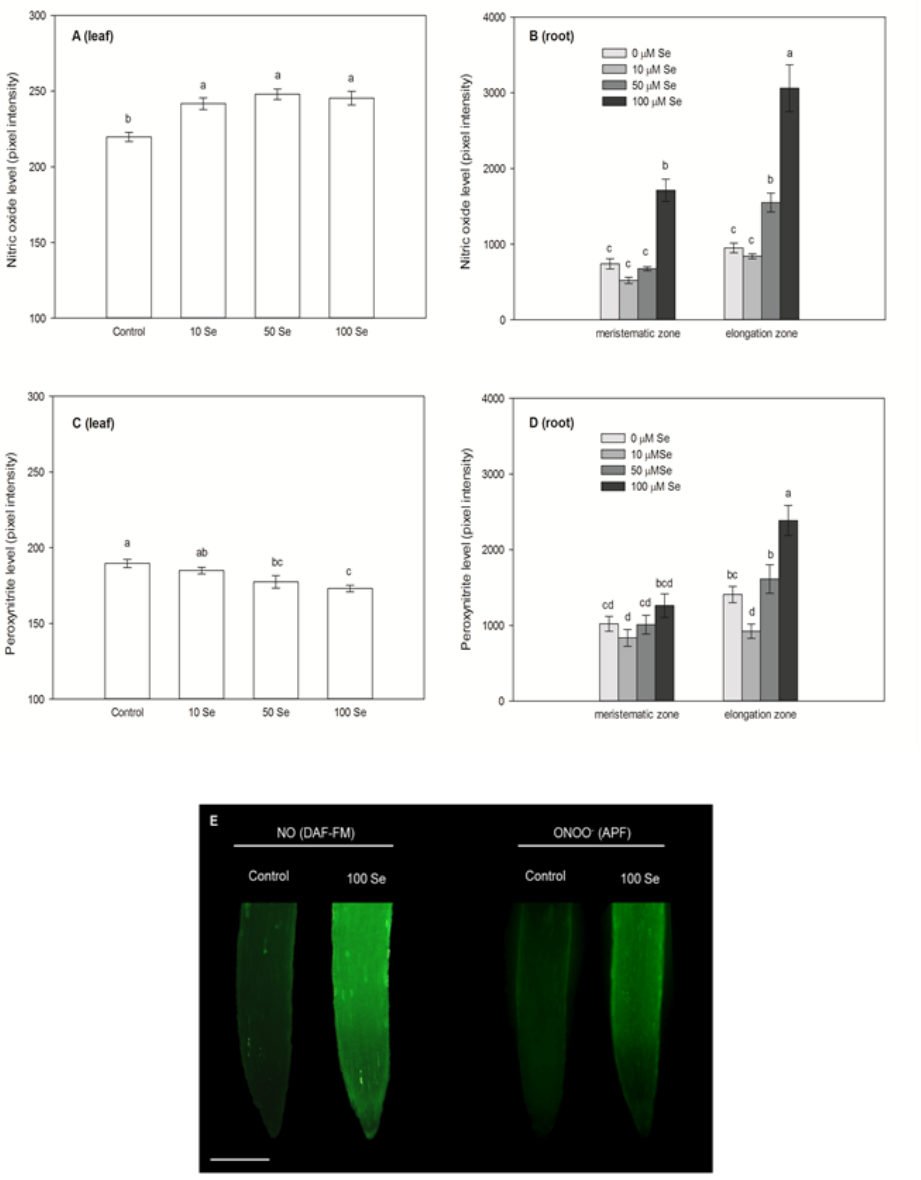
Selenite differently modified the RNS levels and nitroproteome of pea organs

Besides ROS and oxidative stress, the supposed effect of selenite on the formation of reactive nitrogen species and protein nitration was also evaluated by fluorescent microscopy and Western blot analysis, respectively. As shown in Fig. 5A, all selenite concentrations caused a statistically significant but only minor intensification of NO accumulation in the leaf. Within the root tip, the NO content of the meristem was remarkably enhanced by 100 μM, while in the elongation zone both 50 and 100 μM selenite caused NO level increase (Fig. 5B). Regarding peroxynitrite, treatment with 50 and 100 μM selenite significantly decreased its level in the leaf (Fig. 5C), while only the elongation zone of 100 μM selenite-exposed pea root showed intensified $ONOO^-$ formation compared to control (Fig. 5D). Furthermore, 10 μM selenite led to the significant decrease of peroxynitrite level in the elongation zone, while in the meristem no changes were detected relative to control level.



409

410 **Fig. 5** Nitric oxide (pixel intensity of DAF-FM, AB) and peroxynitrite (pixel intensity of APF, CD) in the leaf
411 disks (A and C) and root tips (measured in meristematic and elongation zones, B and D) of control (0) and 10, 50
412 or 100 μM selenite-exposed pea. Different letters indicate significant differences according to Duncan's test
413 (n=10, P≤0.05). (E) Representative fluorescent microscopic images of DAF-FM DA- or APF-stained root tips of
414 control and 100 μM selenite-treated pea. Bar=0.5 mm

415

416 The RNS-dependent posttranslational modification, protein tyrosine nitration was
417 examined by Western blot in the leaf and root of control and selenite-treated pea (Fig. 6). In
418 both organs of untreated plants, several 3-nitrotyrosine-positive protein bands were observed.
419 In the roots, weakening of the immunoreaction was evident as the effect of selenite. In
420 contrast, the protein bands being present also in control leaves (at 200, 75, 50, 37, 25 and 15

KDa) showed intensified immunoreaction in case of 50 and 100 µM selenite exposure, while the lowest applied selenite concentration had no obvious effect on nitration in the leaves.

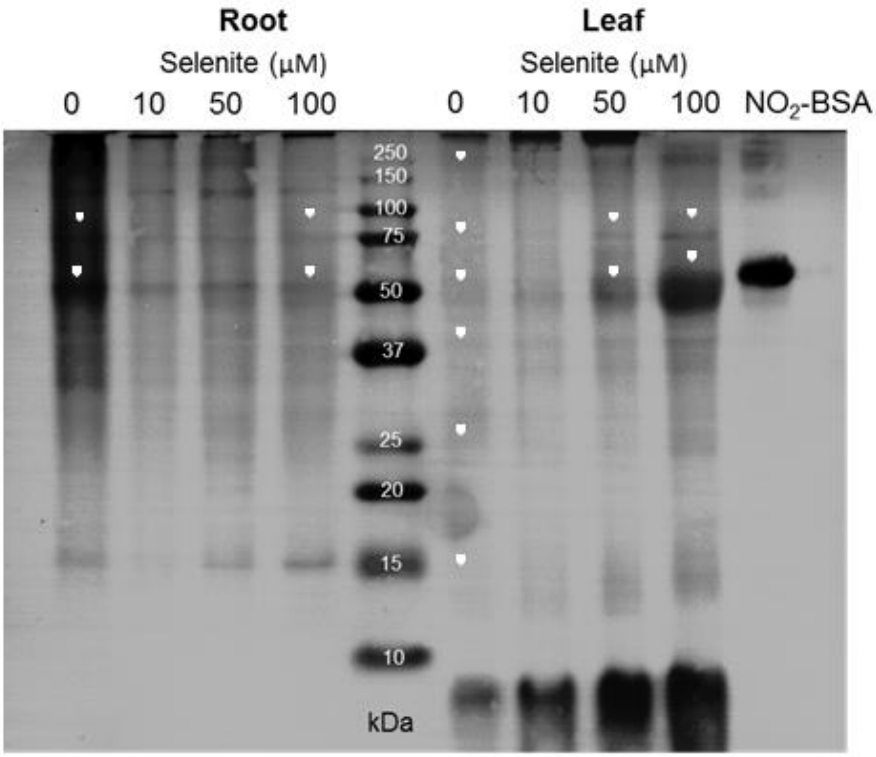


Fig. 6 Representative immunoblots showing protein tyrosine nitration in the root and leaf system of pea under control conditions (C) and during 10, 50 or 100 µM selenite exposure. Root and leaf samples were separated by SDS-PAGE (root: 7.5 µg protein, leaf: 20 µg protein) and analysed by Western blotting with anti-nitrotyrosine antibody (1:2000). Commercial nitrated BSA (NO_2-BSA) was used as a positive control. Representative bands referring to the observed changes are labelled with arrows.

Discussion

Pea plants are able to take up selenite from the external medium (Table 1), although the molecular mechanism of the transport is not well understood. The possibility that selenite and phosphate may use common membrane transporters was proposed (Haug et al. 2007) and later confirmed in rice, where the phosphate transporter OsPT2 seems to be greatly involved in selenite uptake (Zhang et al. 2014). Pea plants showed higher Se accumulation in their root system compared to the leaves, which was demonstrated by the low leaf:root ratios ranging from ~0.2 to 0.08. Indeed, selenite was shown to poorly translocate from the root to the shoot system (Hawrylak-Nowak et al. 2015). It is rather rapidly converted to organic forms (selenocysteine, selenomethionine, methylselenocysteine), which are retained in the root (de Souza et al. 1998; Zayed et al. 1998). Based on the tissue concentrations of total selenium within the leaves, green pea as important forage and crop plant, belongs to the non-accumulator category (Çakır et al. 2012). Selenium competes with the chemically similar sulphur during the uptake and assimilation (Hopper and Parker 1999). Therefore, Se in excess is capable to induce sulphur deficiency response; although Se-exposed pea plants showed enhanced S contents (Table 1). This can be explained by the fact that excess selenium up-regulates the expression of sulphate transporters (SULTR1;1 and SULTR2;1) which consequently lead to S accumulation (Van Hoewyk et al. 2008).

The effect of selenite on growth and development of pea proved to be organ- and concentration-dependent. At the lowest applied concentration, selenite unequivocally promoted plant growth resulting in extensive growth of plant organs (Fig. 1), which may enhance fitness. Indeed, there is increasing evidence regarding the beneficial effects of low Se doses (e.g. 0.5 mg Se L^{-1} in soils, 1.0 mg Se L^{-1} in hydroponics culture or 1.5 mg Se L^{-1} as foliar spraying) in plants presumably originating from its antioxidant, anti-senescent and stress-modulator role (Djanaguiraman et al. 2010; Garcia-Banuelos et al. 2011; Kaur et al. 2014; Hawrylak-Nowak et al. 2015). In contrast, selenite at higher doses remarkably diminished pea growth similarly to other works (reviewed by Kaur et al. 2014). Moreover, root elongation showed more pronounced Se sensitivity compared to shoot growth, since all selenite concentrations inhibited it. Also in other species, such as *Arabidopsis thaliana* or *Brassica napus* root growth was severely reduced by selenite (Tamaoki et al. 2008; Lehotai et al. 2012; Dimkovikj and Van Hoewyk 2014). One reason for this can be, *inter alia*, the disturbances in hormone homeostasis (e.g. auxin, cytokinin, ethylene) and cell viability loss of the primary meristem (Lehotai et al. 2012) and Se-induced alterations in primary metabolism

(Dimkovikj and Van Hoewyk 2014). Besides shoot and root growth, selenium exposure in the form of selenite affected pea development as well, since the higher concentrations of Se (50 and 100 μM) accelerated the reproductive phase (Fig. 1D). Similarly, reproductive parameters such as floral bud development, opening of flowers or podding, were induced by 10 and 20 μM selenate in canola (Hajiboland and Keivanfar 2012); although the underlying mechanisms of Se-triggered flowering are not yet known. Based on these, low selenite concentration promoted vegetative growth of pea, while severe selenite excess resulted in the inhibition of growth together with the acceleration of reproductive events. Regarding the pigment composition of pea leaves (Table 2), the rate of loss was higher in case of chl *a* compared to chl *b*, which resulted in the reduction of chl *a/b* ratios suggesting that the chl *a* pool is more sensitive to excess Se than chl *b*. This is contrasting to the results in other plant species such as spinach or cucumber, where the chl *b* pool was more affected by exogenous selenium (Hawrylak-Nowak et al. 2015; Saffaryazdi et al. 2012, respectively). By all accounts, selenium was shown to interact with sulfhydryl containing enzymes such as 5-aminolevulinic acid dehydratase and porphobilinogen deaminase, resulting in the inhibition of chlorophyll biosynthesis (Padmaja et al. 1990).

Selenium-compounds can evolve pro-oxidant effects disturbing the redox status of animal and plant cells (Spallholz 1994; Van Hoewyk 2013). Several works concluded that selenium triggers the formation of ROS. For instance, in the leaves of selenite-exposed *Arabidopsis* accessions or *Stanleya* species, elevated H_2O_2 and superoxide anion levels were detected (Tamaoki et al. 2008; Freeman et al. 2010) and the root tips of *Arabidopsis* treated with selenite also showed H_2O_2 accumulation (Lehotai et al. 2012). Similarly to these, in both pea organs, the formation of H_2O_2 was induced rather by higher selenite doses (Fig. 2A). Also at higher concentrations, selenite induced changes in the activities of antioxidant enzymes such as CAT or APX, and led to lipid peroxidation in the leaves and roots of pea (Fig. 2BCD) showing correlation to the H_2O_2 levels. In e.g. Se-treated barley, lettuce, *Spirulina* and *Ulva* species the modification of antioxidants and the intensification of lipid peroxidation as the effect of selenium exposure were reported (Akbulut and Cakir 2010; Ríos et al. 2009; Chen et al. 2008; Schiavon et al. 2012). One of the major molecules which maintain the cellular redox homeostasis is glutathione, also having importance in plant growth and development (Gill et al. 2013). Several studies reported the selenite- or selenate-induced depletion of GSH in both root and shoot tissues of e.g. *Arabidopsis thaliana*, *Brassica napus*, *Stanleya pinnata* (Van Hoewyk et al. 2008; Hugouvieux et al. 2009; Tamaoki et al. 2008; Dimkovikj and Van Hoewyk 2014; Freeman et al. 2010). Similarly, in pea leaves, lower selenite concentrations

caused the decrease of total GSH content (Fig. 3A). Although, Dimkovikj and Van Hoewyk (2014) observed elevated GSH concentration in the whole root system of selenite-exposed *Brassica* similarly to pea roots in the present study (Fig. 3A). The reason for the selenite-induced GSH accumulation may partly be the elevation of γ-glutamyl cyclotransferase (GGCT) protein levels as it was shown in *Brassica* root tissues (Dimkovikj and Van Hoewyk 2014). The remarkable up-regulation of the transcript encoding GGCT2; 1 in the roots of selenate-exposed *Arabidopsis* (Van Hoewyk et al. 2008) also supports the involvement of this enzyme in GSH metabolism under Se stress. When the total GSH levels in pea root tips were examined by fluorescent microscopy, their selenite-triggered reduction was observed (Fig. 3B). The difference between the GSH contents measured by the spectrophotometer and the fluorescent staining, may simply originate from the technical dissimilarity of the two methods and suggests that root tips do not represent the whole root system in this case. Similar results were obtained in the root tips of *Brassica napus* treated with selenite (Dimkovikj and Van Hoewyk 2014). Since glutathione is associated with auxin transport and is involved in the maintenance of root growth (Koprivova et al. 2010), its depletion in the root tips may contribute to the notable inhibition of root elongation found in the present work (see Fig. 1A). The activity of glutathione S-transferase as a good stress marker was also modified in selenium-exposed pea plants (Fig. 4AB). From the results obtained for different model substrates it can be concluded that different GST isoforms are responsible for the pNpa conjugation after selenite exposure implicating the role of pNpa GST in the detoxification during selenite exposure in pea. The involvement of GST in selenium stress response is supported by the strong up-regulation of GST gene (At2g02390) in selenate-treated *Arabidopsis* (Van Hoewyk et al. 2008) or *Stanleya* species (GSTF6, Freeman et al. 2010). Glutathione reductase enzyme maintains the reduced status of GSH and acts as a substrate for glutathione S-transferases (Yousuf et al. 2012). The lowest applied selenium concentration dramatically induced GR activity in the root (Fig. 4C) consequently helping to maintain the level of reduced GSH, which in turn can be used as a substrate for GSTs during defence mechanisms. Also in coffee cell suspension, selenite at low concentration was able to notably increase GR activity (Gomes-Junior et al. 2007). In the leaves, GR activity also elevated as the result of selenite exposure suggesting the key role of this enzyme in selenite tolerance. Alternatively, high reduced GSH content may also be used for selenite reduction to selenodiglutathione similarly to animal systems (Wallenberg et al. 2010), although molecular evidence for GR being a rate limiting enzyme in Se metabolism of plants is still lacking (Terry et al. 2000). Consequently, our results confirm the occurrence of selenium-induced

oxidative stress, though this depends on the concentration of Se. As it was suggested by Hartikainen et al. (2000), at low concentration (in this study 10 μM) Se has promoting effect on growth and does not induce oxidative stress, while at higher doses (here 50 and 100 μM) it triggers oxidative stress and deteriorates pea growth. Moreover, our results confirm that glutathione and related enzymes play a crucial role in selenium stress responses.

Besides ROS, the effect of selenite on RNS levels was also monitored and intensive NO generation was observed in the root tips of treated plants (Fig. 5B). The most significant and concentration-dependent selenite-triggered NO formation was detected in the elongation zone of root tips suggesting the tissue specificity of this response. Selenite presumably induces the main NO synthesizing enzyme of the root; nitrate reductase, as it was reported in lettuce (Ríos et al. 2010). Moreover, also in the leaves NR may be the source of selenite-triggered NO (Fig. 5A), since it can contribute to NO synthesis in the aerial plant parts as well (Zhang et al. 2011; Zhao et al. 2009). The effect of selenium on NR can be direct or indirect, since Se-induced S deficiency may increase molybdenum content thus inducing NR (Shinmachi et al. 2010; Yu et al. 2010). Being a highly oxidative and nitrosative agent (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011), peroxynitrite diminution in leaves and roots as the effect of low selenite doses suggests that selenite at low concentration would activate some peroxynitrite detoxification mechanisms. In the leaf, also more severe selenium exposure reduced peroxynitrite levels reflecting a more efficient detoxification in this organ. One possibility of peroxynitrite scavenging is the reaction of it with glutathione leading to the formation of S-nitrosoglutathione and NO (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011). The high GSH content in the selenium-exposed pea together with the NO accumulation may reflect to this $ONOO^-$ detoxification pathway. Additionally, key enzymes in the decomposition of $ONOO^-$ are the glutathione peroxidases and thioredoxin reductases. In animals and humans, these enzymes contain selenocystein (SeCys) being essential for their catalytic activity (Schrauzer 2000). Although, there is no evidence regarding the incorporation of SeCys in proteins in plants, thus the role of selenium in the regulation of enzyme activity in plants is still unknown (Van Hoewyk 2013).

Protein tyrosine nitration as an RNS-dependent posttranslational modification contributes to the evolution of the secondary nitrosative stress. Investigating this posttranslational modification of proteins by Western blot (Fig. 6), it was observed that this PTM being present in unstressed pea plants is a basal mechanism of the regulation of protein activity in green pea. In pea and in other plant species, such as sunflower and pepper nitration

was observed during control circumstances by others (Corpas et al. 2009; Chaki et al. 2009; 2015). Furthermore, as in the work of Corpas et al. (2009) the root proteome of pea proved to be more nitrated compared to that of the leaf, which reflects the organ-specific nature of tyrosine nitration. Besides, the organs differentially responded to selenite exposure. In the root system, the nitration pattern of the proteome was not modified, since new nitrated protein bands were not observed. In contrast, the nitration level of leaf proteome was significantly intensified by selenite similarly to; *inter alia*, salt-stressed olive leaves, cold-treated pea leaves or arsenic-exposed *Arabidopsis* (reviewed in Corpas et al. 2013). In the leaves of pea, the level of nitration well correlated with the exogenous selenite concentrations suggesting the concentration-dependent feature of protein tyrosine nitration. At the same time, modifications of the nitroproteome show no strict correlation to the alterations in the NO and $ONOO^-$ levels which partly can be the reason of the high reactivity of these forms with each other and with other molecules. Also, it is worth mentioning that the nitrogen dioxide radical ($NO_2^{\cdot}$) also possesses a notable nitrating capacity, thus the amount of this molecule may determine the rate of nitration as well (Souza et al. 2008).

Altogether, selenite alters vegetative and reproductive development of pea. At low dose, it promotes growth and does not disturb the cellular ROS and RNS metabolism. Moreover, our results confirmed that severe selenite stress inhibits growth and concomitantly induces oxidative stress. Besides, the presented data first reveals selenite-induced concentration- and organ-dependent nitrosative stress in pea. Since oxidative and nitrosative mechanisms occur in parallel, we urge to consider nitro-oxidative stress as an underlying mechanism of selenium phytotoxicity.

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804 **Figure legends**

805

806 **Table 1** Total selenium (Se) and sulphur (S) concentrations (μg/g dry weight) in the leaves
807 and roots of pea plants treated with 0, 10, 50 or 100 μM selenite. Leaf:root ratios of Se
808 concentrations in control and selenite-treated pea plants. Different letters indicate significant
809 differences according to Duncan's test (n=6, P≤0.05)

810 **Fig. 1** The length (cm, A) and the fresh weight (g, B) of the shoot and root system of pea
811 plants treated with 0, 10, 50, 100 μM selenite. Different letters indicate significant differences
812 according to Duncan's test (n=6, P≤0.05). (C) Representative photographs showing the shoot
813 and root system of control (0 μM Se) and Se-treated pea plants. Bar=5 cm. (D) Representative
814 photographs showing the shoot system of control (0 μM Se), 50 or 100 μM Se-exposed pea.
815 White arrows indicate flowers appeared as the effect of the treatments. Bar=10 cm

816 **Table 2** Concentrations of photosynthetic pigments (μg/g fresh weight) and the chlorophyll
817 a/b ratios in the leaves of control and selenite-treated pea plants. Different letters indicate
818 significant differences according to Duncan's test (n=6, P≤0.05)

819 **Fig. 2** Hydrogen peroxide concentration in pea leaves and root, measured
820 spectrophotometrically (A) and detected by DAB staining (a) in the leaves of pea (from left:
821 control, 10, 50 and 100 μM Se, Bar=1 cm). Activity (μkat/mg protein) of ascorbate
822 peroxidase (B) and catalase (C) in the leaves and roots of pea. (D) The concentration of
823 TBARS in the leaf and root of pea plants treated with 0, 10, 50, 100 μM selenite. Different
824 letters indicate significant differences according to Duncan's test (n=6, P≤0.05)

825 **Fig. 3** (A) Concentration of total glutathione (μmol/g fresh weight) in the leaves and roots of
826 control and selenite-exposed pea. Different letters indicate significant differences according to
827 Duncan's test (n=6, P≤0.05). (B) Representative microscopic images of MBB-stained root tips
828 of control (0 μM Se) and 10, 50, 100 μM selenite-treated pea. Bar=100 μm. (b)
829 Representative microscopic images of MBB-stained root tips treated with water (Control), 1
830 mM GSH or 10 mM CDNB. Bar=100 μm

831 **Fig. 4** Specific activity (μkat/ mg protein) of glutathione-S-transferase in control and 10, 50 or
832 100 μM selenite-treated pea leaves (A) and root (B) determined by using the model substrates
833 CDNB, fluorodifen, pNpa and NBC. (C) Specific activity (μkat/ mg protein) of glutathione
834 reductase in the leaves and roots of pea treated with 0, 10, 50 or 100 μM Se. Different letters
835 indicate significant differences according to Duncan's test (n=5, P≤0.05)

836 **Fig. 5** Nitric oxide (pixel intensity of DAF-FM, AB) and peroxynitrite (pixel intensity of
837 APF, CD) in the leaf disks (A and C) and root tips (measured in meristematic and elongation

838 zones, B and D) of control (0) and 10, 50 or 100 μM selenite-exposed pea. Different letters
839 indicate significant differences according to Duncan's test (n=10, P≤0.05). (E) Representative
840 fluorescent microscopic images of DAF-FM DA- or APF-stained root tips of control and 100
841 μM selenite-treated pea. Bar=0.5 mm
842 **Fig. 6** Representative immunoblots showing protein tyrosine nitration in the root and leaf
843 system of pea under control conditions (C) and during 10, 50 or 100 μM selenite exposure.
844 Root and leaf samples were separated by SDS-PAGE (root: 7.5 μg protein, leaf: 20 μg
845 protein) and analysed by Western blotting with anti-nitrotyrosine antibody (1:2000).
846 Commercial nitrated BSA (NO_2-BSA) was used as a positive control. Representative bands
847 referring to the observed changes are labelled with arrows.