

Selenium Improves Physiological Parameters and Alleviates Oxidative Stress in Shoots of Lead-Exposed *Vicia faba* L. *minor* Plants Grown Under Phosphorus-Deficient Conditions

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Abstract The goal of the study was to investigate the effects of exogenous selenium (Se) on the tolerance of faba bean plants to lead (Pb) stress under P-deficient conditions. The bean plants were grown for 2 weeks on Hoagland solution supplied with Pb (0, 50 μ M) and Se (0, 1.5, or 6 μ M), separately or simultaneously. It was shown that Pb did not affect shoot growth but caused major damage in the leaves, which was accompanied by Pb accumulation in these tissues. The exposure of the shoots to Pb led to significant changes in the biochemical parameters: the MDA content, glutathione peroxidase (GSH-Px), guaiacol peroxidase (GPOX), and catalase (CAT) activity increased. Furthermore, Pb intensified $O_2^{\bullet-}$ and H_2O_2 production. Both the Se concentrations used increased the chlorophyll *b*, chlorophyll *a+b*, and carotenoid content in the faba bean plants. Selenite also generally enhanced CAT, GPOX, and GSH-Px activities and the T-SH level. Our results imply that the degree of disturbances caused by Pb could be partially ameliorated by Se supplementation. Selenite at a lower dose alleviated Pb toxicity by decreased H_2O_2 and $O_2^{\bullet-}$ production and decreased the GSH-Px, GPOX, and CAT activities. The beneficial effect of the higher selenite concentration could be related to reduction of lipid peroxidation in the shoots of the Pb-treated plants. However, the effect of Se on the Pb-stressed plants greatly depended on the selenite dose in the nutrient solution.

Keywords Antioxidant enzymes · Oxidative stress · Growth · Lead · Selenium · *Vicia faba* L. *minor*

Introduction

Plants are able to absorb and accumulate diverse types of metals, from toxic to those with unknown metabolic functions. Heavy metals present in the environment in excess are dangerous for human and animal health. Lead (Pb) belongs to the most common and dangerous heavy metals. Pb binds strongly to sulfhydryl groups of proteins and contributes to distortion of enzymes and structural proteins. Pb accumulation in plant tissues results in numerous disturbances of physiological processes such as alterations in cell membrane permeability, water stress, disturbances in cell division, or inhibition of the electron transport during photosynthesis and respiration (Sengar and others 2008). One of the visible symptoms of Pb toxicity in plants is growth inhibition or leaf chlorosis (Garg and Aggarwal 2011; Sengar and others 2008). Pb induces oxidative stress and ROS production in plant tissues. To overcome the effect of Pb stress, plants can effectively scavenge oxygen species: H_2O_2 , $\bullet OH$, and $O_2^{\bullet-}$. Both enzymatic (catalases and peroxidases) and non-enzymatic antioxidants (GSH, AsA, and *car*) are involved in this process (Verma and Dubey 2003).

Recent studies have reported that Central Italy, France, Germany, and the UK show relatively high, but still within guidelines, concentrations of Pb in agricultural soils. However, the widespread occurrence of Pb in soils indicates the need for strict control of Pb in agricultural land and in the food chain. Lazio, a province in Central Italy, is considered as the most Pb-contaminated region due to volcanic materials (Tóth and others 2016).

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Research attention has been focused on identifying factors that could contribute to reduction of Pb absorption or toxicity in plant organisms. Selenium (Se) is one of the potential antagonists of Pb. Many studies in animals have confirmed the antagonistic effect of Se on Hg, Cd, Zn, Cu, or Ag toxicity (Cabañero and others 2006). For this reason, research on enrichment of crop plants (mustard, onions, garlic, cereal) with Se and their use as an effective source of Se in the human diet are very popular (Guo and others 2014).

The role of Se in plants is not well known and is still controversial. Se is not an essential element for higher plants. However, plants take up and metabolize Se in tissues. Se accumulation differs among plant species. Depending on the amount of accumulated Se, plants have been classified as “non-Se-accumulators” (grasses and most forage and crop plant species), which usually contain less than 25 mg Se kg⁻¹ DW, “Se-hyperaccumulators” having the ability to take up hundreds to several thousand mg of Se kg⁻¹ DW, and “Se-indicators” accumulating up to 1000 mg Se kg⁻¹ DW. Moreover, the boundary between the tolerated and toxic dose of Se is narrow. High Se concentrations are toxic to plants. Se toxicity results from its incorporation into amino acids to substitute sulphur and formation of Se-amino acids. Next, Se-amino acids are incorporated into protein and S–S bonds are replaced by the less stable Se–Se bonds. This is a reason for the modification in the biological activity of the protein (Terry and others 2000). The influence of Se on plants depends both on its chemical form and its concentration in the nutrient solution. Although there is no evidence for Se requirement in non-accumulator plants, numerous studies report that at low concentrations Se exerts a beneficial effect on antioxidant properties, alleviating oxidative stress and promoting plant growth and development (Cartes and others 2005; Djanaguiraman and others 2005; Hartikainen and others 2000; Terry and others 2000; Xue and others 2001). The selenate and selenite doses routinely used under hydroponic conditions to counteract environmental stress are often less than 1 mg L⁻¹ (Feng and others 2013a). Hartikainen and others (2000) showed that selenate at low concentrations behaved as an antioxidant, whereas at higher concentrations it acted as a prooxidant. A low dose of Se in the form of selenite was also associated with a 43 % increase in seed production in *Brassica rapa* L. (Lyons and others 2009). Recent research indicates that addition of Se may protect plants against salt stress (Kong and others 2005), drought (Wang and others 2011), and UV-irradiation (Hartikainen and Xue 2000). Moreover, the still growing number of publications shows that addition of Se may also affect the total content of heavy metals by decreasing their uptake by plants (He and others 2004; Fargašová and others 2006; Feng and others 2013b;

Mroczek-Zdyrska and Wójcik 2012). Several studies have indicated that Se can affect plant tolerance to toxic metals due to not only inhibition of heavy metal uptake and alleviation of oxidative stress but also, because of chloroplasts rebuilding, augmentation of the assimilation pigment and maintenance of cell membrane integrity (Feng and others 2013b).

However, to our knowledge, Pb and Se interactions in plants are still not clear and have been described by only a few authors (Fargašová and others 2006; Mroczek-Zdyrska and Wójcik 2012).

The faba bean (*Vicia faba* L.) is considered one of the oldest leguminous crops in the world. Globally, it is the third most important feed grain legume after soybean (*Glycine max*) and pea (*Pisum sativum* L) (Singh and others 2012). Its highly nutritional seeds are a crucial source of proteins and carbohydrates in food used for human consumption. According to the agricultural requirements, the main fields of interest in faba bean cultivation are yield improvement, disease resistance, abiotic stress tolerance, or seed quality (Gnanasambandam and others 2012).

Addition of phosphate to a Pb-containing growth solution reduced the bioavailability of Pb (Zhang and Ryan 1999). Hence, to prevent precipitation of lead phosphate in the growth solution, we conducted the experiment in P-deficient conditions. We based this on the finding that plants can subsist on the reserves of P stores in the seed for about two (Grant and others 2001) or even several weeks (White and Veneklaas 2012). Moreover, the uptake of Se by plant roots is also influenced by competing anions, such as sulphate and phosphate (Sors and others 2005). Selenite uptake is enhanced by low S and P concentrations or their absence (Winkel and others 2015). Li and others (2008) showed that P starvation resulted in a 60 % increase in selenite uptake by wheat, possibly because P starvation up-regulated the expression of the phosphate transporter genes. Thus, the aim of the present study was to investigate the effect of Pb in combination with Se in P-deficient conditions on *Vicia faba* L. *minor* shoot growth and viability and on selected biochemical parameters (MDA and T-SH content) and antioxidant enzyme activity (CAT, GPOX and GSH-Px). Moreover, the ability of Se to alleviate Pb toxicity in *Vicia faba* L. *minor* shoots was tested and discussed.

Materials and Methods

Plant Material and Experimental Design

Vicia faba L. *minor* (cv. Nadwislanski) plants were grown hydroponically in a laboratory condition. Seeds were

germinated on moist filter paper in plastic containers containing half-strength Hoagland's nutrient solution (with $\text{NH}_4\text{H}_2\text{PO}_4$ to protect the plants from the short-term fluctuations in P supply) in the dark at 25 °C for 7 days. Then, the seedlings were transplanted into 3 L plastic containers (ten plants per one container) with modified full-strength Hoagland's medium (Hoagland and Arnon 1959) containing the following mineral components (POCH): KNO_3 (6 mM), $\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$ (4 mM), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (2 mM), iron citrate (0.85 μM), H_3BO_3 (46 μM), $\text{MnCl}_2 \times 4\text{H}_2\text{O}$ (9 μM), $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ (0.76 μM), $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ (0.32 μM), and $\text{H}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$ (0.11 μM). To prevent precipitation of lead phosphate in growth solution (Zhang and Ryan 1999), $\text{NH}_4\text{H}_2\text{PO}_4$ was removed from the Hoagland's solution in all experimental groups (Wierzbicka and Potocka 2002). Next, the experiment was conducted in P deficiency conditions for 14 days. To determine the effect of Pb and Se, the growth solution was supplemented with 50 μM Pb in the form of $\text{Pb}(\text{NO}_3)_2$ (lead nitrate, Sigma) and/or 1.5 or 6 μM Se in the form of Na_2SeO_3 (sodium selenite, Sigma). We have chosen selenite, which is still not well explored, as a source of Se because both selenite (Terry and others 2000) and Pb (Garg and Aggarwal 2011) are mainly accumulated in the roots and their translocation to shoots is limited. We wanted to check whether selenite can be an effective factor in mitigation of Pb phytotoxicity. The Pb and Se concentrations were chosen on the basis of preliminary experiments and literature data. The 2-week cultivation was performed at 25/20 °C (day/night) under a 16/8 h (day/night) photoperiod at photosynthetic active radiation of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. During the experiment, the nutrient solution was continuously aerated and its losses were supplemented daily with dH_2O . The solution was changed once a week and its pH was kept at 5.0–5.2.

The analyses were performed on plant shoots, fresh or frozen in liquid nitrogen, 14 days after addition of Pb and/or Se. Five plants were selected from each plastic container for the respective analyses and all the measurements were performed in triplicate ($n = 15$). For the analysis of dry weight, the shoots were dried at 70 °C.

Determination of Pb and Se Content

The shoot samples (0.25 g DW) were mineralized in 10 ml HNO_3 using the microwave digestion system "MARS 5" (Varian). After digestion, the residues were diluted to 25 ml with Milli-Q water. Pb and Se contents were determined by the method of atomic absorption spectrometry (AAS) with acetylene flame atomization in the air (FAAS) with the SPEKTR AA spectrometer (Varian). Se content was determined by the method of AAS with hydride generation (AAS-HG). Calibration curves for Pb and Se were done with certified calibration standards (Merck). Analysis

of Se content was verified by certified reference material Standard Reference Material® 2709 San Joaquin Soil. Reference material test precision for Se was 2.1 %.

Histochemical Visualization

Histochemical visualization was determined on the 1st and 2nd pairs of leaves on day 14 of the experiment. Prior to staining, the leaves were washed under tap water and then rinsed three times in dH_2O . After staining, to obtain clear results, the leaves were washed in dH_2O and chlorophyll was removed by boiling in MeOH (POCH) at 70 °C in a water bath (Memmert).

Detection of Pb in plant shoots was performed using the rhodizonic method. After rinsing in dH_2O , the leaves were transferred to a 0.2 % aqueous solution of the reagent containing 1 drop of Tween 20, evacuated three times in a vacuum pump for 10 min, and left for 12 h in the dark. Pb was detected as dark-brown product formed with rhodizonic acid disodium salt (Sigma) (Wierzbicka 1987).

Assessment of leaf viability was performed by trypan blue staining. For this purpose, the leaves were placed in a boiling solution of the dye for 1 min. Trypan blue (Sigma) cannot pass through intact membranes of living cells, whereas it easily penetrates the damaged cell membrane staining sites of necrosis (Belenghi and others 2003).

Detection of H_2O_2 in the leaves was carried out after the leaves were stained in a solution containing 3,3-diaminobenzidine (DAB, 0.1 %, pH 3.8) (Sigma). The leaves were placed in a dye solution, then evacuated three times in a vacuum pump for 10 min and left for 12 h in the dark (Shinogi and others 2003).

Localization of $\text{O}_2^{\bullet-}$ in the leaves was carried out using NBT (Sigma) (0.05 %) in phosphate buffer (0.05 M, pH 7.5) (Shinogi and others 2003). The leaves were placed in a solution of the dye, evacuated three times in a vacuum pump for 10 min, and incubated in the dye for 1 h. NBT led the reaction with $\text{O}_2^{\bullet-}$ forming oxidized NBT, a dark blue insoluble formazan.

Chlorophyll and Carotenoid Determination

The contents of chl *a*, chl *b*, and *car* were determined as described by Lichtenthaler and Wellburn (1983). Fresh leaves (0.5 g) were homogenized in 5 ml of acetone (POCH) (80 %, chilled to 4 °C). The homogenate was filtered and the precipitate was washed with cold acetone until the total chlorophyll extraction. Next, the extract was supplemented with 80 % chilled acetone to 25 ml. The mixture was centrifuged (MPW-350R) at 10,000 $\times g$ at 4 °C for 10 min. The absorbance of the supernatant was measured at 663 nm (chl *a*), 645 nm (chl *b*), 652 nm (chl *a+b*), and 470 nm (*car*) on a spectrophotometer (UV-VIS Helios Gamma).

Lipid Peroxidation Determination

The level of lipid peroxidation was estimated as the content of MDA (Heath and Packer 1968). Frozen shoots (0.5 g) were homogenized in 5 ml of 0.5 % TBA (Sigma) in 20 % TCA (Sigma) with 250 μ l BHT (Sigma). After heating at 95 °C for 30 min, the mixture was quickly cooled in an ice bath and again centrifuged at 15,000 \times *g* for 15 min. The absorbance of the supernatant was read at 532 and 600 nm, the latter for unspecific turbidity which was subtracted. The MDA content was calculated with the extinction coefficient (155 mmol⁻¹ cm⁻¹).

Total SH Content Determination

The total content of SH groups was determined as described by Maas and others (1987) with Ellman's reagent (DTNB) (Sigma). The frozen samples of shoots were homogenized in a cooled mortar in an extraction solution containing 0.15 % ascorbic acid (Sigma), 2 % SSA (Sigma), and 0.037 % Na₂EDTA (Sigma) (5:1, v/w). The homogenate was centrifuged twice at 10,000 \times *g* for 15 min at 4 °C. Supernatant (0.5 ml) was mixed with 0.5 ml 1 M K₂HPO₄–KH₂PO₄ buffer (POCH) at pH 8.0 and 0.1 ml of 10 mM DTNB (Sigma). The absorbance was measured at λ = 412 nm after 1 min with a UV–VIS spectrophotometer (Helios Gamma). The SH group content was read from the standard curve for L-cysteine (Sigma).

Protein and Enzymatic Antioxidant Determinations

Proteins were assayed according to Bradford (1976) using BSA (Sigma) as a standard with a UV–VIS spectrophotometer (Helios Gamma) 15 min after addition of colour dye (Bio-Rad Protein Assay) at λ = 595 \pm 10 nm. The enzymatic assays were performed after the enzymes were extracted in 5 ml (CAT and GPOX) or 2.5 ml (GSH-Px) (Sigma) of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA (Sigma) and 1 % PVPP (Sigma). The homogenate was centrifuged twice at 10,000 \times *g* at 4 °C for 15 min and the supernatant was applied for the assays.

Determination of CAT (E.C. 1.11.1.6) activity was conducted in fresh plant samples (1 g) as described by Aebi (1984). Phosphate buffer (1.5 ml of 50 mM, pH 7.0) and 0.5 ml of 0.15 % H₂O₂ (30 wt%, Sigma) were added to the quartz cuvette. The reaction was initiated by adding 5 μ l of the supernatant to the reaction mixture at 25 °C. The absorbance decrease (Δ Abs/min) was read at λ = 240 nm for 30 s with the UV–VIS spectrophotometer (Helios Gamma). The calculation includes the molar absorption coefficient of H₂O₂ (ϵ = 0.036 mM⁻¹ cm⁻¹).

Measurement of GPOX (E.C. 1.11.1.7.) activity was determined in frozen plant samples (0.5 g) according to

Velikova and others (2000). The reaction mixture contained 2.75 ml of 50 mM phosphate buffer (pH 7.0) containing 1 % guaiacol (Sigma) and 100 μ l of supernatant. The reaction was started by adding 150 μ l 100 mM H₂O₂ (30 wt%, Sigma) to the reaction mixture and absorbance (Δ Abs/min) was read at λ = 470 nm after 30 s with a UV–VIS spectrophotometer (Helios Gamma). The calculation includes the molar absorption coefficient of guaiacol (ϵ = 26.6 mM⁻¹ cm⁻¹).

GSH-Px (E.C. 1.11.1.9.) activity was determined in frozen plant samples (0.5 g) according to Ali and others (2006). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM Na₂EDTA (Sigma), 0.25 U ml⁻¹ GR (EC 1.6.4.2) (Sigma), 1 mM L-GSH (Sigma), 0.15 mM β -NADPH (Sigma), 1 mM NaN₃ (Sigma), and 20 μ l homogenate. The reaction was initiated by adding 1.5 mM t-BOOH (Sigma) at 37 °C and the absorbance decrease was measured with a UV–VIS spectrophotometer (Helios Gamma) at λ = 340 nm. The reaction with t-BOOH allowed measurements of the activity of GSH-Px containing Se (without measuring the activity of GSH-Px without Se). A unit of GSH-Px activity was the amount of the enzyme that catalyses oxidation of β -NADPH to β -NADP⁺ (mM min⁻¹ mg⁻¹ protein) at 25 \pm 2 °C with the molar absorption coefficient for the β -NADPH (ϵ = 6.22 mM⁻¹ cm⁻¹).

Statistical Analysis

To examine the biometric parameters, chlorophyll, and carotenoid contents, five plants were selected and all the measurements were performed in triplicate (*n* = 15). The biochemical analysis (the contents of MDA and T-SH, and the activities of CAT, GPOX, and GSH-Px) was performed in triplicate and all the experiments were run in three time-independent repetitions (*n* = 9). Data are presented as the mean \pm standard errors (SE). Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc analysis for determination interaction significance at *p* < 0.05.

Results

Se and Pb Accumulation and Plant Growth

The Pb concentration in the plant shoots increased successively up to day 14 (data not shown), but finally, the shoots of *Vicia faba* L. *minor* accumulated only about 1.5 % of the total Pb present in the plant. The addition of Se to the Pb-treated plants did not alter Pb accumulation in the shoots. However, in the presence of both Pb and 6 μ M Se, we noted a 34 % decrease in the Se content in comparison to plants treated with 6 μ M Se only (Table 1).

Elongation of the shoots in the Pb-treated plants was at the level of the control plants. The presence of Se without and with Pb stress had no effect on shoot growth, shoot elongation, FW and DW of the shoots, the number of leaves, and the length and width of leaves (Table 2).

Measurements of Chlorophyll and Carotenoids

A significant decrease (2 %) in the chl *a* content was found only in the 1.5 μM Se and 50 μM Pb + 1.5 μM Se-treated plants in comparison to the control. The content of these compounds in all groups increased in comparison to the control at least 20 % (chl *b*) and at least 10 % (chl *a+b* and *car*). The increase in the chl *b* content was accompanied by a proportional decrease in the ratio $R^{a/b}$ (Table 3).

Histochemical Detection of Pb

There were no Pb deposits in the leaves of the control and Se-treated plants. The 14-day exposition of the plants to Pb resulted in distinct colouration of the leaves. On the 1st pair leaves, Pb deposits were observed along the main nerve, whereas on the 2nd pair leaves, dark-brown colour occurred mainly at the top and the edges of the leaf blade. The addition of Se at the concentration of 1.5 and 6 μM to the medium containing Pb decreased accumulation both in the 1st and 2nd pairs of leaves. A more significant effect was found for Se at the concentration of 1.5 μM , which visibly decreased staining intensity in the 1st pair leaves and almost completely reduced it in the 2nd pairs of leaves (Fig. 1).

Histochemical Detection of Viability

The macroscopic observations showed varied and age-dependent viability of the control leaves. After 14 days, a slight blue colour appeared over the entire surface of the

1st pair of leaf blades, whereas intense staining was noted at the top of the 2nd pair of leaves. The decrease in cell viability was visible in the Pb-treated plants as an increase in the blue staining. The characteristic blue colour appeared over the entire surface of the leaf blades but stronger colouration was observed for the 2nd pair of leaves. The addition of Se to the growth medium with 50 μM Pb decreased the staining intensity in the leaves. However, a lower Se concentration (1.5 μM) under Pb treatment improved the viability of leaf blades more clearly, and the light blue staining was observed in the top part of the blades of the 1st pair of leaves, whereas small necrotic changes were observed mainly in the central part of the blades of the 2nd pair leaves. The higher Se concentration (6 μM) was not so effective under Pb treatment. The leaf blades were characterized by the presence of light blue colour in the entire assimilatory parenchyma (Fig. 2).

Analysis of the Oxidative Stress in the Shoots

Histochemical analyses of H_2O_2 and $\text{O}_2^{\bullet-}$ accumulation in the leaves were performed for visualization of the oxidative stress.

On day 14, slight brown colouration indicating H_2O_2 production was observed only near the main vascular bundles in the basal part of all the control leaves. The exposition of the plants to Pb clearly intensified production of H_2O_2 in the leaf blades. A brown colour appeared in the vascular system, as well as in the assimilation parenchyma on the periphery of the leaf blades. In the 1.5 μM Se-treated plants, only the vascular bundles were stained brown, but a stronger reaction was observed in the 1st pair leaf blades. In the 6 μM Se-treated plants, the brown colour occurred mainly in the basal part of the leaf blades. However, in the 50 μM Pb + 1.5 μM Se-treated plants and the 50 μM Pb + 6 μM Se-treated plants, quite strong staining of leaf blades was observed, especially at the bottom of the 1st and 2nd pair leaves (Fig. 3).

A light blue colour indicating the formation of $\text{O}_2^{\bullet-}$ appeared only near the main and the lateral vascular bundles of the control plant leaves. In the 50 μM Pb-treated plants, significantly enhanced production of $\text{O}_2^{\bullet-}$ was found, especially on the 1st pair of leaves. On these leaf blades, an intense blue colour was present in nearly the entire surface of the leaf blades. On the 2nd pair of leaves, strong blue colouration appeared in the some central parts of the leaf blades. The addition of Se to the growth medium with Pb decreased the staining resulting from formation of $\text{O}_2^{\bullet-}$ in the Pb-treated of leaves, both at lower and higher Se concentrations (Fig. 4).

The increase in the accumulation of reactive oxygen species enhanced production of lipid peroxides, which was an effect of oxidative stress. The level of MDA, one of the

Table 1 Lead and selenium contents in *Vicia faba* L. *minor* shoots grown for 14 days in the control solution or in the presence of Pb and/or Se

Pb and/or Se concentration (μM)	(mg kg^{-1} DW)	
	Pb	Se
0	ND	0.42 a
1.5 Se	ND	4.69 b
6 Se	ND	4.67 b
50 Pb	168 a	0.41 a
50 Pb + 1.5 Se	153 a	4.04 b
50 Pb + 6 Se	155 a	3.09 c

Values with different letters in the same column differ significantly from each other at $p < 0.05$

ND not detectable

Table 2 The growth of shoots in *Vicia faba* L. *minor* plants cultivated for 14 days in the control solution or in the presence of Pb and/or Se

Pb and/or Se concentration (μM)	Shoot elongation (cm)	Shoot fresh weight (mg plant^{-1})	Shoot dry weight (mg plant^{-1})	Number of leaves	Length of leaves (cm)	Width of leaves (cm)
0	10.44 \pm 1.03 a	2889.33 \pm 158.86 a	212.03 \pm 11.61 a	2.00 \pm 0.00 a	2.74 \pm 0.13 a	1.78 \pm 0.09 a
1.5 Se	13.23 \pm 0.62 a	2938.67 \pm 152.36 a	209.23 \pm 10.88 a	2.00 \pm 0.00 a	2.89 \pm 0.05 a	1.76 \pm 0.03 a
6 Se	11.73 \pm 1.17 a	2710.00 \pm 123.20 a	208.87 \pm 11.00 a	2.00 \pm 0.00 a	2.59 \pm 0.13 a	1.65 \pm 0.07 a
50 Pb	13.07 \pm 0.52 a	2963.33 \pm 163.96 a	215.19 \pm 13.46 a	1.93 \pm 0.07 a	2.73 \pm 0.13 a	1.86 \pm 0.08 a
50 Pb + 1.5 Se	13.49 \pm 0.90 a	2998.00 \pm 83.83 a	229.07 \pm 5.85 a	1.87 \pm 0.13 a	2.76 \pm 0.16 a	1.67 \pm 0.09 a
50 Pb + 6 Se	11.47 \pm 0.52 a	3064.67 \pm 195.87 a	241.84 \pm 17.35 a	2.00 \pm 0.00 a	2.89 \pm 0.09 a	1.82 \pm 0.07 a

Values are mean \pm SE ($n = 15$). Values with different letters in the same column differ significantly from each other at $p < 0.05$

Table 3 Chlorophyll and carotenoids contents in *Vicia faba* L. *minor* leaves grown for 14 days in the control solution or in the presence of Pb and/or Se

Pb and/or Se concentration (μM)	Chl <i>a</i> (mg g^{-1} FW)	Chl <i>b</i> (mg g^{-1} FW)	Chl <i>a+b</i> (mg g^{-1} FW)	R ^{<i>a</i>/<i>b</i>} (mg g^{-1} FW)	Car (mg g^{-1} FW)
0	1.06 a	0.73 \pm 0.01 a	1.87 \pm 0.01 a	1.45 \pm 0.01 a	0.46 \pm 0.00 a
1.5 Se	1.04 b	0.93 \pm 0.06 bc	2.08 \pm 0.08 bc	1.19 \pm 0.10 b	0.51 \pm 0.02 b
6 Se	1.05 ab	0.92 \pm 0.02 bc	2.09 \pm 0.02 bc	1.14 \pm 0.02 bc	0.51 \pm 0.01 bc
50 Pb	1.06 a	0.88 \pm 0.01 b	2.05 \pm 0.02 b	1.20 \pm 0.02 b	0.51 \pm 0.00 b
50 Pb + 1.5 Se	1.04 b	1.04 \pm 0.01 c	2.21 \pm 0.01 c	1.00 \pm 0.01 c	0.55 \pm 0.00 c
50 Pb + 6 Se	1.05 ab	0.89 \pm 0.01 b	2.06 \pm 0.02 b	1.19 \pm 0.02 bc	0.51 \pm 0.00 bc

Values are mean \pm SE ($n = 15$). Values with different letters in the same column differ significantly from each other at $p < 0.05$

major lipid peroxidation indicators, increased in the 50 μM Pb-treated shoots by 47 % in comparison to the control. Moreover, Se increased lipid peroxidation by 20 and 21 % at 1.5 μM Se and 6 μM Se, respectively. The lower (1.5 μM) Se concentration did not exert an effect on the MDA level in the 50 μM Pb-treated shoots, but the higher (6 μM) Se concentration decreased the MDA level by 22 % in comparison to the plants treated with Pb (Fig. 5).

The T-SH content significantly increased, except for the Pb-treated plants. In the 1.5 and 6 μM Se-treated plants, the T-SH content increased by 32 and 96 %, respectively. In the shoots treated with 50 μM Pb + 1.5 μM Se and 50 μM Pb + 6 μM Se, the T-SH content increased by 129 and 93 %, respectively. The addition of 1.5 and 6 μM Se to the growth medium with Pb intensified synthesis of thiol compounds in the shoots by 42 and 35 %, respectively, in comparison to the 50 μM Pb-treated plants (Fig. 6).

In comparison to the control, a 3-fold increase in CAT activity in the shoots exposed to 50 μM Pb was observed. Se at the concentrations of 1.5 and 6 μM increased CAT activity in the shoots by 74 and 103 %, respectively. The simultaneous treatment with Pb and 6 μM Se increased CAT activity (204 %) in comparison to the control. In the groups exposed to both Pb and Se, CAT activity was significantly lower in comparison to the 50 μM Pb-treated

plants, that is, 68 and 23 % at 1.5 μM Se and 6 μM Se, respectively (Fig. 7a).

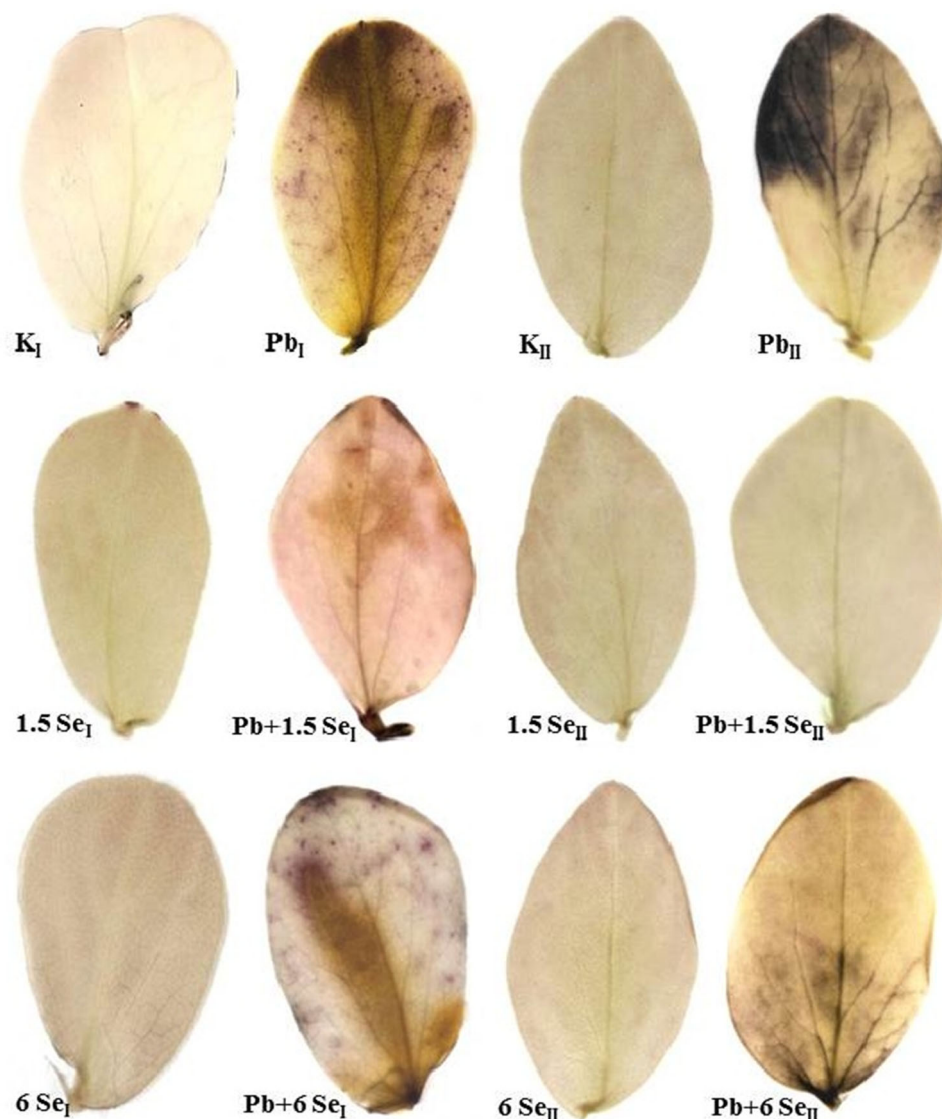
GPOX activity in the shoots treated with Pb, Se, and 50 μM Pb + 6 μM Se increased by 22–32 % in comparison to the control. However, 1.5 μM Se supplementation of Pb-containing growth medium reduced GPOX activity by 51 %, in comparison to the plants treated with 50 μM Pb, to a level that was significantly lower than in the control (Fig. 7b).

In the shoots exposed to Pb or 1.5 or 6 μM Se, an increase in the GSH-Px activity by 72, 93, and 86 %, respectively, was noted in comparison to the control. The addition of 1.5 μM Se to the growth medium with Pb maintained the GSH-Px activity at the control level, which was 40 % lower than in the shoots exposed to the Pb alone. However, addition of 6 μM Se to the growth medium with Pb increased the GSH-Px activity by 93 % (Fig. 7c).

Discussion

Se is a known anticarcinogen and antioxidant compound, which recently is quite frequently supplied in fertilizers to improve its deficiency in soil or to enrich plants and food crops in Se, having ameliorating advantages in food

Fig. 1 Localization of lead (after rhodizonic acid staining) in *Vicia faba* L. *minor* 1st (I) and 2nd (II) pair leaves after 14 days of cultivation in the control solution or in the presence of Pb and/or Se. *Brown colour* indicates lead deposits in leaf blades (Color figure online)



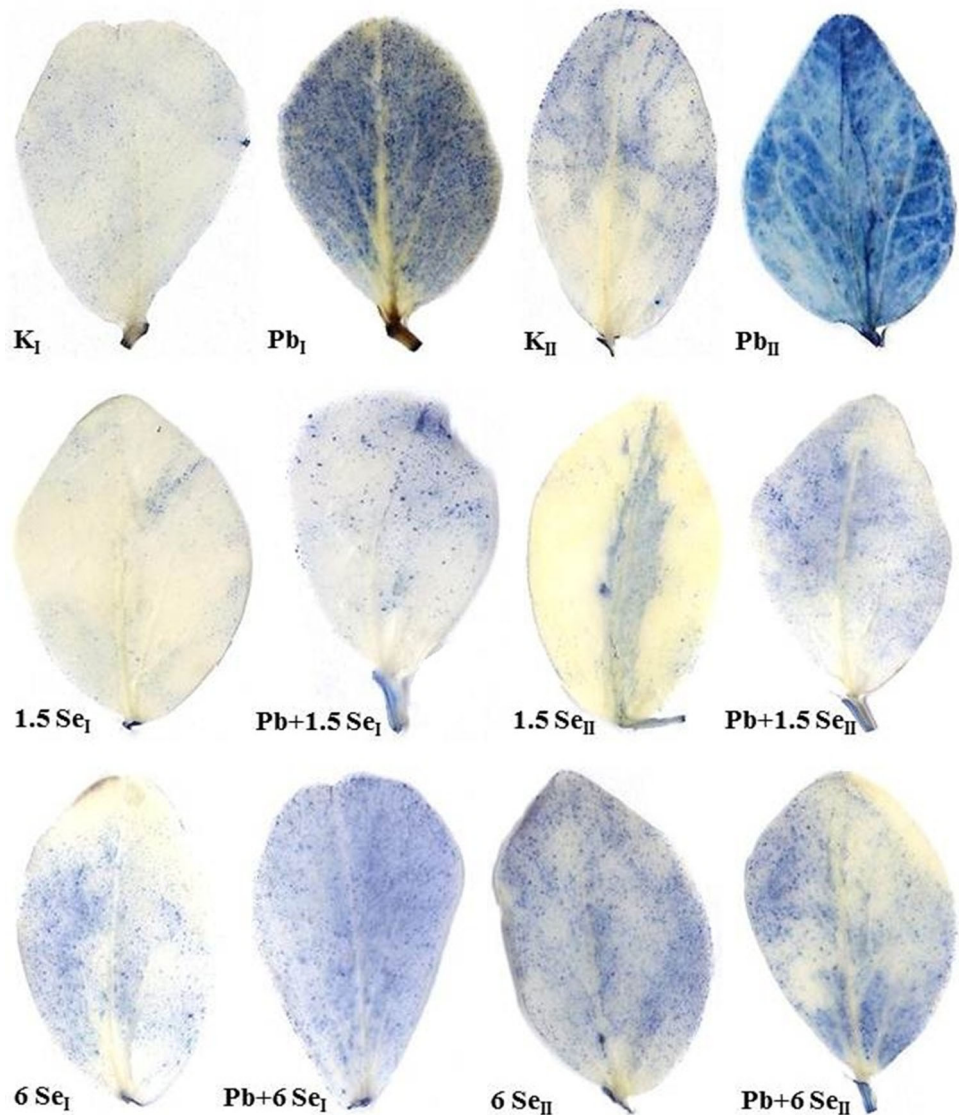
characteristics, like the shelf life (D'Amato and others 2014). The response of plants to Se under Pb exposure has been previously described by a few authors (Fargašová and others 2006; Mroczek-Zdyrska and Wójcik 2012); however, the literature in this field is quite limited and the results obtained are often contradictory, which relates to both the form and the dose of Se or the concentration ratios between the metal and Se applied (Fargašová and others 2006).

The uptake and accumulation of Pb by plants is an important and intensively studied problem. It has been proven that Pb is taken up from the growth medium and accumulated mainly in the roots (Garg and Aggarwal 2011; Mroczek-Zdyrska and Wójcik 2012). Consequently, shoots contain significantly less Pb (Garg and Aggarwal 2011; Sengar and others 2008; Wierzbicka and Potocka 2002). The rhodizonic method used in this study confirmed the

presence of Pb mainly in the vascular bundles and in the mesophyll cells of the leaf blades similarly to the results obtained by Książek and others (1984).

The literature confirms the dose- and form of Se-dependent (selenate, selenite, and organic compounds, SeCys and SeMet) Se root/shoot accumulation (Simojoki and others 2003; Terry and others 2000). Selenite showed approximately 50 % less accumulation of Se in the aboveground parts in comparison with selenate (Klusionova and others 2015; Zhang and others 2007). On the contrary, Lehotai and others (2012) found that selenite-treated plants accumulated more Se in their shoots. One likely explanation of this phenomenon can be that the oxidation process of selenite to selenate may appear during cultivation. However, both Se forms (selenate and selenite) are reduced to selenide, and next they run a common pathway in Se metabolism (Lehotai and others 2012). Moreover, in lower

Fig. 2 Cell death (after trypan blue staining) of *Vicia faba* L. *minor* 1st (I) and 2nd (II) pair leaves after 14 days of cultivation in the control solution or in the presence of Pb and/or Se. Blue colour indicates sites of necrosis (Color figure online)



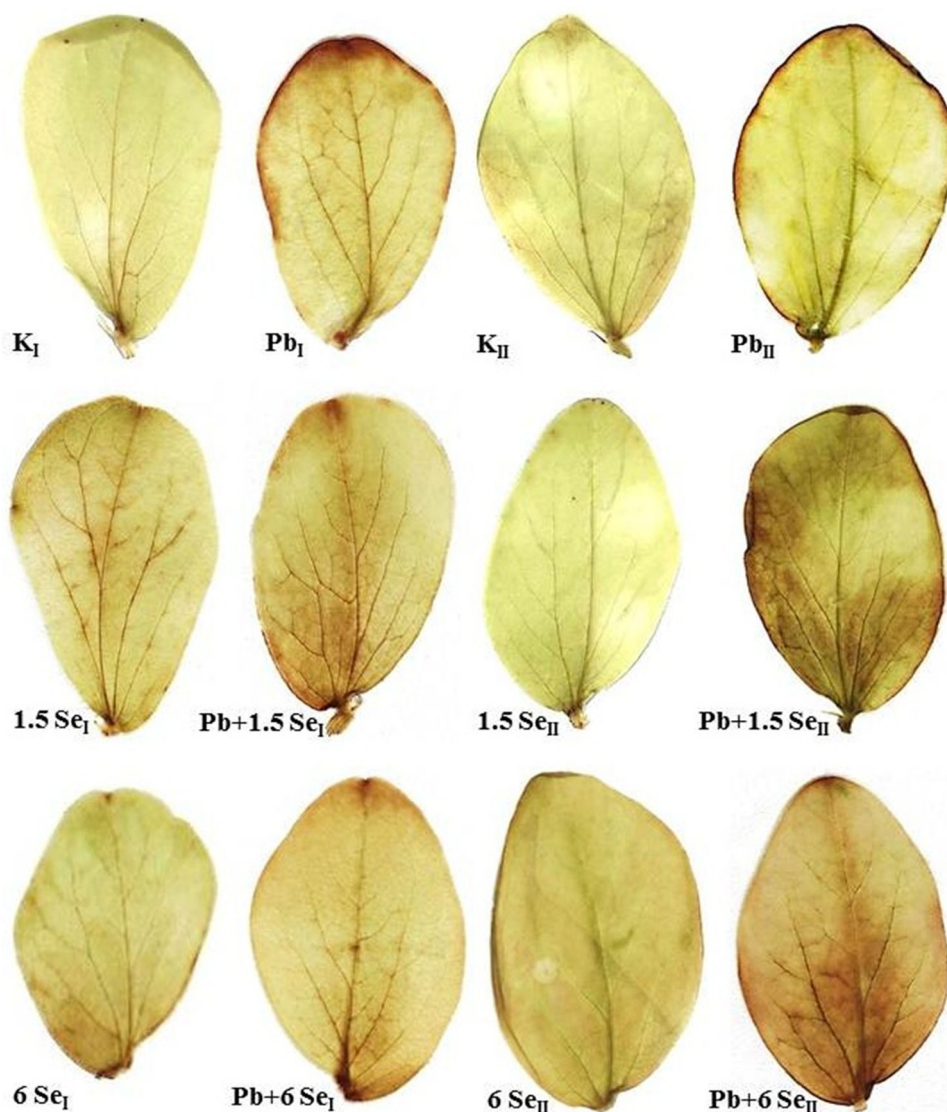
doses, Se was accumulated mainly in the roots, but at higher doses, its content in the roots and shoots was similar (Simojoki and others 2003). Similar to our results for *Vicia faba* L. *minor*, Se applied in the form of selenite is poorly translocated to shoots (De Souza and others 1998) because the majority remains in roots, where it is rapidly transformed into organic Se compounds (Fargašová and others 2006; Mroczek-Zdyrska and Wójcik 2012; Simojoki and others 2003; Terry and others 2000). Furthermore, Se-accumulator plants accumulate selenate in older leaves, whilst organic Se compounds (MeSeCys) are stored in the youngest tissues (Terry and others 2000). However, non-Se-accumulators store Se mainly in roots and only small amounts are located into the stem and leaves (Sors and others 2005).

Se can modify the uptake and accumulation of other elements that are important for plant metabolism. He and

others (2004) found that the uptake of Fe by lettuce was enhanced after Se application. Furthermore, rice grains from seleniferous soil showed significantly higher concentrations of As, Cr, and K and lower concentrations of S and Zn in comparison to rice grown on normal soil (Sharma and others 2014).

Our research proved no Se effect on Pb content in the shoots of *Vicia faba* L. *minor*. The literature provides reports about various reduction levels in heavy metal (Pb, Cd, Cr, Co, Ni, Zn) uptake and accumulation in both the roots and the shoots of plants resulting from the presence of Se in a growth medium (Fargašová and others 2006). This difference could be a result of plant species, age, the part of the plant (leaves or shoots), and the mode of supplementation. Moreover, the effect of Se on heavy metal accumulation in plants depends strongly on the proportion between the elements (Fargašová and others 2006). In a

Fig. 3 Visualization of hydrogen peroxide accumulation (after DAB staining) in *Vicia faba* L. *minor* 1st (I) and 2nd (II) pair leaves after 14 days of cultivation in the control solution or in the presence of Pb and/or Se

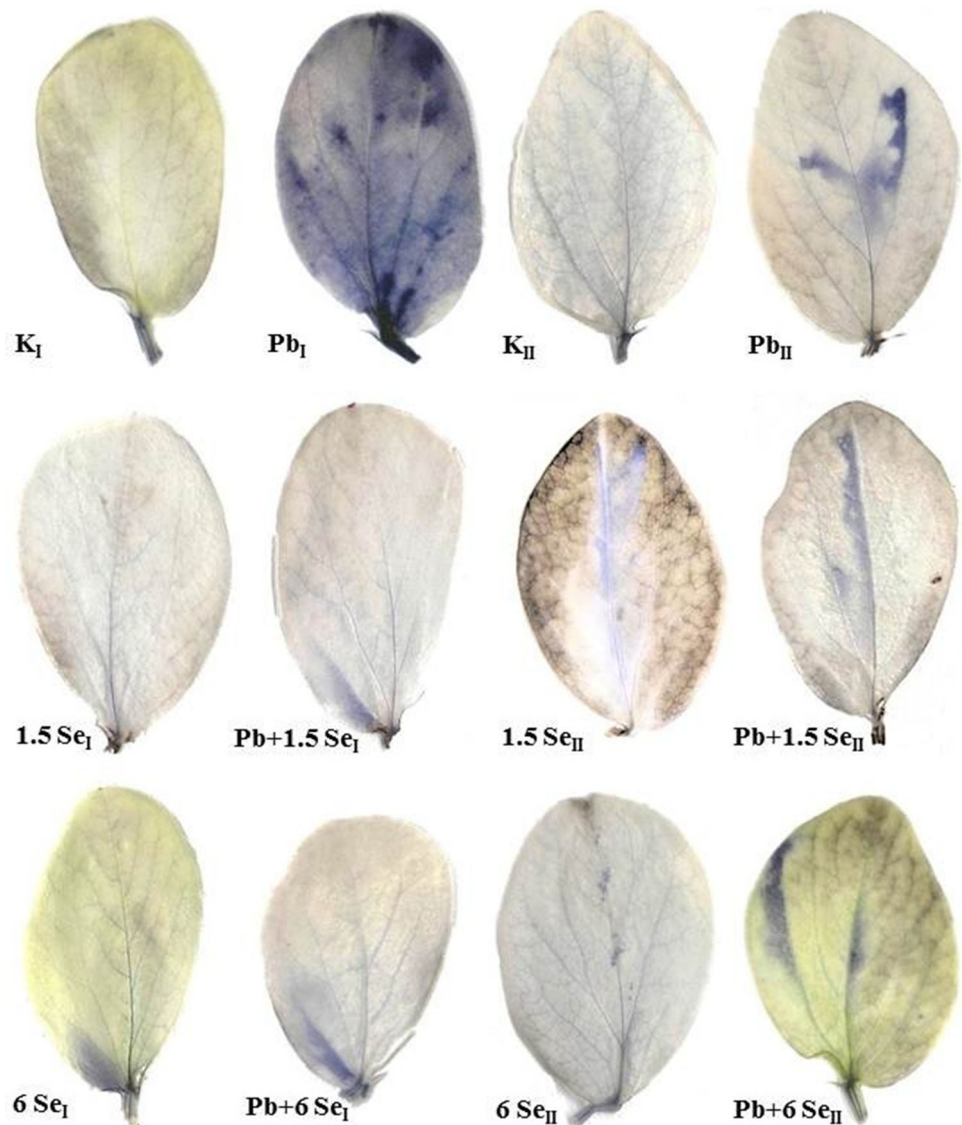


field experiment, the addition of selenite to lettuce subjected to Pb and Cd significantly decreased the accumulation of these heavy metals (He and others 2004). Similarly, Shanker and others (1996) noted that Se supplementation (both as selenite or selenate) to soil contaminated with Cd decreased the uptake and accumulation of Cd in maize; however, the decline in the Cd concentration under Se supplementation was greater in roots than in shoots. The mechanism of heavy metal detoxification by Se might be connected with inhibition of uptake and translocation of heavy metals from roots to shoots and/or formation of non-toxic Se-metal complexes (Feng and others 2013a). Belzile and others (2006) suggested that the mutual detoxification between selenite and Hg might be associated with formation of Se–Hg complexes. Interestingly, at higher Se concentrations, stimulation of heavy metal uptake can also be expected (Fargašová and others 2006; Feng and others 2013a). It was noted that selenite application stimulated the

accumulation of As in *Thunbergia alata* (Bluemlein and others 2009), Al in ryegrass (Cartes and others 2010), or Cd and Cu in wheat and pea (Landberg and Greger 1994). It is assumed that these phenomena can be attributed to an improper dose of Se and/or abnormal S assimilation (Feng and others 2013a).

The Pb dose used in this study had no negative effect on the growth parameters of Se-supplemented and non-Se-supplemented *Vicia faba* L. *minor* plants probably because a predominant amount of Pb was detected in roots (Mroczek-Zdyrska and Wójcik 2012). Such a restricted translocation of Pb from roots to shoots could be explained by effective protection of the photosynthetic apparatus (Vassilev and others 1995) or increased antioxidant defence in shoots (Verma and Dubey 2003). Our results are in agreement with others (Hawrylak-Nowak and others 2014), who reported that under Cd-stress there were no significant differences in the shoot biomass of Se-supplied

Fig. 4 Visualization of superoxide anion accumulation (after NBT staining) in *Vicia faba* L. *minor* 1st (I) and 2nd (II) pair leaves after 14 days of cultivation in the control solution or in the presence of Pb and/or Se



plants, compared to Cd-exposed plants. It is noteworthy that plant growth and biomass stimulation by sublethal doses of toxicants has been reported (Jia and others 2012) and this phenomenon is referred to as hormesis (Kovács-Bogdán and others 2010).

In the present study, there was no significant effect of Se on shoot growth and development, like in other research (Simojoki and others 2003; Xue and others 2001). However, literature data show ambiguous results from induction of shoot growth (Djanaguiraman and others 2005) to inhibition (Xue and others 2001). Trace amounts of Se stimulated growth in a variety of non-hyperaccumulators including ryegrass, lettuce, potato, and duckweed (Pilon-Smits and others 2009). Peng and others (2000) noted that low selenite doses induced the development of hydroponically grown wheat, whereas Se excess inhibited plant growth in a non-linear dose–response relationship.

Although most publications reported a decrease in the assimilation pigment content in the presence of heavy metals (Hawrylak and others 2007), an increase was also observed both under metal treatment (Jia and others 2012) and at a lower Se dose (5 μM) in lettuce leaves (Hawrylak and others 2007) and in olive oil from Se-treated trees (D’Amato and others 2014), which is in accordance with our results. The increase in the assimilation pigment content can be explained by the low activity of chlorophyllase (Gupta and others 2012), the Se role in chlorophyll biosynthesis (Hawrylak and others 2007), protection of photosynthesis against light stress (Seppänen and others 2003), and the efficient removal of ROS by Se-stimulated SOD and GSH-Px (Djanaguiraman and others 2005). It is noteworthy that low-dose stressors increased photosynthetic activity, chl-protein complexes, and chl content, and the mechanism of the stimulation has been characterized in

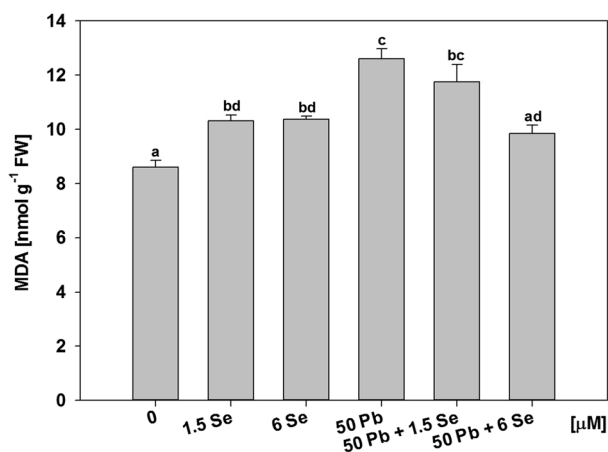


Fig. 5 The level of lipid peroxidation expressed in terms of the MDA concentration in the shoots of *Vicia faba L. minor* plants after 14 days of cultivation in the control solution or in the presence of Pb and/or Se. The bars indicate mean ($n = 9$) \pm SE. Values with different letters differ significantly from each other at $p < 0.05$

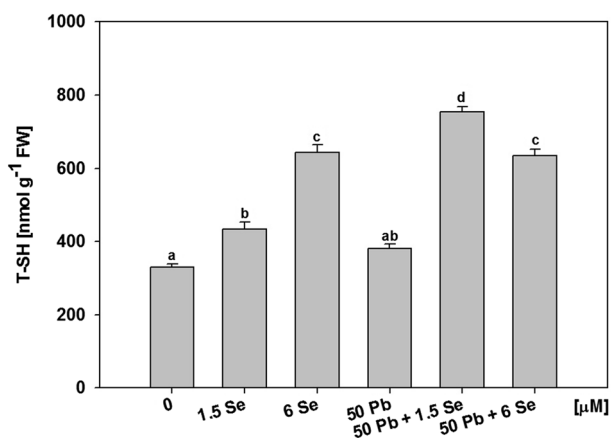


Fig. 6 The total SH content (T-SH) in the shoots of *Vicia faba L. minor* plants after 14 days of cultivation in the control solution or in the presence of Pb and/or Se. The bars indicate mean ($n = 9$) \pm SE. Values with different letters differ significantly from each other at $p < 0.05$

terms of signalling pathways activated via induction of cytokinin synthesis (Kovács-Bogdán and others 2010). In our experiments, Pb stress and/or Se presence also intensified the synthesis of *car* in bean plants analogically to the literature data (Rastgoo and Alemzadeh 2011). Carotenoids are non-enzymatic antioxidant pigments that protect chlorophyll, membrane, and the cell genetic composition against ROS by quenching of triplet chlorophyll, restricting peroxidation, and destruction of the chloroplast membrane (Rastgoo and Alemzadeh 2011).

Moreover, Kong and others (2005) suggest that exogenous Se, in an appropriate dose, can promote the integrity of the membrane system and some cellular organelles (mitochondria, chloroplasts) in the leaf and root tissues.

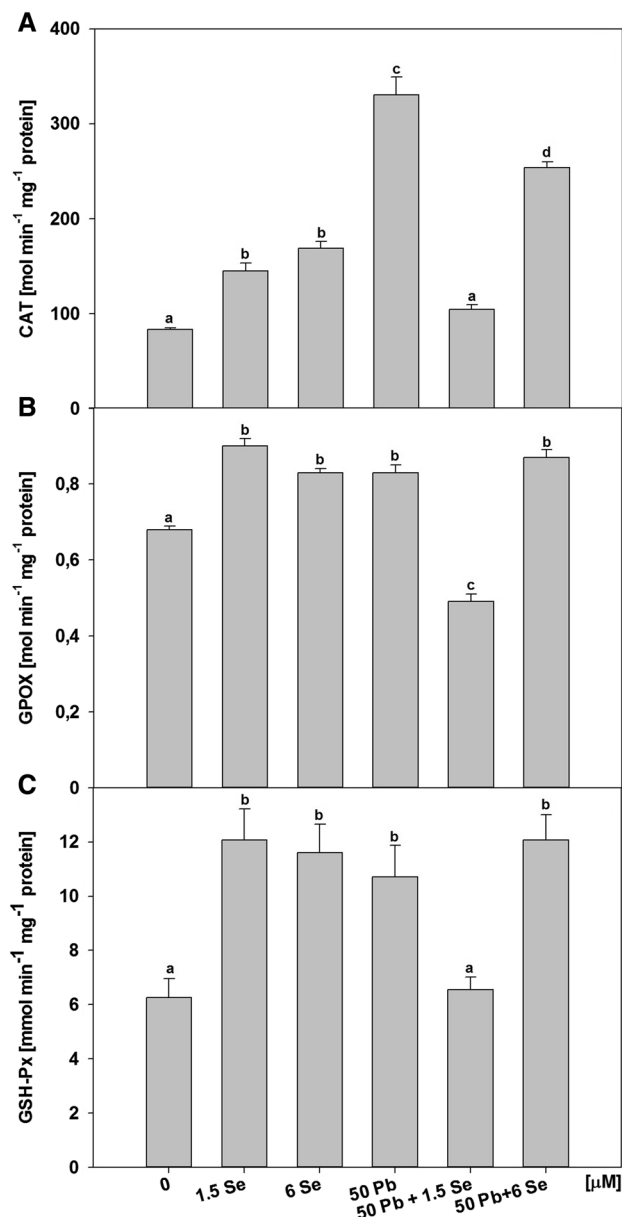


Fig. 7 Antioxidant enzyme activity (mol min mg protein) in the shoots of *Vicia faba L. minor* plants after 14 days of cultivation in the control solution or in the presence of Pb and/or Se. The bars indicate mean ($n = 9$) \pm SE. Values with different letters differ significantly from each other at $p < 0.05$

In our experiment, we did not see characteristic symptoms of P deficiency, for example, decreased plant height, delayed leaf emergence, reduced tillering, secondary root development, dry matter yield (Grant and others 2001), appearance of a dark green colouration, or leaf malformation (Taiz and Zeiger 2002). This is in accordance with the statement that many plants can subsist on the reserves of P stores in the seed for about two (Grant and others 2001) or even several weeks (White and Veneklaas 2012). One exception could alternatively be necrotic spots on the

leaves, which may be caused by short-term P deficiency (Taiz and Zeiger 2002), but we suggest that the local accumulation of Pb in the leaf tissues was the direct cause of cell death (Alkio and others 2005).

The cell membrane is an important barrier in preventing or reducing the metal entry into the cell. The excess of metals can influence plasma membrane integrity and cause disturbances in cell homeostasis (Benavides and others 2005). Thus, the injuries of the plasma membrane detected on the basis of MDA formation may serve as indicators of metal toxicity and lipid peroxidation and are usually connected with an increase in ROS production (Cheeseman 2007; Hanaka and others 2016). In this study, the significant increase in lipid peroxidation in the shoots under Pb treatment was accompanied by increased H_2O_2 and $\text{O}_2^{\bullet-}$ production, as evidenced by the colour reaction with DAB and NBT in leaf blades. The elevation in ROS production in the shoots of abiotically stressed plants has often been described in the literature (Cheeseman 2007; Hanaka and others 2016; Rastgoo and Alemzadeh 2011; Velikova and others 2000) and may be associated with substantial damage caused by Pb stress and induction of cell death (Kawai-Yamada and others 2004). In the *Vicia faba* leaves, the areas of Pb accumulation were accompanied by increased necrotic symptoms. This overlap may suggest that the local accumulation of Pb in the leaf tissues was the direct cause of cell death (Alkio and others 2005).

In our experiment, the MDA level in the shoots was reduced in the presence of the higher Se concentrations introduced to the growth medium with Pb. This indicates that the Se effect on the MDA level depends on the concentration applied, its content in the specific tissue, and the Se form. Consistent with our results, several researchers have also shown inhibition of lipid peroxide accumulation after Se supplementation in the form of selenite (Hawrylak-Nowak and others 2014). These results can be attributed to the antioxidative properties of Se, especially in lower doses (Hartikainen and others 2000). However, for selenate, the lower Se concentrations decreased lipid peroxidation, whereas the higher Se concentration intensified it (Cartes and others 2005).

The presented data show that Pb stress leads to a significant (by 296 %) increase in CAT activity and suggest that this enzyme plays a fundamental role in the response to overproduction of ROS generated by Pb (Verma and Dubey 2003). Moreover, this metal also caused an increase in GPOX and GSH-Px activity (22 and 72 %, respectively) in the shoots of *Vicia faba* L. *minor*. The increase in the activity of the antioxidant enzymes in response to Pb is a common response of plants to stress (Rastgoo and Alemzadeh 2011; Verma and Dubey 2003).

The oxidoreductase enzymes (GSH-Px) are the most sensitive to Se, due to the antioxidant properties of Se

(Hartikainen and others 2000). In the present work, both Se concentrations significantly decreased CAT activity, but only the lower Se concentration significantly decreased GSH-Px and GPOX activity in the shoots exposed to Pb stress.

The use of Se as selenite in combination with Pb clearly intensified T-SH induction in the shoots of the examined bean plants, which is in accordance with other results (Hawrylak and Szymańska 2004). It seems that the differences in the T-SH group content between the roots and the shoots result from the rate of Se translocation to the shoots (Terry and others 2000).

In conclusion, our results indicate that addition of Se to Pb-containing growth medium exerted a protective effect against Pb toxicity, and the different Se concentrations applied had a beneficial effect on different parameters (assimilation pigment content, T-SH and MDA content, antioxidant enzyme activity, or ROS production). The phenomenon of Se action on plants may be associated with reduced Pb accumulation in shoots and a positive effect of Se on necrotic changes in Pb-exposed leaves. The mechanism of the beneficial effect of Se on antioxidant capacity may be direct, via the antioxidant activity of Se compounds, or indirect, via Se-induced upregulation of general stress resistance mechanisms (Pilon-Smits and others 2009). It is assumed that some oxygen radicals may be removed in a non-enzymatic way in the presence of Se (Hartikainen and others 2000). The beneficial influence of Se on Pb-exposed shoots was related to an increase in the assimilation pigment concentration, which suggests that Se addition to a Pb-containing solution can help maintain the integrity of cell membranes in plastids (Hawrylak-Nowak and others 2014). Nevertheless, the effects described above show that different processes have different sensitivities to the presence of Se and its ambivalent character (protection or toxicity) results in beneficial or detrimental effects on plants. The influence of Se supplementation on Pb-treated field bean shoots is dependent on the proportion of these elements in the nutrient solution and the positive effect of Se was more pronounced for the lower Se dose. It seemed that lack of P for 2 weeks did not significantly modify the plant response to Se and/or Pb.

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