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ORIGINAL ARTICLE

In vitro acclimation to prolonged metallic stress is associated with modulation of antioxidant responses in a woody shrub *Daphne jasminea*

Alina Wiszniewska[1] · Ewa Muszyńska[2] · Anna Kołton[1] · Iwona Kamińska[1] · Ewa Hanus-Fajerska[1]

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Abstract

The study aimed at distinguishing antioxidant responses conditioning acclimation status to metallic stress in a shoot culture of a woody plant *Daphne jasminea*. Growth, organogenesis, metal accumulation, oxidative stress level and antioxidant responses were compared in the long-term treated shoots (Lt lines) and short-term treated (St lines) with Cd, Ni and Pb in relation to control shoots. Growth rate and organogenic potential were higher in the long-term treated Lt Cd and Lt Pb lines than in respective short-term treated St lines. Lt Ni line proliferated less effectively than St Ni line, whereas biomass accretion was similar in both Ni lines. All heavy metal (HM)—exposed shoots suffered from oxidative stress, however, it was more severe in St than in Lt lines. In the long-term treated lines three major factors were associated with the acclimation status: activity of peroxidase (POD) and accumulation of anthocyanins and carotenoids. POD activity was 4–1.5-times higher in Lt lines than in the control one. Anthocyanin content nearly tripled, while carotenoid content doubled in Lt lines. Other elements of antioxidant system, CAT and SOD, glutathione, ascorbic acid and proline, complemented the action of the "major players" in long-term treated Lt lines depending on heavy metal type. Short-term treated lines responded by accumulation of proteins and a decline in catalase activity. The study demonstrated that long-term heavy metal treatment can be considered acclimation strategy, during which substantial modulation of the antioxidant system occurs. Due to orchestrated action of cytosolic POD, vacuolar anthocyanins and membrane-bound carotenoids HM toxicity could be counteracted in various cellular compartments.

Key message

Acclimation to heavy metal exposure in *D. jasminea* shoot cultures involves modulation of antioxidant activity and is conditioned by an orchestrated action of cellular peroxidases, vacuolar anthocyanins and membrane-bound carotenoids.

Keywords Acclimation · Heavy metals · Peroxidase · Pigments · Selection · Tolerance

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✉ Alina Wiszniewska
a.wiszniewska@urk.edu.pl

[1] Unit of Botany and Plant Physiology, Institute of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Kraków, Al. 29 Listopada 54, 31-425 Kraków, Poland

[2] Department of Botany, Faculty of Agriculture and Biology, Warsaw University of Life Sciences (SGGW), Nowoursynowska 159, Building 37, 02-776 Warsaw, Poland

Introduction

Heavy metal (HM) contamination of the environment is gradually increasing due to natural processes and anthropogenic activities (Wu et al. 2015; Tóth et al. 2016; Mazurek et al. 2017). Toxic elements abundant in the soil, atmosphere and water seriously affect homeostasis of ecosystems by exerting negative effects on microbiota, animals, and flora. Plants, as immobile organisms, are unable to escape from contaminated areas and are forced to adjust their metabolic performance to counteract toxicity of excessive metals and metalloids. Deleterious effects of heavy metals include disturbances in the function and structure of macromolecules,

especially enzymes, components of cell membranes and DNA (Amari et al. 2017; Hasan et al. 2017). These direct events have negative consequences at the whole plant level and lead to water and nutrient element imbalances, disruption of photosynthesis and overproduction of radicals that causes oxidative stress (Sytar et al. 2013; Amari et al. 2017). Plants subjected to toxic metals need to activate defense machinery and adjust their metabolic responses, particularly the activity and concentration of antioxidant system elements, as well as transport and accumulation of toxic ions (Shahid et al. 2014; Viehweger 2014; Khan et al. 2018). As a result of metabolism remodeling, HM can be either stabilized in the rhizosphere or accumulated in aboveground organs, in various cellular compartments, contributing to plant tolerance towards their toxicity (Vrbová et al. 2013; Rodrigues et al. 2017).

Among numerous plant species, isolated populations have adapted to settle in metalliferous soils and at constant presence of elevated levels of metals (Muszyńska et al. 2013; Arencibia et al. 2016; Bothe and Słomka 2017). Some other species are capable of hyperaccumulating HM ions taken up from the soil. Such ecotypes and species can be a valuable source of tolerance traits in crop breeding as well as a unique material to be exploited in phytoremediation techniques of soil clean-up. Limitations of large-scale use of natural plant populations include their low biomass accretion and slow growth (Muszyńska et al. 2013). Employing heavy metal tolerant trees and shrubs improves phytoremediation of industrial areas, due to enhanced HM accumulation and high biomass production, as well as revitalization of polluted urban areas, where woody ornamentals can be planted (Prasad 2012). However, studies on the mechanisms of HM tolerance in woody ornamentals are still infrequent. Although our knowledge on such mechanisms in herbaceous plants is already vast, it is suggested that the reactions can be more complex in woody species (Luo et al. 2016).

Biotechnological tools, such as genetic transformation and in vitro selection, may facilitate and intensify obtaining HM-tolerant genotypes, lines, cultivars and species, including woody ornamentals (Gantait et al. 2019). In recent years enhanced plant tolerance to heavy metals had been achieved by introduction of transgenes encoding proteins involved in HM detoxification, like metallothioneins (Vrbová et al. 2013) and phytochelatin synthase (Chen et al. 2015), but also proteins regulating stress responses, like members of HSP (*heat shock proteins*) family (Cui et al. 2013) or transcription factors DREB (*drought—responsive element binding*) (Charfeddine et al. 2017). In vitro selection enables not only production of tolerant plantlets but also investigation of the mechanisms of tolerance acquisition in the presence of toxic selecting agents, exerting constant stress pressure, on various levels of organization (Ashrafzadeh and Leung 2015; Bernabé-Antonio et al. 2015; Alfaro-Saldaña et al. 2016; Malik et al. 2016). In recent years the benefits of in vitro culture systems facilitated investigations of the effects exerted by most ubiquitous heavy metals, including Cd, Ni and Pb, on numerous plant species belonging to crops, ornamentals, medicinal plants and extremophytes (Fourati et al. 2019; Milusheva et al. 2019; Charfeddine et al. 2017; Muszyńska et al. 2018a, b).

Reactions associated with tolerance and sensitivity traits can be distinguished via comparative studies on species/genotypes/lines exhibiting different level of tolerance to stress factors (Arencibia et al. 2016; Ferrer et al. 2018; Wei et al. 2018; Koźmińska et al. 2019). Although attempts have been made to elucidate HM tolerance mechanisms using this approach, there is limited information on the efficiency of plant acclimation to HM stress using prolonged, constant exposure to these ions.

In our previous studies we analyzed various aspects of HM response in *Daphne jasminea* Sibth. & Sm. (Thymelaeaceae), a woody shrub of ornamental value (Wiszniewska et al. 2015, 2017a, b, 2018). By prolonged in vitro selection we obtained three shoot culture lines tolerant to Cd, Ni and Pb, respectively. For over 2 years tolerant shoots propagated and spontaneously rooted during constant exposure to HM. The experiment described here involved microshoots from these tolerant lines and microshoots from stock cultures, not exposed previously to HM. We compared growth and several metabolic responses related to antioxidant system functioning, as well as histological distribution of some of its components in plants subjected to long-term (Lt lines) and short-term (St lines) HM treatment. We hypothesized that prolonged treatment with HM ions consistently enhances antioxidant responses of cultured shoots and thus increases their tolerance to metallic stress. Therefore the aim of the study was to compare the effectiveness of antioxidant machinery in *D. jasminea* under both long-term and short-term exposure to three heavy metals: Cd, Ni and Pb, and to distinguish antioxidant responses related to HM acclimation status. We also aimed at evaluating the role of antioxidant machinery in tolerance induction during prolonged HM treatment in this woody species.

Materials and methods

Plant material

Plant material constituted shoot culture lines of *Daphne jasminea* Sibth. & Sm. (Thymelaeaceae). Shoot cultures were multiplicated on optimized basal medium composed of WPM medium salts (Lloyd and McCown 1980) and MS vitamins (Murashige and Skoog 1962), supplemented with growth regulators: 12.3 µM N6-[2-isopentyl]adenine (2iP), 5.37 µM 1-naphthaleneacetic acid (NAA), as well as several

organic compounds: 0.5 g L^{-1} polyvinylpyrrolidone (PVP), 0.5 g L^{-1} 2-N-morpholino-ethanesulfonic acid (MES), 0.6 g L^{-1} activated charcoal, 0.65 g L^{-1} calcium gluconate, and 20.0 g L^{-1} sucrose. Medium pH was adjusted to 5.6 prior to solidification with 0.8% Difco agar (Wiszniewska et al. 2015).Three HM-tolerant lines have been obtained by vitro selection and propagated for over 2 years (over 102 weeks), with regular subcultures (every 8 weeks) to fresh basal medium supplemented with heavy metal ions: (1) Lt Cd line, maintained on the medium containing 5 µM $CdCl_2$, (2) Lt Ni line, maintained on the medium containing 0.05 mM $NiSO_4$, and (3) Lt Pb line, maintained on the medium containing 1.0 mM $Pb(NO_3)_2$. These sub-optimal, but not lethal concentrations of heavy metal salts were chosen during selection experiments accompanying our previous studies on *D. jasminea* (Wiszniewska et al. 2015, 2017a, b, 2018). Shoots of control cultures (C line) were propagated on the same medium without heavy metals. Cultures were maintained in a growth chamber at 22 °C, under 16 h photoperiod (irradiance 80 µmol m^{-2} s^{-1}).

Experimental scheme

In the experimental period, *D. jasminea* microcuttings were treated with heavy metals for 10 weeks. Cultures were established in 250 mL Erlenmeyer flasks, using ten explants per flask. An explant was 5-mm long apical part of the shoot. Explants from long-term treated cultures (Lt Cd, Lt Ni and Lt Pb) were put on fresh media containing respective heavy metal salts. Short-term treated cultures (St Cd, St Ni and St Pb) were established by explanting shoots from the control line onto fresh media supplemented with the same concentrations of heavy metal salts, as in the case of Lt cultures. As a control treatment in this experiment (C line), explants were taken from the control line and transferred onto fresh medium without HM.

After 10 weeks of culture data on growth and biochemical responses were collected. Growth performance was evaluated on the basis of number of produced new adventitious shoots per explant, fresh and dry weight (FW and DW, respectively) of shoots and roots, and percentage of adventitious rooting.

Determination of oxidative stress indicators

Histochemical detection of hydrogen peroxide

Detection of H_2O_2 was performed according to Daudi and O'Brien (2012). Whole microplantlets were placed in a plastic tubes and immersed with 1 mg mL^{-1} DAB (Sigma-Aldrich). Samples were gently vacuum infiltrated for 30 min, and incubated in the dark overnight. After incubation, samples were rinsed with sterile water. Microplantlets were cleared with bleaching solution (ethanol:acetic acid:glycerol = 3:1:1) in a water bath in 95 °C prior examination. Dark brown precipitate was formed in the reaction of DAB with H_2O_2.

Oxidative stress indicators

Malondialdehyde (MDA) content was detected using thiobarbituric acid-reactive substances assay (Du and Bramlage 1992). Concentration of malondialdehyde (MDA) in plant tissue was calculated with the absorbance of solution at 440, 532 and 600 nm and molar extinction coefficient of MDA (157,000). Extraction was conducted with 80% ethanol. Samples with trichloroacetic acid and thiobarbituric acid were incubated in 95 °C for 25 min and cooled on ice.

GSH/GSSG and ASC/DHA ratio—the proportions of reduced and oxidized forms of glutathione and ascorbic acid were calculated on the basis of determined concentrations according to the method of Queval and Noctor (2007) described below.

Determination of non-enzymatic antioxidants and other stress-related compounds

Non-enzymatic antioxidants

Reduced and oxidized glutathione Glutathione pool was measured by the assay based on GR-dependent reduction of DTNB 5,5-dithiobis(2-nitro-benzoic acid) (Queval and Noctor 2007). Extraction was conducted at 4 °C using 0.2 N HCl (extraction medium/FW ratio of 1 mL/100 mg). Samples were centrifuged at 16,000×*g* for 10 min at 4 °C. 0.5 mL aliquots of the supernatant were neutralized with 0.5 M NaOH in the presence of 50 µL of 0.2 M NaH_2PO_4 (pH 5.6) to reach pH between 5 and 6. The method allows to measure total glutathione pool (reduced plus oxidized form) and after pre-treatment of the extract aliquots with 2-vinylpyridine (VPD) only GSSG. To measure total glutathione, aliquots of 30 µL of neutralized extracts were added to 300 µL 0.2 M NaH_2PO_4 (pH 7.5), 30 µL of 10 mM EDTA, 30 µL of 10 mM NADPH, 30 µL of 12 mM DTNB, and 180 µL of distilled water. The reaction was started by the addition of 30 µL of GR (20 U mL^{-1}) and the increase in the absorbance at 412 nm was monitored for 2 min. GSSG fraction was measured in the same routine after incubation of 200 µL of neutralized extract with 3 µL VPD for 30 min at room temperature to complex GSH. Calculations were made on the basis of standard curves plotted simultaneously for GSH and GSSG.

Ascorbate Total ascorbate and the pool of reduced ascorbate (ASC) was determined according to Queval and Noctor (2007). Extraction and neutralization were performed as for

glutathione determination. To assay ASC, 60 µL of neutralized supernatant was mixed with 300 µL of 0.2 M NaH_2PO_4 (pH 5.6) and 225 µL of distilled water and absorbance at 265 nm was read. Then, 15 µL of AO (40 U mL^{-1}) was added to the reaction mixture, samples were shaken and absorbance at 265 nm was read again (1 min after enzyme adding). To assay total ascorbate, 100 µL of neutralized supernatant was added to 140 µL 0.12 M NaH_2PO_4 (pH 7.5) and 10 µL 25 mM DTT, and solutions were incubated for 30 min at room temperature. After incubation absorbance measurements were performed as described for ASC. For calculations standard curves were made with the use of ascorbic acid solutions.

Carotenoids Carotenoids were extracted from plant tissue with 80% acetone. After centrifugation the absorbance of solution was measured with spectrophotometer (Hitachi U-2900, Japan). Results were calculated according to Wellburn equations (Wellburn 1994).

Phenolic compounds Total phenolics, phenylpropanoids, flavonols and anthocyanins were determined using UV/VIS spectrophotometry (Fukumoto and Mazza 2000). Chlorogenic acid (CGA), caffeic acid (CA) and quercetin (QC) were used as standards for total phenolic content (TPC), phenylpropanoids and flavonols, respectively. Anthocyanin content was expressed as the cyanidin (CY), according to its molar extinction. Plant tissue was ground with 80% methanol and centrifuged for 15 min at 4500×*g*. Supernatant was mixed with 0.1% HCl (in 96% ethanol) and 2% HCl (in water) and after 15 min the absorbances at 280, 320, 360 and 520 nm were read (Hitachi U-2900 spectrophotometer). The content of determined compounds was expressed in mg of respective standard equivalents per 100 g FW.

Other stress-related compounds

Proline Plant material was homogenized with 3% aqueous sulfosalicylic acid. After centrifugation an aliquot was mixed with acid ninhydrine and glacial acetic acid and reaction was conducted for 1 h at 100 °C. Reaction was terminated on ice and cold solution was mixed with toluene. Absorbance of toluene phase was read with spectrophotometer (Hitachi U-2900, Japan) at 520 nm according to Bates et al. (1973). Standard curve with proline was prepared.

Total protein content Protein content in plant material was detected with Bradford reagent (Bradford 1976). Standard curve with bovine albumin was prepared.

Activity assays of antioxidant enzymes

Peroxidase (POD)

Activity of peroxidases was expressed as an increase of absorbance of *p*-phenylenediamine oxidized to phenazine by enzyme from plant tissue (Lück 1962). Plant material was homogenized with potassium phosphate buffer (0.05 M, pH 6.2). In reaction mixture were enzyme extract, hydrogen peroxide and *p*-phenylenediamine. The absorbance was read after 1 and 2 min from H_2O_2 addition at 485 nm. One unite (U) of enzyme activity was expressed as the increase of absorbance by 0.1 for 1 min.

Catalase (CAT)

Activity of catalase was expressed as a decrease of H_2O_2 absorbance which was disintegrated by the enzyme from plant tissue (Aebi 1984). Plant material were homogenized with potassium phosphate buffer (0.05 M, pH 7.0). The reaction mixture consisted of the enzyme extract and hydrogen peroxide. The absorbance was read after 1 and 2 min from H_2O_2 addition at 240 nm. Decrease in absorbance by 0.0145 corresponds to decomposition of 1 µmol of H_2O_2.

Superoxide dismutase (SOD)

Total SOD activity was determined as inhibition of the reduction of nitroblue tetrazolium (Hwang et al. 1999). Plant material was homogenized with potassium phosphate buffer (0.05 M, pH 7.5). In reaction mixture were enzyme extract, methionine, nitroblue tetrazolium and riboflavin. Solution was incubated at light condition (two 18 W fluorescent bulbs) at room temperature. The absorbance was measured at 560 nm after 5 and 10 min after starting the reaction. Sample without enzyme was prepared as a control where reaction efficiency reached 100%. One unit of enzyme activity was defined as the 50% inhibition of reaction.

Histochemical detection of CAT and POD activity

Leaf (approx. 3×3 mm^2) and root (approx. 3 mm long) samples were fixed for 2 h in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature under a −0.04 MPa vacuum. After fixation, samples were rinsed 4 times for 15 min in the same buffer. The visualization of the peroxisomal CAT activity was performed according to the protocol described by Frederick and Newcomb (1969). Briefly, samples were pre-incubated for 30 min in 0.05 M Tris–HCl buffer (pH 9.0) at 37 °C and incubated for 1 h at 37 °C in medium consisting of 0.05 M Tris–HCl buffer (pH 9.0), 2 mg mL^{-1}

3,3′-diaminobenzidine (DAB) and 0.06% (v/v) H_2O_2. Afterwards, all samples were washed 4 times in 0.05 M K/Na-phosphate buffer (pH 6.8) for 15 min each. The incubation in the standard DAB solution without H_2O_2 was used in control reaction to confirm the specificity of CAT reaction.

For POD activity localization, the pre-incubation of samples in 0.05 M Tris–HCl buffer (pH 7.6) with the addition of 0.02 M 3-amino-1,2,4-triazole as CAT inhibitor was performed for 30 min at 37 °C. Then, incubation in solution consisting of 0.05 M Tris–HCl (pH 7.6), 0.5 mg mL^{-1} DAB, 0.01% (v/v) H_2O_2, 0.02 M 3-amino-1,2,4-triazole was run for 1 h at room temperature. As a control reaction, confirming POD specificity, samples were incubated in DAB medium without H_2O_2. Afterwards, all samples were washed four times in 0.05 M K/Na-phosphate buffer (pH 6.8) for 15 min each, as described above. Subsequent steps of histochemical detection were the same for both CAT and POD localization. Samples were post-fixed in 1% (v/v) osmium tetroxide for 2 h at 4 °C, dehydrated in an ethanol series and substituted by propylene oxide. Then they were embedded in epoxy resin and polymerized at 60 °C for 24 h, according to the manufacturer's procedure.

Semi-thin sections were prepared with Jung RM 2065 microtome, and specimens were examined without any staining under a light microscope (Olympus-Provis, Japan).

Heavy metal accumulation

Dry plant samples were treated with a mixture of nitric acid and perchloric acid (3:1 v/v). The content of Cd, Ni and Pb was determined using inductively coupled plasma optical emission spectrometry (ICP–OES) on Optima 7300 DV spectrometer (Perkin Elmer) in respective metal treated shoots.

Statistical analyses

The experiment was conducted three times in three replicates, with at least 30 explants/microcuttings (6 flasks) per treatment within one replicate. Statistical analyses were performed using STATISTICA 13.0 software (StatSoft, Tulsa, OK, USA). Regarding data collected during biochemical analyses, for each heavy metal means were compared between C and Lt or St line using t-Student's test. Regarding data on growth parameters and heavy metal accumulation, means of respective Lt and St lines were compared using t-Student's test. Biochemical parameters were also correlated using multidimensional analyses: Principal Component Analysis (PCA)—to distinguish the most impactful factors differentiating responses between respective Lt and St lines, whereas Cluster Analysis for grouping of studied lines.

Results

Growth responses

Shoot regeneration rate amounted to 100% in all Pb- and Cd-treated cultures, while in Ni-treated cultures it was slightly lower, reaching 92 and 95% in St Ni and Lt Ni, respectively (data not shown). Growth performance and adventitious organogenesis manifested by the number of new shoots, their fresh biomass, rooting percentage and root dry biomass were superior in Lt Cd and Lt Pb lines in comparison with respective St lines (Table 1). Lt Cd produced nearly 7 new shoots per explant, while St Cd—4.4 ($P < 0.05$). Fresh weights of shoots and roots were over 1.5 times higher in Lt Cd than in St Cd line, while rooting rate—2.5 times. Number of new shoots in Lt Pb amounted to 6.5, whereas in St Pb—4.2 ($P < 0.05$). Fresh biomass of developed shoots was twofold higher in Lt Pb line than in St Pb, while fresh biomass of roots and rooting percentage—over 7 times higher. Dry

Table 1 Growth and organogenic response of *D. jasminea* long-term treated (Lt) and short-term treated (St) lines

Heavy metal dose	Line	No. of adventitious shoots/explant	Shoot FW (mg)	Shoot DW (% FW)	Adventitious rooting (%)	Root FW (mg)	Root DW (% FW)
5.0 µM $CdCl_2$	St Cd	4.4 ± 0.4	100 ± 20	17.5 ± 0.9	14.2 ± 1.9	45 ± 11	2.2 ± 0.4
	Lt Cd	6.9 ± 0.5*	173 ± 20*	16.1 ± 2.1	36.2 ± 2.1*	73 ± 9*	5.5 ± 0.4*
0.05 mM $NiSO_4$	St Ni	6.1 ± 0.7	167 ± 60	18.1 ± 1.4	23.8 ± 1.7	30 ± 3	10.1 ± 0.6
	Lt Ni	5.5 ± 0.6	144 ± 20	16.6 ± 0.3	24.5 ± 2.4	24 ± 6	8.2 ± 1.7
1.0 mM $Pb(NO_3)_2$	St Pb	4.2 ± 0.3	74 ± 10	19.9 ± 0.7	4.7 ± 1.5	12 ± 8	3.4 ± 0.4
	Lt Pb	6.5 ± 0.4*	152 ± 40*	17.5 ± 1.6	33.6 ± 3.2*	88 ± 10*	8.4 ± 1.5*

Values are means of three replicates ± SE.

Means indicated by the asterisk (*) between St and Lt lines of respective metal treatment significantly differ at $P < 0.05$ according to t-Student's test

biomass of shoots did not differ between the lines (Table 1). Under Ni treatment, growth parameters were the same for Lt and St line ($P > 0.05$) (Table 1). Number of new shoots reached 5.5–6.1 ($P > 0.05$), while adventitious rooting rate amounted to 24%.

Oxidative stress indicators and antioxidant responses

Accumulation of H_2O_2 in developed microplantlets was confirmed by histochemical staining.

In all heavy metal treatments, microplantlets from St lines showed more intensive color in H_2O_2 assays than microplantlets from respective Lt lines (Fig. 1). Slight brown coloration in the control shoots was restricted to the basal part of the microplantlet (Supplementary Material 1).

Biochemical responses of *D. jasminea* shoots depended on treatments. Both long-term treated and short-term treated lines exhibited a number of similar trends (decline, increase or stability) in relation to the control line (Fig. 2). Since these uniform reactions were independent of HM-treatment duration, we summarized them in detail in the Supplementary Material 2. Following the aim of this study, we focused on those responses that varied between the Lt lines and St lines.

Among oxidative stress indicators, MDA content, reflecting the level of lipid peroxidation, increased in all HM-treated shoots (Fig. 2), although to a lesser extent in Lt lines (Supplementary Material 2). Oxidation status differed between the lines treated with various HMs but the response type was generally the same in respective Lt and St lines. As compared with the control, ASC/DHA ratio did not change under HM exposure. Contrary to that, GSH/GSSG ratio increased under Ni treatment, and did not change in the presence of Pb (Fig. 2). Cd treatment was the only case when Lt and St lines exhibited disparate oxidation status (see below).

Cadmium treatment

Cadmium accumulation

Cadmium accumulation in the shoots of both lines was comparable, reaching 14.7 and 12.2 µg g^{-1} DW ($P > 0.05$) in Lt Cd and St Cd, respectively (Table 2).

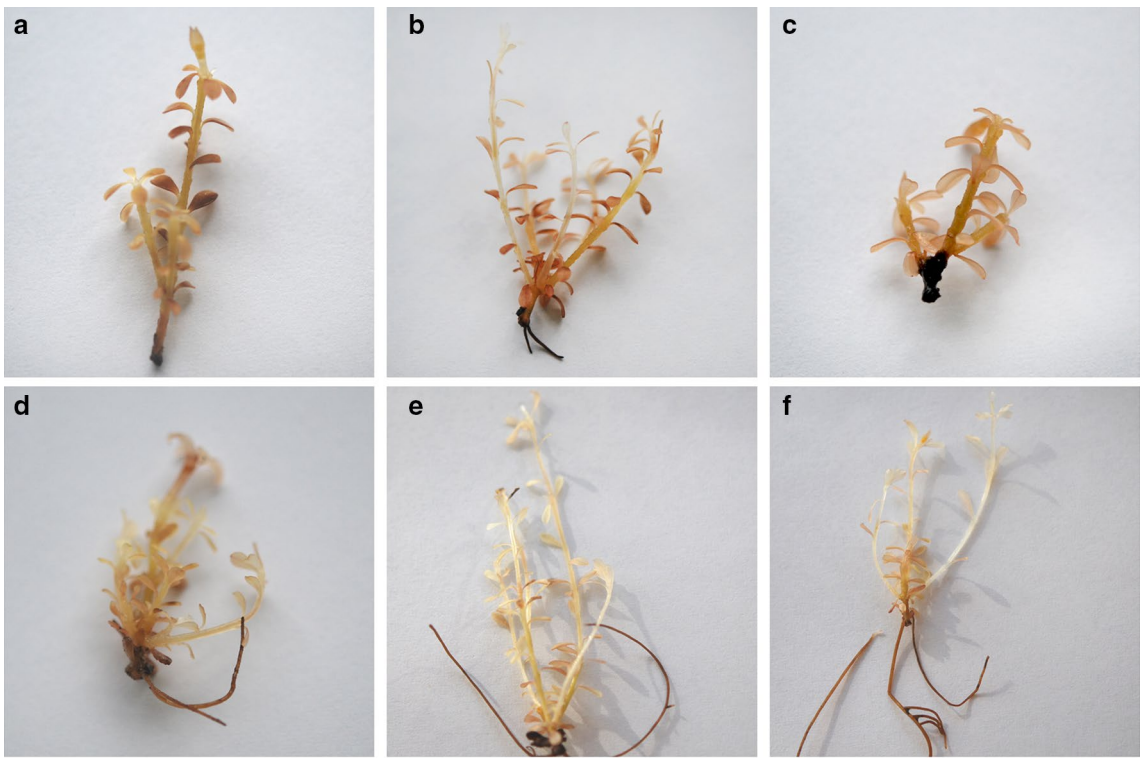


Fig. 1 Accumulation of hydrogen peroxide in *D. jasminea* microplantlets treated with heavy metals. **a**–**c** short-term treated (St) lines: **a** St Cd, **b** St Ni, **c** St Pb; **d**–**f** long-term treated (Lt) lines: **d** Lt Cd, **e** Lt Ni, **f** Lt Pb

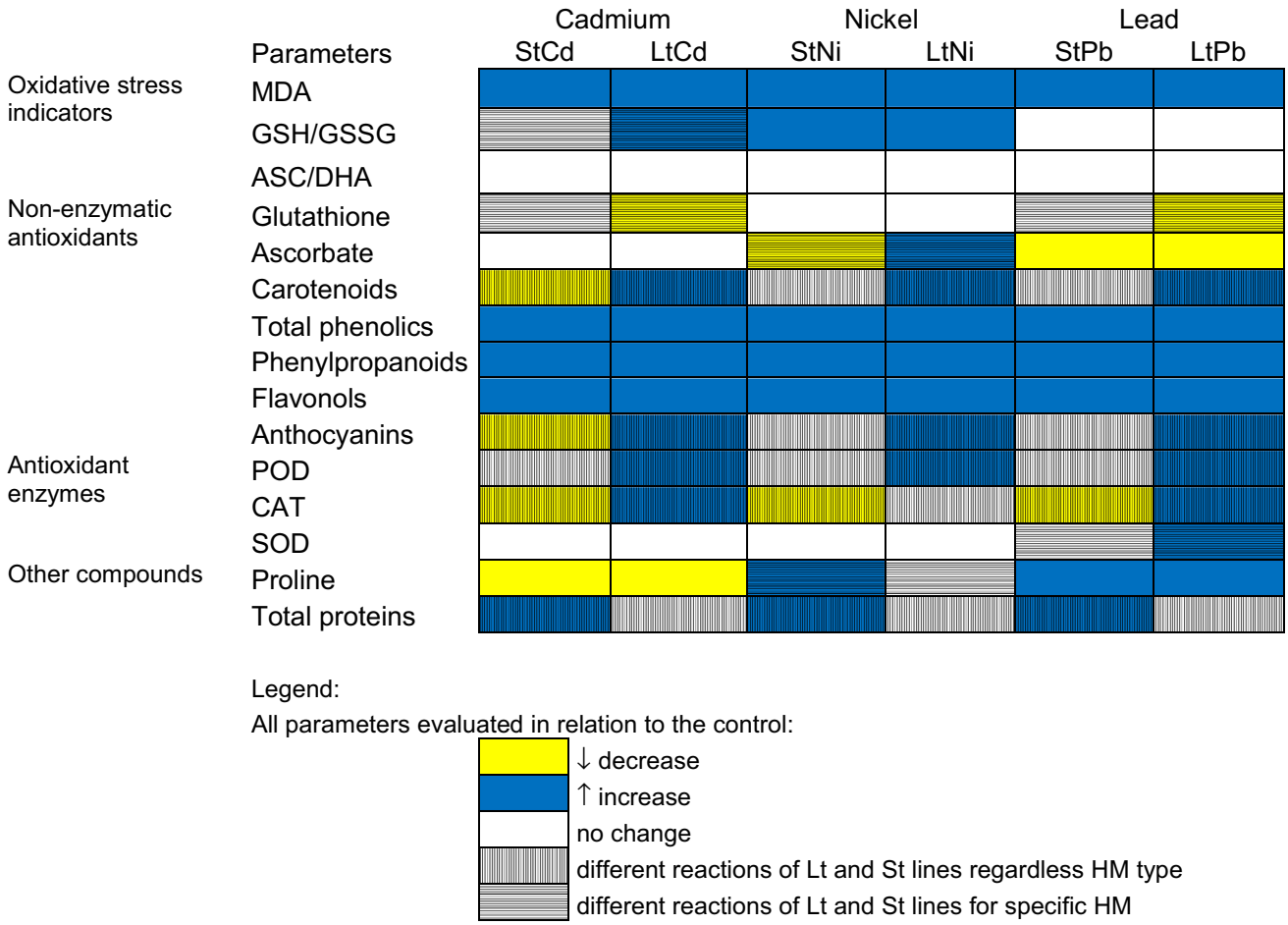


Fig. 2 Heat map visualizing biochemical responses of heavy metal-treated shoot lines of *D. jasminea* versus the responses of the control shoots. *St* short-term treated lines; *Lt* long-term treated lines

Table 2 Heavy metal accumulation in *D. jasminea* long-term treated (Lt) and short-term treated (St) lines exposed to heavy metals

Element	Heavy metal accumulation (μg g^{-1} DW)	
	St line	Lt line
Cd	12.2 ± 2.3	14.7 ± 1.8
Ni	49.8 ± 1.1	50.0 ± 2.6
Pb	194.2 ± 3.7*	49.5 ± 1.3

Values are means of three replications ± SE

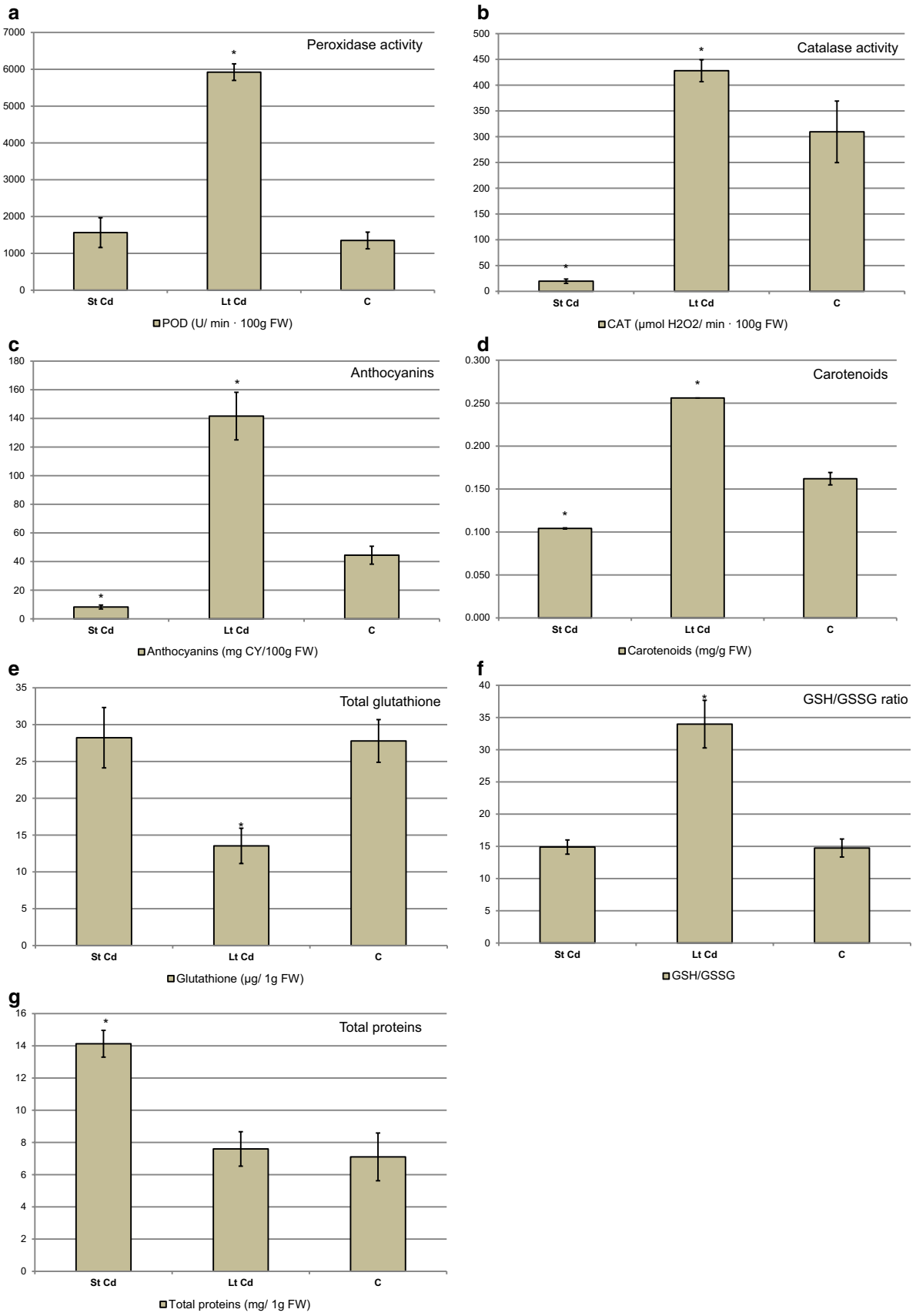
Means indicated by the asterisk (*) between St and Lt lines of respective metal treatment significantly differ at $P < 0.05$ according to t-Student's test

Antioxidant responses distinct in Lt and St Cd lines

Differential responses included primarily the activity of two antioxidant enzymes, POD and CAT. POD activity in Lt Cd line was over four times higher than in the control line. In St Cd line it remained unchanged and did not differ from the control (Fig. 3a). Also CAT activity increased significantly in Lt Cd line (1.5-fold), and decreased significantly in St Cd line (Fig. 3b).

Differences between the lines included also the content of non-enzymatic antioxidants. In Lt Cd line, the concentration of anthocyanins and carotenoids increased by 220% and 58%, respectively, and declined in St Cd line by 82% versus the control (Fig. 3c, d). In contrary, the content of total glutathione in St Cd line was the same as in the control line, while in Lt Cd line it dropped to 48% of the control content (Fig. 3e). Despite that, GSH/GSSG ratio rose in this line due to a low content of oxidized form of glutathione (Fig. 3f).

Total protein content increased in St Cd line, while in Lt Cd it did not change in comparison with the control line (Fig. 3g).


a
Peroxidase activity
7000
6000
5000
4000
3000
2000
1000
0
St Cd
Lt Cd
C
POD (U/ min · 100g FW)
b
Catalase activity
500
450
400
350
300
250
200
150
100
50
0
St Cd
Lt Cd
C
CAT (µmol H2O2/ min · 100g FW)
c
Anthocyanins
180
160
140
120
100
80
60
40
20
0
St Cd
Lt Cd
C
Anthocyanins (mg CY/100g FW)
d
Carotenoids
0.300
0.250
0.200
0.150
0.100
0.050
0.000
St Cd
Lt Cd
C
Carotenoids (mg/g FW)
e
Total glutathione
35
30
25
20
15
10
5
0
St Cd
Lt Cd
C
Glutathione (µg/ 1g FW)
f
GSH/GSSG ratio
40
35
30
25
20
15
10
5
0
St Cd
Lt Cd
C
GSH/GSSG
g
Total proteins
16
14
12
10
8
6
4
2
0
St Cd
Lt Cd
C
Total proteins (mg/ 1g FW)

◂**Fig. 3** Biochemical responses of *D. jasminea* to cadmium treatment different in long-term treated (Lt) and short-term treated (St) lines. **a** peroxidase activity; **b** catalase activity; **c** anthocyanins; **d** carotenoids; **e** total glutathione; **f** GSH/GSSG ratio; **g** total protein content. Values are mean ± SE. Means indicated by asterisk (*) differ from the control treatment according to t-Student's test at $P \leq 0.05$

Histochemical localization of CAT and POD activity in Cd-lines

In Cd-treated leaves CAT activity was detected in epidermal cells, with more intense coloration in Lt line. In this line, the enzyme activity was visible mostly in lower epidermis (Fig. 4a), while in St line in the epidermis of both sides of the leaf (Fig. 4b). In contrast to St Cd line, in Lt Cd one CAT activity was also visualized inside the palisade mesophyll cells and vascular bundle.

The areas of POD activity in the leaves of Cd lines were similar to those exhibiting CAT activity. Thus, POD was visualized in lower epidermis of Lt Cd line (Fig. 4c) and in both epidermal layers of St Cd line (Fig. 4d). Regardless of the line, POD activity appeared in the phloem (Fig. 4c, d). Localization of the enzymes in the control leaves is presented in Supplementary Material 3.

Nickel treatment

Nickel accumulation

The level of Ni accumulation was the same in both Lt and St lines, and reached 50 µg g^{-1} DW (Table 2).

Antioxidant responses distinct in Lt and St Ni lines

Reactions that diversified both Ni-exposed lines included primarily the activity of POD and CAT. In Lt Ni line POD activity increased by 1.5 times over control but did not change in St Ni line (Fig. 5a). CAT activity in Lt Ni line was as high as in the control, while it declined to 15% of the control level St Ni line (Fig. 5b).

Considering non-enzymatic antioxidants, the content of anthocyanins, carotenoids and ascorbate increased by 215%, 47%, and 12%, respectively in Lt Ni line. In St Ni line, the levels of anthocyanins and carotenoids remained unchanged (Fig. 5c, d), while ascorbate content dropped by 14% (Fig. 5e).

The content of other stress-related compounds, i.e. proline and total protein did not differ from the control in Lt Ni line, and surged in St Ni by 366% and 142% vs. control, respectively (Fig. 5f, g).

Histochemical localization of CAT and POD activity in Ni-lines

Localization of CAT activity in Ni-treated leaves differed between the lines. In Lt Ni leaves the enzyme was active only in the phloem (Fig. 6a), while in St Ni line brown coloration appeared in the vascular bundle, in the mesophyll and in the upper and lower epidermis (Fig. 6b).

As for POD activity in Ni-treated leaves, more intense brown coloration was observed in Lt Ni line than in St Ni one. In the leaves of Lt Ni line POD activity was visualized mainly in the epidermis of both leaf sides (Fig. 6c), while in St Ni leaves POD was localized in the phloem (Fig. 6d).

Lead treatment

Lead accumulation

In St Pb shoots Pb accumulation was nearly four times higher than in Lt Pb line. Lead concentrations amounted to 194.2 and 49.5 µg g^{-1} DW in St Pb and Lt Pb line, respectively (Table 2).

Antioxidant responses distinct in Lt and St Pb lines

As in the case of Cd and Ni treatments, differences between Lt Pb and St Pb lines were manifested mainly in the activity of antioxidant enzymes. In Lt Pb line POD activity was 2.7 times higher, while in St Pb one it was comparable with the control line (Fig. 7a). Activity of CAT increased 2.6 times in Lt Pb line, and declined to just 14% of the control in St Pb line (Fig. 7b).

Pb-treated lines differed in their activity of SOD. In Lt Pb line it increased slightly but significantly, and amounted to 117% of the control value ($P < 0.05$). In St Pb line, SOD activity was the same as in the control line (Fig. 7c).

The levels of non-enzymatic antioxidants (anthocyanins, carotenoids and total glutathione) in St Pb line did not differ from the control (Fig. 7d–f). In Lt Pb line anthocyanin and carotenoid contents increased by 227% and 58%, respectively, while total glutathione content declined by 52% in comparison with the control (Fig. 7d–f).

Among other stress-related compounds, total protein content increased in St Pb line by 108%, whereas in Lt Pb line it remained the same as in the control line (Fig. 7g).

Histochemical localization of POD and CAT activity in Pb-lines

CAT in the leaves of both Pb-treated lines was active in every cell type, and no differences appeared between the lines (Fig. 8a, b).

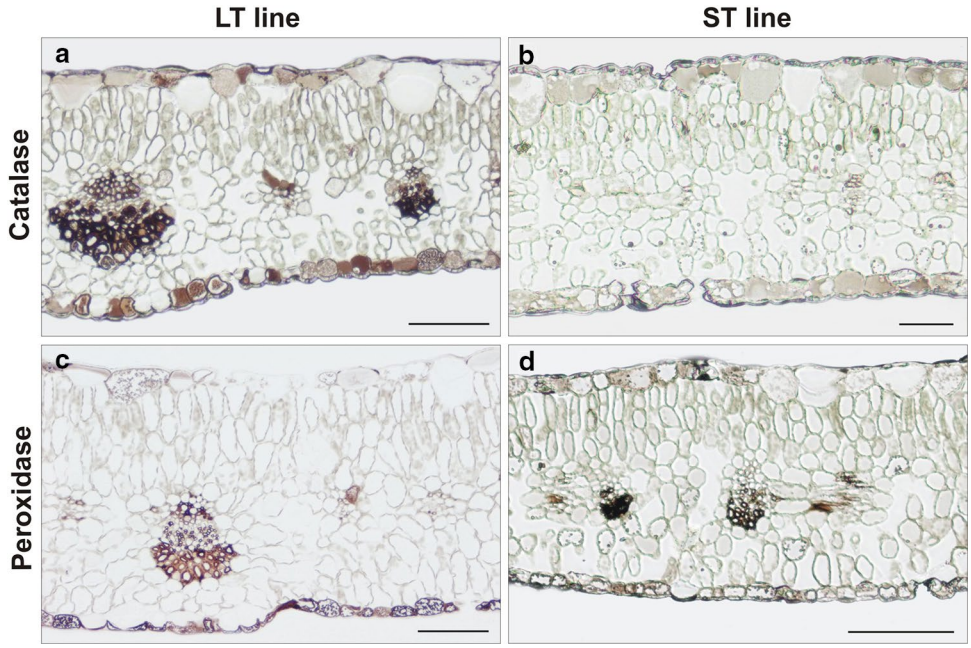


Fig. 4 Histochemical localization of catalase (CAT) and peroxidase (POD) activity in the leaves of *D. jasminea* lines long-term treated (Lt) and short-term treated (St) with cadmium. **a**, **b** CAT; **c**, **d** POD. Bar 100 µm

Considering POD activity, in Lt Pb leaves it was visualised mostly in both epidermal layers (Fig. 8c), while in St Pb leaves it was detected only inside the palisade parenchyma cells (Fig. 8d).

Multidimensional statistical analyses

PCA was performed for each HM treatment separately. Generally, for each HM, two components of variance were distinguished. The first component (for each HM studied) was identified as the one that included responses on HM application itself, irrespective of its duration. The second component was identified as gathering responses differentiating long-term treated (Lt) from short-term treated (St) lines. The variables strongly correlating with the second components were associated with different responses of the studied lines.

In Cd lines, the second component (explaining 44.59% of variance) positively correlated with the activities of POD and CAT, and also the contents of carotenoids and anthocyanins (Supplementary Material 4). Total glutathione content negatively correlated with the second component. In Lt Ni and St Ni lines, the second component (explaining 40.61% of variance) positively correlated with POD activity, as well as the content of anthocyanins, carotenoids and ascorbate. Negative correlation of proline content with the second component was also determined (Supplementary Material 4).

For Pb treatment, differences between the lines significantly correlating with the second component (explaining 40.20% of variance) included the activity of three enzymes: POD, CAT and SOD. The contents of carotenoids and anthocyanins also showed a strong correlation with the second component (Supplementary Material 4).

Cluster analysis using k-mean method assumed that k equaled 2 (reflecting two distinct lines). The first cluster included all Lt lines, and the second cluster encompassed St lines and the control line. We evaluated the influence of each variable on classification of the lines and found that the activity of POD and CAT and the concentration of anthocyanins and carotenoids significantly differentiated the tested lines in each of HM treatment. Figure 9 shows a dendrogram for these four significant variables.

Discussion

General impact of heavy metals in *D. jasminea* shoot cultures

Changes in the functioning of antioxidant machinery are ubiquitous elements of defense reactions in plants exposed to metallic stress (Shahid et al. 2014; Viehweger 2014; Malar et al. 2016; Guo et al. 2017; Gupta et al. 2017). In the current study *D. jasminea* shoots treated with HM experienced oxidative stress, which was reflected in enhanced accumulation of hydrogen peroxide and lipid peroxidation. To counteract it, the tested lines distinctly modulated activity of antioxidant enzymes and accumulation of low-molecular non-enzymatic antioxidants. In comparison with the short-term treated lines, long-term treated ones were capable of decreasing the intensity of oxidative stress. This is in agreement with results of Ding et al. (2016), who observed enhanced ability to reduce oxidative damage in rice cells acclimated to HM stress by long-term in vitro exposure.

In turn, as the antioxidant system was not efficient enough in St lines of *D. jasminea*, additional defense mechanisms were activated. Enhanced protein accumulation suggested that their synthesis was upregulated by a sudden exposure to HM. This response may be aimed at HM detoxification by metallothioneins and phytochelatins (Vrbová et al. 2013; Chen et al. 2015), repair of damaged macromolecules and maintenance of proper protein conformation by stress-expressed proteins: heat shock proteins and other chaperones (Cui et al. 2013; Li et al. 2015). Higher content of proteins may also reflect metabolic shifts, regulated by stress-responsive transcription factors (Charfeddine et al. 2017).

An insight into plant functioning under metallic stress using in vitro culture models has been achieved for representatives of woody plants, including *Cydonia* (Schuch et al. 2010), *Larix* (Bonet et al. 2016), and *Vaccinium* (Manquián-Cerda et al. 2018), as well as herbaceous plants, like *Plantago* (Martins et al. 2013). Recently in vitro culture systems were employed in HM-related studies on extremophytes, such as metallicolous *Dianthus* and *Alyssum* ecotypes (Muszynska and Hanus-Fajerska 2017; Muszynska et al. 2018a, c), and halophytic *Sesuvium* (Fourati et al. 2019).

This indicates that shoot culture can be exploited as a model system to investigate mechanisms of HM responses, tolerance and survival strategies. Advantageously, uptake of toxic ions and their transportation to aboveground tissues are facilitated in shoot cultures due to the lack of barriers formed in the roots. All shoots are exposed to HM in the same way, as variations resulting from distinct patterns of rhizogenesis are reduced.

In our study the uptake of Cd and Ni from the medium was the same in the long-term treated and short-term treated lines. In contrary, Pb uptake was restricted in Lt line, and promoted in St line. This is surprising, since Pb is considered a much less mobile element than Cd or Ni. We suppose that in Lt Pb line extracellular barriers could be responsible for restricted Pb penetration to the shoots. This may be supported by the fact that POD was mainly active in the epidermal layers of the leaf, suggesting the existence of mechanisms protecting mesophyll tissues from Pb toxicity. Also high content of phenolic compounds in this line may be related to cell wall modifications aimed at Pb immobilization.

Long-term exposure as an acclimation strategy?

A novel approach in our study involved a comparison of heavy metal-induced responses between shoots non-acclimated (St) and acclimated (Lt) to growth in the presence of toxic metals.

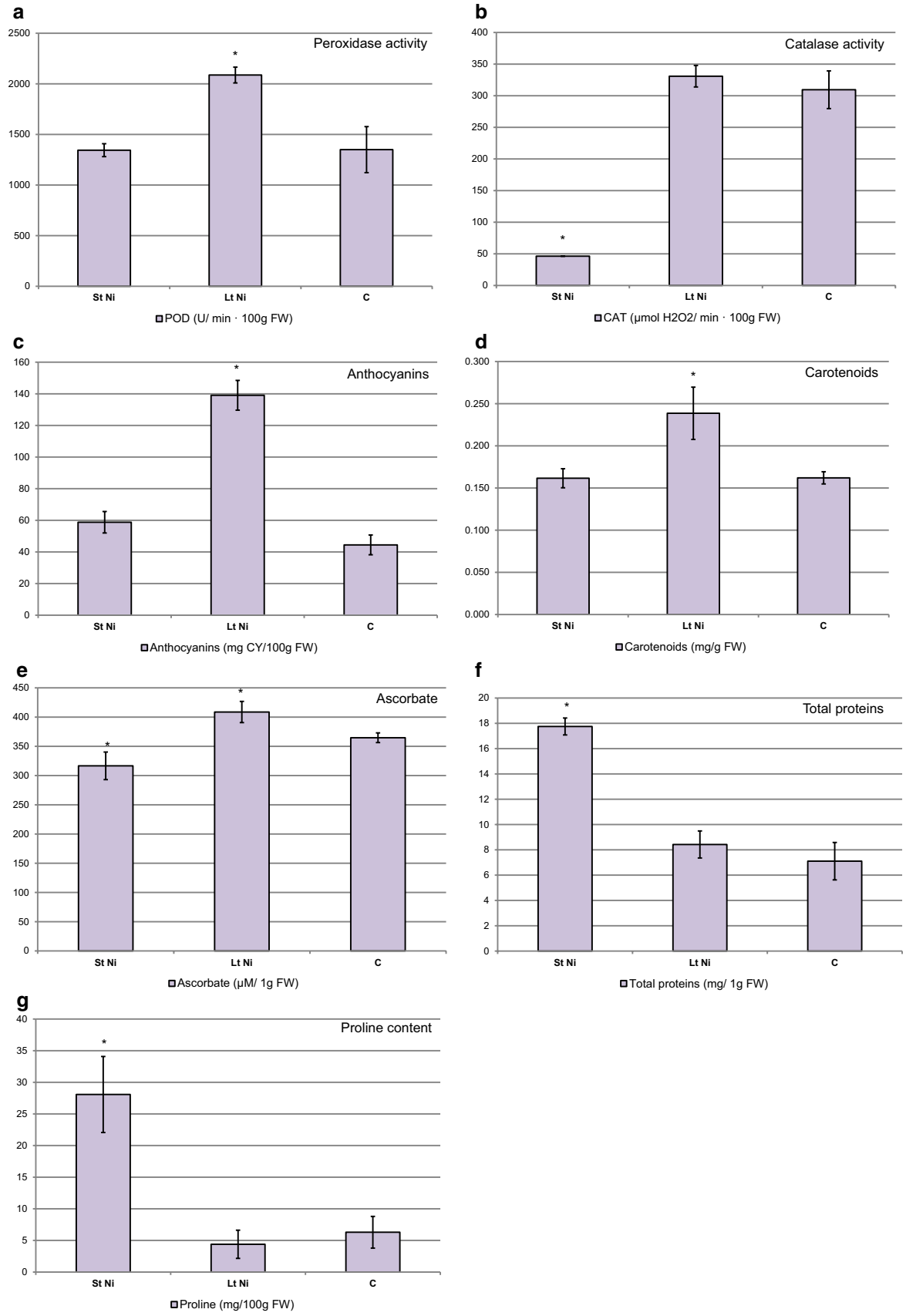
Long-term exposure to HM, especially Cd and Pb, facilitated acclimation of *D. jasminea* to unfavorable conditions. This finding is substantiated by the fact that several responses related to the functioning of antioxidant system differed between acclimated (long-term treated) and non-acclimated (short-term treated) lines, contributing to diverse intensity of oxidative stress, similarly as reported by Ding et al. (2016). Higher antioxidant efficiency ameliorated growth and proliferation of Lt shoots. These responses reflect HM tolerance and suggest that during long-term exposure *D. jasminea* plants developed effective mechanisms of coping with chronic metallic stress (Potters et al. 2007; Ding et al. 2016).

Major players of heavy metal acclimation in *D. jasminea*

Studies on metal-tolerant and metal-sensitive plant genotypes, ecotypes and species revealed that tolerant variants often exhibit constitutively higher antioxidant activity and accumulation of antioxidative compounds (Giannakoula et al. 2010; Martins et al. 2013; Zhou et al. 2017; Muszyńska et al. 2018b; Ferrer et al. 2018). In *D. jasminea* treated with HM, irrespectively of treatment duration, antioxidant machinery was significantly reorganized. However, three elements of antioxidant system were blocked in each St line, and activated in Lt lines. These elements, evidently associated with acclimation status, included POD activity and the synthesis of anthocyanins and carotenoids. It seems that these are the main components responsible for tolerance to HM developed during long exposure in *D. jasminea* and their importance is discussed below.

Peroxidases are multifunctional enzymes, involved also in defense reactions (Passardi et al. 2005). They counteract ROS overproduction and regulate the level of ROS for signaling purposes (Das and Roychoudhury 2014; Cuypers et al. 2016). In *D. jasminea* shoots of all long-term treated lines POD was localized in leaf epidermis and phloem. Its activity was substantially elevated, identifying POD as a crucial enzyme in Lt shoots that determined their tolerance to heavy metals. In Cd-acclimated rice cells activity of this enzyme was also elevated, supported by enhanced activity of CAT (Ding et al. 2016). POD localization in *D. jasminea* may be associated with transportation of HM ions via symplast to the most external cells of the shoot to avoid damage of mesophyll cells. In contrary, in the St Pb shoots the highest POD activity was detected in leaf palisade cells, indicating that mesophyll cells were not efficiently protected from metal accumulation.

Enhanced synthesis of anthocyanins was another reaction related to HM acclimation status. These compounds belong to flavonoids and are often synthesized under metallic stress (Landi and Tattini 2015). They may also mitigate HM toxicity when applied exogenously (Glińska et al. 2007; Dai et al. 2012a). Anthocyanins are potent antioxidants (Sytar et al. 2013), efficient chelators of HM ions and they take part in


a
Peroxidase activity
2500
2000
1500
1000
500
0
*
St Ni
Lt Ni
C
POD (U/ min · 100g FW)
b
Catalase activity
400
350
300
250
200
150
100
50
0
*
St Ni
Lt Ni
C
CAT (µmol H2O2/ min · 100g FW)
c
Anthocyanins
160
140
120
100
80
60
40
20
0
*
St Ni
Lt Ni
C
Anthocyanins (mg CY/100g FW)
d
Carotenoids
0.300
0.250
0.200
0.150
0.100
0.050
0.000
*
St Ni
Lt Ni
C
Carotenoids (mg/g FW)
e
Ascorbate
450
400
350
300
250
200
150
100
50
0
*
*
St Ni
Lt Ni
C
Ascorbate (µM/ 1g FW)
f
Total proteins
20
18
16
14
12
10
8
6
4
2
0
*
St Ni
Lt Ni
C
Total proteins (mg/ 1g FW)
g
Proline content
40
35
30
25
20
15
10
5
0
*
St Ni
Lt Ni
C
Proline (mg/100g FW)

◂**Fig. 5** Biochemical responses of *D. jasminea* to nickel treatment different in long-term treated (Lt) and short-term treated (St) lines. **a** peroxidase activity; **b** catalase activity; **c** anthocyanins; **d** carotenoids; **e** ascorbate; **f** total protein content; **g** proline content. Values are mean ± SE. Means indicated by asterisk (*) differ from the control treatment according to t-Student's test at $P \leq 0.05$

their detoxification in the vacuoles (Dai et al. 2012b). In *D. jasminea*, irrespectively of high accumulation of phenolic compounds in response to HM-treatment, anthocyanin content was reduced in non-acclimated St lines. This could be caused by a switch in metabolic reactions under sudden metallic stress and utilization of carbohydrates rather in the primary metabolic pathways than in anthocyanin synthesis. This reaction could also result from the alterations in the activity of enzymes catalyzing anthocyanin synthesis from other flavonoid precursors (Liu et al. 2012). Considering the above, accumulation of anthocyanins in Lt lines could be acclimation strategy, developed after adjustment of primary metabolism to chronic metallic stress. Similar mechanism as in *Azolla imbricata* (Dai et al. 2012b) can be proposed: high amounts of anthocyanins chelate metal ions more efficiently and detoxify them in vacuoles of leaf epidermal cells.

In the long-term treated *D. jasminea* shoots concentrations of carotenoids were elevated, while in the short-term treated shoots they were similar to or lower than in the control line. HM tolerant plants have constitutively higher content of carotenoids than the sensitive ones (Wiszniewska et al. 2017a; Ferrer et al. 2018). Recent concepts of carotenoid synthesis in response to environmental stimuli highlight the regulatory role of H_2O_2 produced during chronic, moderate stress (Fanciullino et al. 2014). The main function of carotenoids is protection from photooxidative stress (Takahashi and Badger 2011). However, as in vitro cultured shoots were not exposed to intense light, this aspect could be marginalized in the present study. Carotenoid action was more likely directed at scavenging of ROS overproduced under metallic stress (Schmidt et al. 2016). Since carotenoids are lipophilic and bound in chloroplastic membranes in lipid-protein complexes, they stabilize membrane lipids and protect membrane surface from hydrophilic oxidizers (Fanciullino et al. 2014). Lipid peroxidation in Lt lines was less pronounced than in St lines (Supplementary Material 2). In this way membrane-bound carotenoids in Lt lines could complement antioxidant action of vacuolar anthocyanins and cytosolic peroxidases to counteract oxidative stress in various cellular compartments.

Other components involved in acclimation to respective heavy metals

Our study pointed out a few other antioxidants related to acclimation status to respective heavy metals. They included catalase activity, which was stimulated in the lines long-term treated with Cd and Pb and inhibited in the short-term treated lines. The modulation of CAT activity depends on the metal type and its dose. Moreover, in metal-treated plants it is highly variable among species (Anjum et al. 2016). We did not find any convincing differences in CAT localization between long-term and short-term treated lines, although the enzyme activity varied, similarly as in Cd-acclimated rice cells cultured in suspension (Ding et al. 2016). Therefore, the explanation of CAT role in acclimation to HM in *D. jasminea* requires further studies on identification and quantification of the enzyme isoforms, as well as their precise localization in the cells. We noticed that CAT activity

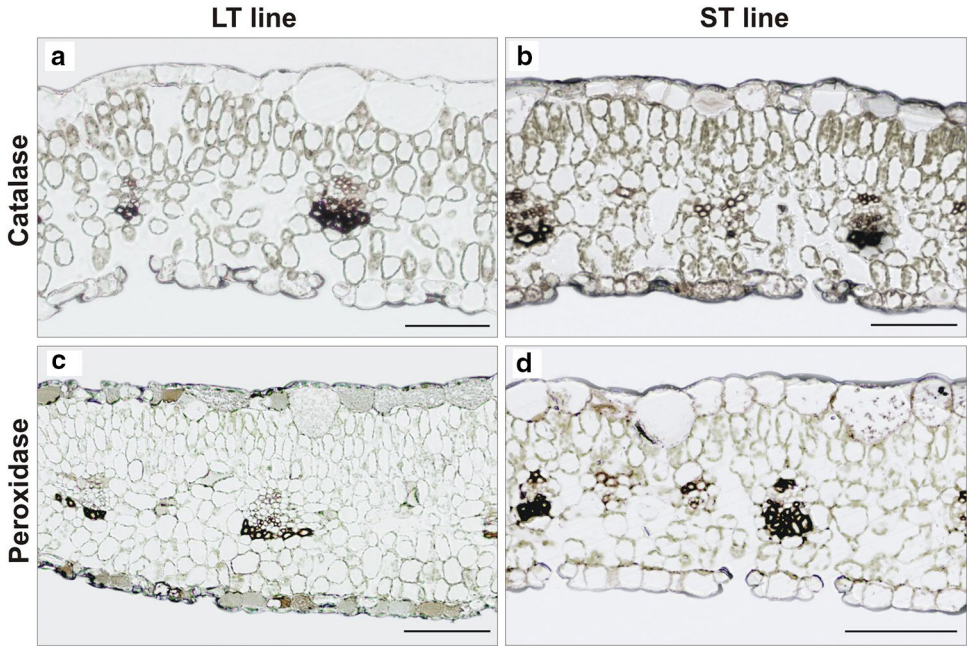
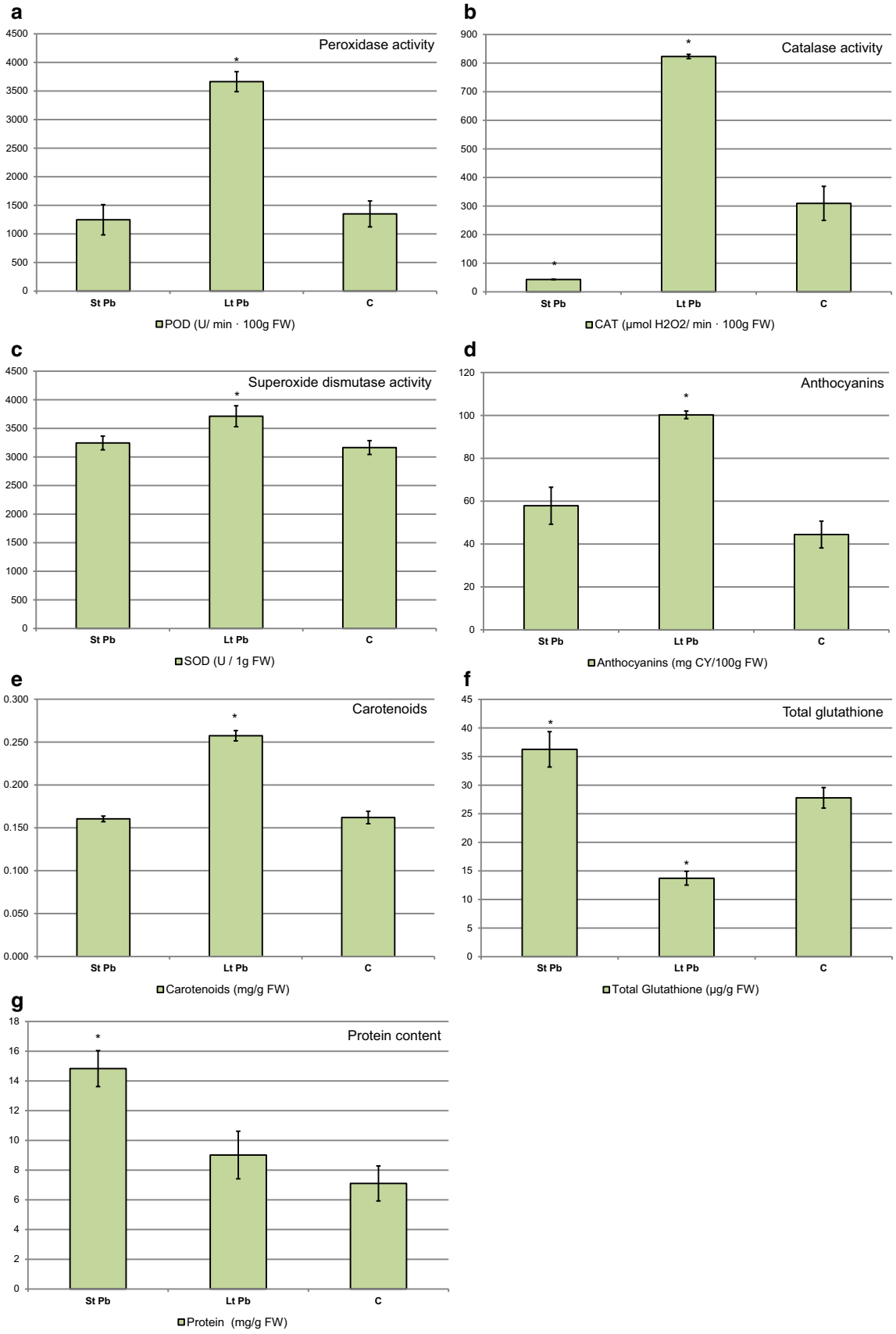


Fig. 6 Histochemical localization of catalase (CAT) and peroxidase (POD) activity in the leaves of *D. jasminea* lines long-term treated (Lt) and short-term treated (St) with nickel **a**, **b** CAT; **c**, **d** POD. Bar 100 µm


a
Peroxidase activity
4500
4000
3500
3000
2500
2000
1500
1000
500
0
St Pb
Lt Pb
C
POD (U/ min · 100g FW)
b
Catalase activity
900
800
700
600
500
400
300
200
100
0
St Pb
Lt Pb
C
CAT (µmol H2O2/ min · 100g FW)
c
Superoxide dismutase activity
4500
4000
3500
3000
2500
2000
1500
1000
500
0
St Pb
Lt Pb
C
SOD (U / 1g FW)
d
Anthocyanins
120
100
80
60
40
20
0
St Pb
Lt Pb
C
Anthocyanins (mg CY/100g FW)
e
Carotenoids
0.300
0.250
0.200
0.150
0.100
0.050
0.000
St Pb
Lt Pb
C
Carotenoids (mg/g FW)
f
Total glutathione
45
40
35
30
25
20
15
10
5
0
St Pb
Lt Pb
C
Total Glutathione (µg/g FW)
g
Protein content
18
16
14
12
10
8
6
4
2
0
St Pb
Lt Pb
C
Protein (mg/g FW)

◂**Fig. 7** Biochemical responses of *D. jasminea* to lead treatment different in long-term treated (Lt) and short-term treated (St) lines. **a** peroxidase activity; **b** catalase activity; **c** superoxide dismutase activity; **d** anthocyanins; **e** carotenoids; **f** total glutathione; **g** total protein content. Values are mean ± SE. Means indicated by asterisk (*) differ from the control treatment according to t-Student's test at $P \leq 0.05$

usually declined in St lines. The inhibition of CAT activity in comparison with the control line was rather due to protein deactivation than degradation, as total content of proteins in St lines was greater than in control. Higher sensitivity to protein inactivation during multi-metal stress was recently recognized as an attribute of non-tolerant shoot line of *Alyssum montanum* (Muszyńska et al. 2019).

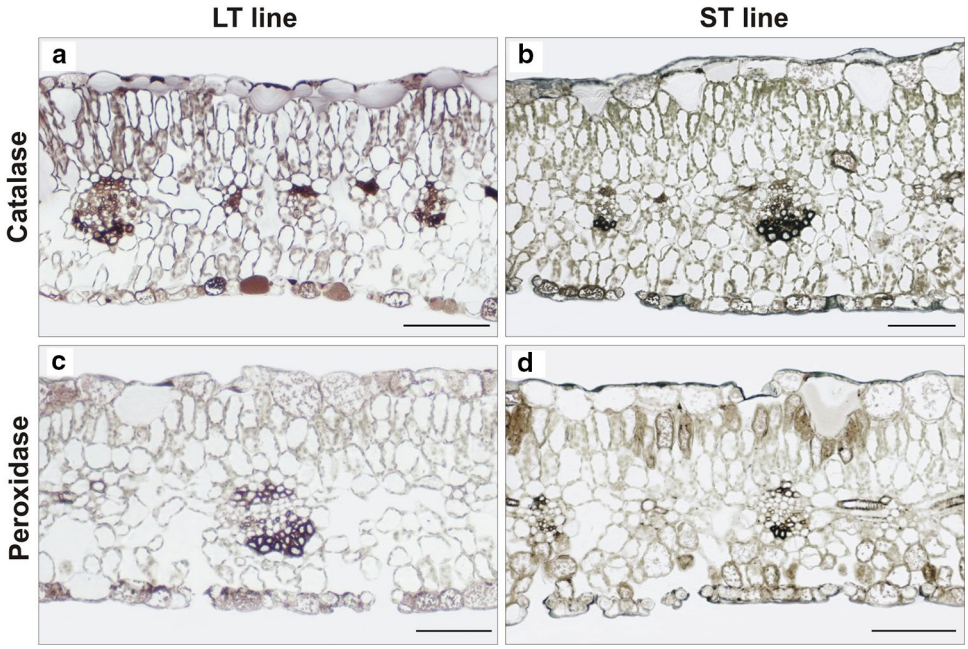


Fig. 8 Histochemical localization of catalase (CAT) and peroxidase (POD) activity in the leaves of *D. jasminea* lines long-term treated (Lt) and short-term treated (St) with lead. **a**, **b** CAT; **c**, **d** POD. Bar 100 µm

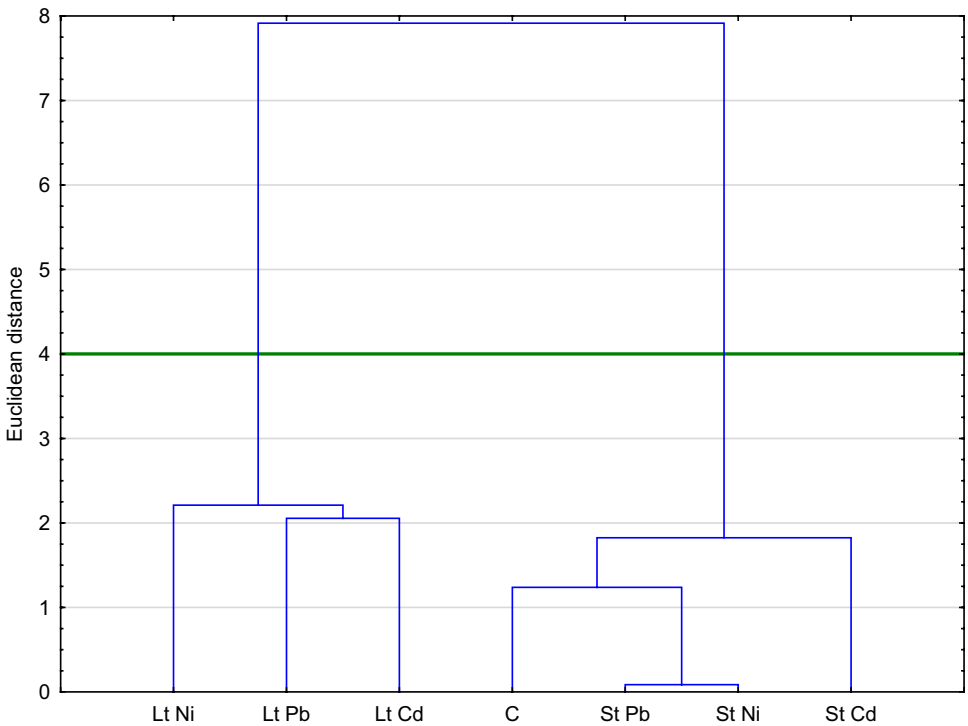


Fig. 9 Dendrogram constructed by cluster analysis showing similarities between *D. jasminea* shoot lines long-term treated (Lt) and short-term treated (St) with heavy metals. C—the control line

Enzymatic machinery of Lt Pb shoots was supported by enhanced activity of SOD. Plant pretreatment with a priming agent, such as salicylic acid, modulates the occurrence and activity of SOD isoforms under Pb exposure (López-Orenes et al. 2014). Considering long-term exposure an acclimation strategy, similar mechanisms may be responsible for higher SOD activity in Lt Pb line. It is yet unknown what compounds, process or pathway can be involved in potential priming effect during long-term HM exposure, and this requires further studies.

Long-term treated lines differed also from the other in the content of components constituting cellular redox buffering system, i.e. glutathione and ascorbate. In Lt Ni line, the content of ascorbate was elevated in comparison with the control, contributing to low oxidation status of the shoots. Surprisingly, in Lt Cd and Lt Pb lines glutathione content decreased, while in their respective St variants its level did not change. Notwithstanding, oxidation status in the Lt Cd and Pb shoots remained low. This unusual phenomenon can be explained by a switch between ROS-processing pathways (Noctor et al. 2018). It seems that glutathione does not play a role in HM acclimation of *D. jasminea*, and its action is efficiently substituted by other antioxidant components. This finding opens new questions on functioning of glutathione-ascorbate cycle in *D. jasminea* subjected to chronic metallic stress.

Specific response of the Lt Ni shoots was a stable content of proline, in contrast to the St Ni shoots, in which it was greatly elevated. This amino acid is accumulated under HM stress as a compatible solute and antioxidant (Lefèvre et al. 2014; Fourati et al. 2019). Results obtained in St Ni line are consistent with our previous observations of Ni-induced physiological disorders related to osmotic stress in *D. jasminea* (Wiszniewska et al. 2018). Stable content of proline in Lt Ni line may be an indicator of effective control of osmotic homeostasis and may reflect reduced Ni toxicity during prolonged exposure (Zou et al. 2013; Islam et al. 2014).

Conclusion

In this study we showed that metallic stress affects antioxidant system in the woody plant *D. jasminea*. Some responses were the same in the long-term and short-term treated lines, however, the specific reactions seem more interesting concerning mechanisms of plant acclimation and tolerance to heavy metals. We showed that long exposure to HM significantly remodeled antioxidant system in *D. jasminea* shoots. Several responses were uniform in all long-term treated lines, regardless of the applied heavy metal. These included the activity of POD, and accumulation of low-molecular antioxidants: anthocyanins and carotenoids. The orchestrated action of three major players: cytosolic peroxidases, vacuolar anthocyanins and membrane-bound carotenoids conditioned normal growth and proliferation during chronic metallic stress. Some other antioxidant elements, like CAT, SOD, glutathione and ascorbate complemented the action of major players in a HM-dependent way. We therefore concluded that long-term exposure to toxic metals can be regarded as the acclimation strategy. Further studies are necessary to identify whether any "priming agent" is responsible for acclimation status acquired during prolonged exposure to HM. New questions arising from the current study, related primarily to the mechanisms of enzyme activity regulation, should also be addressed. It is also important to find a link between the role of molecules involved in energy dissipation during photosynthesis, like anthocyanins and carotenoids, and heavy metal acclimation.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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