JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

TISSUE-SPECIFIC RESPONSES IN SOYBEAN PLANTS EXPOSED TO CADMIUM

*Marcel Roszival, Viktória Lukácsová, Kristína Muráňová, Roman Bíro, Nikola Grmanová, Patrik Mészáros**

Address(es): doc. RNDr. Patrik Mészáros, PhD.,
Constantine the Philosopher University in Nitra, Faculty of Natural Sciences, Department of Botany a Genetics, Nábrežie mládeže 91, 949 74 Nitra, Slovakia, +421 3764 087 44.

*Corresponding author: pmeszaros@ukf.sk

https://doi.org/10.55251/jmbfs.5614

ARTICLE INFO

Received 8. 12. 2021
Revised 5. 5. 2022
Accepted 10. 5. 2022
Published 1. 8. 2022

Regular article

OPEN ACCESS

ABSTRACT

Cadmium seriously affects plant growth, development, homeostasis and yield production. The effect of two doses of cadmium (10 and 100 $mg.kg^{-1}$ Cd^{2+}) on plants of sensitive soybean variety (*Glycine max* L. cv. Kyivska 98) were studied after short treatment in a pot experiment. Several parameters were determined in roots and leaves, including cell viability, the content of malondialdehyde and proline, and activity of defense proteins (catalases, guaiacol peroxidases, ascorbate peroxidases, superoxide dismutases, chitinases, β-1,3-glucanases). Changes caused by cadmium depended both on the concentration of cadmium, as well as on the type of plant organ and its developmental stage. Soybean tissues generally showed low levels of oxidative stress and reduced proline content likely due to activity or depletion by different mechanisms (respectively). The activity of catalases and guaiacol peroxidases was inhibited by cadmium concentrations only in younger leaves. The enzymatic activities of superoxide dismutases, chitinases and β-1,3-glucanases were highly variable depending on the cadmium concentration as well as the soybean organ analyzed. Our experiments revealed differences in defense strategies and regulatory mechanisms between plant organs as well as developmental stages of leaves in response to cadmium toxicity.

Keywords: cadmium, proline, catalase, peroxidase, superoxide dismutase, β-1,3-glucanase, chitinase

INTRODUCTION

Anthropogenic activities, rapid industrialization and modern agricultural practices over the last few decades have contributed to the rapidly increasing concentration of heavy metals in the environment (**Ma *et al.*, 2019**; **Feng *et al.*, 2021**). Easy absorption and subsequent metal accumulation in plants have a serious impact on crop growth and productivity. Contamination of the environment with heavy metals is a serious problem especially in the long term due to non-degradable nature of metals. Their occurrence in agricultural land represents a particularly serious risk after entering the food chain (**Feng *et al.*, 2021**), therefore research on heavy metal toxicity on organisms remains constantly relevant.

Plants have developed a wide range of sophisticated mechanisms to cope with heavy metals (**Kazan and Lyons, 2014**), which are administered through biochemical adjustments. Metals typically attack the plasma membrane (and the membranes of organelles) by generating reactive oxygen species (ROS) and subsequent peroxidation of lipids. Peroxidation of membrane lipids (polyunsaturated fatty acids - PUFA) occurs on double or unsaturated bonds between two carbon atoms as well as ester bonds between fatty acid and glycerol in phospholipids (**Sharma *et al.*, 2012**). Secondary products of PUFA peroxidation such as malondialdehyde (MDA) (**Choudhary *et al.*, 2019**) are considered a suitable biomarker of membrane damage (**Davey *et al.*, 2005**). PUFA peroxidation products can also indirectly oxidize proteins (**Yamauchi *et al.*, 2008; Sharma *et al.*, 2012**) and DNA molecules (**Das and Roychoudhury, 2014**).

Plants control the degree of oxidation through effective enzymatic and non-enzymatic defense mechanisms. Non-enzymatic antioxidants include polyphenols, reduced glutathione, L-ascorbate, α-tocopherol or β-carotene. Antioxidant enzymes, which have received considerable attention in recent years, include in particular: I) superoxide dismutases (SOD) - metalloenzymes (Fe-SOD, Mn-SOD, Cu / Zn-SOD) that catalyze the decomposition of superoxide radicals to form hydrogen peroxide and oxygen (**Pan *et al.*, 2020**); II) iron containing catalases (CAT) that cleave hydrogen peroxide to form water and oxygen (**Mhamdi *et al.*, 2012**); III) ascorbate peroxidase (APX) that reacts with H_2O_2 in the presence of ascorbate to form water (**Comparot *et al.*, 2002**); IV) guaiacol peroxidase (GPX) - that oxidizes guaiacol and also ascorbate to convert H_2O_2 to water (**Van Doorn and Ketsa, 2014**).

Plant tolerance to metal toxicity is based on neutralizing toxic metals in the protoplast with subsequent sequestration from the cell and/or limiting metal mobility in tissues (**Krzesłowska, 2011**). When a toxic metal enters a cell, chelation occurs through special cysteine-rich ligands such as phytochelatins (**Yadav, 2010**) and metallothioneins (**Cherian and Kang, 2006**). The amino acid proline may also be involved in chelation, the accumulation of which during stress conditions represents one of the most studied perspectives of plant defense reactions (**Liu *et al.*, 2017a**). Proline is an important osmolyte, participates in antioxidant defense and also acts as a signaling molecule (**Hayat *et al.*, 2012**). Plant-based defense processes against the toxic effects of heavy metals also involve the synthesis of PR proteins, with attention often focused on chitinases and β-1,3-glucanases.

Chitinases, poly - [1,4-(N-acetyl-β-D-glucosamine)] glycan hydrolases are developmentally regulated, tissue-specific enzymes (**Mészáros *et al.*, 2013**), which play multiple roles in plants. In addition to their roles in normal growth and metabolism (cell wall modifications, programmed cell death, signaling etc.) (**Metwally *et al.*, 2005; Wu *et al.*, 2012; Liu *et al.*, 2017b**), they are important primarily in the defense against pathogens (**Xu *et al.*, 2007;**) but are also activated by abiotic stress, for example due to UV radiation, high temperatures or excessive salinity (**Grover *et al.*, 2012**). Their activity has been reported in different plant species exposed to cadmium, lead and arsenic (**Békésiová *et al.*, 2008, Gálusová *et al.*, 2015**). Activity levels and/or dynamic of chitinase action have been correlated with metal tolerance (**Metwally et al., 2005; Mészáros *et al.*, 2013**).

Similarly, β-1,3-glucanases (glucan endo-1,3-glucosidases) are hydrolytic enzymes that inhibit pathogen spreading, but cleave β-1,3-glucan (callose) that commonly occurs in plant tissue (**Ebrahim *et al.*, 2011**). Their activities have been firmly linked with the regulation of water and mineral distribution in tissues (**Zavaliev *et al.*, 2011**). In addition, they play an important role in many physiological processes such as microsporogenesis or embryogenesis (**Kasprzewska *et al.*, 2003**), but also in defense against abiotic stressors, including heavy metals (**Piršelová *et al.*, 2011, 2012**).

PRs may act alone or cooperate with other defense proteins (**Balasubramanian *et al.*, 2012**). During pathogen infection, β-1,3-glucanases and chitinases appear to be coordinated to act in a similar way (**Jongedijk *et al.*, 1995; Cheong *et al.*, 2000**). In contrast, under abiotic stress like drought they seem to act opposingly (**Gregorová *et al.*, 2015**), but responses under conditions of abiotic stress (including toxic metals) have not yet been sufficiently elucidated.

This study reports on activity profiles of selected defense enzymes upon plant exposure to cadmium. We present their variable activities in different organs and also in similar tissues of different developmental stage, and correlate their action with tissue damage rate as well as other defense responses. Our work brings novel data on the interplay of some PRs with antioxidative defense and put them into the context of plant tolerance to metal toxicity.

MATERIAL AND METHODS

Seed preparation and pre-germination

Mature seeds of the metal sensitive soybean variety (*Glycine max* L. cv. Kyivska 98) were sterilized with 0.47% (w/v) sodium hypochlorite (10% v/v commercial Savo, Bochemie, Bohumín, Czech Republic) for 15 minutes with occasional mixing. The seeds were then rinsed three times with sterile distilled water and transferred to a triple layer of filter papers moistened with sterile distilled water in sterilized plastic containers. The seeds germinated in dark at a temperature of approximately 22 °C for 2 days.

Growing of plant material and application of cadmium

Germinated seeds with 5 - 10 mm long roots were planted in pots (Ø 27 cm, 5 L) containing 1.7 kg of a commercially available universal substrate (pH 5.0 - 6.5; 70% combustible substances Mountfield, Mnichovice, Czech Republic), watered with distilled water (1 L) and grown in a growth chamber at 22 °C, 60% relative air humidity, 12/12 h photoperiod and 7000 lx light intensity for 4 weeks. In the following phases of the experiment, the plants were regularly watered twice a week with 500 mL tap water. After 4 weeks (third trifoliar leaf stage), the plants were watered with cadmium nitrate tetrahydrate (Centralchem, Bratislava, Slovak Republic) solution containing 10 and 100 $mg.kg^{-1}$ Cd^{2+} (respectively). After three days of exposure to cadmium, plant material was collected. Roots, the mature and the youngest trifoliate leaves were analyzed from three independent plants of each variant of the experiment.

Determination of lipid peroxidation rate

The rate of membrane lipid peroxidation was determined according to the protocol of **Karabal *et al.* (2003)** based on the content of malondialdehyde (MDA) in the tested tissues. The concentration of the formed MDA-TBA complex was calculated using extinction coefficient of 155 $mM.cm^{-1}$ and expressed in $mol.g^{-1}$ of fresh weight.

Determination of cell viability with Evans blue

Root and leaf cell viability was determined according to **Baker and Mock (1994)**. Viability was expressed in relative values based on the absorbance value.

Determination of proline content

The plant material (0.2 g fresh weight) was homogenized with mortar and pestle in 1.5 mL of 95% ethanol (Centralchem, Bratislava, Slovak Republic) and the homogenate was transferred to microtubes. The mixture was centrifuged for 10 minutes at 4000g. 500 µL of the obtained supernatant was mixed with 1 mL of 1% ninhydrin (Sigma-Aldrich, St. Louis, USA) in 60% acetic acid (Centralchem, Bratislava, Slovak Republic) and 20% ethanol, and the reaction mixture was incubated in a thermoblock at 95 °C for 20 minutes. After incubation, the mixture was cooled for 5 minutes on ice and then centrifuged at 10,000 g for 1 min. The absorbance of the mixture was measured on a spectrophotometer at 520 nm. As a blank, we used a mixture of 1 mL of the ninhydrin reaction mixture and 500 µL of 95% ethanol. The proline content was calculated using a calibration curve constructed on a commercially available standard L-proline (Sigma-Aldrich, St. Louis, USA) and expressed in $\mu g.mL^{-1}$.

Protein isolation

The crude protein extracts were isolated from roots and leaves using liquid nitrogen. 200 mg of tissue was ground into powder in a mortar, mixed with 500 µL of a cooled extraction solution composed of 0.1 M sodium acetate (Slavus, Bratislava, Slovak Republic) pH 5.0 and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, USA), mixed on a vortex and centrifuged three times at 14,000 g, 4 °C, for 15 minutes.
The concentration of isolated proteins was determined according to **Bradford (1976)**. Bovine serum albumin (BSA) (ThermoScientific, Rockford, USA) was used as a standard. Aliquots of samples were stored at -80 °C for determination of the enzyme activities.

Spectrophotometrical determination of the activity of antioxidative enzymes

The activities of chosen antioxidative enzymes – catalases (CAT), guaiacol peroxidases (GPX), ascorbate peroxidases (APX) – were determined according to **El-Tayeb (2006)** and **Kováčik (2012)**.

In-gel determination of the individual enzyme fraction activities

Electrophoretic detection of enzyme activities was performed in 1-D Mini-Protean TetraCell electrophoresis apparatus (BioRad, Hercules, USA).

The activity of superoxide dismutases (SOD) was detected according to **Weydert and Cullen (2010).** Protein extracts (50 mg) were separated on 1.5 mm thick 12% native polyacrylamide gels. After electrophoresis, SOD was detected using riboflavin (Sigma-Aldrich, St. Louis, USA) and nitrobluetetrazolium chloride (Serva, Heidelberg, Germany) dyes.
Enzymatic activity was determined for isoforms of chitinases (**De Bolle *et al.*, 1991; Gálusová *et al.*, 2015**) and β-1,3-glucanases (**De Bolle *et al.*, 1991; Michalko *et al.*, 2013**). Aliquots of protein samples (20 µg) isolated from roots and leaves were separated on SDS-containing or native polyacrylamide gels. After electrophoresis, the enzyme activities were visualized using a photodocumentation device (UVP Bio Doc-It System, Ultra-VioletProducts Ltd, Cambridge, UK for SOD and chitinases, or Epson Perfection V600, Epson, Jakarta, Indonesia, for β-1,3-glucanases). The activities of individual bands on created images were quantified using the software Scion Image 4.0.2 (www.scioncorp.com) based on the average density of the fractions (in pixels) on the adjusted background and expressed in relative values to the control sample.
Enzyme activities were visualized in heat maps prepared using BAR HeatMapper Plus Tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

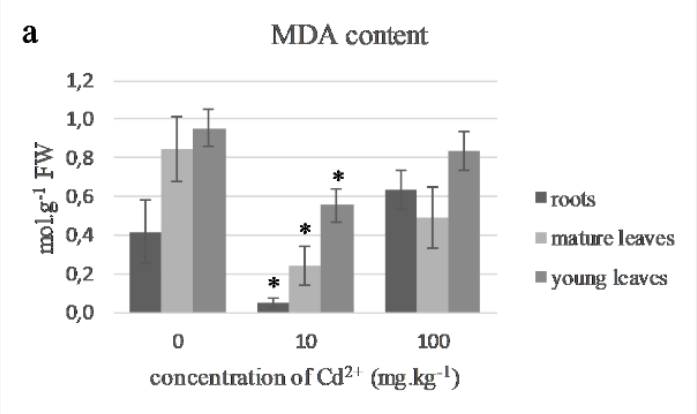
Statistical evaluation of results

Each experiment was performed in at least three independent replicates. The obtained data were subjected to statistical analyzes using MS Excel. The statistical significance of the differences between the control and stressed samples was tested by Student's t-test. Two-way ANOVA was performed to test the effects of cadmium concentration and type of tissue on the tested parameter. Data are expressed as the mean ± standard deviation. A P-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Basic indicators of ongoing stress

The rate of the ongoing stress in the tissues of experimental soybean plants was evaluated based on membrane lipids peroxidation rate, the extent of cell damage in tissues, as well as of accumulation rate of the multifunctional proline. Both cadmium concentration and plant tissue type had a significant effect on these three parameters. Content of MDA in tissues changed upon exposure to both concentrations of cadmium. While at 10 $mg.kg^{-1}$ Cd^{2+} we noted significant drop of MDA content (up to 42-88%) in all tested organs, at higher Cd concentration the membrane peroxidation only slightly increased in roots but declined in leaves (Fig 1a).
The extent of membrane damage affects cell viability. Cadmium caused a decrease in the number of viable cells in the root tissues (significantly at higher Cd concentration, P≤0.05), but not in any developmental stage of soybean leaves (Fig 1b). Our data further showed that Cd stress at both concentrations significantly reduced the accumulation of proline by up to 54% in both roots and mature leaves. Interestingly, in young leaves such reduction was observed at low Cd concentration only (Fig 1c).



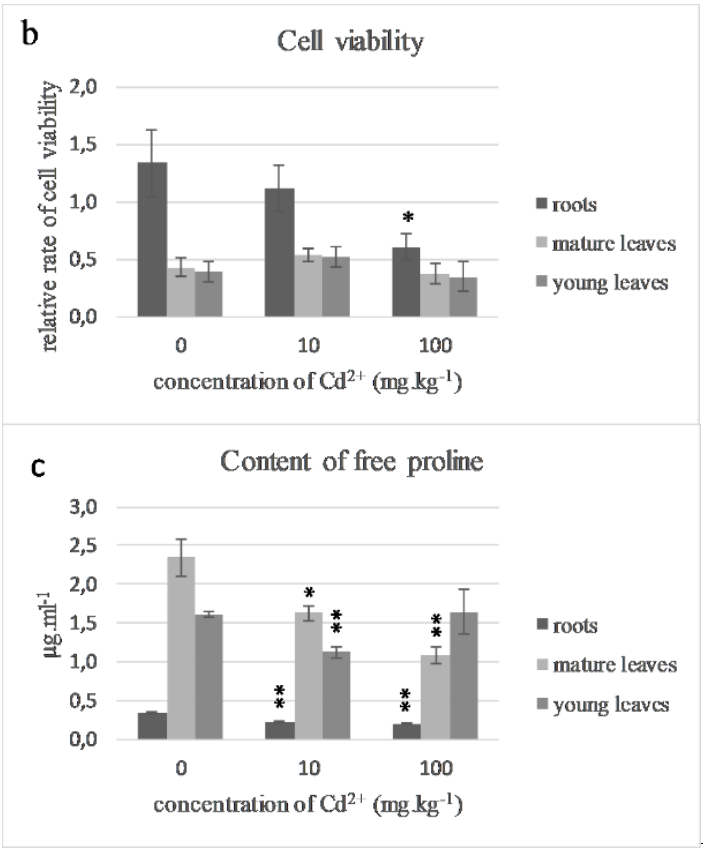


Figure 1 Content of MDA (a), cell viability (b) and content of free proline (c) in soybean tissues under cadmium stress. Bars indicate standard deviation of the mean from three independent biological replicates. Significant effect of Cd compared to control is indicated with asterisks: *P≤0.05, **P≤0.01.

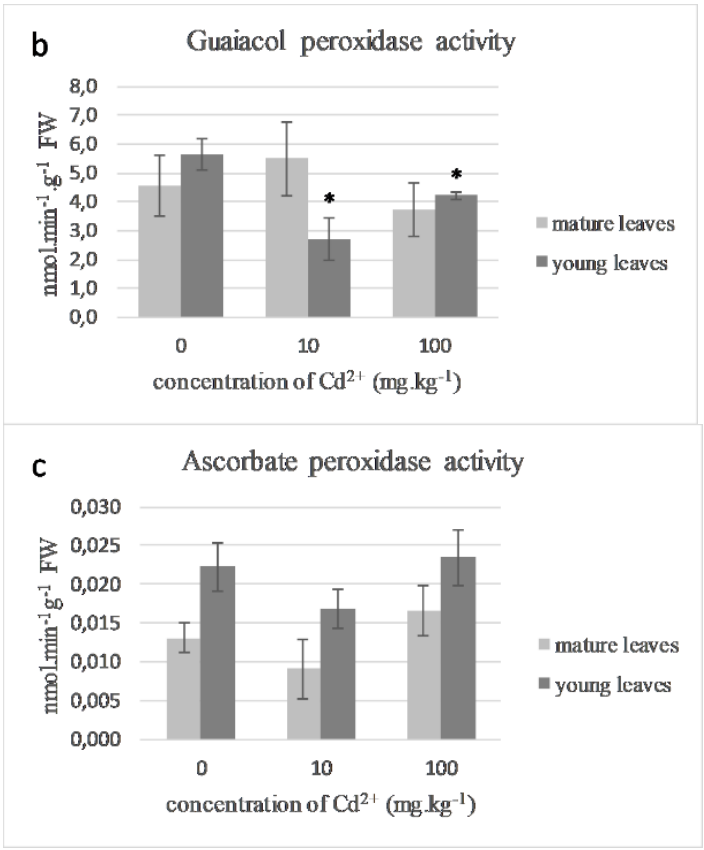
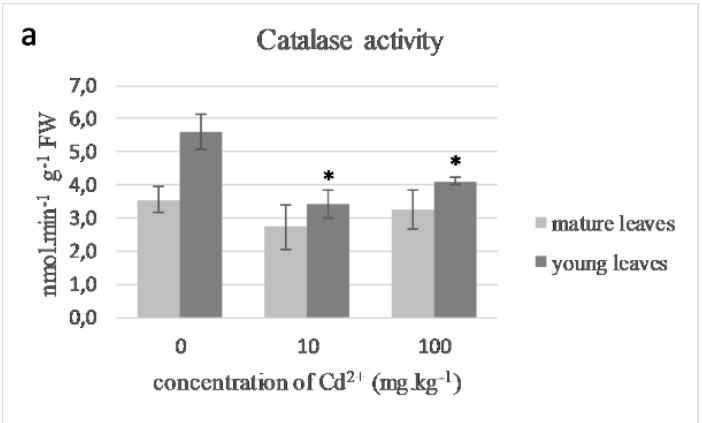


Figure 2 Activity of catalases (a), guaiacol peroxidases (b) and ascorbate peroxidases (c) in soybean leaves under cadmium stress. Bars indicate standard deviation of the mean from three independent biological replicates. Significant effect of Cd compared to control is indicated with asterisks: *P≤0.05.

Activation of antioxidative enzymes in leaves in response to cadmium toxicity

The total activity of catalases (CAT), guaiacol peroxidases (GPX) and ascorbate peroxidases (APX) were evaluated spectrophotometrically in soybean leaves to estimate and compare the antioxidative enzyme activation in different tissues. We observed significant effect of the Cd concentration as well as the leaf type on the activity of CAT and GPX (two-way ANOVA) (Fig 2a-c). Compared to control samples, pronounced changes appeared only in the developmentally younger leaves, where the activity of CAT and GPX decreased at both applied concentrations of cadmium (Fig 2a and 2b). Noteworthy, the inhibition effect was more obvious at low Cd concentration.



The enzymatic activity of superoxide dismutases was analyzed more in detail at the level of individual isoforms. Analyses revealed peculiar reactions to cadmium stress. Six SOD isoforms were detected in polyacrylamide gels (Fig 3a), and we noticed quantitative differences in the SOD profile between the tested samples, depending on developmental stage. The effect of the tissue on the activity of isoforms SOD 1-4 was clearly evident. While the activity of several isoforms tended to decrease in mature leaves, in young leaves they predominantly increased (Fig 3b). For example, the SOD isoforms 1- 4 were significantly (P≤0.05) inhibited in mature leaves at both Cd concentrations (usually stronger at 100 Cd). In contrast, the same-sized enzyme fractions were considerably activated in young leaves (especially at higher Cd concentrations). The most noticeable reaction was recorded for the isoform SOD 4 at 100 mg.kg^{-1} Cd^{2+}, the activity of which was inhibited by 41.5% in mature leaves but enhanced in young leaves by 30.4%. Isoforms 5 and 6 don't seem as involved in response to cadmium toxicity (Fig 3b).

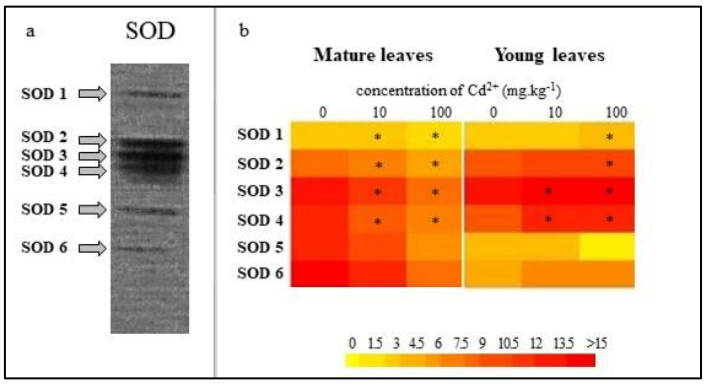


Figure 3 Detected enzymatic profile of SOD isoforms in polyacrylamide gel (a) and heat map of SOD activity (b) in leaves of tested cultivar exposed to Cd^{2+}. The intensity of colour expresses the rate of enzyme accumulation and is expressed in relative values. Significant effect of Cd^{2+} compared to the corresponding control (0) is indicated with asterisks: *P≤0.05.

Chitinase activity in soybean tissues under cadmium stress

Chitinases are important components of plant defense against various stressors, including metals like cadmium. They represent a second-line, non-specific defense

response against metal toxicity (**Békésiová *et al*., 2008; Mészáros *et al*., 2013**). Here we determined the activities of chitinase fractions separated by size as well as charge (total, acidic/neutral and basic/neutral isoforms). Their activities were affected in a variable dose and tissue dependent manner. No chitinase was synthetized *de novo* in response to cadmium. Of the 3 total chitinase fractions (CHIT) with molecular weights of 21, 42 and 66 kDa (Fig 4a), each was affected by the presence of Cd. The CHIT 66 kDa and CHIT 21 kDa were inhibited in roots by both Cd concentrations. This effect was weaker in leaves, since these fractions appeared inhibited only by high Cd concentration in mature leaves. Opposingly, the CHIT 42 kDa chitinase fraction was induced in each tissue type at high Cd concentration (Fig 4b).

Chitinase enzymes were separated also according to their charge and molecular weight on native polyacrylamide gels. Of the four acidic/neutral chitinases (AcCHIT) detected in roots, the isoforms ***a-c*** were significantly induced by Cd^{2+}, but the isoform ***d*** was inhibited in this tissue. Similarly, four AcCHIT were induced also in leaves, though isoforms ***c*** and ***d*** were absent in mature leaf tissues. Observed induction depended on leaf type and/or Cd^{2+} concentration (Fig 4c-d).

Two basic/neutral chitinase isoforms (BasCHIT) were detected in roots, but did not respond to cadmium toxicity. On the other hand, up to three BasCHIT isoforms were detected in leaves and their activity varied depending on the developmental stage of the leaves. Interestingly, their activity was induced by Cd^{2+} in mature leaves, whereas suppressed in young leaves (at least at one Cd concentration) (Fig 4e-f).

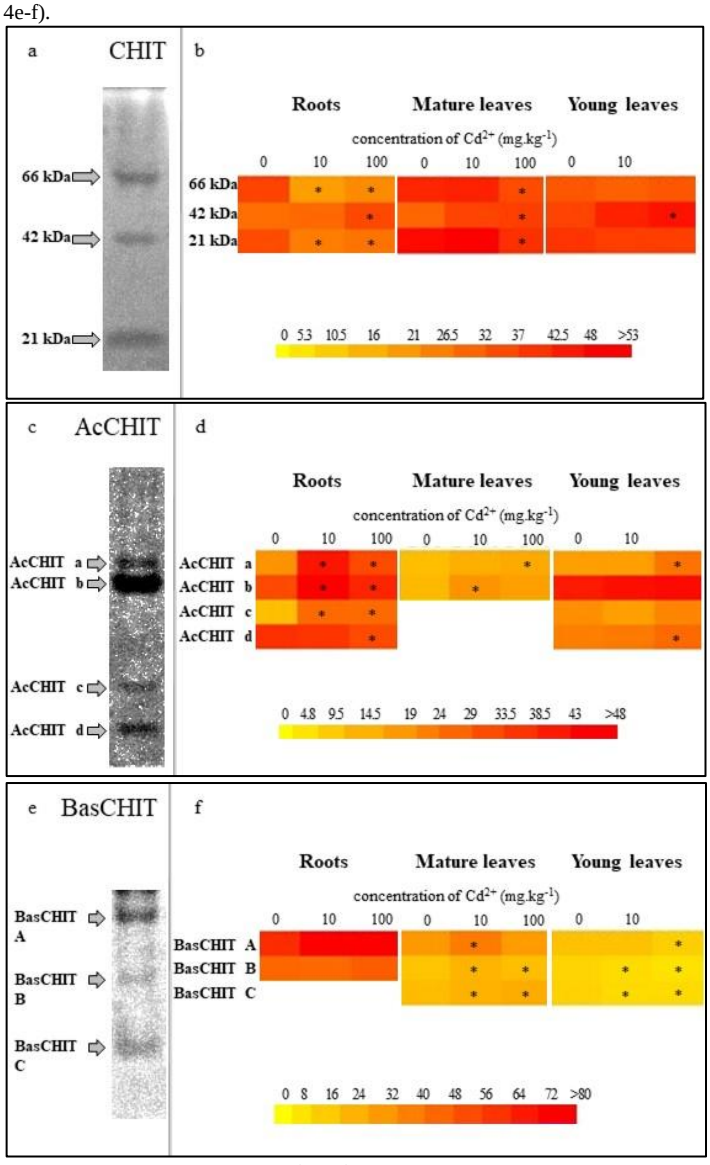


Figure 4 Detected enzymatic profile of chitinase (a), acidic/neutral chitinase (c) and basic/neutral chitinase (e) isoforms in polyacrylamide gel and heat map of their activity in the tested soybean tissues exposed to Cd^{2+} (b, d, f). The intensity of colour expresses the rate of enzyme accumulation and is expressed in relative values. Significant effect of Cd^{2+} compared to the corresponding control (0) is indicated with asterisks: *P≤0.05.

β-1,3-glucanase activity in soybean leaves under cadmium stress

The activity of β-1,3-glucanases was determined electrophoretically in polyacrylamide gels based on their ability to hydrolyze laminarin, a linear (1,3)-β-D-glucan that was added to polyacrylamide gels as substrate. We detected 5 fractions of β-1,3-glucanases ranging in sizes of 12 - 100 kDa and 4 acidic/neutral isoforms in both soybean leaf types (Fig 5a,c). Due to insufficient resolution of the protein bands their activity in roots could not be quantified.

Of the β-1,3-glucanases (GLU) in leaves with a molecular weight of ~ 12, 16, 50, 65 and 100 kDa only the last isoform (100 kDa) showed metal-induced activity in both mature and young leaves. As for the other protein fractions, we generally observed their inhibition in mature soybean leaves (up to -37.5%, P≤0.05), but increased accumulation in young soybean leaves (up to 60%, P≤0.05) (Fig 5b).

All the acidic/neutral β-1,3-glucanase isoforms (AcGLU) were mostly induced in the presence of metal, in both the leaf types (up to 221.2%, P≤0.05) (Fig 5d). The observed activity changes depended on the concentration of Cd as well as the developmental stage of the leaf, however, a clear relationship between the activity of isoforms could not be identified in correlation analyses (Tab 1,2,3).

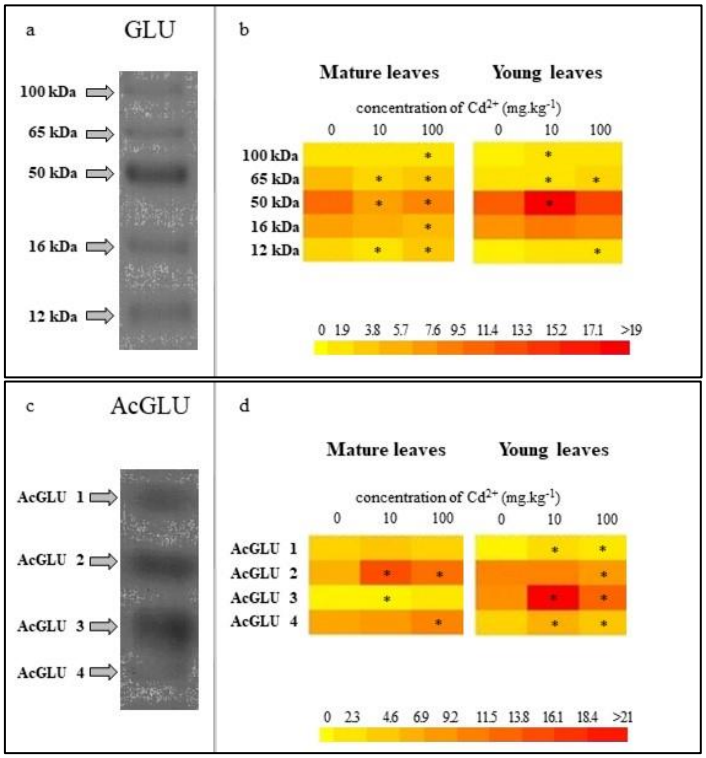


Figure 5 Detected enzymatic profile of glucanase (a) and acidic/neutral glucanase (c) isoforms in polyacrylamide gel and heat map of their activity in the tested soybean tissues exposed to Cd^{2+} (b, d). The intensity of colour expresses the rate of enzyme accumulation and is expressed in relative values. Significant effect of Cd^{2+} compared to the corresponding control (0) is indicated with asterisks: *P≤0.05.

DISCUSSION

Cadmium is one of the most toxic metals negatively affecting all biotic components of ecosystems (**Gong *et al*., 2017**). It is considered to be the only metal that poses health risks to humans and animals in concentrations that can be found in plant tissues without an apparent phytotoxic effect (**Ismael *et al*., 2019**). The toxicity of Cd on plants depends on its amount and concentration, absorption rate by plants, duration of exposure, species but also variety (**Szőllősi *et al*., 2009; Bardáčová *et al*., 2017; Morkunas *et al*., 2018**). Against this toxicity, plants have developed a sophisticated and interconnected network of defense strategies to prevent or tolerate heavy metal intoxication (**Emamverdian *et al*., 2015; Ghori *et al*., 2019**), which apparently varies during plant ontogeny and also within a single plant (**Gálusová *et al*., 2015**). In our work, we analyzed the effects of two concentrations of cadmium on soybean roots and leaves. We applied cadmium in concentrations, which are much higher than the natural contents in the surface horizon of soils in Slovakia (0.002−1.450 $mg.kg^{-1}$) (**Makovníková *et al*., 2006**). The concentration of about 10 $mg.kg^{-1}$ Cd most often represents mild stress for plants, and may even have a positive effect on certain morphological and physiological parameters of plants (**Figlioli *et al*., 2019; Demecsová *et al*., 2020**). On the other hand, higher concentrations of Cd can induce demonstrably harmful stress for plants and their fitness. Similar concentrations of cadmium were also tested in works that evaluated plant responses to cadmium stress (**Piršelová *et al*., 2011; Konotop *et al*., 2012; Figlioli *et al*., 2019; Holubek *et al*., 2020**). The tested soybean cultivar Kyivska 98 is considered to be relatively sensitive to cadmium toxicity (**Mészáros *et al*., 2014; Socha *et al*., 2015**).

Basic indicators of ongoing stress

We evaluated the physiological and oxidative state of experimental plants based on the degree of lipid peroxidation and cell viability. At lower Cd concentration, we observed a reduced MDA content in roots as well as leaves (Fig 1a) indicating mild stress and/or efficient antioxidant system that suppresses ROS formation and lipid peroxidation. With stronger stress, the MDA content tended to increase (yet not significantly), which might signal less efficient elimination of ROS and membranes damage (**Szőllősi *et al*., 2009; Holubek *et al*., 2020**).
Though membrane damage often causes cell death, cell viability was lowered only in roots at 100 Cd (Fig 1b). A gradient is observable at the whole plant level pointing to the sensitive balancing of defense. This, at least partly, is supported by levels of proline and other enzymes. **Metwally *et al*. (2005)** even suggest that cytoplasmic membrane integrity and root growth inhibition do not depend directly on membrane lipid peroxidation. Other studies suggest that cell viability decreases only at high cadmium concentrations (**Ortega-Villasante *et al*., 2005; Fusconi *et al*., 2007**).
Our measurements revealed significant reduction in proline content in Cd-stressed soybean roots and leaves, in contrast to results of other authors (**Khatamipour *et al*., 2011; Ahmad *et al*., 2015**). Low proline-content might suggest its utilization as an antioxidant, chelator, source of energy, or material for newly synthetized defense proteins rich in proline (**Berglund *et al*., 1995; Kaur and Asthir, 2015; Asrorov *et al*., 2017**). The organ- and developmental dependence of these indicators have already been described by **Cechin *et al*. (2006, 2010)**.

Activity of defense enzymes

Oxidative damage to cells depends mainly on the effectiveness of antioxidant components. The general endogenous antioxidant system consists of enzymatic antioxidants such as SOD (superoxide dismutase), CAT (catalase), GPX (guaiacol peroxidase), and non-enzymatic antioxidants that include vitamins or their analogs (vitamin A, C, E, coenzyme Q10 and flavonoids), minerals (selenium and zinc) and other metabolites (melatonin) (**Powell, 2000; Hasanuzzaman *et al*., 2010; Karuppanapandian *et al*., 2011; Han *et al*., 2017**). In our work, we investigated the activity of four important antioxidant enzymes, such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX) and guaiacol peroxidases (GPX). They efficiently degrade toxic ROS, such as superoxide anion and hydrogen peroxide, differing in addition to function and effect mainly by localization in cells. SOD is found in various compartments of the cell, making it the primary defense against ROS (**Sharma *et al*., 2012**). Catalases are localized mainly in peroxisomes, while APX in chloroplasts and cytosols of the cell. GPXs are found in the intracellular as well as in the extracellular space of the cell, rendering them the key enzymes of the antioxidant network in plants (**Karuppanapandian *et al*., 2011**).
We observed only few significant changes after spectrophotometric measurements of enzyme activities. Applied concentrations of cadmium significantly suppressed CAT and GPX activity only in young soybean leaves. Several works suggest that young leaves may be more susceptible to environmental stress than mature leaves (**Cechin *et al*., 2010; Rankenberg *et al*., 2021**). On the other hand, in this leaf stage, we observed an increased accumulation of up to four SOD isoforms, while their significant suppression occurred in the developmentally mature leaves. The effect of cadmium on the activity of key enzymes involved in ROS detoxification has been observed in several studies and Cd has been shown to affect the level of antioxidant enzymes by inhibiting or stimulating their activity (**Hasan *et al*., 2009; Rana, 2015**). The high level of antioxidant capacity in plants is likely responsible for their increased tolerance to environmental stressors, but may vary between plant species and their genotypes as well as the type, extent and duration of stress (**Vitória *et al*., 2001; Ferreira *et al*., 2002; Singh *et al*., 2005; Sharma *et al*., 2012; Hasanuzzaman *et al*., 2020**). According to **Drazkiewicz *et al*. (2003)**, studies on the age-dependent response of *Zea mays* leaves to Cd toxicity have shown that Cd affects mature leaves more intensively than younger ones. Increased accumulation of antioxidant components signals active expression of the relevant genes for effective elimination of ROS. On the other hand, the suppressed activity of antioxidant enzymes may be a sign of a strong toxic effect of cadmium in the form of enzymatic inhibition or denaturation. Therefore, low levels of oxidative stress (as determined by MDA content) appear to be provided by other components of the soybean antioxidant system. A decrease in enzyme activity under Cd-stress may occur in plants due to the depletion of the reducing agents (glutathione, guaiacol) with a consequent decrease for example in the ascorbate glutathione cycle (**Rana, 2015**). The decrease in SOD activity may be related to gradual degradation of enzymes by excessive free radical formation, to metabolic changes associated with oxidative stress, or to the lack of cofactors Zn, Mn or Fe due to Cd (**Cho and Kim, 2003; Rana, 2015**). It is more than evident that the regulation of antioxidant enzymes in plants is extremely complex and the individual components of the antioxidant system are involved depending on various internal and external factors.

Table 1 Correlation matrix between the reaction of tested parameters on applied Cd concentrations in the roots of selected soybean cultivar.

roots	MDA	viability	proline	CHIT 66 kDa	CHIT 42 kDa	CHIT 21 kDa	BasCHIT A	BasCHIT B	BasCHIT C	AcCHIT a	AcCHIT b	AcCHIT c	AcCHIT d
MDA	1,00												
viability	-0,57	1,00											
proline	-0,01	0,83	1,00										
CHIT 66kDa	0,56	0,36	0,82	1,00									
CHIT 42 kDa	0,73	-0,98	-0,70	-0,16	1,00								
CHIT 21 kDa	0,44	0,49	0,89	0,99	-0,30	1,00							
BasCHIT A	0,16	-0,90	-0,99	-0,73	0,79	-0,82	1,00						
BasCHIT B	0,80	-0,95	-0,61	-0,04	0,99	-0,18	0,72	1,00					
BasCHIT C													
AcCHIT a	-0,33	-0,59	-0,94	-0,97	0,41	-0,99	0,88	0,30		1,00			
AcCHIT b	-0,65	-0,26	-0,75	-0,99	0,06	-0,97	0,65	-0,07		0,93	1,00		
AcCHIT c	0,28	-0,95	-0,96	-0,64	0,86	-0,74	0,99	0,80		0,81	0,55	1,00	
AcCHIT d	-0,61	1,00	0,80	0,31	-0,99	0,44	-0,88	-0,96		-0,55	-0,21	-0,93	1,00

Legend: Data represents Pearson correlation coefficients between the variables. Coloured cells indicate a strong positive (green cells, $r \geq 0,7$) or negative (brown cells, $r \leq -0,7$) relationship between the given variables. MDA = malondialdehyde, CHIT = total chitinase, BasCHIT = basic chitinase, AcCHIT = acidic chitinase.

Table 2 Correlation matrix between the reaction of tested parameters on applied Cd concentrations in the mature leaves of the selected soybean cultivar.

mature leaves	MDA	viability	proline	CAT	GPX	APX	SOD1	SOD2	SOD3	SOD4	SOD5	SOD6	CHIT 66	CHIT 42	CHIT 21	BasCHIT A	BasCHIT B	BasCHIT C	AcCHIT a	AcCHIT b	AcCHIT c	AcCHIT d	GLU 100 kDa	GLU 65 kDa	GLU 50 kDa	GLU 16 kDa	GLU 12 kDa	AcGLU 1	AcGLU 2	AcGLU 3	AcGLU 4
MDA	1,00																														
viability	-0,56	1,00																													
proline	0,65	0,27	1,00																												
CAT	0,97	-0,74	0,45	1,00																											
GPX	-0,44	0,99	0,39	-0,65	1,00																										
APX	0,43	-0,99	-0,40	0,64	-1,00	1,00																									
SOD1	0,88	-0,11	0,93	0,75	0,03	-0,04	1,00																								
SOD2	0,84	-0,01	0,96	0,68	0,12	-0,13	1,00	1,00																							
SOD3	0,58	0,35	1,00	0,37	0,47	-0,48	0,89	0,93	1,00																						
SOD4	0,44	0,49	0,97	0,21	0,61	-0,62	0,81	0,86	0,99	1,00																					
SOD5	-0,69	0,99	0,10	-0,84	0,95	-0,95	-0,27	-0,18	0,19	0,34	1,00																				
SOD6	-0,18	0,91	0,63	-0,41	0,96	-0,96	0,30	0,39	0,70	0,80	0,83	1,00																			
CHIT 66kDa	0,21	0,70	0,88	-0,04	0,79	-0,79	0,64	0,71	0,92	0,97	0,57	0,93	1,00																		
CHIT 42 kDa	-0,67	-0,24	-1,00	-0,47	-0,37	0,38	-0,94	-0,97	-0,99	-0,96	-0,08	-0,62	-0,87	1,00																	
CHIT 21 kDa	-0,83	0,93	-0,11	-0,94	0,87	-0,87	-0,47	-0,38	-0,02	0,14	0,98	0,70	0,38	0,13	1,00																
BasCHIT A	-0,77	0,96	-0,01	-0,90	0,92	-0,91	-0,38	-0,29	0,08	0,24	0,99	0,77	0,47	0,03	0,99	1,00															
BasCHIT B	-0,99	0,68	-0,53	-1,00	0,57	-0,57	-0,80	-0,74	-0,45	-0,30	0,79	0,32	-0,05	0,55	0,90	0,85	1,00														
BasCHIT C	-0,74	-0,15	-0,99	-0,55	-0,28	0,29	-0,97	-0,99	-0,98	-0,93	0,02	-0,54	-0,81	1,00	0,23	0,13	0,63	1,00													
AcCHIT a	-0,01	-0,82	-0,76	0,24	-0,89	0,90	-0,47	-0,55	-0,82	-0,90	-0,72	-0,98	-0,98	0,75	-0,56	-0,64	-0,15	0,68	1,00												
AcCHIT b	-0,89	0,13	-0,92	-0,76	0,00	0,01	-1,00	-0,99	-0,88	-0,80	0,29	-0,28	-0,62	0,93	0,49	0,40	0,82	0,96	0,45	1,00											
AcCHIT c																															
AcCHIT d																															
GLU 100 kDa	-0,37	-0,56	-0,95	-0,14	-0,67	0,67	-0,76	-0,82	-0,97	-1,00	-0,41	-0,85	-0,98	0,94	-0,22	-0,31	0,23	0,90	0,93	0,75			1,00								
GLU 65 kDa	1,00	-0,50	0,70	0,95	-0,38	0,37	0,91	0,87	0,64	0,50	-0,64	-0,11	0,27	-0,72	-0,78	-0,72	-0,98	-0,78	-0,08	-0,92			-0,44	1,00							
GLU 50 kDa	1,00	-0,57	0,64	0,97	-0,46	0,45	0,88	0,83	0,57	0,43	-0,70	-0,19	0,19	-0,65	-0,83	-0,78	-0,99	-0,73	0,01	-0,89			-0,36	1,00	1,00						
GLU 16 kDa	0,67	0,24	1,00	0,47	0,37	-0,38	0,94	0,97	0,99	0,96	0,07	0,61	0,86	-1,00	-0,14	-0,04	-0,55	-1,00	-0,75	-0,93			-0,94	0,72	0,66	1,00					
GLU 12 kDa	0,64	-0,99	-0,16	0,81	-0,97	0,97	0,21	0,12	-0,25	-0,40	-1,00	-0,87	-0,62	0,14	-0,96	-0,99	-0,75	0,04	0,76	-0,23			0,47	0,59	0,66	-0,14	1,00				
AcGLU 1	-0,71	0,98	0,08	-0,86	0,95	-0,94	-0,30	-0,21	0,16	0,32	1,00	0,82	0,54	-0,05	0,98	1,00	0,81	0,04	-0,70	0,32			-0,39	-0,66	-0,72	0,05	-1,00	1,00			
AcGLU 2	-0,99	0,43	-0,75	-0,92	0,31	-0,30	-0,94	-0,91	-0,70	-0,57	0,58	0,03	-0,35	0,77	0,73	0,66	0,96	0,83	0,15	0,95			0,51	-1,00	-0,99	-0,77	-0,52	0,60	1,00		
AcGLU 3	0,53	-1,00	-0,30	0,72	-0,99	0,99	0,07	-0,02	-0,38	-0,53	-0,98	-0,93	-0,72	0,28	-0,92	-0,95	-0,65	0,18	0,84	-0,10			0,59	0,47	0,54	-0,27	0,99	-0,97	-0,40	1,00	
AcGLU 4	-0,29	-0,63	-0,92	-0,05	-0,73	0,74	-0,70	-0,77	-0,95	-0,99	-0,49	-0,89	-1,00	0,91	-0,30	-0,40	0,14	0,86	0,96	0,69			1,00	-0,35	-0,27	-0,90	0,55	-0,47	0,43	0,66	1,00

Legend: Data represents Pearson correlation coefficients between the variables. Coloured cells indicate a strong positive (green cells, $r \geq 0,7$) or negative (brown cells, $r \leq -0,7$) relationship between the given variables. MDA = malondialdehyde, CAT = catalase, GPX = guaiacol peroxidase, APX = ascorbate peroxidase, SOD = superoxide dismutase, CHIT = total chitinase, BasCHIT = basic chitinase, AcCHIT = acidic chitinase, GLU = total β-1,3-glucanase, AcGLU = acidic β-1,3-glucanases.

Several researches suggest that increased SOD activity often correlates with increased plant tolerance to environmental stressors (**Sharma *et al.*, 2012**). In our results, increased SOD activity in young leaves may indicate a higher ability of young tissues to tolerate cadmium. A sign of such tolerance may also be the co-induction of SOD with several isoforms of β-1,3-glucanases, which are also characterized by increased accumulation, especially in young leaves and, conversely, reduced in mature leaves (Fig 5).

Table 3 Correlation matrix between the reaction of tested parameters on applied Cd concentrations in the young leaves of the selected soybean cultivar.

young leaves	MDA	viability	proline	CAT	GPX	APX	SOD1	SOD2	SOD3	SOD4	SOD5	SOD6	CHIT 66	CHIT 42	CHIT 21	BasCHIT A	BasCHIT B	BasCHIT C	AcCHIT a	AcCHIT b	AcCHIT c	AcCHIT d	GLU 100 kDa	GLU 65 kDa	GLU 50 kDa	GLU 16 kDa	GLU 12 kDa	AcGLU 1	AcGLU 2	AcGLU 3	AcGLU 4
MDA	1,00																														
viability	-0,85	1,00																													
proline	0,93	-0,98	1,00																												
CAT	0,91	-0,55	0,70	1,00																											
GPX	0,98	-0,73	0,84	0,97	1,00																										
APX	0,89	-1,00	0,99	0,62	0,78	1,00																									
SOD1	0,46	-0,86	0,75	0,05	0,27	0,81	1,00																								
SOD2	-0,31	-0,23	0,05	-0,68	-0,50	0,15	0,70	1,00																							
SOD3	-0,82	0,40	-0,56	-0,98	-0,92	-0,48	0,13	0,80	1,00																						
SOD4	-0,58	0,06	-0,25	-0,87	-0,73	-0,15	0,46	0,96	0,94	1,00																					
SOD5	-0,48	-0,05	-0,13	-0,80	-0,65	-0,03	0,56	0,98	0,89	0,99	1,00																				
SOD6	-0,78	0,99	-0,95	-0,45	-0,64	-0,98	-0,91	-0,34	0,29	-0,05	-0,17	1,00																			
CHIT 66kDa	-0,25	-0,30	0,12	-0,63	-0,44	0,22	0,75	1,00	0,76	0,93	0,97	-0,41	1,00																		
CHIT 42 kDa	-0,77	0,32	-0,48	-0,96	-0,88	-0,39	0,22	0,85	1,00	0,97	0,93	0,20	0,81	1,00																	
CHIT 21 kDa	0,94	-0,98	1,00	0,72	0,86	0,99	0,73	0,02	-0,59	-0,27	-0,16	-0,95	0,09	-0,51	1,00																
BasCHIT A	-0,28	0,74	-0,61	0,15	-0,08	-0,68	-0,98	-0,82	-0,32	-0,62	-0,71	0,81	-0,86	-0,40	-0,58	1,00															
BasCHIT B	0,40	0,14	0,05	0,75	0,58	-0,05	-0,63	-1,00	-0,85	-0,98	-1,00	0,25	-0,99	-0,90	0,07	0,77	1,00														
BasCHIT C	0,73	-0,26	0,43	0,95	0,85	0,34	-0,28	-0,88	-0,99	-0,98	-0,95	-0,14	-0,85	-1,00	0,45	0,46	0,92	1,00													
AcCHIT a	0,07	-0,58	0,42	-0,35	-0,14	0,51	0,92	0,93	0,51	0,77	0,84	-0,67	0,95	0,59	0,40	-0,98	-0,89	-0,64	1,00												
AcCHIT b	-0,97	0,71	-0,83	-0,98	-1,00	-0,77	-0,25	0,52	0,93	0,75	0,67	0,62	0,46	0,89	-0,84	0,05	-0,60	-0,86	0,16	1,00											
AcCHIT c	0,38	-0,81	0,69	-0,03	0,19	0,76	1,00	0,76	0,21	0,53	0,62	-0,87	0,80	0,30	0,67	-0,99	-0,69	-0,36	0,95	-0,17	1,00										
AcCHIT d	-0,02	-0,51	0,34	-0,44	-0,22	0,43	0,88	0,96	0,58	0,83	0,89	-0,60	0,97	0,66	0,31	-0,95	-0,92	-0,70	1,00	0,25	0,91	1,00									
GLU 100 kDa																															
GLU 65 kDa	0,70	-0,97	0,91	0,34	0,54	0,95	0,96	0,46	-0,17	0,18	0,29	-0,99	0,52	-0,08	0,90	-0,88	-0,37	0,02	0,76	-0,52	0,93	0,70		1,00							
GLU 50 kDa	-1,00	0,89	-0,96	-0,87	-0,96	-0,93	-0,53	0,24	0,77	0,51	0,41	0,83	0,17	0,71	-0,97	0,36	-0,33	-0,67	-0,15	0,95	-0,46	-0,06		-0,76	1,00						
GLU 16 kDa	-1,00	0,86	-0,94	-0,90	-0,98	-0,90	-0,48	0,30	0,81	0,57	0,47	0,79	0,23	0,76	-0,95	0,30	-0,39	-0,71	-0,08	0,97	-0,40	0,00		-0,71	1,00	1,00					
GLU 12 kDa	-0,82	0,40	-0,56	-0,98	-0,92	-0,48	0,13	0,80	1,00	0,94	0,89	0,29	0,76	1,00	-0,58	-0,32	-0,85	-0,99	0,51	0,93	0,21	0,59		-0,17	0,77	0,81	1,00				
AcGLU 1	-0,94	0,62	-0,75	-1,00	-0,99	-0,68	-0,13	0,62	0,97	0,82	0,75	0,53	0,56	0,94	-0,77	-0,07	-0,69	-0,92	0,28	0,99	-0,05	0,36		-0,42	0,91	0,93	0,97	1,00			
AcGLU 2	-0,58	0,92	-0,84	-0,19	-0,41	-0,89	-0,99	-0,59	0,02	-0,33	-0,43	0,96	-0,64	-0,08	-0,82	0,94	0,51	0,14	-0,85	0,38	-0,97	-0,80		-0,99	0,65	0,60	0,01	0,27	1,00		
AcGLU 3	-0,99	0,90	-0,97	-0,86	-0,95	-0,94	-0,56	0,21	0,76	0,49	0,38	0,85	0,14	0,69	-0,97	0,38	-0,30	-0,65	-0,18	0,94	-0,48	-0,09		-0,77	1,00	1,00	0,75	0,90	0,67	1,00	
AcGLU 4	-1,00	0,86	-0,94	-0,91	-0,98	-0,90	-0,47	0,31	0,82	0,57	0,47	0,79	0,24	0,76	-0,95	0,29	-0,40	-0,72	-0,08	0,97	-0,39	0,01		-0,71	1,00	1,00	0,82	0,94	0,59	0,99	1,00

Legend: Data represents Pearson correlation coefficients between the variables. Coloured cells indicate a strong positive (green cells, $r \geq 0,7$) or negative (brown cells, $r \leq -0,7$) relationship between the given variables. MDA = malondialdehyde, CAT = catalase, GPX = guaiacol peroxidase, APX = ascorbate peroxidase, SOD = superoxide dismutase, CHIT = total chitinase, BasCHIT = basic chitinase, AcCHIT = acidic chitinase, GLU = total β-1,3-glucanase, AcGLU = acidic β-1,3-glucanases.

β-1,3-glucanases and chitinases belong to the PR-proteins, which present an important component of plant defense against various environmental stresses (**Békésiová *et al*., 2008; Konotop *et al*., 2012; Zur *et al*., 2013; Gregorová *et al*., 2015**), and also play an important role in normal growth and development (**Kumar *et al*., 2018**). Many authors have analyzed stress factors that induce β-1,3-glucanase expression in plants (**Zhu *et al*., 1994; Suo and Leung, 2001; Balasubramanian *et al*., 2012; Torres *et al*., 2015; Zieliński *et al*., 2021**). In most of the previous works, the anti-pathogenic functions of these enzymes have been studied. Recently, however, several studies have demonstrated the importance of β-1,3-glucanases in the defense of plants against abiotic stresses, such as e.g. drought (**Gregorová *et al*., 2015**), low temperature (**Goñi *et al*., 2011; Zur *et al*., 2013**) and heavy metals (**Piršelová *et al*., 2011; Bardáčová *et al*., 2016; Su *et al*., 2016**). We detected 5 isoforms of total β-1,3-glucanases and 4 isoforms of acidic/neutral β-1,3-glucanases in soybean leaves. Moreover, we noted differences in the activity of these isoforms under the effect of cadmium between mature and young leaves. In general, we observed an increase in β-1,3-glucanase activity in young soybean leaves and contrariwise, a predominant decrease in β-1,3-glucanase activity in mature leaves. The number and activity of β-1,3-glucanase isoforms in individual plant parts depends on many factors, mainly on plant species, cultivar, sensitivity, developmental stage as well as the type, concentration and duration of exposition to the toxic metals and multifunctional character of individual isoforms in plant tissues (**Piršelová *et al*., 2011; Piršelová *et al*., 2012; Bardáčová *et al*., 2016**). Variability in β-1,3-glucanase activity may be related to plant diversification due to domestication, natural hybridization, and allopolyploidy (**Moravčíková *et al*., 2016**). A higher number of β-1,3-glucanase isoforms in the later developmental stages of plants may be related to reversible callose deposition and growth degradation (**Gregorová *et al*., 2015; Moravčíková *et al*., 2016**).

In our research, we identified three fractions of chitinases when size-separated, and some further isoforms when separated based on charge. These included two basic/neutral chitinases in roots and three in leaves as well as two acidic/neutral fractions in mature leaves and four such isoforms in roots and young leaves. Their activity showed much greater variability in response to the cadmium concentrations and also depending on the tested tissue, and was, therefore, less homogenous than the activity of SOD or β-1,3-glucanases. The overall activity of chitinases in cadmium-stressed plants is generally increasing (**Metwally *et al*., 2005; Békésiová *et al*., 2008**), but little is known about how individual isoforms behave and why their accumulation differs due to heavy metal in different organs of the same plant. Similar chitinase pattern has already been observed by **Gálusová *et al*. (2015)** in two soybean genotypes exposed to cadmium and arsenic. Higher total activity of acidic/neutral chitinase isoforms have already been identified in more sensitive pea cultivar (**Metwally *et al*., 2005**) and, interestingly, higher number of these isoforms was identified in soybean cultivar Kyivska 98 plant sprouts (**Mészáros *et al*., 2014**). In the work of **Gálusová *et al*. (2015)** the enzymatic profile of chitinases in soybean consisted of five individual isoforms, differing between organs and between leaves of different ages. They pointed out that the chitinase activity in leaves tends to decrease upward to the top of the plant, which confirms our observations of generally lower (or even suppressed) chitinase activity in young leaves compared to mature leaves.

Our results may indicate that younger leaves are better protected either by activation of several defense proteins (Fig 2a, 2b, 3b, 4b, 4d, 5b) and/or restriction of metal accumulation (**Gálusová *et al*., 2015**). In mature leaves, many enzymes are suppressed because they appear to be inhibited, degraded, or are totally missing (Fig 3b, 4b, 4d, 5b). The differences between mature and young leaves are probably related to the acceleration of leaf senescence in mature leaves due to stress and degradation of many compounds as well as loss of scavenging potential against ROS (**Kanojia *et al*., 2020; Guo *et al*., 2021; Rankenberg *et al*., 2021**). Strong correlations are observable between many of the tested parameters. Interesting relationships are shown between the activities of individual enzyme isoforms, however there are large differences (a lot of contradictory tendencies) between organs (Tab 1,2,3); the data indicate the interplay between certain defense components and possible involvement of common regulatory mechanisms.

CONCLUSION

Plants exert differences in defense strategies against Cd depending on plant organs as well as developmental stages. PR proteins and other components of plant defense are subject to regulation depending on the concentration of metal but apparently also by internal signals. Several of these enzymes seem to act oppositely as observed under abiotic stress for these enzymes previously. These might link the defense strategy to the availability of resources and/or developmental related cues (e.g. senescence). The single time point in single tissue provides a narrow view on plant defense activities; a more detailed analysis at the whole plant level might reveal the nature of components (timing of their action) that are crucial for tolerance. It is obvious that we cannot evaluate the stress response of plants on the basis of one specific time and tissue, but we have to take into consideration also the developmental stage of the plant, its physiological state and many other internal and external factors. From the point of view of correctness and accurate interpretation, this is especially important when comparing stress responses between varieties.

Acknowledgments: This work was supported by the Slovak Research and Development Agency under the contract No. APVV-18-0154 and by grant from the Scientific Grant Agency of the Ministry of Education of Slovak republic VEGA No. 1/0073/20.

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