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Variable accumulation of cadmium in flax (*Linum usitatissimum* L.)

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Abstract

Given the potential use of flax in metal-contaminated soil remediation programs, the uptake and accumulation of cadmium was studied in five varieties of flax (*Linum usitatissimum* L.) including var. Belinka, Escalina, Jitka, Marina and Krasnoder. Early stage plants in hydroponics were exposed to Cd to assess their tolerance by means of growth parameters and content of photosynthetic pigments. All varieties exerted tolerance indexes in the range of 63 – 89 %, while oxidative stress was not detected in either variety. Chitinase enzymes were analyzed in leaf protein extracts since activities of these enzymes have previously been correlated with plant tolerance to Cd. However, total enzyme activities remained unchanged in presence of Cd in all flax varieties. A more detailed analysis of these enzymes identified up to 3 chitinase isoforms upon separation of leaf protein extracts in polyacrylamide gels, and their quantification confirmed responsiveness to Cd for each of them. The obtained data were interpreted in light of metal uptake rate, which we measured using gammaspectrometry in growth media spiked with ^{109}Cd and in the plant tissue. The variety Jitka showed the most sensitive to Cd and accumulating fast and the highest amounts of metal. In contrast, the variety Belinka appeared most tolerant, accumulating the least Cd in a slow rate. Activation of chitinase isoforms correlated with more sensitive varieties and suggests activation of general defense mechanisms. The obtained data suggest the variety Jitka as most promising for phytoremediation programs and the var. Belinka as the most suitable when avoidance of potential health risk is of interest.

Introduction

Crop plants grown in heavy metal (HM) contaminated areas represent a considerable health risk after entering the food chain (Chen *et al.* 2016a). Even in areas with low contamination, metals can cause phytotoxicity and consequently yield losses, affecting livestock or people by consuming large quantities of the produced crops (Guo *et al.* 2020). Though data from the LUCAS Topsoil Survey assign the immense majority of European agricultural land as adequately safe for food production, a continent-wide survey highlighted large areas where precautionary measures are needed, and estimated that 6.24 % of the agricultural land needs local

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assessment and eventual remedial action (Tóth *et al.* 2016). Remediation of soils is, however, extremely costly therefore phytoremediation is currently emphasized as a new, noninvasive and publicly acceptable technology that removes pollutants from the environment or to render them harmless by stabilization. The plants usable for phytoremediation should have very high metal removal rate that depends on the plant biomass harvested and metal concentration in harvested biomass (Sumiahadi and Acar 2018).

Attempts to reduce the Cd pollution evoked deeper research on plant species that can well tolerate and accumulate metals in their tissues. Hyperaccumulating plants have the capacity to concentrate more than 100 $mg.kg^{-1}$ of dry matter (DM) in harvested organs compared to usual species that accumulate 0.05 – 0.2 mg of Cd kg^{-1} of their dry leaves. According to the Global Hyperaccumulator database (http://hyperaccumulators.smi.uq.edu.au/), few species within up to 7 plant families are known to hyperaccumulate Cd. Most of these slow growing plants produce a low biomass in contrast to some other metal-accumulating crop species such as sunflower, maize, tobacco or fast growing trees (poplar, willow). The phytoextraction potential and high biomass production renders the latter interesting in the phytoremediation concept to overcome the mass limitation of hyperaccumulators (Vassilev *et al.* 2002).

Flax (*Linum usitatissimum* L.) has been suggested in phytoremediation strategy by several authors (Bjelková *et al.* 2011; Guo *et al.* 2020) as this species exerted better Cd accumulation and tolerance capacity than some other crops such as cotton and hemp (Angelova *et al.* 2004; Shi and Cai 2009). Furthermore, flax as an annual crop species can remove considerable quantities of heavy metals from the soil every year, has a well elaborated large-scale agrotechnology and plethora of industrial uses (Griga and Bjelková 2013).

Although a protein map of mature flax seeds from the Chernobyl remediation area in Ukraine was prepared (Klubicová *et al.* 2011) and changes in flax protein profiles during growth in the presence of cadmium ions are known (Szalata *et al.* 2009, 2014), the profile and activity of specific groups proteins and enzymes in flax remains unclear. Tolerance of flax to heavy metals (cadmium) is attributed to phytochelatins (Najmanova *et al.* 2012), cadmium-binding proteins (Oomah *et al.* 2007), but also sorption processes of cellulose fibers (Rezić 2013). Furthermore, chitinase enzymes have been shown to respond flexibly to the presence of different types of metals and contribute to tolerance (Metwally *et al.* 2005; Mészáros *et al.* 2013). Identifying biomarkers which could help to identify varieties that are tolerant at the same time accumulate high amounts of metal (cadmium) would be useful for bioremediation programs. The present work was therefore devoted to studying a set of flax varieties bred in Europe and compare their tolerance/Cd uptake rate with previously published results.

Experimental

Plant material and experimental setup

Different varieties of flax (*Linum usitatissimum* L.) – Jitka, Marina, Belinka, Krasnoder and Escalina were provided by the Gene Bank of Slovakia (NAFC, Research Institute of Plant Production, Piešťany, Slovakia). Seeds were surface-sterilized for 5 min. in 0.5 % (v/v) sodium hypochlorite and washed twice in distilled sterile water. After germinating on wet filter paper for 5 days the plants were cultivated in hydroponic conditions in plastic containers with 500 mL of 1/4 Hoagland medium (pH 5.6) (Hoagland 1920) in a growth chamber (KBWF 720, Binder) under 16h/8h photoperiod, photosynthetic photon flux (PPF) level 120 $\mu mol.m^{-2}.s^{-1}$, at 28 ºC (day)/ 18 ºC (night) temperature and 60 % relative humidity. After 10 days a set of plants was exposed to 50 $mg.L^{-1}$ Cd^{2+} (in a form of $CdCl_2$) spiked with ^{109}Cd for the period of 72 h. Another set of plants was further grown under the same conditions in media without Cd. The experiment was repeated 3-times and for each flax variety.

Physiological measurements and oxidative stress

Shoots were removed from roots with scalpel and their length and weight were determined. Tolerance indexes were calculated as average value

of the stressed plant character to average value of the non-stressed plant character and expressed in %.
The content of photosynthetic pigments was determined according to Lichtenthaler and Wellburn (1983). The level of occurring oxidative stress was measured through the level of malondialdehyde (MAD) according to Karabal *et al.* (2003).

Analyses of chitinase enzymes

Crude protein extracts were isolated from 0.2 g flax shoots. The material was homogenized in presence of liquid nitrogen using a laboratory tissue lyzer (Retsch MM 400). To the powderized tissue was added 320 μL of cold extraction buffer consisting of 0.1 mol.L^{-1} sodium acetate (pH 5.2) with 100 mM fenylmethylsulfonylfluorid (Sigma). The samples were vortexed 3-times for 15 s, while during brakes (min. 15 s) keeping on ice. The samples were gradually centrifuged twice at 12,000 rpm at 4 °C for 15 min. The crude extracts were measured for protein content using standard Bradford method, aliquoted and stored at -80 °C.
Total chitinase activity in samples was measured fluorimetrically (Fluoroskan II microtiterplate reader, TITERTEK, Finland) in 20 μL protein extracts mixed with 30 μL of 300 μM 4-methylumbelliferyl-β-D-*N*,*N*´,*N*´´-triacetylchito-trioside in 0.1 M sodium citrate buffer (pH 3.0). After incubation at 37 °C for 1 h the reactions were stopped with 150 μL of 0.2 M Na_2CO_3. The measurement was performed using excitation and emission filters 355 nm/450 nm and the enzyme activity was expressed as picomoles of methylumbelliferone generated per μg of soluble protein per hour.
Chitinase isoforms were detected in protein aliquots (20 μg). Samples were separated in 12.5 % (w/v) SDS-containing polyacrylamide slab gels with 0.01 % glycol chitin as enzyme substrate (Gálusová *et al.* 2015) at a constant voltage of 120 V at 8 °C for 2 h. The samples were not heat-treated prior to loading. The separated proteins in gels were re-naturated in 1 % (v/v) Triton X-100 in 50 mM sodium acetate buffer (pH 5.0) overnight. The gels were washed in sodium acetate buffer without detergent for 1 h and stained with 0.01 % (w/v) Fluorescent Brightener 28 (Pan *et al.* 1991) and UV-illumination. Enzyme profiles were digitally photographed (UVP BioDoc-It System) and quantified using Scion Image software (http://www.scioncorp.com) (Gálusová *et al.* 2015).

Gammaspectrometry

Cadmium uptake and content in plant tissue was measured by gammaspectrometry using a radioisotope ^{109}Cd. The isotope was used to spike the Cd solution in liquid samples of culture medium (Bq.mL^{-1}) and to measure the assimilated metal in washed and dried plant biomass (Bq.g^{-1} tissue). The measurements were implemented using a scintillation gammaspectrometer type 76BP76/3 (Scionix, The Netherland) with well type NaI(Tl) crystal. Calibration of the instrument and calculation of radioactivity were realized using a library of analyzed radionuclides and the program ScintiVision-32 (Ortec, USA).

Results and Discussion

Several fibre flax and linseed varieties have been shown to tolerate Cd in soil containing up to 1 g of metal per kg of soil, while exerting no visible symptoms of toxicity or with minimal growth reduction or developmental aberrations in the stems (Bjelková *et al.* 2011). The plantlets of assayed flax varieties were grown in hydroponic conditions and exposed to short-term Cd stress. We observed no visible symptoms of metal toxicity such as necrosis or chlorosis. The tolerance indexes expressed based of FW data ranged between 63 – 85 % (Fig. 1A), similarly to previous studies with adult plants of some other flax varieties (Shi and Cai 2009; Bjelková *et al.* 2011; Douchiche *et al.* 2012). In contrast, doses of 10, 20 and 40 mg $CdCl_2$ per kg of soil were toxic but sublethal and inhibited flax growth (Hosman *et al.* 2017). The reduction in plant growth in the presence of cadmium is attributed to reduce water absorption due to its osmotic effect, lack of nutrition as a result of ion imbalance, and to decline in many metabolic activities due to its toxicity (Singh *et al.* 2016). Most tolerant plants to Cd appeared here the variety Krasnoder, the growth of which was

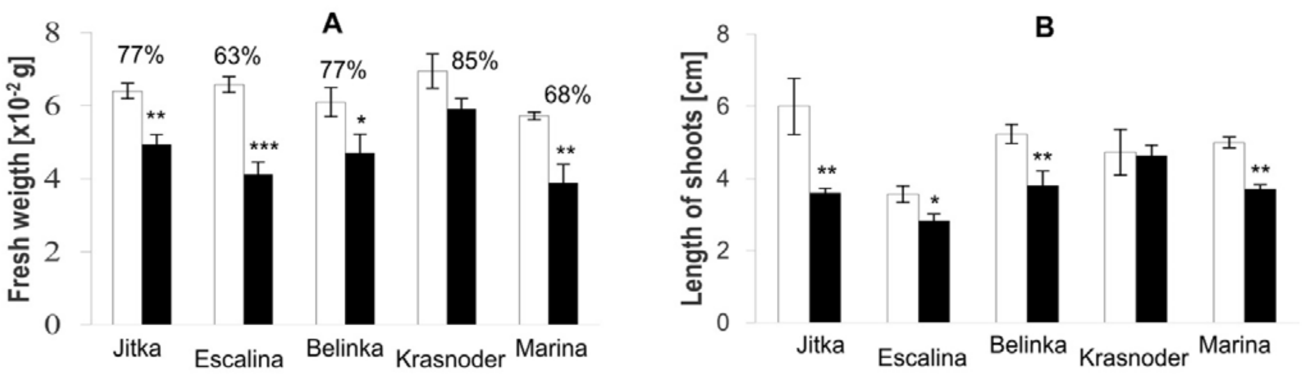


Fig. 1. Effect of cadmium on growth parameters of the tested flax varieties by means of fresh weight (**A**) and length of shoots (**B**). Values above columns indicate tolerance indexes. Data are given for control (white columns) and Cd-treated plants (black columns) and represent means ± SE (n = 3). Asterisks indicate significant effect of the metal at $P \leq 0.05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***).

unaffected also in regard of shoot length (Fig. 1B). On the contrary, the variety Escalina appeared the least tolerant to Cd (Fig. 1A), though the shoot length dropped most obviously for the variety Jitka (Fig. 1B). Genotype-dependent sensitivity to Cd has been described for flax (Bjelková *et al.* 2011; Saastamoinen *et al.* 2013; Praczyk *et al.* 2015) but also other species (Socha *et al.* 2015).

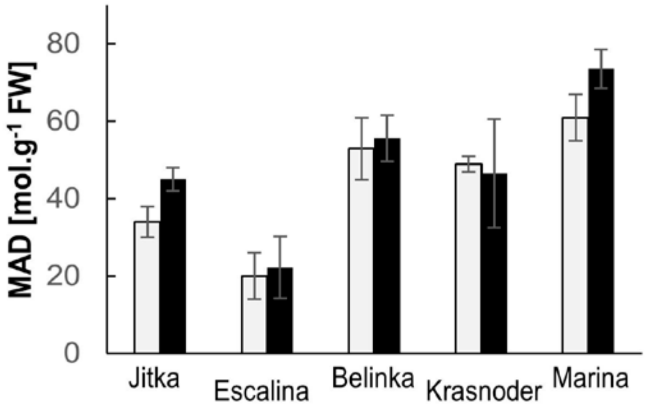
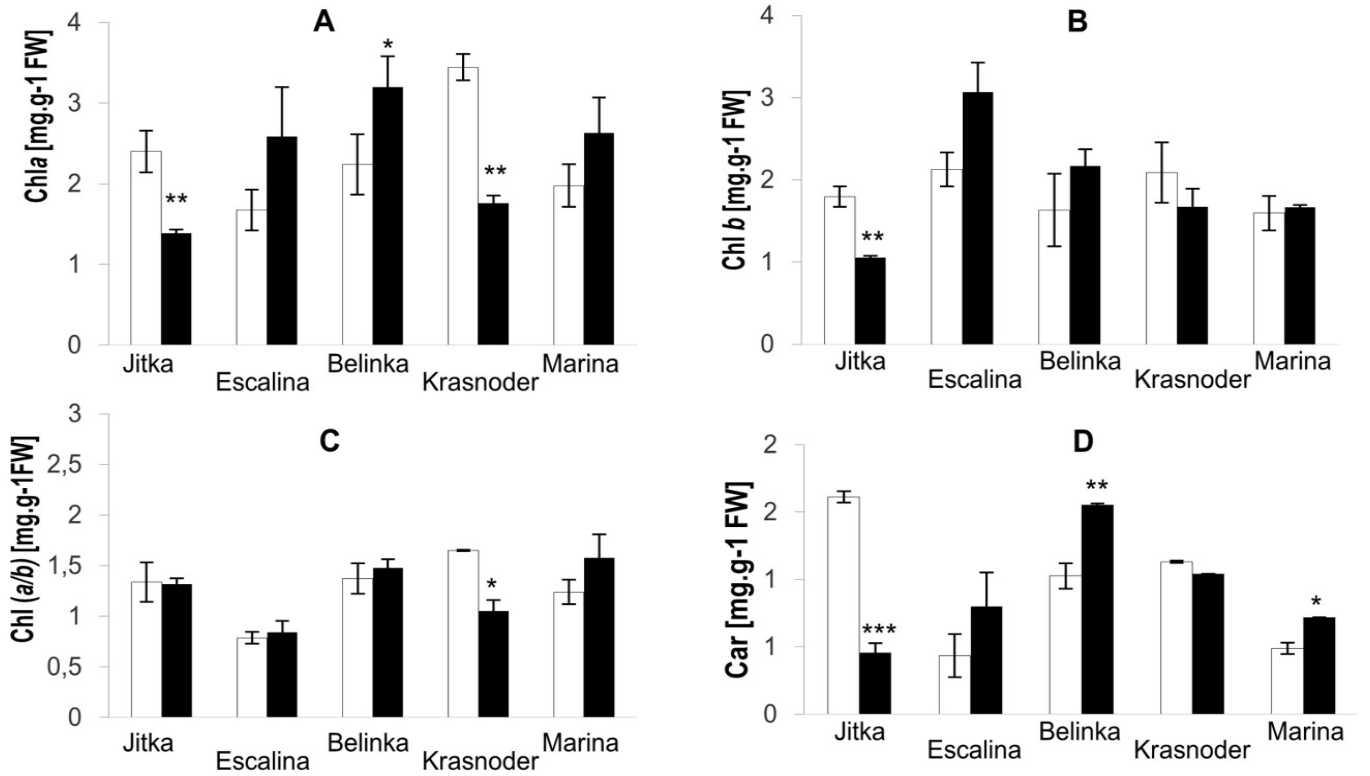


Fig. 2. Effect of cadmium on content of malondialdehyde as indicator of oxidative stress in the tested flax varieties. Data are given for control (white columns) and Cd-treated plants (black columns) and represent means ± SE (n = 3).

The shoots were analyzed for content of MAD as measure of ongoing oxidative stress. Basic levels significantly differed among the varieties (Fig. 2). It has been speculated that higher basic levels of peroxidation presume an a priori more efficient detoxification mechanism against metal stress and/or might lead to a ‘‘standby’’ state of defense for a faster and/or stronger response in the more tolerant cultivars (Mészáros *et al.* 2013). The data, however, revealed minor alterations in MAD levels in either of the flax cultivars, indicating to efficient antioxidative system and/or tolerance to Cd. Noteworthy, MAD content and rate of membrane peroxidation have been reported to depend on Cd concentration in the flax var. Vicking (Belkadhi *et al.* 2010).

Photosynthetic pigment contents

The contents of pigments in the (control) leaves of flax varieties varied (Fig. 3). According to Mitchell and Bakker (2014), the emergence of such variability is driven by plasticity and plant ontogenesis rather than adaptation to the environment. The mentioned authors confirmed the intraspecific variability of the content of photosynthetic pigments in *Hypochaeris radicata*, as well as its impact on the resistance to abiotic stress such as drought or light. The importance of intraspecific variability in plant functional properties in structuring communities and managing ecosystem processes is becoming increasingly important, but the mechanisms governing the ongoing changes are still unknown. The most significant negative impact of Cd on chlorophyll *a* content was in the variety Krasnoder (Fig. 3A); it points to damage to photosystems I and II. In contrast, the stressed variants in the Belinka and Escalina varieties contained more chlorophyll *a* than the corresponding controls (P < 0.05). Increased chlorophyll levels have previously been observed as a consequence of mild drought (Avramova *et al.* 2012), and by some others as a consequence



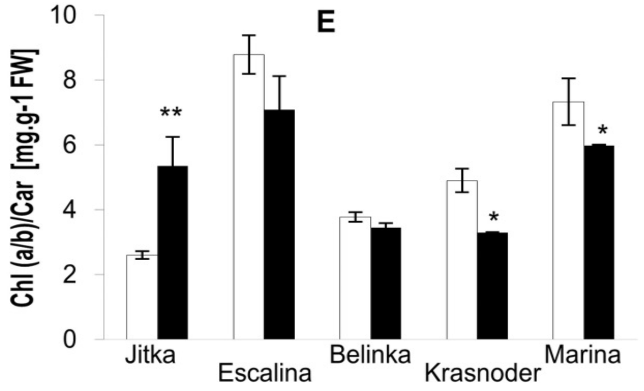


Fig. 3. Effect of cadmium on photosynthetic parameters of the tested flax varieties. Indicated are data for content of chlorophyll *a* (**A**), chlorophyll *b* (**B**), their ratio Chl*a*/Chl*b* (**C**), content of carotenoids (**D**) and ratio of Chl*a*/Chl*b* to carotenids (**E**). Data are given for control (white columns) and Cd-treated plants (black columns) and represent means ± SE (n = 3). Asterisks indicate significant effect of the metal at $P \leq .05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***).

of short drought (Parida *et al.* 2007; Nikolaeva *et al.* 2010). In most cases, however, chlorophyll loss occurs in the leaves when exposed to heavy metals (Dobroviczká *et al.* 2013; Maglovski *et al.* 2017). The effect of Cd on the chlorophyll *b* content was recorded only in the Jitka variety (Fig. 3B). This type of pigment is part of the PSII light collection antenna system. Unchanged content of Chl *b* under metal stress was also observed in other plant species like soybean (Dobroviczká *et al.* 2013) and wheat (Maglovski *et al.* 2017).
The ratio of chlorophylls *a*/*b*, which is an indicator of the functionality and light adaptation of the photosynthetic apparatus, was influenced by cadmium in the variety Krasnoder (Fig. 3C). Such a decrease results from faster degradation of Chl *a* than Chl *b* (Kummerová *et al.* 2010) due to disorganization of chloroplast membranes (Feng *et al.* 2010) and changes in their sizes and densities (Sun *et al.* 2012). Unchanged values of Chl *a*/*b* in the other flax varieties (Fig. 3C) indicate good plant condition and relative tolerance to cadmium.
The content of carotenoids in individual varieties of flax was also variable, while the highest content was detected in the variety Jitka (Fig. 3D). This parameter is genetically determined and may indicate a better defense against stress (Chen *et al.* 2016b); this assumption, however, is not supported here by growth parameters. Under Cd stress, we recorded an increased content of *Car* in the varieties Belinka (P < 0.01), Marina (P < 0.05) and Escalina (P < 0.05), in contrast to

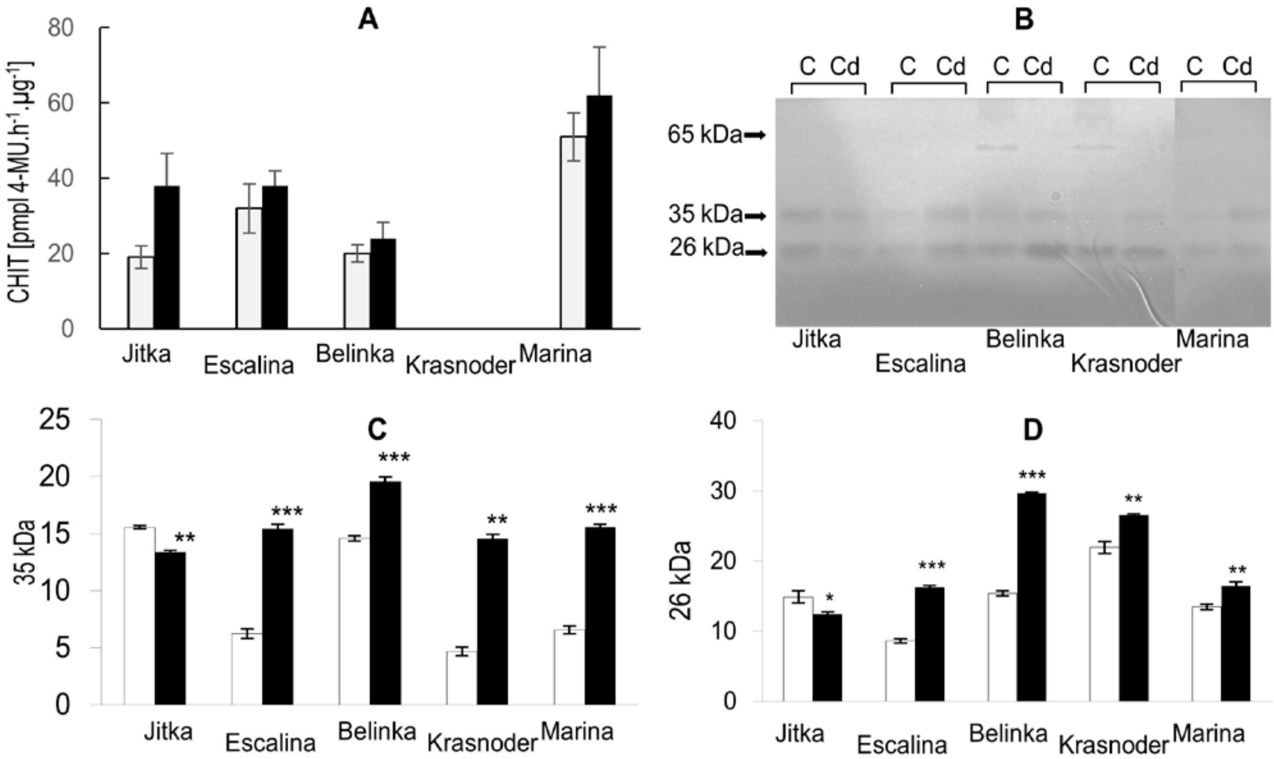


Fig. 4. Effect of cadmium on chitinase enzymes in the tested flax varieties. Total chitinase activity (CHT) was measured fluorimetrically (**A**). The activity of isoforms separated in polyacrylamide gels (**B**) was quantified. Integrated densities of bands were obtained for the isoform of 35 kDa (**C**) and 26 kDa (**D**). Data are given for control (white columns) and Cd-treated plants (black columns) and represent means ± SE (n = 3). Asterisks indicate significant effect of the metal at $P \leq 0.05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***).

the variety Jitka in which the content of *Car* decreased (Fig. 4D). Increased *Car* levels have also been reported by Chaneva *et al.* (2010) and may reflect the involvement of compensation mechanisms with a likely short effect in order to obtain resources. Nevertheless, a decrease in *Car* content in presence of heavy metals has been reported more frequently (Gálusová *et al.* 2015).
The carotenoid content was not affected in the individual varieties to the same extent as the chlorophyll contents, in agreement with the observations of Stoeva *et al.* (2005). The weight ratio of chlorophylls *a* and *b* to total carotenoids $(a + b)/(x + c)$, as an indicator of the "greenness" of the plant, was deceased by Cd in the Krasnoder and Marina varieties (both at $P < 0.05$). Conversely, increasing the ratio in the Jitka variety ($P < 0.01$) is likely a form of a compensatory mechanism for obtaining resources under stress (Fig. 3E).
Though changes in pigment concentrations and their ratios are a good indicator of stress effects on plants (Maglovski *et al.* 2019), their contents are affected by a number of internal factors as well, including genotype and leaf type (Dobroviczká *et al.* 2013), age and type of leaf tissue (Hédiji *et al.* 2010). The differences caused by cadmium are attributed to variability in the accumulation of glutathione and phytochelatins, which chelate and/or detoxify the metal (Gaudet *et al.* 2011).

Activities of defense enzymes – chitinases

The total activity of chitinase enzymes was different for individual varieties of flax (Fig. 4A), as already described for peas (Metwally *et al.* 2005) or soybeans (Socha *et al.* 2015). However, we did not notice any significant changes in the presence of Cd in any of the varieties. Since the total activity of chitinases is the result of the action of several enzyme isoforms, we separated the flax protein extracts in polyacrylamide gels. The chitinase enzyme profile in flax has not been studied in more detail in relation to the response to heavy metals. We detected the presence of two protein fractions

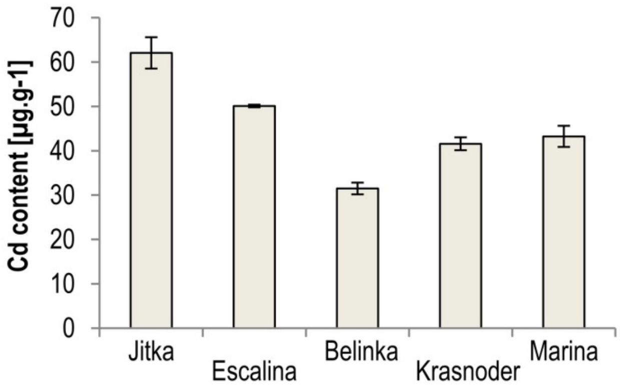


Fig. 5. Content of cadmium in the roots of the tested flax varieties measured by gammaspectrometry using spiked ^{109}Cd. Data represent means ± SE (*n* = 3).

with chitinolytic activity of ~ 26 and 35 kDa, in all tested flax varieties (Fig. 4). In addition to them, we also detected an isoform of chitinases of ~ 65 kDa in the Belinka and Krasnoder varieties (Fig. 4B). By quantifying the intensity of the activities of the individual fractions, we found that all isoforms were significantly responsive to cadmium ions, but not in the same way or to the same extent.

The ~ 35 kDa chitinase activity was inhibited in the Jitka variety by Cd, while in all other varieties it was induced (Fig. 4C). This induction was most pronounced (almost threefold) in the Krasnoder variety. The trend of the activities of the isoform ~ 26 kDa was the same (Fig. 4D), but the most significant induction was observed in the variety Belinka (almost double). The ~ 65 kDa isoform was detectable only in control plants of the Belinka and Krasnoder varieties, but due to cadmium its activity fell below the level of detectability (Fig. 4D). The obtained results confirmed the high responsiveness of flax chitinases to the given concentration of cadmium, but we did not notice any change in their profiles (Békésiová *et al.* 2008), nor differences in profiles between varieties (Mészáros *et al.* 2013).

Dynamics of uptake and accumulation of Cd

We observed differences in the accumulation of Cd in the dry matter of young plants of different varieties of flax. The highest amounts were determined after 10 days of cultivation in medium with ^{109}Cd in the variety Jitka and Escalina, on the contrary the least in the variety Belinka (Fig. 5). Previously, variable Cd accumulation in four soybean cultivars has been reported (Bardáčová *et al.* 2017) and shown to coincide with tolerance to metal stress, nevertheless, metal accumulation and tolerance should be considered as independent traits (Goolsby and Mason 2015).

The high Cd intake of these varieties is relatively fast during the first three days. On the contrary, the low metal accumulation of the Belinka variety is associated with the relatively slowest intake

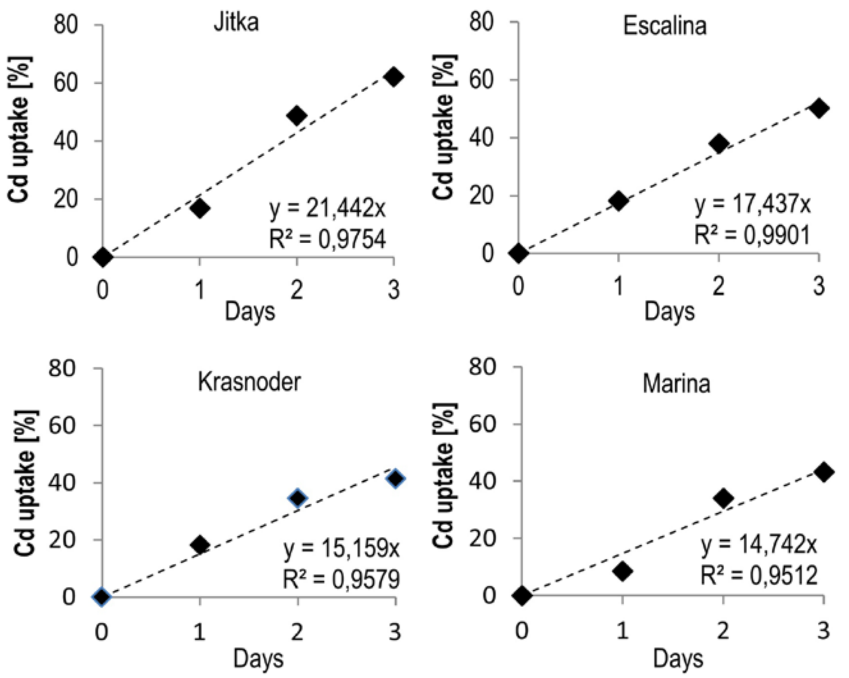


Fig. 6. Uptake of cadmium (%) by roots of the tested flax varieties during first three days of experiment. Data were measured by gammaspectrometry using spiked ^{109}Cd and are presented as average values (*n* = 3).

(Fig. 6). Linear Cd depletion rate from media has been described previously especially at low concentrations of metals and likely refers to combination of both apoplastic Cd flow towards the xylem through channels or transporters and symplastic uptake by roots (Hu *et al.* 2013). However, metal uptake by roots may show specific kinetics and rates depending on the concentration of metal in the environment, soil characteristics (e.g. presence of various adsorbents or chelating agents), but also on plant defense and sequestration strategies (Pipíška and Remenárová 2014; Bardáčová *et al.* 2017).

Plant strategies in metal uptake and allocation are key to their survival. High metal intake in the Jitka variety correlated with suppressed growth (Fig. 1) and reduced content of individual pigments (Fig. 3). These results are consistent with a good transfer of Cd to the aboveground parts of this variety (Bjelková *et al.* 2011). At the same time, both detected isoforms of chitinases were inhibited in the Jitka variety (Fig. 4). This may indicate that metabolic processes (including enzyme synthesis) have been adversely affected by metal (Békésiová *et al.* 2008). Therefore, we evaluate the Jitka variety as the most sensitive to Cd among the tested flax varieties.

In contrast, the Belinka and Krasnoder varieties accumulated relatively low amounts of Cd, had less inhibited growth or pigment content (Fig. 1 and 3, respectively) and were characterized by a strong induction of both chitinase enzymes (Fig. 4). Interestingly, complete inhibition of the 65 kDa isoform was also detected in these varieties. This suggests the specificity of regulation, as well as the different roles of individual chitinases in the defense of flax against cadmium toxicity. The Belinka and Krasnoder varieties are the most tolerant within the selected set of flax varieties. In this study, they accumulated even less Cd than the Escalina variety, which in a previous study (Bjelková *et al.* 2011) accumulated up to 4 to 5-fold lower amount Cd (per ha per season as related to particular soil Cd concentration) than the other 10 varieties, including the var. Jitka.

The differences detected among varieties were relatively stronger compared to studies performed on field trials in naturally contaminated soils which rarely exceeded 0.5 – 1 mg Cd kg^{-1} soil (Grant *et al.* 2000). For example Guo *et al.* (2020) examined 18 different flax and linseed cultivars in a field trial for Cd concentrations in plant tissues but they recognized very small differences. Though it is assumed that genotype effects turn more obvious with high concentration of Cd in soil, they still were lower than expected also in soil containing more than 10 mg Cd kg^{-1} (Bjelková *et al.*, 2011). This suggests higher sensitivity of young plants. At the same time, bioavailability of the contaminating metal depends on many soil factors which likely restrict the uptake kinetics, and provides time to activate defense mechanisms to cope stress despite of long exposure and higher total uptake. In addition, seasonal changes have to be considered as well since all these factors are believed to over the effect of genotype itself (Marquard *et al.* 1990; Grant *et al.* 2000; Guo *et al.* 2020).

Conclusions

Of the flax varieties examined we recognized the variety Jitka as best Cd accumulator, confirming the results of previous studies. In addition to impact of Cd on pigment content, activity of chitinase isoforms appears to coincide with plant tolerance/sensitivity to Cd. Though genotype differences tend to dismiss under field trial, laboratory experiments still can provide a good mean for pre-selection of varieties promising for phytoremediation programs. On the other hand, phloem transport is the basis for Cd redistribution in shoots and phloem is the productive part of flax processed into linen textiles, therefore the safety of raw material reutilization has to be considered. From this point of view the variety Belinka appeared as the most appropriate.

Conflict of Interest

The authors declare that they have no conflict of interest.

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