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# Changes in the contents of amino acids and the profile of fatty acids in response to cadmium contamination in spinach

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## ABSTRACT

Changes of amino acid (AAs) contents (glutamic acid – Glu, aspartic acid – Asp) and fatty acids profile (FAs) in spinach under cadmium (Cd) soil contamination (Cd1 = 30, Cd2 = 60, Cd3 = 90 mg/kg soil) are reported here. Spinach plants were sampled 25, 40, 55 and 75 days after sowing. Growing Cd soil contamination was associated with the strong inhibition of above-ground biomass (23.5–6.3 g dry matter per pot) and with the enhancement of Cd content (0.60–72.38 mg/kg dry matter) in leaves. During 55 days of plant growing the increase of Glu and Asp content was associated with the enhancement of Cd content. The highest accumulation of AAs was determined on Cd3 treatment after 55 days of cultivation. Strong decreases of both AAs were confirmed in the last sampling period for Cd treatments (reduction of Glu content of Cd3 treatment to ca. 64% and Asp content to ca. 72% in contrast to control). The content of saturated fatty acids increased (mainly palmitic acid) and the content of unsaturated fatty acids decreased in spinach aboveground biomass with increasing Cd concentration. Results of multivariate analysis of variance MANOVA showed the significant effect of Cd contamination for FAs metabolism, but the most significant effect was confirmed for plant growing period.

**Keywords**: abiotic stress; heavy metals; peroxidation of lipids; *Spinacia oleracea* L.

Cadmium (Cd) is a heavy metal released into the environment by thermal power and heating plants, metal industries, urban traffic, sewage sludge and phosphate fertilizers (Pavlíková et al. 2002a, Vollmann et al. 2015). Plants have no metabolic requirement for Cd, however it is relatively easy available to plants. A frequent outcome following exposure to Cd is the overproduction of reactive oxygen species, potentially causing oxidative damage in plant cells and thus requiring the intervention of antioxidant defense systems (Sandalio et al. 2001). Cadmium induces oxidative stress in plants by blocking essential functional groups in biomolecules and by indirect mechanisms such as interaction with the antioxidant defense system, disruption of the electron transport chain or induction of lipid peroxidation (Cuypers et al. 2010). It decreases water stress tolerance of plants. The accumulation of Cd in plant tissues caused damages to the photosynthetic apparatus; it inhibited photosynthesis by increasing stomatal and mesophyll resistance to carbon dioxine uptake (Gallego et al. 2012). The reduction in photosynthetic rate led to a limited supply of metabolic energy and therefore to nitrogen (N) assimilation restriction. Nitrogen flow through amino acids can change in response to Cd stress. Plants that were exposed to toxic elements have also been shown to ac-

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cumulate specific amino acid (AAs), which may have beneficial functions and play various roles in plants (Xu et al. 2012b, Pavlíková et al. 2014a).

The visual symptoms of Cd toxicity in plants are chlorosis and necrosis of leaves, browning of roots and cell apoptosis. The chlorosis may be due to Fe deficiency, because Fe binding in spinach is affected by Cd accumulation (Pavlíková et al. 2002b, Martin et al. 2012). Cadmium is chemically similar to certain metal elements, including Fe, Zn, Mn, Mg and Ca, and, therefore, can displace these elements from metalloproteins (Verbruggen et al. 2009, Lux et al. 2010). The elements, such as Fe, Zn and Mn are cofactors of metalloenzymes (for example superoxide dismutase, cytochrome P450) and their contents related to plant defense against oxidative stress (Cakmak 2000).

Cadmium stress induced leaf senescence. During senescence activity of the plant hormones are changed and chlorophyll and proteins are degraded. Declining photosynthesis and continued active export of sugars combine to make senescing tissues increasingly carbon-starved. Amino acids derived from proteolysis are an important source of carbon skeletons. Glucogenic AAs give acetyl-CoA to stress metabolism. Simultaneously lipids are degraded into glycogen and free fatty acids (FAs), from which acetyl-CoA arises by ß-oxidation. Acetyl-CoA is the initial substrate for synthesis of FAs and several AAs. For this reason the aim of this study was to characterize changes in metabolism of transfer AAs and FAs composition in spinach in relationship to plant growing period and to Cd stress.

## MATERIAL AND METHODS

Adaptation of spinach (*Spinacia oleracea* L. cv. Matador) plants to excessive Cd levels in soil was investigated in pot experiment repeated for two years. For this experiment, 20 spinach seeds were sown into plastic pods containing soil mixture as specified below. The plants (10 plants per pot) were cultivated from April to June under natural light and temperature conditions at the experimental hall of the Czech University of Life Sciences Prague, Czech Republic. The water regime was controlled and the soil moisture was kept at 60% MWHC (maximum water-holding capacity).

For cultivation of spinach plants, 5 kg of Chernozem soil ($pH_{KCl}$ = 7.2, $C_{ox}$ = 1.83%, CEC (cation exchange capacity) = 258 $mmol_+$/kg) was thoroughly mixed with 0.5 g N, 0.16 g P, and 0.4 g K applied in the form of ammonium nitrate and potassium hydrogen phosphate for control treatment and with the same amount of nutrients plus cadmium (applied in $Cd(NO_3)_2 \cdot 4\,H_2O$) for treated variants. Three concentrations of Cd (Cd1 = 30, Cd2 = 60, Cd3 = 90 mg/kg) were applied. Each treatment was performed in three replications every year. The presented data are the average of both experimental years. Spinach plants were sampled 25, 40, 55 and 75 days after sowing.

The free amino acids from methanol + $H_2O$ extracts from mature leaves were determined using EZ-faast amino acid analysis procedure (Phenomenex, Santa Clara, USA). Samples were analyzed for AAs contents by GC-MS using the Hewlett Packard 6890N/5975 MSD (Agilent Technologies, Torrance, USA). Samples were separated on a ZB-AAA 10 m × 0.25 mm AA analysis GC column using the constant carrier gas (He) flow (1.1 mL/min) (Pavlík et al. 2012).

For analyses of Cd contents plant samples were decomposed using the dry ashing procedure. The ash was dissolved in 1.5% $HNO_3$. Aliquots of the certified reference material RM NCS DC 73350 poplar leaves (purchased from Analytika, Czech Republic) were mineralized under the same conditions for quality assurance. The Cd concentrations were analyzed by ICP-OES (Varian VistaPro, Varian, Mulgrave, Australia).

Overall content of fatty acids (free and derived from various lipids) was determined after their conversion to respective methylesters (FAMEs). Samples of fresh biomass (~0.2 g) were extracted by 2 mL of $CH_3OH$ + $CHCl_3$ (3:2, v/v) on a shaker for 24 h. Acid catalysed transesterification of FAs with acetylchloride according to the method of Stránský and Jursík (1996) was carried out. The content of FAMEs was measured by GC-MS (Thermo Scientific DSQ II Single QuadrupoleGS-MS, Thermo Fisher Scientific, Waltham, USA) with a nonpolar column Zebron ZB-5 30 m × 0.25 mm × 0.25 μm (Zemanová et al. 2015). FAs were determined in biomass sampled after 25 and 55 days of plant growing. For the lack of biomass it was not possible to determine FAs in Cd3 treatment. Individual FAMEs were identified by their mass spectra fragmentation as well as their coelution with synthetic standards (Supelco 37). The percentage of saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs) was compared for control and treated plants.

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The statistical analyses were performed using multivariate analysis of variance (MANOVA) with multivariate *F*-value (Wilks' lambda). A MANOVA was applied to identify the effect of treatments and growing period and their interactions as independent variables, and contents of Cd, yield of biomass, free AAs and FAs as dependent variables. A MANOVA was followed by the post-hoc comparison Tukey's test ($P < 0.05$). All analyses were performed with Statistica 12.0 software (StatSoft, Tulsa, USA).

## RESULTS AND DISCUSSION

The results of the pot experiment revealed the toxic effect of Cd on spinach plants. Plant response to excessive Cd content in soil was assessed on the basis of a decreased spinach dry matter and increased concentrations of this elements in the aboveground biomass (Figures 1 and 2). Growing Cd doses (from 30–90 mg Cd/kg soil) were associated with strong inhibition of the aboveground biomass (23.5–6.3 g per pot after 75 days) and with enhancement of Cd content (0.60–72.38 mg/kg after 75 days) in leaves. Compared to the untreated control, the biomass yield of Cd3 treatment was reduced to ca. 27% while the Cd content in aboveground biomass was enhanced up to 120-fold. No significant differences in the biomass yields and Cd contents in plants were observed between individual experimental years. Our data correspond with those by Pavlík et al. (2010), Pavlíková et al. (2014a) who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. The damage caused by Cd led to senescence and to the bleaching of chlorophylls at Cd3 treatment. Magnesium in chlorophyll is replaced with Cd (Küpper et al. 1998). The visual symptoms of Cd toxicity – chlorosis and necrosis of leaves were confirmed for example by Pavlíková et al. (2008) and Martin et al. (2012).

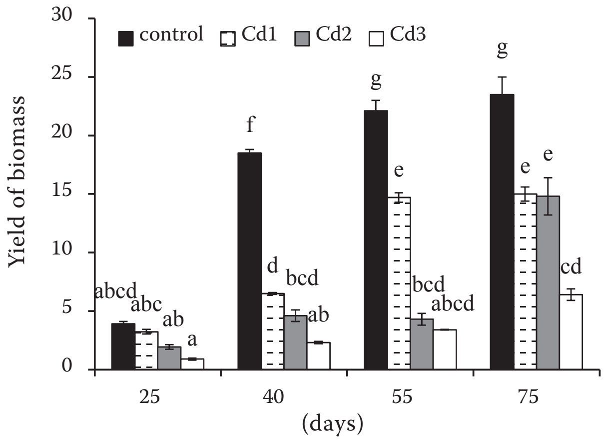

Figure 1. Aboveground biomass yield (g dry matter per pot) of spinach aboveground biomass. Explanation for Figures 1–4: Spinach was grown under varying Cd concentrations (0, 30, 60, 90 mg Cd/kg). Plants were harvested after 25, 40, 55 and 75 days of spinach cultivation. Data represent means ± standard error of three replicates every year ($n = 6$). Different letters indicate significantly different values ($P < 0.05$) between treatment × growing period calculated by MANOVA

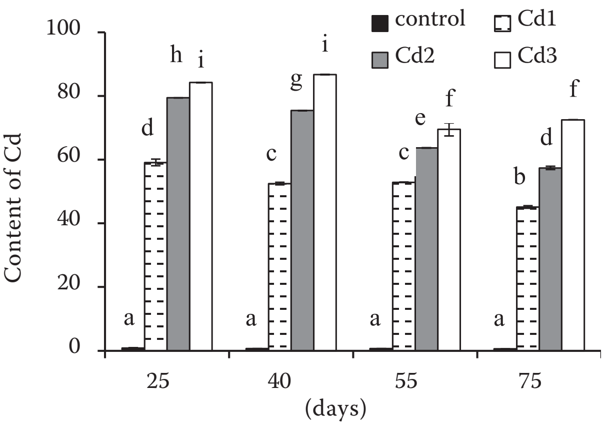

Figure 2. Cadmium (Cd) accumulations in aboveground biomass of spinach (mg/kg dry matter)

Plants exposed to toxic metals accumulated specific AAs, which may have beneficial functions and play various roles (Xu et al. 2012a, 2012b, Pavlíková et al. 2014a,b). Chaffei et al. (2004) suggested that an increase in the proportion of high N:C by AAs, is a protective strategy in plants. Consistent with this hypothesis, our analyses indicated the accumulation of a large amount of glutamic acid (Glu) and aspartic acid (Asp) in Cd treatments in 55th day of plant cultivation (Figures 3a,b). The highest accumulations of both AAs were determined on Cd3 treatment after 55 days of cultivation. Glu and Asp are used to transfer N from source organs to sink tissues and to build up reserves during periods of N availability for subsequent use in growth, defense, and reproductive processes. Strong decrease of both AAs were confirmed in the last sampling period for Cd treatments (after 75 days of plant growing). Glu content of Cd3 treatment was reduced to ca. 64% of the control treatment. Asp content was decreased to ca. 72%. This decrease related to the interaction of onto-

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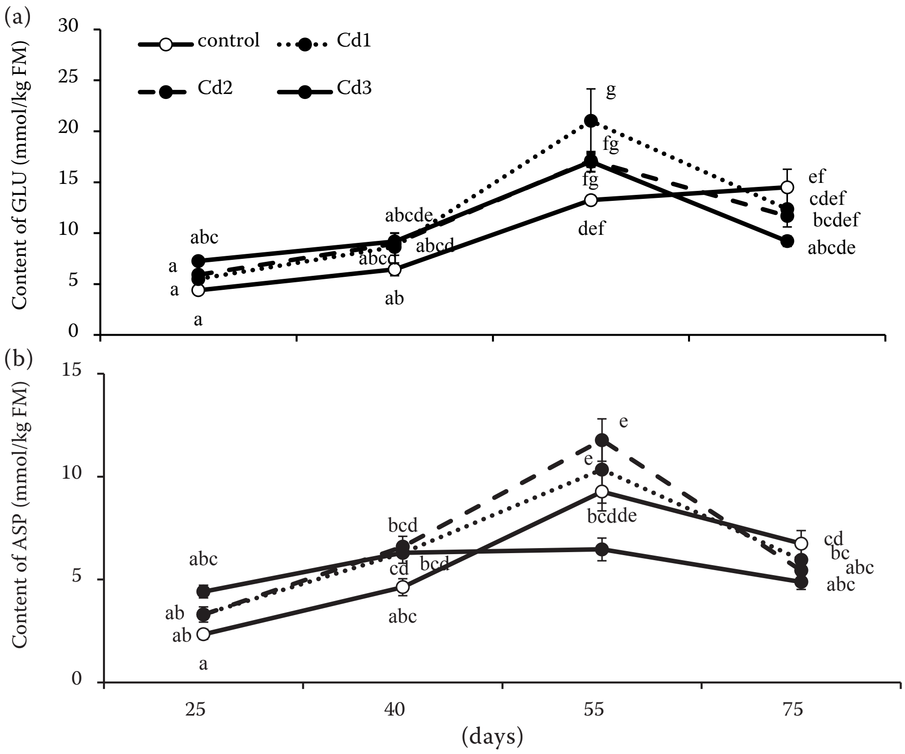

Figure 3. The concentrations of (a) free glutamic acid (GLU) and (b) free aspartic acid (ASP) in the aboveground biomass of spinach (mmol/kg fresh matter (FM))

genetic period and effect of Cd stress. Changes of Asp contents were less significant in contrast to Glu, because Glu is used in the synthesis of glutathione and phytochelatins in plant cells (Vitória et al. 2001). According to Zemanová et al. (2013) the declines of contents of both AAs can be caused by intensive syntheses of plant defense elicitors. Pavlík et al. (2012) confirmed that contamination of heavy metals caused depletion in the pools of free Glu and Asp in lettuce plants growing for 75 days.

As it was calculated using the multivariate analysis of variance MANOVA, contents of Cd, Asp, Glu and yield of biomass were significantly affected by treatments (Wilks' lambda 0.003, $F$ = 226.2, $P$ = 0.00*), growing period (Wilks' lambda 0.33, $F$ = 77.6, $P$ = 0.00*) and treatments × growing period (Wilks' lambda 0.044, $F$ = 17.33, $P$ = 0.00*). The results showed the most significant effect of treatments.

The comparison between treatments showed significant differences of FAs composition related to Cd stress only in 25th day of plant cultivation (Figure 4). The Cd contamination the increased the content of saturated fatty acids (by 44% for Cd1 and 94% for Cd2) compared to control. Our results from the 55th day of plant cultivation confirmed increase only by 16% for both Cd treatments. SFA contained in plants – palmitic acid (16:0) was detected in all treatments and in both sampling periods. Arachidic acid (20:0) was only detected in control treatment in day 25 of plant cultivation (Figure 5).

Analyses of unsaturated fatty acids have confirmed 7,10,13-hexadecatrienoic (16:3n-3), 9,12-octadecadienoic (linoleic acid, 18:2n-6) and 9,12,15-octadecatrienoic (α-linolenic acid, 18:3n-3)

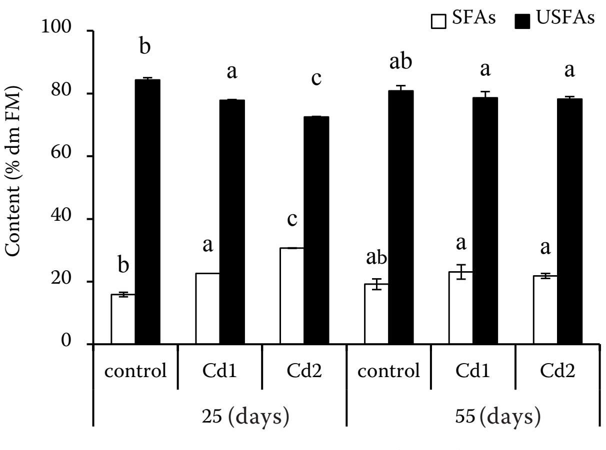

Figure 4. Total saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs) contents of spinach. Explanation for Figures 4 and 5: Spinach was grown under varying Cd concentrations (0, 30, 60, 90 mg Cd/kg). Plants were harvested after 25 and 55 days of spinach cultivation. Data represent means ± standard error of three replicates every year ($n$ = 6). Different letters indicate significantly different values ($P$ < 0.05) between treatment × growing period calculated by MANOVA

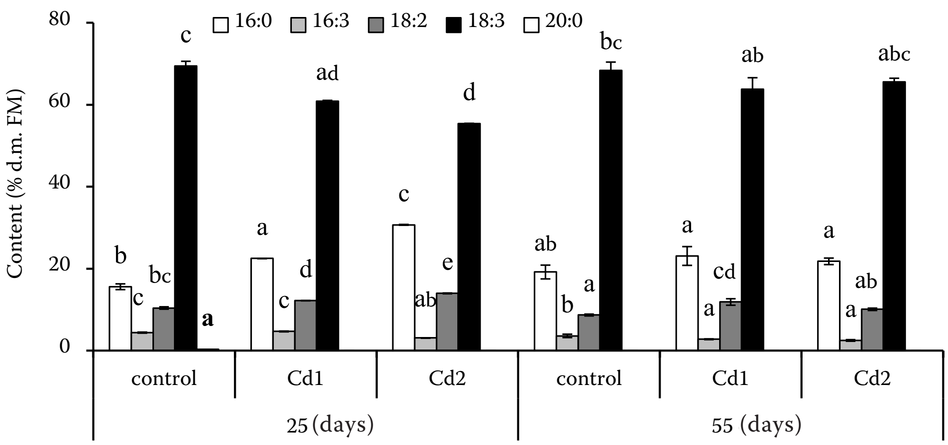

Figure 5. Profile saturated fatty acids and unsaturated fatty acids contents of spinach

acids in all treatments. Decreases of USFAs percentage were detected for 16:3n-3 and 18:3n-3 FAs in both Cd treatments. 16:3n-3 USFA is the product of desaturases from 16:0 and it is used as substrate for biosynthesis of different USFAs (Zemanová et al. 2015). For acclimation to oxidative stress plants mainly need USFAs – 18:2n-6, 18:3n-3. The percentage of 18:2n-6 FA increased in plants with Cd contamination. In accordance with our results Verdoni et al. (2001) published a significant decrease of linolenic acid (18:3) and increase of 18:1 and 18:2 FAs in primary leaves of tomato. Upchurch (2008) reviewed that in stress tolerant plants the degree of membrane lipid unsaturation, principally linolenic acid decreases in response to heavy metal stresses. Our results supported this finding. For plant growing under oxidative stress linolenic acid is substrate rapidly degraded into metabolites, which lead to the production of oxylipins, for example jasmonic acid regulating growth and plant development (Savchenko et al. 2014).

As it was calculated using the multivariate analysis of variance MANOVA, contents of SFAs and USFAs were significantly affected by variants (Wilks' lambda 0.067, $F = 35.53$, $P = 0.00^*$), growing period (Wilks' lambda 0.201, $F = 49.2$, $P = 0.00^*$) and variants × growing period (Wilks' lambda 0.119, $F = 23.60$, $P = 0.00^*$). The most significant effect was confirmed for plant growing period.

Zemanová et al. (2015) clearly showed importance of a relationship between Cd accumulation and the FAs composition in Cd hyperaccumulator *Noccaea caerulescens*. According to these results SFAs decrease and USFAs increase in biomass of *N. caerulescens* with increasing Cd concentration is a typical feature of plants resistant to Cd stress. An opposite trend of FAs content was determined in spinach biomass – non hyperaccumulating plant. The results of Nouairi et al. (2006) indicated similar changes of FAs in leaves of *Brassica juncea* grown under Cd stress. The comparison between hyperaccumulator and spinach showed significant differences of FAs composition related to Cd chronic stress. The number of identified FAs in spinach biomass was very low compared to the hyperaccumulator. Saturated very-long-chain fatty acids (VLCFAs) were found only in hyperaccumulating plants. Biosynthesis of VLCFAs decrease the amount of energy necessary for plant growth and development. Catabolic processes of these FAs decreased plant sensitivity to environmental stress. This finding reflects that hyperaccumulator in contrast to spinach has an efficient defense strategy relating to changes in FAs composition.

The results of our experiment showed changes in transfer AAs contents (the highest accumulation of AAs on Cd3 treatment after 55 days of cultivation) and in FAs composition (significant increase of palmitic acid) in spinach in relationship to growing Cd soil contamination. Multivariate analysis of variance confirmed a significant effect of growing period of plants to these changes. For this reason investigation of changes in the plant metabolism is necessary to test in long-term conditions.

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