The changes of contents of selected free amino acids associated with cadmium stress in *Noccaea caerulescens* and *Arabidopsis halleri*

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ABSTRACT

Changes of free amino acid (AA) contents (glutamic acid, glutamine, aspartic acid, asparagines, proline, hydroxyproline) in *Noccaea caerulescens* and *Arabidopsis halleri* under cadmium soil contamination (Cd1 = 30, Cd2 = 60, Cd3 = 90 mg/kg soil) are reported. Results of the pot experiment confirmed different effect of Cd on *N. caerulescens* in contrast to *A. halleri* and the higher stress adaptation of *A. halleri*. Total free AA contents in both plant species were not significantly modified by Cd contamination. The glutamic acid and glutamate contents in plant biomass were decreased under Cd2 and Cd3 stress. The declines of contents of both AA can be caused by intensive syntheses of plant defense elicitors, but declines in *A. halleri* were significantly lower in contrast to *N. caerulescens*. The content of aspartic acid was increased in *N. caerulescens* under Cd stress, but in *A. halleri* its changes were not observed. The different pathways of nitrogen utilization of tested plants were confirmed: the major AA forms used for nitrogen transport are glutamate for *N. caerulescens* and asparagine for *A. halleri*. The increase of proline content was determined only in *N. caerulescens* growing under Cd stress in the beginning of growing period.

Keywords: heavy metals; nitrogen-transport amino acids; *Thlaspi caerulescens*

Hyperaccumulators of heavy metals are plants which actively take up exceedingly large amounts of heavy metals from soil. Heavy metals are not retained in the roots but they are translocated to the shoots and accumulated in aboveground organs, especially in leaves, at concentrations 100–1000-fold higher than those found in non-hyperaccumulating species. After the uptake of heavy metals by plant roots, their translocation to shoots and detoxification within the storage sites are two critical steps. This is achieved by chelation, transport, trafficking, and sequestration by organo-ligands at a subcellular level (Clemens et al. 2002). The potential ligands are grouped into three major classes: oxygen donor ligands (e.g. carboxylates), sulphur donor ligands (e.g. metallothioneins and phytochelatins), and nitrogen donor ligands (e.g. amino acids) (Bhatia et al. 2005).

Two Brassicaceae species, *Arabidopsis halleri* (*AH*) and *Noccaea caerulescens* (*NC*) (formerly *Thlaspi caerulescens*), have become popular models for the study of heavy metal hyperaccumulation (Hanikenne and Nouet 2011). In *AH*, Cd is mostly accumulated in mesophyll cells (Zhao et al. 2000) and high metal concentrations were also observed in the trichomes (Sarret et al. 2009). In the leaves of *NC*, Cd is more concentrated in the epidermis, but the mesophyll is still the major storage compart-

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ment due to its larger volume (Vogel-Mikuš et al. 2008). In leaf cells of hyperaccumulators, metals are generally sequestered in vacuoles (Huguet et al. 2012). This vacuolar compartmentalization is considered as the major metal detoxification pathway, limiting possible interferences between toxic elements and cell metabolism (Verbruggen et al. 2009).

Amino acids (AA) are the precursors to proteins and also their constituents and they play an important role in metabolism and development (Ježek et al. 2011). Plants that were exposed to toxic metals have also been shown to accumulate specific AA, which may have beneficial functions. The AA which are accumulated under heavy metal stress, play various roles in plants, including acting as signaling molecules, and osmolytes, regulating ion transport and facilitating detoxification (Xu et al. 2012). Reports of the role of AA in the hyperaccumulation of metals (including Cd) by plants are limited; therefore the present investigation aims to determine the changes and differences in accumulation of selected free AA in *NC* and *AH* associated with Cd soil contamination. Asparagine and glutamic acid as well aspartic acid and glutamine are involved in N-assimilation, transport and transamination processes of vascular plants, therefore the changes of these amino acids were investigated in detail. Accumulation of Cd and content of selected free AA were measured to test the ability of these plants to tolerate Cd contamination.

MATERIAL AND METHODS

The effect of Cd concentration on the levels of free amino acids was investigated in the pot experiment. Two species of Brassicaceae family were selected: *Noccaea caerulescens* (formely *Thlaspi caerulescens* J. & C. Presl, FK Mey) ecotype 'Ganges' (southern France) and *Arabidopsis halleri* (O'Kane and AL Shehbaz) (northern France). The plants were planted into plastic pots (two plants per pot) containing 3 kg of soil (Table 1). Soil was thoroughly mixed with 0.3 g N, 0.10 g P, and 0.24 g K applied in the form of ammonium nitrate and potassium hydrogen phosphate for control treatment and with the same amount of nutrients plus Cd in the form of $Cd(NO_3)_2 \cdot 4\ H_2O$ in concentrations: 0 (control), 30 (Cd1), 60 (Cd2) and 90 (Cd3) mg/kg, for treated variants. The plants were cultivated under natural light and temperature conditions at the experimental hall. Plants were harvested 30 and 90 days after Cd application. Samples were kept frozen in liquid nitrogen for transport and then at –30°C until extraction procedure.

For determination of Cd, plant samples were decomposed using the dry ashing procedure (Miholová et al. 1993). The concentrations of Cd were determined by ICP-OES with axial plasma configuration (VarianVistaPro, Varian, Mulgrave, Australia).

Total free AA compounds were determined using an EZ-faast amino acid analysis procedure (Phenomenex, Santa Clara, USA). Samples were analyzed for AA contents by the gas chromatography coupled with mass spectrometry detection using a HP 6890N/5975 instrument (Agilent Technologies, Torrance, USA; as described by Pavlík et al. (2012)).

The statistical analyses were performed using hierarchic analyses of variance (ANOVA) with inteactions at 95% ($P < 0.05$) significance level with subsequent Tukey's *HSD* test and linear correlation (R^2). All analyses were performed by using Statistica 9.1 software (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

Results of the pot experiment revealed the different effect of Cd on *NC* and *AH*. Aboveground biomass of *NC* decreased with increasing Cd2 and Cd3 rates (Table 2). The higher Cd rates significantly reduced aboveground biomass in both sampling periods (41% and 64% decrease of yield for Cd3 treatments compared to the control). The lowest Cd rate stimulated the dry aboveground biomass yield of *AH* (42% and 52% increase compared to control). On the other hand, the Cd3 rate reduced the yield (33% and 22% decrease compared to

Table 1. Basic characteristics and total element contents in experimental soil

Soil type	pH	C_{org} (%)	CEC ($mmol_+$/kg)	Cd (mg/kg)
Modal Chernozem	7.2 ± 0.1	1.83 ± 0.01	258 ± 0.1	0.42 ± 0.05

CEC – cation exchange capacity

Table 2. Effect of Cd on aboveground biomass yield and Cd content of *Noccaea caerulescens* (*NC*) and *Arabidopsis halleri* (*AH*). I. – 30 days; II. – 120 days from planting

Sampling period	Control		Cd1		Cd2		Cd3	
	NC	*AH*	*NC*	*AH*	*NC*	*AH*	*NC*	*AH*
Yield of aboveground biomass (g dry matter per pot)								
I.	0.63 ± 0.03	0.67 ± 0.11	0.53 ± 0.10	0.95 ± 0.07	0.41 ± 0.02	0.85 ± 0.13	0.37 ± 0.03	0.45 ± 0.10
II.	2.50 ± 0.21	2.30 ± 0.09	2.54 ± 0.21	3.50 ± 0.25	2.10 ± 0.18	2.20 ± 0.20	0.90 ± 0.12	1.80 ± 0.16
Cd content (mg/kg)								
I.	41 ± 0.2^{aA}	121 ± 0.3^{aA}	1656 ± 1.8^{aB}	990 ± 0.4^{aB}	2860 ± 0.6^{aC}	1447 ± 0.9^{aC}	3773 ± 1.2^{aD}	2141 ± 0.4^{aD}
II.	26 ± 0.4^{cA}	66 ± 0.3^{bA}	726 ± 1.2^{cB}	452 ± 0.4^{cB}	1639 ± 0.6^{cC}	1194 ± 3^{cC}	2629 ± 0.9^{cD}	1022 ± 0.5^{cD}

Data are means ± S.E. (n = 3). Different letters (a) indicate significantly different values (P < 0.05) between samples of plant species. Different letters (A) indicate significantly different values (P < 0.05) between treatments of plant species. Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

control). Visual toxicity symptoms with necrosis were observed on neither of plant species. This finding corresponded with the results of Rascio and Navari-Izzo (2011).

In comparison to *NC*, Cd contents in aboveground biomass of *AH* were lower at all Cd treatments (Table 3), but they were above the 100 mg/kg Cd threshold that defined Cd hyperaccumulation in the natural environment (Cosio et al. 2005). The opposite effects were observed in control variants – higher Cd contents in *AH* in contrast to *NC* were determined. Significant negative linear correlation between contents of Cd in the aboveground biomass and biomass yield was calculated (R^2 = 0.99–1 for *NC*; R^2 = 0.92–1 for *AH*).

Our data correspond with those by Pavlíková et al. (2002, 2008) and Procházková et al. (2012) who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. Reduced growth observed at contaminated treatments may be partly due to lower net photosynthetic rate, but not exclusively, since it was argued that the reduced growth might be also due to increased tissue permeability. It might also result from inhibition of cell division (Redondo-Gómez et al. 2011). Reduction in growth can be linked to the high trace elements accumulation, as in this case plants have to spend extra energy to cope with the high trace element concentrations in the tissues (Israr et al. 2006).

The Cd soil contamination did not significantly modify the total contents of free AA in the aboveground biomass of both species (Figure 1). Nevertheless, contents of total free AA were decreased in the aboveground biomass of *NC* at Cd treatments in comparison with control. The total contents of free AA in aboveground biomass of *NC* were two times higher in contrast to *AH*.

The major free AA determined in *NC* and *AH* were glutamic acid (Glu), glutamine (Gln), asparagine (Asn) and aspartic acid (Asp) (Table 3). In all higher plants, inorganic N is at first reduced to ammonia prior to its incorporation into organic form. Ammonia is assimilated into organic form as Glu and Gln, which serve as the N donors in the biosynthesis of essentially all amino acids and other nitrogen-containing compounds (Sánchez-Pardo et al. 2012). The free Glu content in the aboveground biomass of *NC* was stimulated under Cd1 treatment (24% increase compared to the control), but Cd2 and Cd3 treatments decreased its content. The significant changes of free Glu were observed for Cd3 treatment (66% decrease compared with control). Our findings indicated the decrease of Glu concentration under Cd2 and Cd3 treatments also for *AH*, but declines of Glu concentrations were significantly lower compared to *NC*. Sharma and Dietz (2006) and Pavlík et al. (2010) published opposite results for plants grown under trace elements stress – increase of Glu concentration in stressed plants. The Glu decline in *AH* and *NC* biomass can be caused by intensive syntheses of plant defence elicitors. The significant relationships between Glu concentrations in both plants and biomass yield, and also between Glu concentrations and Cd content in biomass were confirmed using linear regression (R^2 = 0.99 for both sampling periods).

Table 3. The concentrations of selected free amino acids in aboveground biomass during growth of *Noccaea caerulescens* and *Arabidopsis halleri* (μmol/kg fresh weight ± S.E.; *n* = 3). I. – 30 days; II. – 120 days from planting

	Control		Cd1		Cd2		Cd3	
	I.	II.	I.	II.	I.	II.	I.	II.
Noccaea caerulescens								
Glu	244 ± 8^{aA}	2228 ± 4^{bA}	303 ± 9^{aB}	1476 ± 13^{bB}	182 ± 8^{aC}	1237 ± 41^{bC}	51 ± 3^{aD}	833 ± 11^{bD}
Gln	9520 ± 8^{aA}	$14\,356 \pm 5^{bA}$	6715 ± 24^{aB}	$18\,970 \pm 13^{bB}$	6641 ± 8^{aC}	4042 ± 20^{bC}	5940 ± 12^{aD}	2242 ± 19^{bD}
Pro	6944 ± 8^{aA}	6480 ± 4^{bA}	$16\,919 \pm 13^{aB}$	8566 ± 25^{bB}	$16\,449 \pm 9^{aB}$	5707 ± 8^{bC}	$10\,360 \pm 17^{aC}$	7216 ± 12^{bD}
Hyp	N.D.	23 ± 3^{A}	N.D.	55 ± 2^{B}	N.D.	18 ± 1^{C}	N.D.	6 ± 1^{D}
Asp	2632 ± 6^{aA}	7071 ± 6^{bA}	3274 ± 12^{aB}	5816 ± 13^{bB}	2708 ± 9^{aA}	3460 ± 1^{bC}	2990 ± 39^{aC}	6470 ± 12^{bD}
Asn	2840 ± 6^{aA}	3306 ± 5^{bA}	2533 ± 8^{aB}	1748 ± 13^{bB}	1781 ± 9^{aC}	1372 ± 20^{bC}	1459 ± 14^{aD}	2478 ± 12^{bD}
Arabidopsis halleri								
Glu	723 ± 4^{aA}	1535 ± 9^{bA}	736 ± 15^{aA}	3150 ± 19^{bB}	1265 ± 27^{aB}	1767 ± 31^{bC}	985 ± 5^{aC}	1447 ± 4^{bD}
Gln	5917 ± 5^{aA}	5033 ± 5^{bA}	3226 ± 8^{aB}	1172 ± 26^{bB}	1596 ± 25^{aC}	1403 ± 5^{bC}	2089 ± 5^{aD}	1357 ± 14^{bC}
Pro	258 ± 49^{aA}	429 ± 3^{bA}	224 ± 2^{aB}	278 ± 1^{bB}	389 ± 12^{aC}	232 ± 1^{bC}	196 ± 1^{aB}	360 ± 9^{bD}
Hyp	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Asp	915 ± 6^{aA}	1732 ± 10^{bA}	1122 ± 4^{aB}	6082 ± 9^{bB}	1225 ± 8^{aC}	1617 ± 9^{bC}	1593 ± 4^{aD}	1308 ± 6^{bD}
Asn	3155 ± 12^{aA}	6364 ± 14^{bA}	2324 ± 14^{aB}	$18\,170 \pm 90^{bB}$	3468 ± 12^{aC}	2582 ± 9^{bC}	3985 ± 10^{aD}	5440 ± 15^{bD}

Different letters (a) indicate significantly different values ($P < 0.05$) between samples of plant species. Different letters (A) indicate significantly different values ($P < 0.05$) between treatments of plant species. N.D. – not detected. Glu – glutamic acid; Gln – glutamine; Pro – proline; Hyp – hydroxyproline; Asp – aspartic acid; Asn – asparagine (Asn). Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

Gln is dominant free AA in biomass of *NC*. Gln concentrations in *AH* were significantly lower in contrast to *NC*. These results showed a different pathway of nitrogen utilization of both plants. Cd treatments resulted in a decrease of Gln contents compared with control (for Cd3 treatment – by 85% for *NC* and by 73% for *AH*). Results confirmed the significant relationship between contents of Gln, biomass yield and Cd content in biomass ($R^2 = 0.99$ for both sampling periods). Gln is not only the major AA used for N transport in *NC*, but also a key metabolite that acts as an amino donor to other free amino acids, primarily catalyzed by glutamate synthase. This pathway interacts with carbohydrate metabolism or the energy status of the plant leaves (Hodges et al. 2003). Gln and Glu can be used to form Asp and Asn, and these four AA are used to translocate

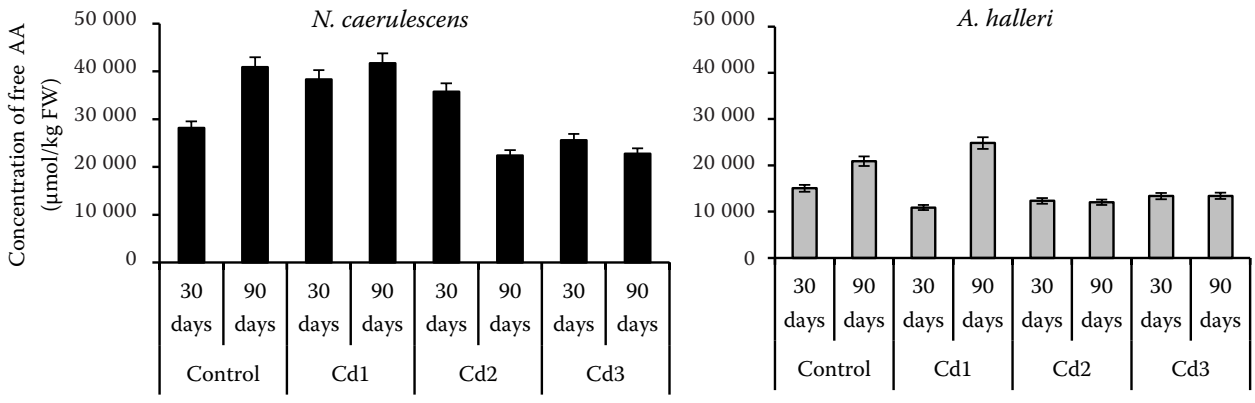

Figure 1. Total contents of free amino acids in aboveground biomass of *Noccaea caerulescens* and *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Values are the means (± S.E.) of 3 replicates. AA – amino acid; Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

organic N from sources to sinks (Mokhele et al. 2012). Our results indicated the increase of free Asp concentrations under Cd stress in *NC*. All Cd treatments increased accumulation of free Asp contents (24%, 29% and 11% increase compared to the control) in the aboveground biomass. The changes of Asp in *AH* were not significant. The strong linear relationship between Asp and Glu was calculated: for *AH* (R^2 = 0.90), and for *NC* (R^2 = 0.68). Aspartate amino-transferase plays a central role in both Asp synthesis and catabolism. According to Pandey et al. (2004), aspartate amino-transferase activity decreased considerably during stress, and the reduction was greater with increased stress.

Asparagine is an AA used to store and transport N from sources to sinks. Our determinations showed a decrease of Asn concentration in biomass of *NC* only in the 2nd sampling period (by 25–59% in contrast to control). *AH* did not shown significant changes under Cd stress. This could be associated with the remobilization of assimilated nitrogen as proteins and other substance. According to Zhang et al. (2013) Asn is a major form of N transported to sink tissues in *Arabidopsis* mutant. Our results confirmed their findings: free Asn was the dominant free AA in *AH*. Substantial changes in xylem Asn level can occur under certain stress conditions often associated with reciprocal changes in Asp levels (Antunes et al. 2008).

Proline accumulation was reported in tissues/organs of plants subjected to various abiotic stresses including risk element toxicity for many plants (Zengin and Munzuroglu 2005, Mistra and Dubey 2006 etc.). Our results did not confirm this trend. The significant increase of free Pro contents in Cd treatments of *NC* in contrast to control was determined in the 1st sampling period. Pro increases in the 2nd sampling period were lesser compared to the 1st sampling period. The free Pro accumulation in aboveground biomass was affected not only by Cd soil contamination, but also by process of plant adaptation to chronic stress and plant growing period (Pavlíková et al. 2008). *AH* showed similar Pro content in biomass in both sampling periods (Table 3). The content of free Pro in aboveground biomass of *AH* was about 15–75 times less in comparison to *NC*. According to García-Ríos et al. (1997), Pro inhibition depends on Glu concentration. This finding confirms the ability of plant chronically stressed by toxic elements to obtain adequate Glu concentration for synthesis of phytochelatins. The relationship between free Pro and Cd contents in biomass was confirmed using linear correlation and the most significant relationship was calculated for the 2nd sampling period (R^2 = 0.65–1).

Hydroxyproline (Hyp) is a major AA in plant cell wall hydrolysates (Deepak et al. 2010). Considerably more hydroxyproline is found in the protein of rapidly proliferating tissue than in proteins of slowly proliferating tissue. The free Hyp was found only in aboveground biomass of *NC* during the 2nd sampling period (Table 3). The lowest concentration of Cd increased the content of free Hyp. Opposite effect – decrease of free Hyp content was found for Cd2 and Cd3 treatments. These results showed linear relationship between contents of free Pro and contents of free Hyp (R^2 = 0.33–1) in *NC*. The Hyp content was associated with the beginning of plant senescence. Hyp in *AH* biomass was below detection limit of gas chromatography (GC).

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