Effects of cadmium stress on growth and amino acid metabolism in two Compositae plants

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ARTICLE INFO

ABSTRACT

Cadmium, a high toxic heavy metal, is one of the most serious contaminants in soil and a potential threat to plant growth and human health. Amino acid metabolism has the central role in heavy metal stress resistance of plants. In this paper, a pot experiment was carried out to study the effects of different concentrations of cadmium (0, 3, 6, 12, 30 mg kg^{-1}) on the growth, Cd accumulation and amino acid metabolism in two Compositae plants (*Ageratum conyzoides* L. and *Crassocephalum crepidioides*). The results showed that under cadmium stress, *C. crepidioides* accumulated more Cd in its shoot and was tolerant to Cd, whereas its low Cd-accumulating relative, *A. conyzoides*, suffered reduced growth. The Cd content in the aerial part of *C. crepidioides* exceeded the threshold of Cd-hyperaccumulator. Furthermore, the bioaccumulation factor (BCF) and biological transfer factor (BTF) values for Cd in *C. crepidioides* were > 1. Thus, *C. crepidioides* can be regarded as Cd-hyperaccumulator. The comparison between both studied plants indicated that Cd stress resulted in a differential but coordinated response of amino acid levels, which are playing a significant role in plant adaptation to Cd stress. Glu, Gln, Asp, Asn, Gaba, Val and Ala dominated the major amino acids. Higher Cd tolerance and Cd accumulation in *C. crepidioides* was associated with greater accumulation of free amino acids, especially for Gln and Asn, in *C. crepidioides* than in *A. conyzoides*.

1. Introduction

Increasing environmental pollution caused by heavy metals, originating mainly from industrial processes and urban activities, as well as the widespread application of pesticides, fertilizers, manure and sewage sludge, has posed a serious problem for safe food production and become a potential agricultural and global environmental problem (Bonet et al., 2014; Daş et al., 2016; Zhu et al., 2017a). Among the various heavy metals, Cadmium (Cd) occupies the top position in terms of hazardous effects posed to plants and human health, due to its high toxicity, mobility, and availability for all living organisms (Ali et al., 2015; Clemens et al., 1999; Wael et al., 2015). The deleterious effects following exposure to Cd in humans has been associated with cancers of the prostate, lungs, and testes, renal dysfunction, rhinitis, emphysema, and bone fractures (Jarup and Akesson, 2009). The accumulation of Cd also inhibits growth, development and productivity of plants via impaired amino acid biosynthesis, inhibition of enzyme activities, induction of oxidative stress, interference with mineral nutrition and metabolic imbalances (Liu et al., 2015; Nagajyoti et al., 2010). The visual symptoms of Cd toxicity in plants are chlorosis and necrosis of leaves, browning of roots and cell apoptosis (Zemanová et al., 2015b). However, plants have evolved a variety of adaptive mechanisms to protect against Cd stress. This is achieved by cellular exclusion, sequestration, chelation osmotic adjustment, metabolic utilization and production of antioxidant systems, etc. (Kushwaha et al., 2016; Li et al., 2016; Rahman et al., 2017; Sytar et al., 2013; Zhang et al., 2015).

Nitrogen metabolism is central in the plant response to heavy metals; it has been shown that Cd may interfere with nitrogen metabolism in plants (Chaffei et al., 2004). Upon exposure to metals, plants often synthesize a set of diverse low-molecular weight substances, particularly specific free amino acids (FAA), which are known as compatible solutes, and have been shown to serve as signaling molecules and play an important role in plants varied from acting as osmolyte, radical scavenger, regulation of ion transport, modulating stomatal opening to detoxification of heavy metals (Pavlíková et al., 2014; Sharma and Dietz, 2006; Xu et al., 2012). The changes appearing in free amino acids

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as a response to different stress factors are important for plant metabolism as they are not only known as precursors and functional components of proteins but also, for most of them, as precursors of other nitrogen containing compounds such as nucleic acids (Rai, 2002). The accumulation of Cd in plant organs cause damages to the photosynthetic apparatus (Gallego et al., 2012). The reduction in the photosynthetic rate results in a limited supply of metabolic energy and therefore to nitrogen assimilation restriction. Nitrogen flow through amino acids can thereby change in response to Cd stress (Zemanová et al., 2015a). Amino acids also affect synthesis and activity of some enzymes, gene expression and redox-homeostasis (Chaffei-Haouari et al., 2009; Islam et al., 2009). Furthermore, amino acids rich in carboxyl, amino, thiol and phenolic groups are involved in the synthesis of glutathione and phytochelatin, which are able to form complex metal cations diminishing its reactivity with other molecules at cellular level, and serving as long-distance metal-chelating compounds (Dave et al., 2013; Ghnaya et al., 2010; Irtelli et al., 2009; Richau et al., 2009).

Previous studies have focused attention on the role of the amino acids in Cd tolerance of plants. It has been recorded that Cd accumulation leads to the accumulation of proline in mung, wheat and barley (Leskó and Simon-Sarkadi, 2002; Vassilev and Lidon, 2011; Zhang et al., 2000). However, Costa and Morel (1994) found that Cd induced no accumulation of proline in lettuce but induced specific increases in the levels of asparagine, methionine and lysine. Zoghlami et al. (2011) showed that, in the roots of tomatoes after Cd exposure, asparagine, glutamine and branched chain amino acids (valine, isoleucine, phenylalanine and tryptophane) significantly accumulated; in contrast, few modifications occurred in the leaves in response to Cd, except for tyrosine. Xu et al. (2012) detected that a higher accumulation of proline in *S. nigrum* supports the observed higher Cd tolerance in S. *nigrum* than in *S. torvum*; a high accumulation of hydroxyproline in *S. torvum* roots may play a protective role in preventing Cd translocation from the roots to the aerial parts of the plant. Zemanová et al., (2013, 2014) reported that the major amino acid forms used for nitrogen transport are asparagine and histidine for the higher stress adaptation of *A. halleri* and glutamate for *N. caerulescens*. An increase of phenylalanine, threonine, tryptophan, ornithine and a decrease of alanine and glycine were observed in the responses of the two *Noccaea* metallophytes species to Cd stress (Zemanová et al., 2017). According to the data available in literature, amino acid metabolisms are differently affected by heavy metal treatments, plant species, genotypic difference, and even by different parts of the plant.

Phytoremediation technology is defined as the use of plants to remove contaminants from soils or to render them harmless, and is regarded as a cost-effective, environmental-friendly method for reclaiming soils contaminated by toxic metals (Lasat, 2002; Mahar et al., 2016; Marrugo-Negrete et al., 2016). Compositae plants have been shown to be excellent candidates for phytoremediation purposes due to their rapid growth, high biomass, strong breeding ability, adapted to growing in soils polluted with heavy metals, and low impact on the food chain and human health (Hernández and Pastor, 2008; Peng et al., 2006). We have already studied the content, subcellular distribution and chemical forms of heavy metals in three types of Compositae plants (*Artemisia lavandulaefolia, Ageratum conyzoides* L.*, Crassocephalum crepidioides*) from one lead-zinc tailings area, and demonstrated that *C. crepidioides* demonstrated the basic characteristics of a Cd-hyperaccumulator, cell wall binding, vacuolar compartmentalization and distribution mainly in lower active chemical forms were supposed to be the main tolerance mechanisms to heavy metals (Zhu et al., 2017b, 2018). Free amino acids have been shown to have functional roles in metal tolerance of plants. However, there is little information on the free amino acid metabolism in Compositae plants under heavy metal stress. Therefore the present study was conducted to (1) confirm the identification of Cd hyperaccumulator for *C. crepidioides* by pot-culture upon exposed to various gradient of Cd stress, and (2) characterize the changes and differences in accumulation of free amino acids of two Compositae species with different Cd enrichment abilities -*Ageratum conyzoides* L. and *Crassocephalum crepidioide*s, growing in pot experiment under Cd stress.

2. Materials and methods

2.1. Experimental design

The pot experiment was carried out in the greenhouse at the institute of geochemistry, Chinese Academy of Sciences, Guiyang, China. Topsoil (0–20 cm) was collected from a local abandoned vegetable garden, and its soil properties were as follows: a soil pH of 6.29; an organic matter content of $65.87 \, g \, kg^{-1}$; a cation exchange capacity (CEC) of $19.86 \, cmol \cdot kg^{-1}$; total Cd concentrations of $0.62 \, mg \, kg^{-1}$ and available Cd concentrations of $0.25 \, mg \, kg^{-1}$. The soil was air-dried, mixed thoroughly and sieved through 1 cm mesh. About 5.0 kg portions of the soil were transferred to plastic pots (30 cm in diameter and 25 cm in height). Cadmium was added at a rate of 0, 3, 6, 12, 30 $mg \, kg^{-1}$ dry soil as an aqueous solution of $CdCl_2 \cdot 2.5H_2O$ for five different soil treatments and designated as CK, Cd3, Cd6, Cd12 and Cd30, respectively. After two months of the Cd addition, soil basal fertilizers were applied at $80 \, mg \, P \, kg^{-1}$ of dry soil and $100 \, mg \, K \, kg^{-1}$ of dry soil as KH_2PO_4. Additional N was added at $100 \, mg \, kg^{-1}$ of dry soil as CO$(NH_2)_2$.

The seeds of *Ageratum conyzoides* L. and *Crassocephalum crepidioide*s were initially grown on artificial, non-polluted soil. After the first pair of healthy tender leaves appeared, the seedlings were thinned to four plants per pot and grown for ten weeks. Plants were kept in an ambient temperature of $22 \pm 2 \, °C$, and a 16 h photoperiod of approximately $300 \, mE \, m^{-2} \cdot s^{-1}$ intensity, and at 60% average relative humidity. Each pot was watered twice a week and the moisture level of the soil was maintained at 60–70% WHC. Each treatment was performed in three replicates.

2.2. Sample collection and chemical analysis

At the harvest, plants were gently removed from the pots and the fresh weights of the individual plant were subsequently determined. Roots, stems and leaves were further separated, then rinsed with tap water and carefully washed with deionized water later. Near half of the samples were dried by at 105 °C for 30 min, then oven dried at 65 °C to constant weight (around 3 days). The dried plant samples were milled to a fine powder and passed through a 2 mm sieve. The plant samples were digested with a 4:1 ratio of concentrated HNO_3-$HClO_4$. The residuals were re-dissolved by HNO_3 (2%) and diluted with distilled water. Water used for dilution and dissolution was purified using a Millipore deionizing system at 18.2 MΩ. HNO_3 and $HClO_4$ were superpure reagents. The solutions from the digested samples were stored at 4 °C until analysis. Cadmium concentration was determined by using inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC-e, PerkinElmer company, US) in the Center for Environmental Remediation, Institute of Geographic Sciences and Natural Resources Research (IGSNRR), Chinese Academy of Sciences. The quality control included triplicate analyses, blanks and two standard reference plants.

The remaining half of the samples were frozen in liquid nitrogen and immediately lyophilized for free amino acid extraction. For total soluble amino acids extraction, the procedure was performed according to Xu and Xiao (2017). Briefly, about 150 mg plant sample (200 μL, 1 nmol·μL^{-1} α-aminobutyric acid and sarcosine added as internal standards) was homogenized with 1.8 mL of trifluoroacetic acid (TFA) 10% (v/v) under sonication for 5 min at 4 °C. The homogenate was centrifuged at 12,000 rpm for 15 min (4 °C), and the supernatant solution was collected. To recover the maximum amount of amino acids from the powder samples, the remaining sample was reextracted using 2 mL TFA (10% v/v) in the same manner. The supernatants were combined and filtered through a 0.22 mm membrane, and the collected solution

was purified using 4 mL Amicon Ultra 3 K centrifugal filter tubes (Millipore, US). The purified supernatant was used for the determination of free amino acids by High performance liquid chromatography (HPLC, 1260, Agilent company, US) in the State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences. Twenty different FAAs were identified and quantified: asparagine (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), γ-amino butyric acid (Gaba), tyrosine (Tyr), cystine (Cys), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), proline (Pro), citrulline (Cit), leucine (Leu).

2.3. Data analysis

The bioaccumulation factor (BCF) and the biological transfer factor (BTF) were calculated to investigate the accumulation and translocation ability of trace metals in the soil-plant system. The bioaccumulation factor refers to the ratio of the metal concentration in the plant shoot and the corresponding concentration of metal in the soil; the biological transfer factor is defined as the ratio of the metal concentration in the plant shoot and the metal concentration in the plant root (Buscaroli, 2017; Pandey et al., 2016).

Statistical analyses in this study were performed using Microsoft Excel (Ver. 2013, US) and SPSS (Ver. 22.0, US) software. Data were analyzed by one-way ANOVAs and least significant difference (LSD) tests to compare the significant differences between treatments ($P < 0.05$).

3. Results

3.1. Effects of Cd stress on the plant growth

In the concentration gradient experiments, all the tested plants grew well, and there were no visual signs of phytotoxicity under any of the treatments. The total biomasses yield of the two tested plants under different Cd treatments are shown in Fig. 1. In comparison to *A. conyzoides*, *C. crepidioides* recorded a higher biomass yield at all Cd treatments. The fresh weight of *C. crepidioides* was 3-fold or more higher than that of *A. conyzoides*. For *A. conyzoides*, the biomasses yield growing in the soils spiked with 3, 6 and 12 mg kg^{-1} Cd were not decreased significantly ($p < 0.05$) compared with the control (CK), though the yield reduced significantly ($p < 0.05$) under higher Cd concentrations of 30 mg kg^{-1} Cd spiked, which implied that there was a tolerance limitation for the plant to Cd. However, there was no significant difference in the average total biomass yields of *C. crepidioides* among the different treatments. Increasing Cd doses were not associated with strong reduction of the biomass yield, only exhibiting small reduction under Cd30 treatment, indicating a strong Cd tolerance.
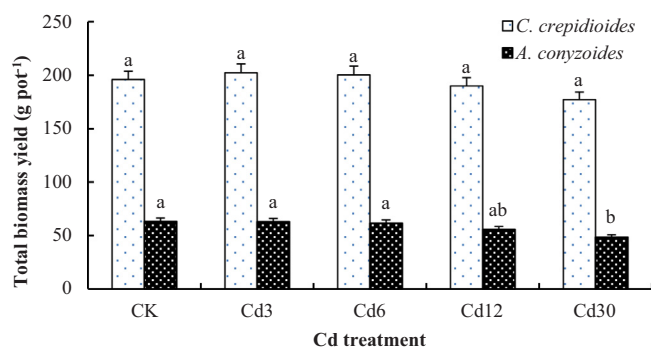

Fig. 1. Effects of Cd treatments on total biomass of the two studied plants, different letters (a, b) indicate significantly different values between treatments of plant species ($P < 0.05$).
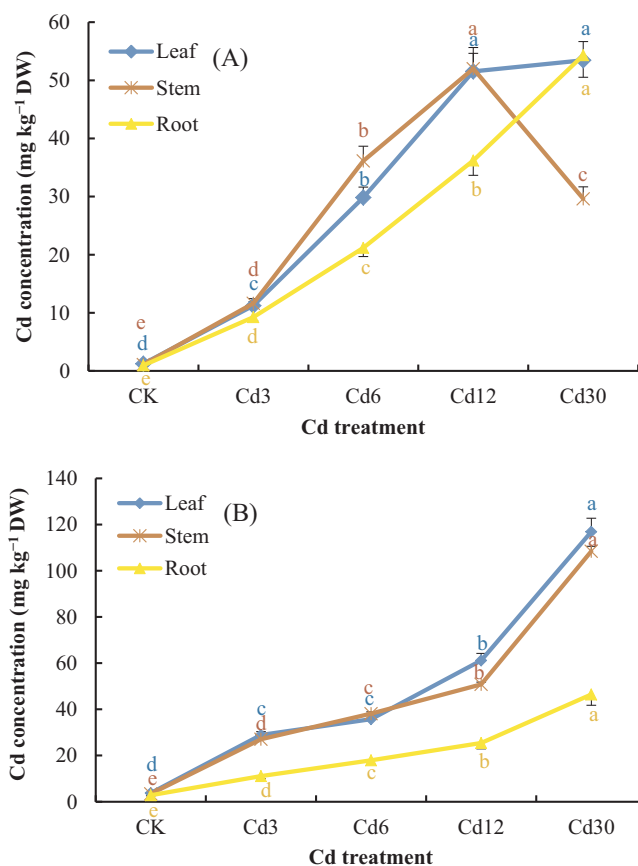

Fig. 2. Effects of Cd treatments on Cd concentrations in different tissues of *A. conyzoides* (A) and *C. crepidioides* (B), DW dry weight, different letters (a, b, c, d, e) indicate significantly different values between treatments of plant species ($P < 0.05$).

3.2. Cadmium concentrations in different organs of plants

Cadmium concentrations in the different parts of the studied plants under different treatments are shown in Fig. 2. As expected, the accumulation of Cd generally displayed the following sequence in both plants: leaf ≈ stem > root (except the value for *A. conyzoides* under Cd30 treatment). Both tested plants can concentrate relatively high concentrations of Cd in the shoots under the Cd treatments, indicating that the two species are Cd accumulative. The higher Cd content was determined for all Cd treatments of *C. crepidioides* compared to Cd treatments of *A. conyzoides*. Considering the total biomass yield, *C. crepidioides* had a greater ability to endure high Cd stress and extracts a higher amount of Cd from Cd-contaminated soils.

For *A. conyzoides*, under the treatment by CK~Cd12, the Cd concentrations in the shoots and roots increased with increasing Cd concentration in the soils, and the BCF and BTF values were greater than 1. However, after exposure to 30 mg kg^{-1} of Cd, the content of accumulated Cd in stem and leaf was 43.1% lower and 3.7% higher than those after Cd12 treatment, suggesting that excess Cd supply might inhibit Cd transport from root to shoot. As for *C. crepidioides*, increasing Cd contents were clearly observed with increasing soil Cd concentrations, thus indicating that the Cd enrichment characteristic of plants exhibited a positive correlation with the Cd concentrations in soil. The highest Cd concentration was 116.9 mg kg^{-1} in the leaf and 108.4 mg kg^{-1} in the stem of *C. crepidioides* grown in 30 mg kg^{-1} Cd soil, respectively, and these values exceeded the Cd hyperaccumulator critical threshold values, fixed at 100 mg kg^{-1}. Moreover, the BCF value for Cd was 3.8 and the BTF value was 2.4. Therefore, considering tolerance, accumulation, BCF and BTF properties, *C. crepidioides* possessed the characteristics of a
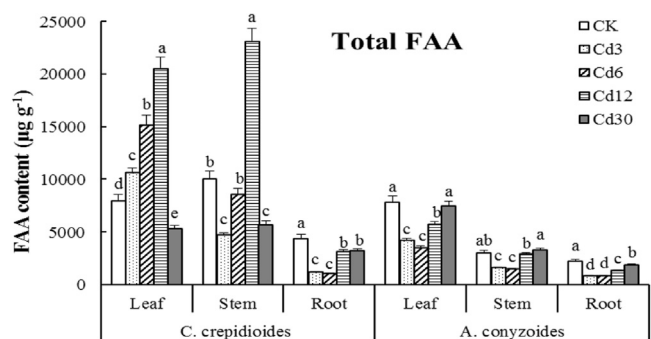
Fig. 3. Effects of Cd treatments on total free amino acid concentration in different tissues of studied plants, different letters (a, b, c, d, e) indicate significantly different values between treatments of plant species (P < 0.05).

Cd-hyperaccumulator (Antoniadis et al., 2017; Baker and Brooks, 1989; Mahar et al., 2016). Further validation experiments based on the concentration gradient are needed to obtain the maximum capacity for Cd uptake by this plant species.

3.3. Effects of Cd on amino acid profile

The data regarding the effect of Cd stress on the concentrations of

FAA in leaves, stems and roots are shown in Figs. 3 and 4. In summary, the total FAA concentrations in *C. crepidioides* were higher than that in *A. conyzoides* (Fig. 3). As for the specific amino acid, the accumulation of Glu, Gln, Ala, Gaba and Leu were greater in *C. crepidioides* than in *A. conyzoides*, while Cys and Asp showed a higher accumulation in *A. conyzoides*. FAA contents varied with plant organ showing higher contents in the aerial parts and lowest contents in the roots. The major FAA in all plant organs were Glu, Gln, Asp, Asn, Gaba, Val and Ala, representing approximately 91% of the total FAA in leaves, 92% in stems and 79% in roots for *C. crepidioides*. These FAAs accounted for 74% of the total amino acid in leaves, 66% in stems and 76% in roots for *A. conyzoides*, respectively. The addition of Cd to the two study plants altered clearly the FAA composition and concentration. However, the changes of FAA were not consistent with cadmium stress.

In control leaves of *C. crepidioides*, the most abundant FAA was Gln (41% of total FAA), followed by Glu (17%), Gaba (8%), Asp, Asn and Ala (6% each). Under Cd stress, these amino acids remained the major FAA. The content of total FAA elevated at lower Cd doses (Ck ~ 12 mg Cd kg^{-1} soil) in the leaves of *C. crepidioides*. Compared to the untreated control, concentrations of total FAA were enhanced up to 1.9-fold and 2.6-fold, respectively. The amount of Gln, Asn, Val and Arg clearly increased, especially for Gln and Asn, whose contents rose to a maximum fraction of 63% and 22% of the total FAA, respectively. In contrast, a considerable reduction was observed for Glu, Gaba, Ala and
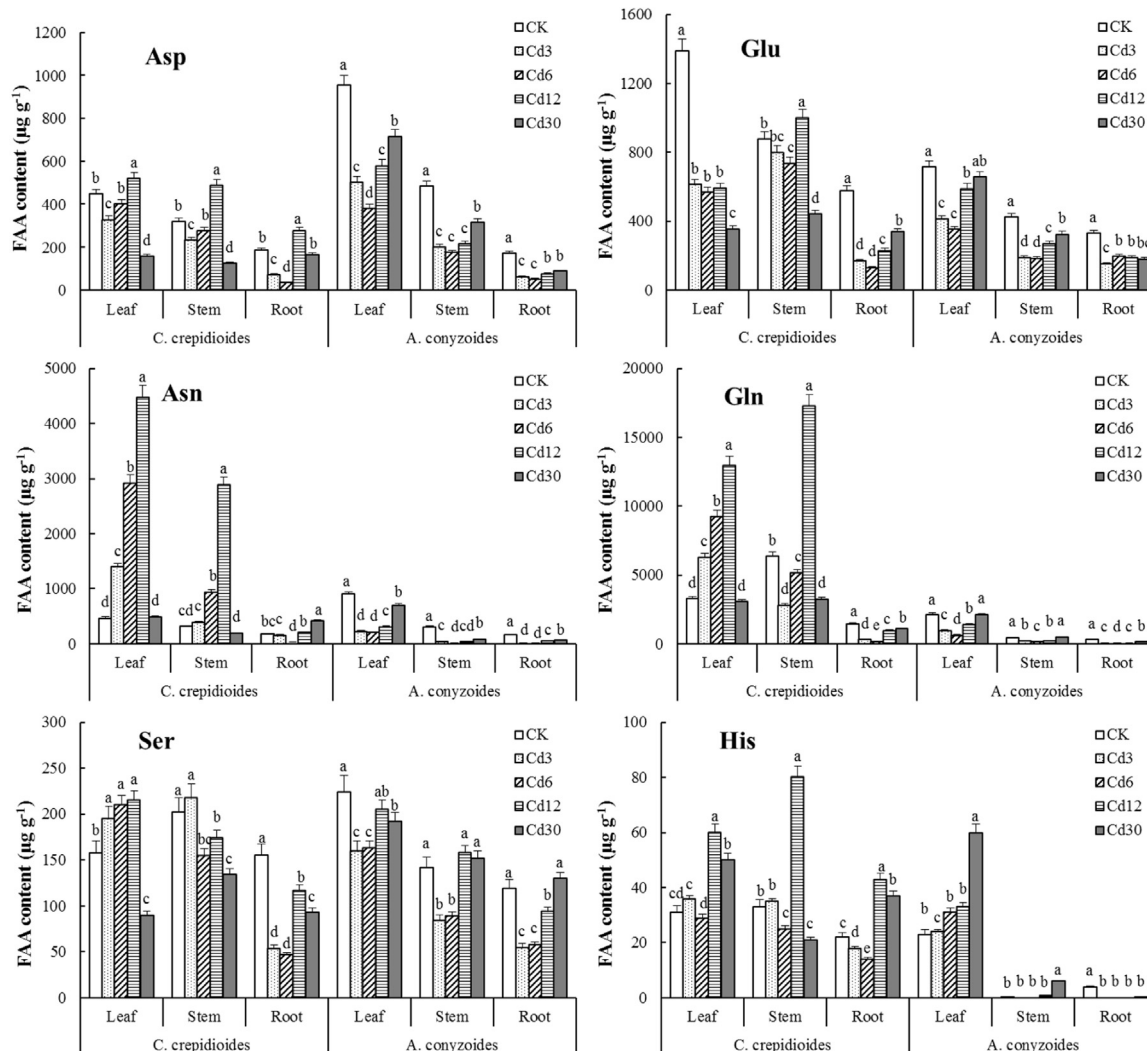

Fig. 4. Effects of Cd treatments on individual free amino acid concentration in different tissues of studied plants, different letters (a, b, c, d) indicate significantly different values between treatments of plant species (P < 0.05).
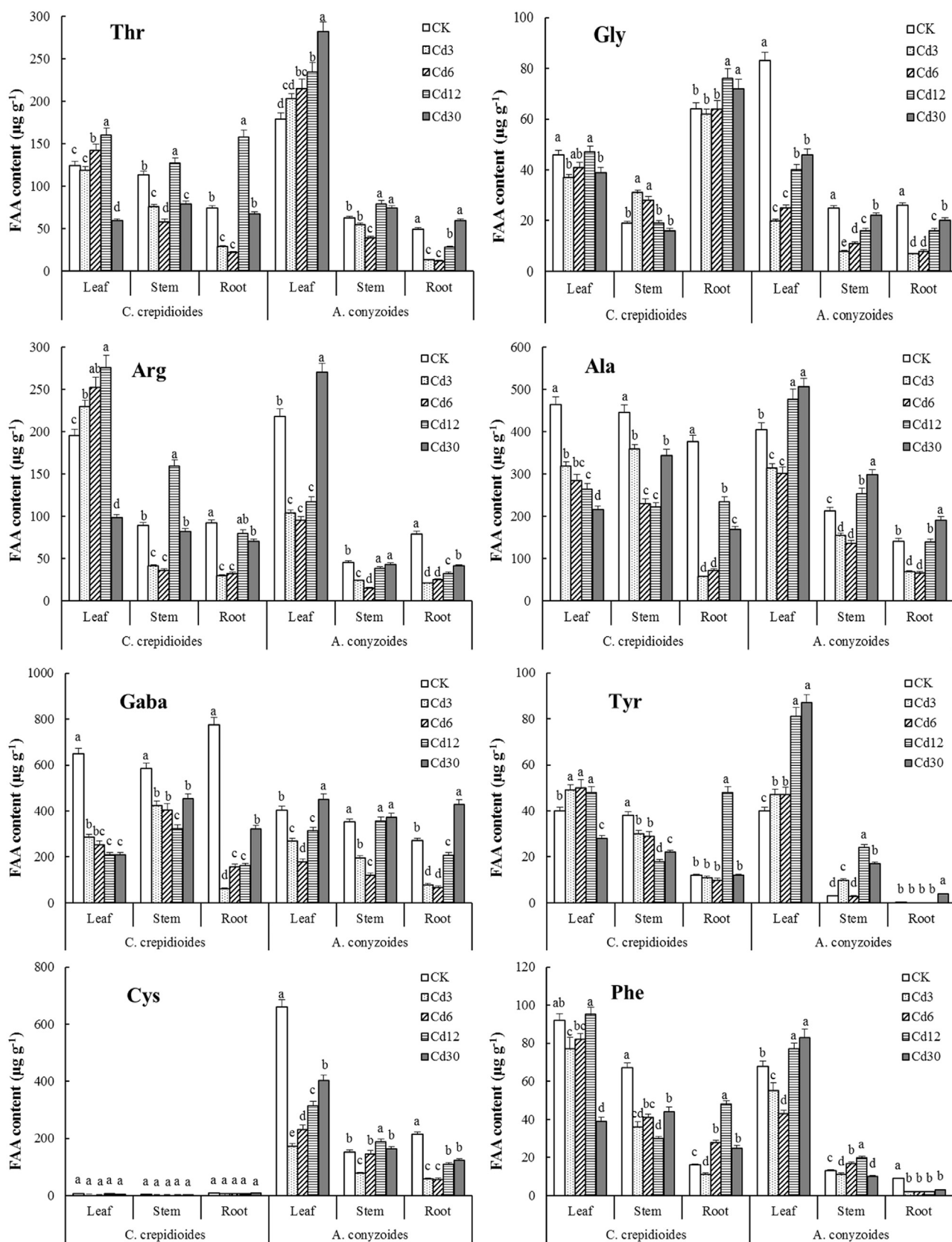
Fig. 4. (*continued*)

Pro. When treated with the highest amount of Cd (30 mg kg^{-1}), *C. crepidioides* exhibited a decrease for all of the FAA contents in the leaves, resulting in a decline of 74% for total FAA content compared with that under Cd12 treatment. Gln represented 64% of the total FAA

in control stems of *C. crepidioides* and increased to 74% under Cd12 treatment, and decreased to 57% under Cd30 treatment. The effects of Cd on Asp, Glu, Asn, and Val in stems were similar to that on Gln. Cd at a lower concentration (CK ~ Cd6) also reduced the FAA content to a
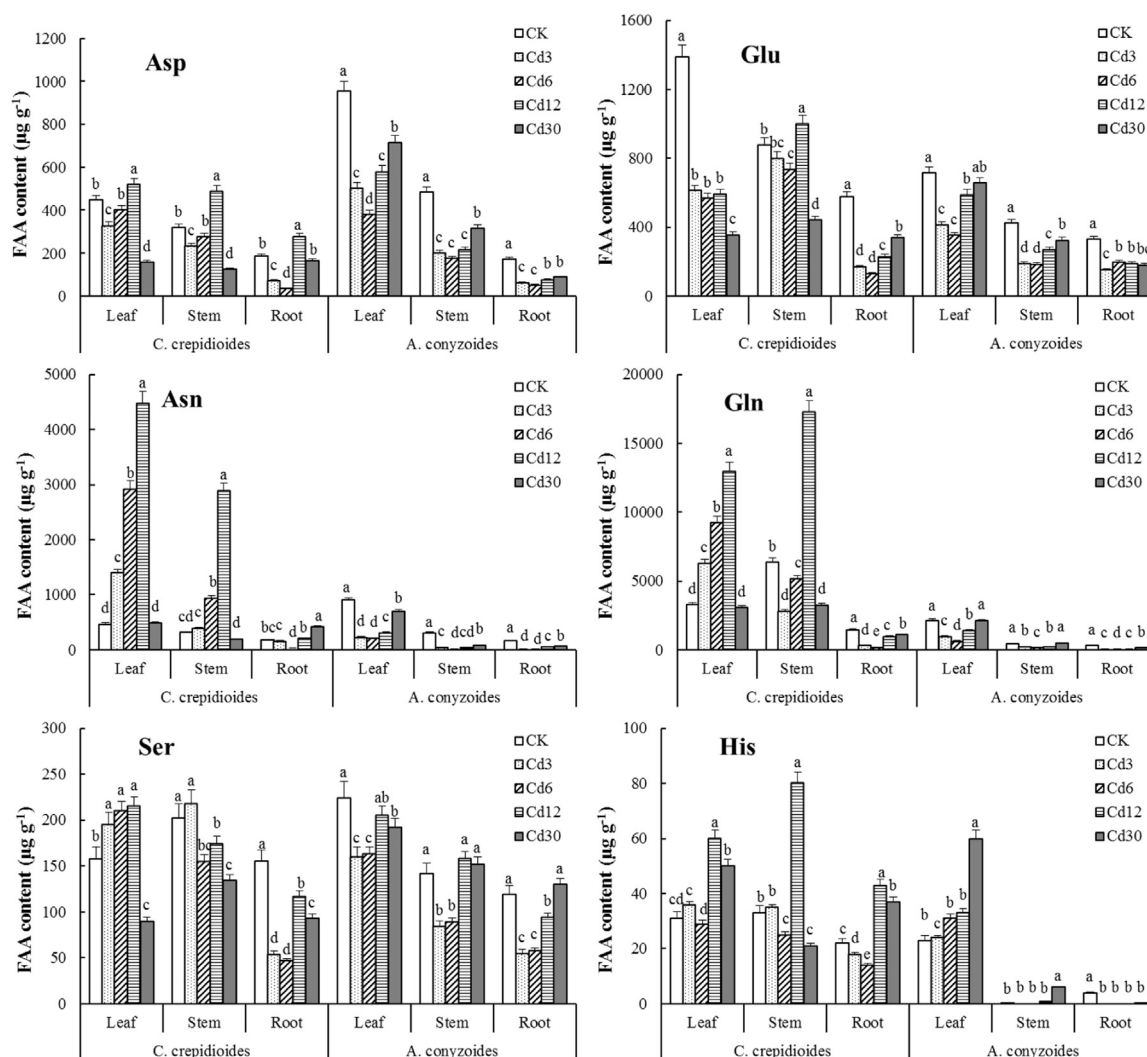
Fig. 4. (*continued*)

higher degree in the roots of *C. crepidioides* compared to the stems, whereas at higher Cd concentrations (Cd6 ~ Cd30), the increase of the FAA content was more apparent in the roots than in the stems.

In the case of *A. conyzoides*, Gln, Asp, Asn, Glu, Gaba and Val were still the main FAA encountered in all organs. Note that Cys was also a main FAA in *A. conyzoides*, whose amount varied from 5% to 10% of the total FAA among the Cd treatments of plant organs. However, Cys was barely determined in *C. crepidioides*. Gln dominated the most abundant FAA in the control plant, whereas, there was a significant difference in the contents of Gln between the two plants. Lower Gln contents were confirmed for *A. conyzoides* treatments than in *C. crepidioides* treatments. A 35–76% lower content of Gln was determined for *A. conyzoides* compared to the *C. crepidioides* controls, and correspondingly, a 51–88% lower content of Gln was determined under Cd treatments. Hence, in comparison with the low Cd accumulator *A. conyzoides*, the Cd accumulator *C. crepidioides* tended to contain higher concentrations of Gln, especially when it was grown in the presence of Cd. Upon Cd treatment, the majority of FAA and total FAA contents in *A. conyzoides* organs exhibited a decrease with increasing external Cd concentrations at lower doses of Cd (CK ~ Cd6). The opposite effect was observed in the presence of higher Cd doses (Cd6 ~ Cd30). A maximum reduction was observed for Asn (77%) followed by Gln (70%), Asp (60%) and Glu (51%) in comparison to the control leaves. Decreases of the total FAA pool were apparent in plant organs growing under Cd6 treatments (leave by 55%, stem by 51% and root by 61% compared to control). The

exposure of $30 \, \text{mg Cd kg}^{-1}$ in soil induced a maximum increase of 234% for Asn, followed by Gln (229%) and Gaba (152%), compared with the Cd6 treatment.

4. Discussion

Tolerance property, enrichment factor property and high biomass production are considered as the three key factors for using plants in phytoremediation strategies (Antonkiewicz and Para, 2016; Mahar et al., 2016). The tolerance characteristics show that there is no significant decrease in the shoot biomass compared with the control; enrichment factor property means that plants with high metal accumulation capacities also translocate metals effectively from root to the shoot. Several Cd-hyperaccumulator plants have low biomass production and growth rates which determine the extended use in soil remediation (Mahar et al., 2016). *A. conyzoides* and *C. crepidioides*, which are annual herb Compositae plants with fast growth and spread rates, occur widely throughout tropical and subtropical China. The results presented in this paper show that these two Compositae plants were identified as Cd accumulator plants, due to relatively high concentrations of Cd in the aerial parts and because their associated BCF were higher than 1, in addition to their associated BTF. However, the comparison of *C. crepidioides* and *A. conyzoides* considering biomass production and Cd accumulation, the former shows a higher growth tolerance under high Cd conditions. *C. crepidioides* can be basically used as

a Cd-hyperaccumulator. Additionally, triple cropping would be possible within one year for *C. crepidioides*, which is a significant superiority over other previously reported Cd accumulators. Accordingly, *C. crepidioides* represents a promising candidate for phytoextraction of Cd from polluted soils. It's necessary to conduct field trials to evaluate the possible use of this plant on a large scale.

Cadmium toxicity perturbed amino acid metabolism in plants, and the changes of amino acid levels can play a significant role in the mechanism of plant adaptation to Cd stress (Zemanová et al., 2014; Zoghlami et al., 2011). Studying the qualitative and quantitative changes in the content of individual amino acids can thus be informative. Amino acid homeostasis is essential for growth, development and defense of plants against environmental stress. The homeostasis is regulated by de novo biosynthesis, uptake/translocation, and protein synthesis/degradation (He et al., 2013). Chaffei et al. (2004) indicated that an increase in the proportion of high N:C amino acids is a protective strategy in plants for preserving roots as a nutritional safeguard organ to ensure future recovery. The results presented in this paper demonstrated an accumulation of large amounts of amino acids in Cd-tolerant *C. crepidioides* compared to *A. conyzoides* (Fig. 3), which was consistent with hypothesis explained above. The amino acid accumulation in high Cd accumulator *C. crepidioides* also suggested that the Cd-chelating molecules are highly active in binding Cd and forming a complex that can be translocated from the roots to the shoots.

Glu, Gln, Asp, and Asn, the main amino acids presented in the present study, are involved in nitrogen assimilation and transport processes within the plants. Furthermore, they are used to build up reserves during periods of nitrogen availability for subsequent use in growth, defense, and reproductive processes (Zemanová et al., 2015a). In all higher plants, inorganic N is at first reduced to ammonia prior to its incorporation into organic compound. Ammonia is assimilated into organic compound as Glu and Gln, which serve as the N donors in the biosynthesis of all essential amino acids and other nitrogen-containing compounds (Sánchez-Pardo et al., 2013). Asp is synthetized by transamination of oxaloacetate and also feeds into the synthesis of Asn, Lys, Met, Thr, and Ile as well as the conversion of Thr into Gly (Angelovici et al., 2009). In the present experiment, a decline in Asp and Glu content was observed in both studied plants at lower Cd doses, as well as an increase at higher Cd doses. According to Zemanová et al. (2013) the declines of both amino acids can be caused by intensive syntheses of plant defense elicitors. These two amino acids are quickly transformed into the required products or incorporated into a protein without increased accumulation in plant when exposed to Cd stress. The accumulation of Glu and Asp at higher Cd doses may be a consequence of buffering effects through the modulation of Pro/His/Orn and Asn/Lys/Thr/Ile/Met (Xu et al., 2012). Gln, with high N/C ratio, is known to be highly reactive and to serve as the major nitrogen transport form in plants. Asn, which is also an amide showing a high N/C ratio, is less reactive than Gln and can be used by the plant as a nitrogen storage compound (Chaffei et al., 2004). In addition, a Cd-Gln and Cd-Asn complex may reduce Cd toxicity. Moreover, asparagine shows the same effect by acting as a ligand towards Cd (Sharma and Dietz, 2006). At lower Cd exposure, increasing Cd doses were associated with clearly increasing Gln and Asn contents in *C. crepidioides* shoots and with decreasing Gln and Asn contents in *A. conyzoides*. These results showed a different pathway of nitrogen utilization of both plants. Note a higher accumulation of Glu, Glu, Asp and Asn in the root of *C. crepidioides* when exposed to highest Cd stress, which supports the observed higher Cd tolerance and Cd accumulation in *C. crepidioides* than in *A. conyzoides*.

Pro and GABA are well known to be the abiotic and biotic stress indicator and protector in plants (Xu et al., 2012; Zemanová et al., 2017). The induction of free Pro in response to heavy metal exposure has been reported (Sharma and Dietz, 2009; Vassilev and Lidon, 2011; Xu et al., 2009). The functions of Pro seem to be manifold. It plays a major role in adjustment to osmotic stresses, maintaining the water

balance as it stabilizes the subcellular structures, and also function as a hydroxyl radical scavenger (Sharma andDietz, 2006). However, Pro exhibited opposite abundance trends between the two tested plants under Cd stress, which indicated that two different stress defense pathways exist in the two species. Pro is a precursor in the Hyp biosynthesis pathway. Thus, increased consumption of Pro for Hyp biosynthesis under Cd stress could be the reason for the observed decrease in Pro content in *C. crepidioide* shoots. The result corresponds with those by Yi and Kao (2003) who reported that no Pro accumulation was observed in Cd-tolerant plant leaves. Higher accummulation of Pro in *A. conyzoides* shoots was detected when exposed to Cd stresses, which can be attributed to its protective role in reducing membrane and protein damages, and acting as an osmo-protectant in stressed plants (Pavlík et al., 2010). Besides being the major component of the free amino acid pool, different roles have been designated for GABA. Induction of signaling pathway via Ca^{2+}, C: N balance via primary metabolism, developmental role via cell wall modification, participation to nutrient uptake and stimulation of cell death are among these different roles in plants (Michaeli and Fromm, 2015). Excessive GABA levels in response to different abiotic and biotic environmental stresses are commonly found in several plants (Pavlíková et al., 2014; Shelp et al., 2012). The opposite trend observed in our study may be a consequence of increased inhibition of glutamate decarboxylase (GAD) activity results in a decline of GABA synthesis from Glu under Cd stress.

Ala, a branched chain amino acid, has been reported to play a functional role in intracellular pH regulation, and it typically accumulates in response to various stresses (Limami et al., 2008; Rocha et al., 2010). Our data demonstrated that increasing Cd concentrations at lower Cd doses resulted in a decline in the content of free Ala in both plants, and can be linked to the increasing use of Ala for the biosynthesis of proline/alanine-rich protein kinases or histidine- and alanine-rich proteins (Komatsu et al., 2009; Mori et al., 2013). When stressed with high Cd amounts, a reduction in the rate of protein synthesis and an increased Ala synthesis due to disturbance of alanine aminotransferase reactions resulted in an increase of free Ala (Hjorth et al., 2006). As another branched chain AA, Val is pivotal in balancing the fluxes between different amino acids pathways. The accumulation of Val may serve to promote stress-induced protein synthesis and to maintain amino acids homeostasis (Joshi et al., 2010). A stimulating effect of Cd in soil on the content of free Val in the leaves of both studied species was observed through the experiments, while an opposite effect was shown in the stems, which may be a direct biological response to the stress conditions.

His was found to play an important role in regulation of biosynthesis of other amino acids, in chelation and transport of metal ions, and in plant reproduction and growth (Stepansky and Leustek, 2006). This experiment showed that the content of His significantly increased with increasing Cd concentrations, while His was below the detection limit in the roots and stems of *A. conyzoides*. Thus, the synthesis of His seems to be associated with Cd stress. Gly and Cys are involved in the biosynthesis of phytochelatines and antioxidant metabolites, and are also found in glycine-rich proteins that affect the growth and function of cell walls (Zemanová et al., 2017). Significantly decreased Gly contents in *A. conyzoides* must be seen as the activation of adaptation processes to the toxic effect of Cd, while in our hyperaccumulator species Gly accumulation was relatively stable. Cys was a major FAA in *A. conyzoides*, while its content was barely determined in *C. crepidioides*. Xu's et al. (2012) previous transcriptome analysis revealed that a cysteine desulfurase gene, NFS, which can catalyze Cys to Ala, showed higher expression in the Cd accumulators *S. nigrum*. The undeterminable Cys concentration in *C. crepidioides* may be a result of the conversion of Cys to Ala.

Trp, Tyr and Phe, which are aromatic amino acids, plays a major role in the regulation of plant development and defense responses. Moreover, they represent the precursors for the synthesis of protein and hormone auxin (Liu et al., 2011; Sanjaya et al., 2008). In the present

study, biosynthesis of Trp and Tyr was induced by Cd stress in the roots of plants and an increased content of Trp and Tyr have been found in shoots under Cd stress. Accumulation of free Trp and Tyr in *A. conyzoides* stems under Cd12 treatment is more than 70-fold and 10-fold higher in contrast to Cd6 treatment. These observations indicated that Trp and Tyr may be involved in Cd resistance and accumulation by reducing oxidative damage in the studied plants. Phe is also a substrate for the phenylpropanoid pathway, which produces a wide range of antioxidative metabolites and phenolic compounds (Sanayama et al., 2011). A fluctuating content of free Phe was observed in our study indicating their responsiveness during Cd stress.

Acknowledgements

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