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Responses to Cd Stress in Two Noccaea Species (*Noccaea praecox* and *Noccaea caerulescens*) Originating from Two Contaminated Sites in Mežica, Slovenia and Redlschlag, Austria

Veronika Zemanová[1] · Milan Pavlík[2] · Daniela Pavlíková[1] · František Hnilička[3] · Stanislava Vondráčková[1]

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Abstract The two *Noccaea* species—*Noccaea praecox* originating from Mežica, Slovenia (Me) (Pb, Zn, Cd pollution) and *Noccaea caerulescens* from Redlschlag, Austria (Re) (high levels of Ni, Cr, Mg)—were studied to compare Cd accumulation and tolerance. After 120 days of plant cultivation in Cd-contaminated soil (90 mg Cd kg^{-1} soil), gas-exchange parameters (e.g. net photosynthetic rate, transpiration rate, stomatal conductance, and intercellular CO_2 concentration), fatty acids, and selected macro- and microelements were determined in addition to N utilization by plants. The comparison between ecotypes showed that Cd stress resulted in similar changes in gas-exchange parameters. Contrasting responses of plants to Cd contamination were confirmed by the macro- and microelement contents and fatty acid and amino acid metabolism. Significantly higher accumulations of Cd and strong decreases in the levels of K, Ca, Na, and Fe were observed in the Me plants in contrast to the Re plants. The higher Re plant ability to take in some cations is a result of selective pressure due to contamination. Different ion uptake by plants affected the activities of metalloenzymes. Significant increases in the glutamic acid/proline ratio resulted from higher adaption of the Me in contrast to the Re plants.

✉ Daniela Pavlíková
pavlikova@af.czu.cz

[1] Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 16521 Prague, Czech Republic

[2] Isotope Laboratory, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague, Czech Republic

[3] Department of Botany and Plant Physiology, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 16521 Prague, Czech Republic

Cadmium is a heavy metal that is released into the environment by thermal power and heating plants, metal industries, cement factories, urban traffic, sewage sludge, and phosphate fertilizers (Gratão et al. 2005). High concentrations of Cd in the soil can be toxic to plants. In leaves, concentrations of Cd that are higher than 5–10 μg g^{-1} DW are toxic to most plants (White and Brown 2010; Lux et al. 2011). However, some species can hyperaccumulate Cd to concentrations in excess of 100 μg g^{-1} DW in their leaves without showing any negative symptoms (Baker et al. 2000).

Cadmium can enter the plant through nonspecific cation channels as well as through different divalent cation transporters, competing with essential mineral nutrients for absorption (Verbruggen et al. 2009; Lux et al. 2011). Therefore, the uptake and distribution of essential mineral nutrients, such as Fe, Ca, Mg, and K, can be severely disturbed in the presence of high levels of cadmium (Martin et al. 2012).

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). Plants exposed to Cd had elevated levels of mitochondrial ROS production, indicating that this organelle had become dysfunctional (Heyno et al. 2008). This element has been shown to be one of the most effective inhibitors of photosynthetic activity (Gallego et al. 2012). It can enter chloroplasts and disturb chloroplast function by inhibiting

the enzymatic activities involved in chlorophyll biosynthesis, pigment–protein complexes, the O_2-evolving reactions of photosystem II, electron flow around photosystem I, and chloroplast structure (Ying et al. 2010; Molins et al. 2013 etc.). Cd reduced the photochemical processes more in older leaf segments than in younger ones, but the functional status of the dark phase of photosynthesis was more strongly diminished in younger ones (Drążkiewicz and Baszyński 2005). According to Perfus-Barbeoch et al. (2002), stomatal closure, damage to the photosynthetic machinery, and interference with pigment synthesis cause a general depression of photosynthetic efficiency, lowering the effective quantum yield. Moreover, by inhibiting enzymes involved in CO_2 fixation, Cd decreases carbon assimilation. Cadmium at low concentrations induced net photosynthetic rate (P_N), content of chlorophyll and carotenoids in hyperaccumulator *Lonicera japonica* (Jia et al. 2015). The results of Dias et al. (2013) indicated that high Cd concentration strongly inhibited P_N. This inhibition was followed by a decrease in transpiration rate (E) and stomatal conductance (g_s). According to Deglinnocenti et al. (2014), the reduction in P_N observed in Cd-treated plants *Lycopersicon esculentum* was not linked to stomatal limitation, because it also was indicated by the unchanged CO_2 intracellular concentration (C_i). Nwugo and Huerta (2008) found high C_i value for rice seedlings (*Oryza sativa*) exposed to high Cd concentrations. High C_i indicated that the inhibition of photosynthesis also was due to an inhibition of Calvin cycle enzymes and/or an inhibition of the photosynthetic electron transport chain. The increase of C_i may be explained by modifications of RuBisCO activities of plant (Redondo-Gómez et al. 2011). According to Leitenmaier and Küpper (2011), hyperaccumulator plants have to store the excess metal in such a way that it does not harm important enzymes and especially not photosynthesis. It has been shown that high amounts of metals are stored specifically in the vacuoles of large epidermal cells (Küpper et al. 1999, 2001; Frey et al. 2000), where no chloroplasts are located so that photosynthesis cannot be inhibited.

The comparison between hyperaccumulators and non hypeaccumulating plants showed significant differences of fatty acids (FAs) composition related to Cd chronic stress. Lipid changes in *Brassica juncea*, the well-known Cd-hyperaccumulator specie, revealed more stability of its cellular membranes to cadmium-stress compared with Cd-sensitive species, *Brassica napus*. The levels of polyunsaturated FAs mainly C18:3, C16:3 and C16:1t declined in *B. napus* (Nouairi et al. 2006). The number of identified FAs in spinach biomass was very low compared with hyperaccumulator *Noccaea caerulescens*. Saturated very-long-chain fatty acids (VLCFAs) have been found only in hyperaccumulating plants. Biosynthesis of VLCFAs decreases amount of energy necessary for plant growth and development. Catabolic processes of these FAs decrease plant sensitivity to environmental stress (Zemanová et al. 2015a, b).

Several studies have focused attention on the role of the amino acids in metal tolerance of plants. The amino acids that accumulate under heavy metal stress play various roles in plants, including acting as signaling molecules, acting as osmolytes, regulating ion transport, and facilitating detoxification. Heavy metals in xylem sap are transported almost entirely complex with amino acids (Liao et al. 2000). Histidine, aspartic acid, glutamic acid, and asparagine are related to the long-distance transport of xylem. Krammer et al. (1996) reported that histidine accumulation is responsible for nickel hyperaccumulation in *Alyssum*. Salt et al. (1999) observed the presence of a Ni-histidine complex in the xylem sap of *N. caerulescens*. Proline (Pro) played a role in the alleviation of Cd toxicity by detoxifying ROS, thereby increasing the glutathione concentration and protecting antioxidative enzyme activities in *Solanum nigrum* seedlings (Xu et al. 2009). A higher accumulation of Pro in *S. nigrum* supports the observed higher Cd tolerance in *S. nigrum* than in *Solanum torvum*. Hydroxyproline is an important component of the Casparian band. A high accumulation of Hyp in *S. torvum* roots may play a protective role in preventing Cd translocation from the roots to the aerial parts of the plant (Xu et al. 2012a, b).

Hyperaccumulators of toxic elements are highly attractive model organisms, because they have overcome major physiological problems that limit metal accumulation in biomass and have toxic element tolerance (Verbruggen et al. 2009; Ueno et al. 2011). In the presented study, two *Noccaea* species—*N. caerulescens* and *Noccaea praecox*—were cultivated to investigate the effect of strong Cd-polluted soil on plant metabolism. Internal transcribed spacer (ITS) rDNA sequences from *N. praecox* populations from Slovenia showed 99 % similarity and formed a sister group to *N. caerulescens*. Evolutionary development of extraordinary Cd hyperaccumulation abilities in particular *N. praecox* populations may be closely related to the levels of this element in the soil (Likar et al. 2010). Similarly studies of *N. caerulescens* showed that ecotypes growing naturally in low Cd-containing soils have much lower hyperaccumulation capacity compared with the ecotypes growing in high Cd-containing soils (Gonneau et al. 2014).

The purpose of this study was to characterize changes in nitrogen metabolism, elements content, FAs, and gas-exchange parameters of two *Noccaea* species—*N. praecox* and *N. caerulescens* (from serpentine group)—growing under strong Cd stress. Our objectives are: (1) to confirm differences among tested parameters for hyperaccumulating plants growing on Cd contaminated and

noncontaminated soil; (2) to show that the regulation of Pro biosynthesis from Glu is dependent on the Glu:Pro ratio and is determined by phenotypic variability; and (3) to assess phenotypic variability for tested parameters between two *Noccaea* species (with 99 % genotypic similarity) growing in pot experiment under strong Cd stress.

Materials and Methods

Plant Material and Cultivation Conditions

In the pot experiments, *N. praecox* (formerly *Thlaspi praecox* Wulfen*)* from Mežica, Slovenia (Me) and *N. caerulescens* (formerly *Thlaspi caerulescens* J. & C. Presl, FK Mey*)* from Redlschlag, Austria (Re) were used. The Mežica mining district source area is characterized by the presence of ore minerals of geogenic/technogenic origin (cerussite, sphalerite, smithsonite, and galena). The environs of Mežica are strongly polluted with Pb and Zn. Because Cd is found as a trace element in sphalerite and smithsonite, its content correlates with that of Zn (Gosar and Miler 2011). The bedrock of Redlschlag is composed of serpentine, which contains large amounts of Ni and Cr and some Zn and Co. Because the soil pH is neutral (approx. pH 6.55), the main problems for plants are low concentrations and availability of micronutrients, although Mg is abundant (46,400 mg Mg kg^{-1}—Puschenreiter et al. 2005).

For the cultivation of *Noccaea* plants (2 plants per pot), 3 kg of soil (from the nonpolluted site Prague–Suchdol, Chernozem—pH 7.2, CEC = 258 $mmol_{(+)}$ kg^{-1}, C_{org} = 1.8 %, Cd_T = 0.42 mg kg^{-1}) was thoroughly mixed with nutrients (0.3 g N, 0.10 g P, and 0.24 g K applied in the form of NH_4NO_3 and K_2HPO_4) as the control treatment and with the same amount of nutrients plus Cd [$Cd(NO_3)_2 \bullet 4H_2O$] at 90 mg Cd kg^{-1} for treated variants. The water regime was controlled, and the soil moisture was kept at 60 % maximum water-holding capacity (MWHC). Each treatment was performed in five replications. Plants were harvested 120 days after Cd application. Samples were kept frozen in liquid nitrogen for transport and then at −30 °C until extraction.

Analyses

Determination of Gas-Exchange Parameters

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured in the leaves in situ using the portable gas-exchange system LCpro + (ADC BioScientific Ltd., Hoddesdon, Great Britain) from 10:00 to 11:30 Central European summer time. The irradiance was 595 µmol m^{-2} s^{-1} photosynthetically active radiation, the temperature in the measurement chamber was 22.7 °C, and the duration of the measurement of each sample was 15 min after the establishment of steady-state conditions inside the measurement chamber (Pavlíková et al. 2014).

Analysis of Free Amino Acids in Plant Biomass

The amino acids in methanol + H_2O extracts were determined using the EZ-faast amino acid analysis procedure (Phenomenex, U.S.A.). Amino acid content was analyzed by GC–MS using a Hewlett Packard 6890 N/5975 MSD (Agilent Technologies, USA). Samples were separated on a ZB-AAA 10 m × 0.25 mm amino acid analysis GC column using constant carrier gas (He) flow (1.1 ml min^{-1}). The oven temperature program was as follows: initial temperature 110, 30 °C min^{-1} ramp to 320 °C. The temperature of the injection port was 280 °C. A total of 1.5–2 µl sample was injected in split mode (1:15, v/v). The MS conditions were as follows: MS source 240 °C, MS quad 180 °C, auxiliary 310 °C, electron energy 70 eV, scan m/z range 45–450 and sampling rate 3.5 scan s^{-1} (Pavlík et al. 2012).

Determination of Fatty Acids

Samples of fresh biomass (~0.2 g) are extracted in 2 ml of methanol + chloroform (3:2, v/v) on a shaker for 24 h. Transesterification of FAs was performed in the supernatant according to method of Stranský and Jursík (1996a, b). The content of methyl esters of FAs was measured by GC–MS (Thermo Scientific DSQ II Single Quadrupole GS–MS, Thermo Fisher Scientific) with a nonpolar column Zebron ZB-5 30 m × 0.25 mm × 0.25 µm. The injection volume was 1 µl of sample in a splitless mode. The carrier gas was helium (He, purity 5.0) with a constant flow rate of 1 ml/min. The temperature program of oven: initial temperature 50 °C (for 2 min); 8 °C min^{-1} ramp to a temperature of 320 °C (for 10 min); inlet temperature 250 °C and transferline temperature 260 °C.

Analyses of Cadmium and Additional Elements in Plant Biomass

Plant samples were decomposed using the dry ashing procedure as follows: an aliquot (~1 g) of the dried and powdered biomass was weighed in a borosilicate glass test tube and decomposed in a mixture of oxidizing gases (O_2 + O_3 + NO_x) at 400 °C for 10 h in a Dry Mode Mineralizer Apion (Tessek, Czech Republic). The ash was dissolved in 20 ml of 1.5 % HNO_3 (v/v) (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass

tubes until analysis. Aliquots of the certified reference material RM NCS DC 73350, poplar leaves, (purchased from Analytika, CZ) were mineralized under the same conditions for quality assurance. The Cd and macro- and microelement concentrations were determined by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia).

Water extractable-Cd contents in leaves were measured in 0.2 g dried biomass suspended in 50 ml deionized water and shaken for 2 h at 20 °C. The suspension was filtered at 0.2 μm porosity with a cellulose nitrate filter and then acidified with HNO_3. Cd concentrations were measured by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia) (Perronnet et al. 2000).

Statistical analyses were performed using hierarchic analyses of variance (ANOVA) considering interactions at the 95 % ($P < 0.05$) significance level with subsequent Tukey's HSD test and correlation (R^2). All analyses were performed with Statistica 9.1 software (StatSoft, USA).

A principal component analysis (PCA), in the CANOCO 4.5 (ter Braak and Šmilauer 2002) software, was used to evaluate multivariate data. We used standardisation of species data because data of different character and units were analysed together. The PCA was used to make visible correlations between all the analysed data and similarity of the different treatments. Obtained results were visualised in the form of a bi-plot ordination diagram created by CanoDraw program.

Results

Yield of Aboveground Biomass and Cd Content

The yield of aboveground biomass, although similar in both varieties under Cd stress, indicated greater reduction of Re–Cd (50 % reduction in contrast to control) than Me–Cd (39 % reduction compared with control; Fig. 1).

The biomass Cd contents showed significant differences between *Noccaea* sp. The highest Cd content was found in the biomass of Me-Cd plants (257-fold greater Cd content in Me–Cd than Me). The Cd contents of Re were significantly lower than in Me, and increase in Cd content under Cd stress was only 68.5-fold (Table 1). Similar differences in water-extractable Cd were determined between treatments (Table 1). The highest proportion of water-extractable Cd was measured for Me and Me–Cd. There was a close *negative* relationship between the yield of the aboveground biomass of all tested plants and plant Cd content ($R^2 = -0.71$). There was no relationship between yield and water-extractable Cd ($R^2 = -0.29$). This result is caused by the different Cd accumulation in proteins or pectins isolated after hydrolyses of the plant cytoskeleton.

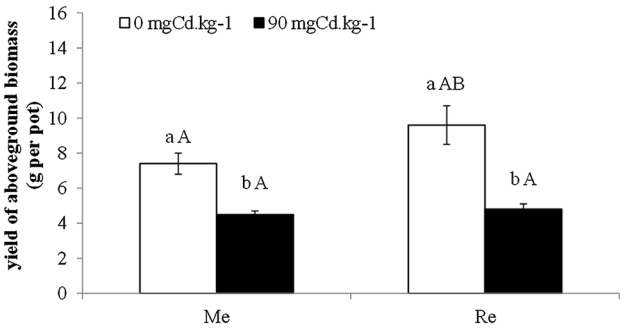

Fig. 1 The yield of aboveground biomass (mg kg^{-1}). Values represent the means of data obtained in the experiment ($n = 5$, i.e., 5 replications per each treatment). Cd concentration was either 0 or 90 mg Cd kg^{-1} soil. *Letters* refer to significantly different values that are significant ($P \leq 0.05$). *A*, *B* Comparison between both species; *a*, *b* comparison between treatments in each species

Plant Gas-Exchange Parameters

To compare the Cd adaptation of the two species, the effect of soil contamination (90 mg Cd kg^{-1}) on gas-exchange parameters was analyzed. As shown in Table 2, Cd treatment inhibited the photosynthetic rate (P_N), but significant differences between species were not observed. The P_N also was similar in the both control treatments. Cd soil contamination caused only 8–12 % P_N reduction. There was a close *negative* relationship between P_N and Cd content in plant biomass ($R^2 = -0.69$). Both the transpiration rate (E) and stomatal conductance (g_s) increased under Cd stress (Table 2). Both species had 3.5-fold greater E in Cd treatment in contrast to controls. Similar but nonsignificant trends were found for g_s and intracellular CO_2 concentration (C_i). The water-use efficiency (WUE) was estimated from P_N and E. The WUE of Me–Cd and Re–Cd compared with the controls declined by 25.3–26.4 %.

Plant Element Contents

The element contents of Me and Re were different; however, the trends of the changes were similar (Table 1). The content of the tested elements in the aboveground biomass was affected by Cd supply. The Mg contents of Cd treatments were not affected by Cd contamination. Reductions in K, Ca, and Na contents were observed in both Cd treatments. The highest reductions in these elements were determined for Me–Cd (45 % K, 36 % Ca, and 64 % Na reduction). The most significant relationship was between Ca and Cd contents (and water-soluble Cd) ($R^2 = 0.77$ and 0.86, respectively). Reductions in K, Ca and Na affected WUE ($R^2 = 0.52$–0.58). Cd reduced Fe in both treatments. There was a close relationship between C_i and the Fe contents ($R^2 = 0.69$–0.95).

Table 1 Element contents in the aboveground biomass of plants

Treatment	Me	Me-Cd	Re	Re-Cd
Cd_T (mg kg^{-1})	28.0 ± 1.6^{aA}	7201 ± 15.9^{bA}	2.0 ± 0.1^{aB}	137 ± 9.6^{bB}
Cd_w (mg kg^{-1})	12.5 ± 0.4^{aA}	1300 ± 9.7^{bA}	0.2 ± 0.0^{aB}	1.0 ± 0.1^{bB}
K (%)	5.99 ± 0.23^{aA}	3.29 ± 0.15^{bA}	4.95 ± 0.19^{aB}	4.55 ± 0.11^{bA}
Mg (%)	0.22 ± 0.03^{aA}	0.22 ± 0.04^{aA}	0.17 ± 0.02^{aB}	0.18 ± 0.01^{bB}
Ca (%)	2.18 ± 0.09^{aA}	1.40 ± 0.10^{bA}	2.77 ± 0.07^{aB}	2.47 ± 0.15^{aB}
Fe (mg kg^{-1})	227 ± 26^{aA}	176 ± 18^{bA}	474 ± 31^{aB}	441 ± 27^{bB}
Na (mg kg^{-1})	277.8 ± 15.0^{aA}	99.6 ± 8.9^{bA}	126.0 ± 12.1^{aB}	78.4 ± 8.7^{bB}

Values represent the means of data obtained in the experiment ($n = 5$, i.e., 5 replications per each treatment). Letters refer to significantly different values that are significant ($P \leq 0.05$)

[A,B] Comparison between both species

[a,b] Comparison between treatments in each species

Table 2 Effect of Cd contamination on CO_2 intracellular concentration (C_i), net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) of *Noccaea* sp. ($n = 5$)

Treatment	P_N (μmol CO_2 m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (vpm)	WUE
Me	8.68 ± 0.9^{aA}	0.433 ± 0.03^{aA}	0.322 ± 0.015^{aA}	354 ± 15^{aA}	20.05
Me–Cd	7.97 ± 1.1^{aA}	1.505 ± 0.11^{bA}	0.989 ± 0.026^{bA}	360 ± 21^{aA}	5.30
Re	8.50 ± 0.6^{aA}	0.263 ± 0.02^{aB}	0.361 ± 0.021^{aA}	332 ± 17^{aA}	32.30
Re–Cd	7.49 ± 0.7^{bA}	0.917 ± 0.04^{bB}	0.917 ± 0.076^{bA}	367 ± 19^{bA}	8.17

From these data, the water-use efficiency was estimated (WUE = P_N/E). Values represent the means of data obtained in the experiment ($n = 5$, i.e., 5 replications per each treatment). Cd concentration was either 0 or 90 mg Cd kg^{-1} soil. Letters refer to significantly different values that are significant ($P \leq 0.05$)

[A,B] Comparison between both species

[a,b] Comparison between treatments in each species

Free Amino Acids

Table 3 includes amino acids related to assimilation, transport and accumulation of nitrogen in plants (Asn, Asp, Glu, and Gln). Minor changes in Asp and Glu contents as a result of Cd contamination were observed in both species. Identical changes in Asn (decrease by 58 % in Me–Cd and 50 % in Re–Cd) were found for all Cd-treated plants. The opposite trend was observed for Gln, primarily for Me–Cd (increase by 90 %). These AA showed a close correlation with WUE ($R^2 = 0.80$), and an effect of Fe content on amino acids (AA) formation was observed ($R^2 = 0.93$).

The contents of proline (Pro) and serine (Ser) increased in both species grown under Cd stress, but the content of

Table 3 Concentrations of selected free amino acids in the aboveground biomass of plants

Free amino acid	Me (μmol kg^{-1})	Me–Cd	Re	Re–Cd
Glutamic acid (Glu)	9110 ± 109^{aA}	9037 ± 65^{aA}	$12{,}295 \pm 98^{aB}$	$13{,}990 \pm 86^{bB}$
Aspartic acid (Asp)	5248 ± 87^{aA}	5323 ± 7^{aA}	5382 ± 115^{aA}	4244 ± 93^{bB}
Glutamine (Gln)	$13{,}398 \pm 99^{aA}$	$25{,}477 \pm 49^{bA}$	$13{,}176 \pm 82^{aB}$	$27{,}545 \pm 79^{bB}$
Asparagine (Asn)	7288 ± 95^{aA}	3051 ± 101^{bA}	$30{,}386 \pm 84^{aB}$	$15{,}179 \pm 96^{bB}$
Proline (Pro)	951 ± 62^{aA}	1220 ± 69^{bA}	3338 ± 73^{aB}	5299 ± 65^{bB}
Serine (Ser)	2502 ± 22^{aA}	5102 ± 35^{bA}	6168 ± 37^{aB}	8664 ± 42^{bB}
Hydroxyproline (Hyp)	297 ± 38^{aA}	329 ± 19^{aA}	302 ± 26^{aA}	417 ± 31^{bB}

Values represent the means of data obtained in the experiment ($n = 5$). Letters refer to significantly different values that are significant ($P \leq 0.05$)

[A,B] Comparison between both species

[a,b] Comparison between treatments in each species

Table 4 Glu:Pro amino acid ratio, which affects the regulation of Pro biosynthesis

Treatment	Me	Me–Cd	Re	Re–Cd
GLU/PRO	9.58	7.41	3.68	2.64

free Pro was significantly higher in both the control and Cd treated Re than in Me. The regulation of Pro biosynthesis from Glu is dependent on Glu:Pro ratio (Table 4). The results confirmed a higher Glu:Pro ratio in both Me treatments than in Re. Plants with a higher Glu:Pro ratio are better adapted to Cd stress, because Glu can be used for the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine and is used in the synthesis of glutathione and phytochelatins in plant cells. A significant correlation between these AA (Pro and Ser) and Asn, Asp, Glu, and Gln was found ($R^2 = 0.76$ and 0.93, respectively). The correlation between Pro or Ser and water-soluble Cd ($R^2 = 0.88$) confirmed the direct effect of Cd on their contents.

Increased Cd contents of plants increased the content of Hyp, a major AA in plant cell wall hydrolysates (Hyp increase of 11 % in Me-Cd and 38 % in Re–Cd). The correlation between Cd and Hyp ($R^2 = 0.75$) confirmed the direct effect of Cd on this AA.

Fatty Acids

Increased peroxidation of FAs is associated with Cd soil contamination and Cd detoxification processes in the plant cell (Fig. 2). The content of saturated and unsaturated FAs in control plants did not significantly differ (saturated FAs = 42.7–48.3 % of total FAs; unsaturated FAs 51.7–57.3 %). A significant decline in saturated FAs (36 %) and increase in unsaturated FAs (33 %) was observed for Me–Cd. Linear correlations confirmed close relationships between saturated and unsaturated FA contents and Cd content in Me–Cd biomass ($R^2 = 0.99$). Cd soil contamination did not significantly modify the total contents of saturated and unsaturated FAs in Re biomass.

Results of Principal Component Analysis

In the PCA performed on all the plant parameters, the first axis of the PCA analysis explained 47 %, the first two axes 79 %, and the first four axes together—96 % of the variability of all analysed data (Fig. 3). The first ordination axis divided individual pots into the *N. caerulescens* group on the left side and *N. praecox* on the right side of the diagram. This indicates a large effect of used plant species on yield of aboveground biomass, content of elements, concentrations of selected free AA, and plant gas-exchange parameters. For both plant species, marks for treatments (control, Cd) were located in the different parts of the diagram, which indicates a high effect of treatments on all the recorded data. The length and direction of the vectors of the studied parameters indicate links among themselves with respect to the treatments and plant species. The concentrations of free AA (Asn, Pro, Gln, Glu, Ser, Hyp), total concentrations of Ca and Fe were accumulated more in *N. caerulescens*. On the other hand, concentrations of Asp, total concentrations of K, Mg, and Cd_T, as well as concentrations of water extractable-Cd (Cd_w) were accumulated more in *N. praecox*. The concentrations of Ser and Hyp were accumulated more in *N. caerulescens* in Cd treatment. The concentrations of Asp were accumulated more in *N. praecox* in control treatment. The yield was positively correlated with WUE as indicated by an angle

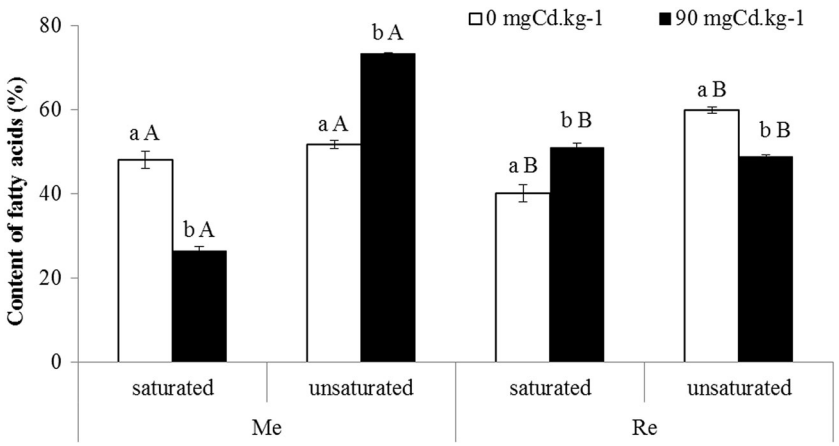

Fig. 2 Total content of saturated and unsaturated fatty acids in *N. caerulescens* and *N. praecox* leaves. Values represent the means of data obtained in the experiment ($n = 3$, i.e., 3 replications per each treatment). Cd concentration was either 0 or 90 mg Cd kg^{-1} soil. *Letters* refer to significantly different values that are significant ($P \leq 0.05$). *A*, *B* Comparison between both species; *a*, *b* comparison between treatments in each species

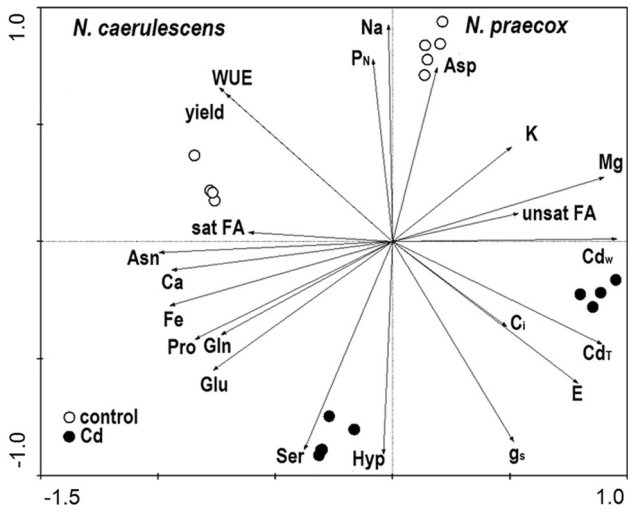

Fig. 3 Ordination diagram showing the results of PCA analysis with selected parameters in *N. caerulescens* and *N. praecox*. Treatment abbreviations: *Cd* treatment 90 mg Cd kg^{-1}. Parameters abbreviations: *yield* yield of aboveground biomass, *sat FA* total content of saturated fatty acids, *unsat FA* total content of unsaturated fatty acids, P_N net photosynthetic rate, C_i intercellular, CO_2 concentration, E transpiration rate, g_s stomatal conductance, *WUE* water-use efficiency, C_T total content of Cd, C_w water extractable-Cd, *Ca* total content of Ca, *Fe* total content of Fe, *K* total content of K, *Mg* total content of Mg, *Na* total content of Na, *Asp* concentration of free aspartic acid, *Asn* concentration of free asparagine, *Pro* concentration of free proline, *Glu* concentration of free glutamic acid, *Gln* concentration of free glutamine, *Ser* concentration of free serine, *Hyp* concentration of free hydroxyproline

between the vectors for them of <90° and was negatively correlated with Cd_T as the angle between vectors for yield/WUE and Cd_T was >90°. Two vectors did not positively correlated, if the angle between them is larger than 90°. A long vector for particular parameters indicates a strong effect on the results of the analysis, and vice versa.

Discussion

The yields of both *Noccaea* species were reduced by Cd contamination (90 mg Cd kg^{-1}), but greater reductions of Re–Cd than Me–Cd were found. We previously published similar results for *N. caerulescens* (ecotype "Ganges") (Zemanová et al. 2013, 2014). Our data agree with those of 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. Selective pressure of contaminated soil has affected quantitative characteristics of plants (Snustad and Simmons 2009). This pressure resulted in higher adaptation of plants from Mežica (Me).

The accumulation of Cd in plant tissues damaged the photosynthetic apparatus, which is generally protected from Cd contamination by reduction of free heavy-metal ion concentration in the cytoplasm by complexation with S- and O-ligands and sequestration in the vacuoles (Wójcik et al. 2005). Ueno et al. (2005) showed that Cd is complexed with malate and stored in vacuoles in *N. caerulescens* leaves. In our experiment, Cd supplementation inhibited P_N in plants and increased C_i, E and g_s. The significant difference between species was observed only for E. An excess of Cd may decrease the activity of enzymes involved in C fixation; thus, the increase of intercellular CO_2 concentration found in plants exposed to Cd may be explained by alterations in RuBisCO activity. According to Shi and Cai (2008), the increase in C_i suggests that the enzymatic dark reactions of photosynthesis were affected. The increase in C_i/C_a determined by Dias et al. (2013) suggests that nonstomatal limitation strongly contributes to the reduction of P_N. The increase in g_s may be related to an alteration in the K:Ca ratio in guard cells and/or alterations in the concentration of abscisic acid, which controls stomatal movement. The decreased leaf photosynthetic rate resulting from the highest decreases in K and Na supply (mainly in the Me) was due to high C_i. C_i indicated that the inhibition of photosynthesis was due to an inhibition of Calvin cycle enzymes and/or an inhibition of the photosynthetic electron transport chain. The increase of C_i may be explained by modifications of RuBisCO activities of plant (Redondo-Gómez et al. 2011). Potassium supply also may affect photosynthesis through changes in leaf morphology and anatomy because the specific leaf area as well as leaf thickness and density may depend on K content (Zhang et al. 2006; Battie-Laclau et al. 2014). Eker and Uysal (2013) found that enhanced K supply played a crucial role in the protection of spinach against Cd-induced oxidative stress by decreasing lipid peroxidation and enhancing the antioxidant defense system.

One of the crucial factors affecting the influence of Cd on plant metabolism and physiological processes is its relationship with other mineral nutrients (Lux et al. 2011; Dias et al. 2013). In this study, plant exposure to Cd decreased the plant content of several elements such as Ca, K, Na, Fe. Differences in element reduction in the tested *Noccaea* plants were observed. The ability of plants to take in elements was affected by the selective pressure of the differently contaminated sites. Cadmium can enter the plant through nonspecific cation channels as well as through different divalent cation transporters, competing for absorption with mineral nutrients (Verbruggen et al. 2009; Lux et al. 2011). Therefore, the uptake and distribution of mineral nutrients, such as Fe, Ca, Mg and K, can be severely disturbed (Martin et al. 2012). Cadmium is chemically similar to certain metal elements, including Fe, Zn, and Ca, and, therefore, could displace these elements from metalloproteins (Verbruggen et al. 2009). Mg in

chlorophyll also can be displaced by Cd. The Cd–Chl complex is highly unstable and decays shortly after formation (Küpper et al. 2007). Soil Cd contamination did not decrease the Mg contents in plants in our experiment. Our results showed no significant increase in Mg content in plants originating from Redlschlag, a place with high Mg content in soils. Lower decline of cation content in Re in contrast to Me is in line with the results of Gonneau et al. (2014). According to their findings *N. caerulescens* from serpentine soil with Mg excess has adapted to nutritionally poor environments by increasing their cation uptake and allocating more energy to cation absorption. The energy cost of ion uptake is high; the supplementary uptake of cations could cause a trade-off with plant growth. Cd ion uptake occurs via the same transmembrane carriers used to uptake Ca^{2+}, Fe^{2+}, and Mg^{2+} (Papoyan et al. 2007). Rivelli et al. (2012) found that Cd can compete with several essential nutrients (e.g. Ca, K), altering their concentration in tissues. The effect of Cd on osmotic potential could be ascribed to dysfunctions of membrane integrity caused by displacement of Ca from the cell surface by Cd or, as suggested by Poschenrieder and Barcelò (2004), by the increase of solutes in cells, probably in the vacuoles, that store Cd-complexes. According to Candan and Tarhan (2005), the peroxidation of polyunsaturated FAs in membranes increased with decreasing Ca^{2+} concentrations during the plant growth period. Our results of Me specie confirmed a decrease in the content of saturated FAs not only in relation to increasing Cd content but also with declining Ca content (correlation between Ca content and unsaturated FAs $R^2 = 0.66$). The increased content of unsaturated FAs in plants growing under Cd stress is caused by increased desaturase activities (Wang 2004; Upchurch 2008).

Several studies have shown that Cd toxicity led to Fe deficiency in plants (Martin et al. 2012). Our results confirmed the decrease in Fe content in the presence of high levels of Cd. Lower antioxidant defenses caused by Cd-induced Fe and Ca deficiency can also contribute to ROS production and lipid peroxidation in Cd-stressed plants (Rodríguez-Serrano et al. 2009). There was a significant correlation between Fe and water-soluble Cd content ($R^2 = 0.80$). According to Gonneau et al. (2014) negative correlation between Cd and Fe was found in non-metallicolous *N. caerulescens*. This suggests that under strong Cd stress, Fe transport pathway may play a major role.

The reduction in photosynthetic rate led to a limited supply of metabolic energy and therefore to N assimilation restriction (Fig. 4). Nitrogen flow through amino acids can change in response to Cd stress. The decline in the free amino acid level may be a consequence of decreased nitrate reductase activity. This decrease in trace metal-treated plants also may reflect a decrease in photosynthesis because sugars are essential for nitrate reductase expression (Cambell 1999). Moreover, the decline in the activities of nitrate reductase and nitrite reductase is likely to be a consequence of a direct interaction between the metal and—SH groups at the active site of the enzymes. Inhibition of photosynthesis and nitrate or nitrite reductase is reflected in the assimilation of C and N in the AA.

The Cd contents of tested plants increased the content of Hyp, a major AA in plant cell wall hydrolysates. Hydroxyproline-rich glycoproteins secreted by plant cells are believed to have a broad range of functions, ranging from providing structural integrity to mediating cell–cell interactions and communication (Wu et al. 2001) and are formed in oxidative stress conditions (De Graaf et al. 2001). Free amino acids are formed by the catabolism of

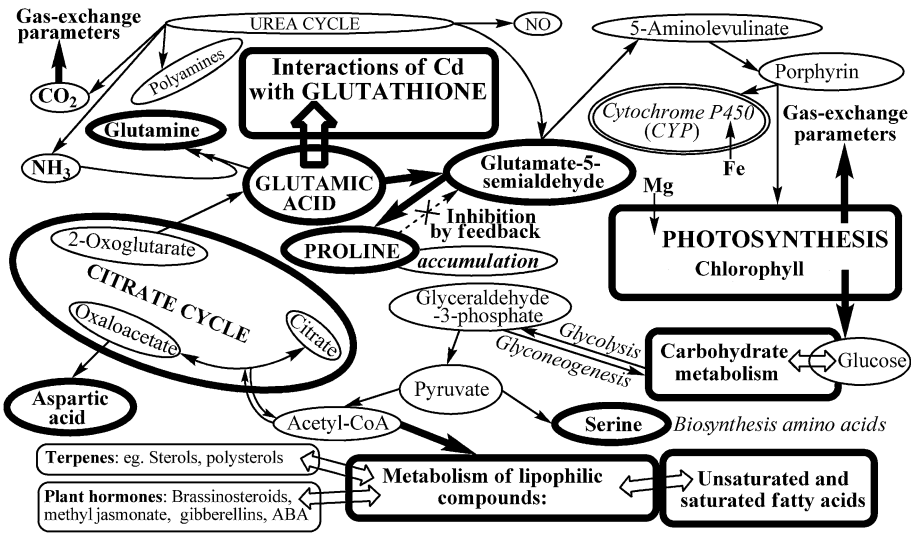

Fig. 4 Effect of Cd stress on plant metabolism

these compounds, and they are transported to developing leaves (Feller et al. 2008). The highest Hyp content was determined in Re–Cd plants and significant differences between Me, Me–Cd, and Re were not confirmed. This finding highlights the lower tolerance of Re to Cd stress. Our results showed that senescence was induced and destruction of proteins was higher in Re than Me. According to Schaller (2004), plant senescence increases N mobilization.

Glu, Gln, Asp, and Asn are used to transfer nitrogen from source organs to sink tissues and to build up reserves during periods of nitrogen availability for subsequent use in growth, defense, and reproductive processes. According to Pi et al. (2014), an Asp decrease could affect K deficiency. Our finding confirmed a correlation between Asp and K ($R^2 = 0.57$). The changes in Asp and Glu contents in tested plants showed effect of Cd contamination to both species. A decrease in Asp and Glu contents as a result of Cd contamination also was observed in our previous work (Zemanová et al. 2013). In our experiment, the significant decrease of Asp was confirmed only for Re–Cd which highlights the lower tolerance of Re to Cd in contrast to Me.

Levels of Glu, which is required for the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine and is used in the synthesis of glutathione and phytochelatins in plant cells (Vitória et al. 2001), are related to the allosteric regulation of glutamate kinase activity by free Pro. Our results showed significantly lower Pro accumulation in Me in contrast to Re. Me plants are able to inhibit the formation of Pro from Glu by feedback by a relatively lower level of accumulated Pro than Re. According to García-Ríos et al. (1997), the regulation of Pro biosynthesis from Glu is dependent on the Glu:Pro ratio and is determined by genotype-environment interaction. Plants with a higher Glu:Pro ratio are better adapted to Cd stress. Our results confirmed this finding and different physiological behavior of Me was shown in contrast to Re was shown.

A significant increase in Gln content was determined for both species. Zemanová et al. (2013) confirmed this finding for the ecotype "Ganges." Gln is not only the major amino acid used for nitrogen transport but also is a key metabolite that acts as an amino donor for other free amino acids, a reaction that is primarily catalyzed by glutamate synthase. This pathway interacts with carbohydrate metabolism and the energy status of the organ (Hodges et al. 2003). A decrease in Gln is reflected in the biosynthesis of purine bases from which nucleic acids, ATP, and plant hormone cytokinins are formed (Pavlík et al. 2012). Decrease of Gln content confirmed different physiological behavior of no hyperacculumating plants in contrast to hyperaccumulators.

Asn is an AA used to store and transport N from sources to sinks. According to Zhang et al. (2013), Asn is the major form of N transported to sink tissues in *Arabidopsis* mutants. Our observations showed a decrease in Asn concentration in the biomass of all plants treated with Cd. Pavlík et al. (2010) confirmed similar results for spinach growing under arsenic stress. However, Lea et al. (2007) published opposite results; cadmium induced an almost tenfold increase in the asparagine concentration of tomato roots but only a fourfold increase in the leaves.

Conclusions

Comparison of *N. caerulescens* and *N. praecox* showed that Cd stress resulted in similar changes in gas-exchange parameters. Contrasting responses of plants to Cd contamination were observed in element contents and FA and amino acid metabolism. Significantly higher accumulation of Cd and strong declines of K, Ca, Na, and Fe were determined in the Me plants in contrast to the Re plants. The increased ability of Re plants to take in some cations is a result of the selective pressure of growth in a contaminated area. The significant increase in the glutamic acid/proline ratio was determined for the hyperaccumulating specie from Slovenia.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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