

# Physiological mechanisms of adaptation of *Dianthus carthusianorum* L. to growth on a Zn-Pb waste deposit - the case of chronic multi-metal and acute Zn stress

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## Abstract

**Aims** This study investigates the response of metallophilous (M) and nonmetallophilous (NM) ecotypes of *Dianthus carthusianorum* L. to chronic multi-metal and acute Zn stress.

**Methods** Plants were cultivated on the Zn-Pb waste heap substrate and under Zn excess in hydroponics. Growth parameters as well as accumulation of organic acids and thiol peptides were determined as a function of metal accumulation.

**Results** When grown on the metalliferous substrate, the M plants showed less phytotoxicity symptoms, lower foliar metal (Zn, Pb, Cd) accumulation, higher malate and citrate but lower glutathione content than the NM plants. When exposed to Zn excess in hydroponics, the M ecotype was also more tolerant but accumulated more Zn in comparison with the NM ecotype, accompanied by greater malate and citrate concentrations in the shoots, which were however not affected by increasing Zn doses. No phytochelatin were detected under any experimental conditions.

**Conclusions** Both constitutive and adaptive tolerance was found in *D. carthusianorum*. Under chronic metal

stress, enhanced tolerance results from restricted metal uptake to the shoots and probably from detoxification by organic acids; however, under acute Zn stress it is not related to diminished metal uptake or organic acids. Glutathione and phytochelatin are not implicated in adaptive metal tolerance.

**Keywords** Adaptive and constitutive tolerance · Glutathione · Organic acids · Pseudometallophyte · Zn tolerance

## Introduction

Zinc (Zn) is widespread in the environment, due to both natural and anthropogenic sources (Broadley et al. 2007; Sinclair and Krämer 2012). The total Zn concentrations in soils usually range from 10 to 300 mg kg<sup>-1</sup> with an average of approximately 50–66 mg kg<sup>-1</sup> (Broadley et al. 2007; Kabata-Pendias and Mukherjee 2007). However, they can reach thousands of mg kg<sup>-1</sup> in severely Zn-contaminated soils, and are particularly high in waste deposits left after Zn ore mining and smelting. Zinc concentrations in such deposits in southern Poland are as high as 7.5–17.2 % of dry substrate matter, and are accompanied by substantially elevated concentrations of lead (Pb) (9,190–22,265 mg kg<sup>-1</sup>) and cadmium (Cd) (558–1,464 mg kg<sup>-1</sup>) (Wójcik et al. 2014). Due to the extremely high metal content, along with other adverse growth conditions, the deposits themselves constitute harsh habitats for settlement of vegetation. Therefore, usually only a limited number of species are able to

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thrive in such areas. Plant adaptation to metalliferous habitats usually results from a long and gradual evolutionary process, although under strong selection pressure, as found on such artificially created waste deposits, the adaptive tolerance may be acquired within a few years or even a single generation (Remon et al. 2007). On the other hand, some pseudometallophytes may also exhibit constitutive tolerance, i.e., inherent tolerance to metals present in all populations of a species, even growing without any selective pressure in uncontaminated habitats (Bert et al. 2000).

Numerous studies have been performed in order to compare the level of metal tolerance and the physiological basis of the resistance of metallicolous and nonmetallicolous plant populations (Schat et al. 2002; Załęcka and Wierzbicka 2002; Dresler et al. 2014; Wójcik and Tukiendorf 2014). Among them, intraspecies investigations of Zn tolerance have also been conducted on several species occurring in both metalliferous and non-metalliferous habitats (Thurman and Rankin 1982; Harmens et al. 1993, 1994; Köhl 1997; Bert et al. 2000; Sun et al. 2005; Deng et al. 2007; Olko et al. 2008; Colzi et al. 2014). These studies were mainly focused on plants exposed to acute short-term metal stress in hydroponic cultures. Although such investigations are useful for fast screening of metal resistance between the populations/ecotypes and provide valuable information on the molecular basis of metal tolerance mechanisms, they may not accurately predict the plant response to sublethal, chronic metal stress, to which plants are exposed in heavily metal-polluted environments. Therefore, it is important to study not only the impact of a single pollutant (even a dominant one) under controlled hydroponic conditions but also to mimic more complex plant-multi-metal polluted soil interactions to gain a full insight into plant metal tolerance mechanisms.

Plants have acquired various mechanisms to tolerate heavy metals (Maestri et al. 2010; Viehweger 2014). Principally, most plant species inhabiting metalliferous habitats avoid metal uptake from the substrate (Wójcik et al. 2014). Detoxification of excess metal ions entering plant cells is achieved by excretion or cytosolic chelation followed by vacuolar sequestration. Of the potential chelating ligands, glutathione (GSH), phytochelatins (PC), and organic acids (malate and citrate) have gained particular interest in terms of metal tolerance and detoxification in plants. In spite of numerous studies, the mechanisms of Zn tolerance are still not clear (Clemens

2010; Sinclair and Krämer 2012). According to Mathys (1977), increased Zn tolerance might be due to increased activity of a Zn-malate shuttle system that transports Zn from the cytosol into the vacuole. While in the vacuole, citrate, exhibiting higher affinity for Zn, might be the main ligand for Zn (Broadley et al. 2007). In line with this hypothesis, a positive correlation between Zn treatment and the concentration of these organic acids was found in *Arabidopsis halleri* (Zhao et al. 2000) and *Thlaspi caerulescens* (Tolrá et al. 1996; Wójcik et al. 2006), and the existence of Zn-malate and Zn-citrate complexes was confirmed in these species (Sarret et al. 2002; Monsant et al. 2011). Since such complexes display low stability constants at cytosolic pH, their role emerges mainly in vacuolar sequestration and long distance translocation of Zn in the xylem (Clemens 2010; Kutrowska and Szlag 2014). Whether organic acids are responsible for Zn tolerance of non-hyperaccumulators remains elusive based on the studies available. In comparison to organic acids, GSH has higher affinity for Zn at cytoplasmic pH values (Clemens 2010). Although in *Sedum alfredii* an increase in GSH was consistent with Zn accumulation (Sun et al. 2005), no contribution of GSH in enhanced Zn tolerance has been demonstrated so far. The synthesis of PCs, GSH-derived metal binding peptides, represents a major detoxification mechanism for Cd and arsenic (As), but accumulation of these peptides was also observed in some plant species treated with Zn (Harmens et al. 1993; Schat et al. 2002; Tennstedt et al. 2009; Sofó et al. 2013), suggesting their contribution to Zn detoxification. In agreement with this, Tennstedt et al. (2009) reported hypersensitivity towards Zn in PC-deficient *cad1-3* and *cad1-6* mutants of *Arabidopsis thaliana*. However, until now no evidence exists to support the role of PC in naturally selected Zn tolerance in plants.

The pseudometallophyte *Dianthus carthusianorum* is capable of growth in habitats containing extremely high concentrations of heavy metals, and it is often the dominant species in these sites (Wójcik et al. 2013). Therefore, the question arises whether this is a result of constitutive expression of metal resistance or rather adaptive responses that occurred to enable the species to cope with adverse environmental conditions. In our previous studies, we demonstrated that the elevated Pb and Cd tolerance of the metallicolous ecotype of *D. carthusianorum* was not related to increased production of thiol peptides and organic acids (Wójcik and Tukiendorf 2014; Wójcik et al. 2015). In these

investigations, we have included Zn tolerance and detoxification mechanisms. Although an interpopulational variation in Zn tolerance was reported in this species (Załęcka and Wierzbicka 2002), the physiological impact of elevated concentrations of Zn still remains unknown. In the present study, metal tolerance mechanisms were compared in plants of metallicolous (M) and nonmetallicolous (NM) ecotypes of *D. carthusianorum* exposed to acute Zn stress in hydroponics (short-term exposure to high concentrations of Zn) and also in plants exposed to chronic stress (long-term exposure to low metal concentrations) when cultivated on soil taken from the sites of origin of the two ecotypes. The specific objects were: i) to check, using reciprocal soil experiments, if the NM ecotype grows equally well as the M ecotype on the substrate from the Zn-Pb waste deposit, thus demonstrating constitutive tolerance of this species to adverse mineral conditions; ii) to determine Zn phytotoxicity thresholds (by the root elongation test, EC<sub>100</sub>) and sensitivity to Zn (by analysis of growth parameters) in the two ecotypes subjected to a range of Zn concentrations in hydroponics; iii) to examine the possible relationship between the level of metal tolerance and the accumulation of thiol peptides and organic acids in plants both under chronic and acute Zn stress.

## Materials and methods

### Plant material, growth conditions, and treatments

Seeds of *Dianthus carthusianorum* L. (*Caryophyllaceae*) were collected from plants spontaneously inhabiting a waste deposit created by mining and processing of Zn-Pb ores in Bolesław, southern Poland (metallicolous ecotype, M) and from a non-contaminated area in Pliszczyn, southern-east Poland (nonmetallicolous ecotype, NM). The detailed description of the study sites was provided previously (Wójcik et al. 2013; Wójcik et al. 2014). The seeds were germinated on a double autoclaved commercial garden soil and the seedlings were transplanted singularly to plastic pots and cultivated on the same soil for 4 or 6 weeks before the soil or hydroponic experiments, respectively. Plants were grown under uniform conditions to ensure that the differences in the responses to subsequent experimental conditions were due to genotypic differences between the ecotypes. Before and during both experiments, the plants were cultivated in a

controlled environment chamber under a 16/8 (day/night) photoperiod with photosynthetic active radiation of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , at 24/18 °C (day/night) and relative humidity of 60–70 %.

### Soil experiments

Four-week old plants of both ecotypes of uniform size were transferred singularly to pots filled with soil originating from either contaminated or uncontaminated sites of origin of both ecotypes of *D. carthusianorum*. The soil was collected from the top 15 cm and was sieved to <3 mm prior to use. The contaminated soil from the waste heap in Bolesław contained Zn, Pb, and Cd in concentrations ca. 64,000, 4,900, and 324.2 mg kg<sup>-1</sup>, respectively, whereas the non-contaminated soil from Pliszczyn contained Zn, Pb, and Cd in concentrations 22, 67, and 0.94 mg kg<sup>-1</sup>, respectively (Wójcik et al. 2013). The mean CaCl<sub>2</sub> extractable concentrations of metals in the waste heap substrate were (in mg kg<sup>-1</sup>): Zn – 73.39, Pb – 1.52, Cd – 2.05 (Wójcik et al. 2014), and in the unpolluted soil the Cd concentration was 0.06 mg kg<sup>-1</sup>, whereas the Zn and Pb concentrations were below detection limits (unpublished data). The pH (determined in H<sub>2</sub>O) of the polluted and unpolluted soils was 7.2. The plants were watered with distilled water when necessary to keep constant soil moisture and analysed after five weeks of growth in different substrate conditions.

### Hydroponic experiments

After gentle and thorough washing of soil particles from the roots, six-week old plants of both ecotypes were transferred singularly to plastic pots filled with 0.5 L Hoagland nutrient medium (pH app. 5.6) containing macroelements (in mM): 1 NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 6 KNO<sub>3</sub>, 4 Ca(NO<sub>3</sub>)<sub>2</sub>, 2 MgSO<sub>4</sub>; microelements (in  $\mu\text{M}$ ): 46 H<sub>3</sub>BO<sub>3</sub>, 9 MnCl<sub>2</sub>, 0.76 ZnSO<sub>4</sub>, 0.32 CuSO<sub>4</sub>, 0.11 H<sub>2</sub>MoO<sub>4</sub>; and 85  $\mu\text{M}$  Fe as Fe (III) citrate. After two days, the nutrient solution was renewed and supplemented with 0, 50, 250, or 1,000  $\mu\text{M}$  of Zn (ZnSO<sub>4</sub> • 7 H<sub>2</sub>O). Five-seven pots (plants) were used for each treatment for each ecotype. The nutrient medium was renewed and supplemented as previously after seven days of plant cultivation and it was continuously aerated during the experiment. The plants were analysed after 14 days of the metal treatment.

## Analysis of metal content in plants

The shoots of plants growing in hydroponics were washed with distilled water and their roots were incubated in ice-cold 10 mM CaCl<sub>2</sub> for 30 minutes to remove surface-adsorbed metal. The shoots of plants from soil cultures were washed with tap water followed by distilled water. The plant samples were dried at 105 °C and wet digested in HNO<sub>3</sub>:HClO<sub>4</sub> (4:1, v/v). Zinc concentrations in plants from the hydroponic experiments and additionally Pb and Cd concentrations in plant shoots from the soil experiments were determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500CE, Santa Clara, CA, USA).

## Metal tolerance assays

### *Plant growth parameters*

Fresh weight of roots and shoots of plants from hydroponics and that of shoots of plants from the soil experiments was determined. The lowest Zn concentration totally inhibiting root growth (effect of concentration, EC<sub>100</sub>) was determined after four days of plant growth in hydroponics at increasing Zn concentrations varying by 250 μM in the range 1,000–4,500 μM Zn. Plant roots were stained black with activated charcoal at the beginning of the treatment and the concentration at which no root elongation was found (no white apical fragments beyond the charcoal-coated black zone were visible) was recorded as EC<sub>100</sub>. Root viability was analysed using the modified double staining (FDA/PI) method described by Ishikawa and Wagatsuma 1998.

### *Measurements of thiol peptides and organic acids*

Thiol peptides were determined by HPLC as described previously (Wójcik et al. 2005). The chromatograms were recorded and analysed using 32 Karat 7.0 software (Beckman, Fullerton, USA). Organic acids were analysed spectrophotometrically as described by Olko et al. (2008) (for the hydroponic experiment) and by capillary electrophoresis (CE) as described by Dresler et al. (2014) (for the soil experiment).

### *Statistical analysis*

Statistical analysis was carried out using SigmaStat 3.5 (Systat Software, Inc.). To detect differences between

treatments and/or ecotypes as well as Cd-treatment x ecotype interactions, the data were analysed by two-way ANOVA (at a significance level of  $p < 0.05$ ). To meet the requirements of homogeneity of variance and normality, data obtained in hydroponic experiments for GSH and organic acid concentrations were log-transformed. A principal component analysis (PCA) was performed to detect key variables contributing to data variability. In soil experiments, all plants per treatment were randomly divided into 5 groups (5–7 plants in each) and their analyses were treated as replications. In hydroponic experiments, data for growth parameters were obtained from 5–8 independent time series (5–10 plants per treatment), and for chemical analyses from 3–5 independent time series (a composite sample of roots or shoots from at least 5 plants per treatment in each time series was used as replication). The EC<sub>100</sub> parameter was determined based on 2 replications (5–10 plants in each) and the images of root viability were collected from at least 10 preparations per treatment.

## Results

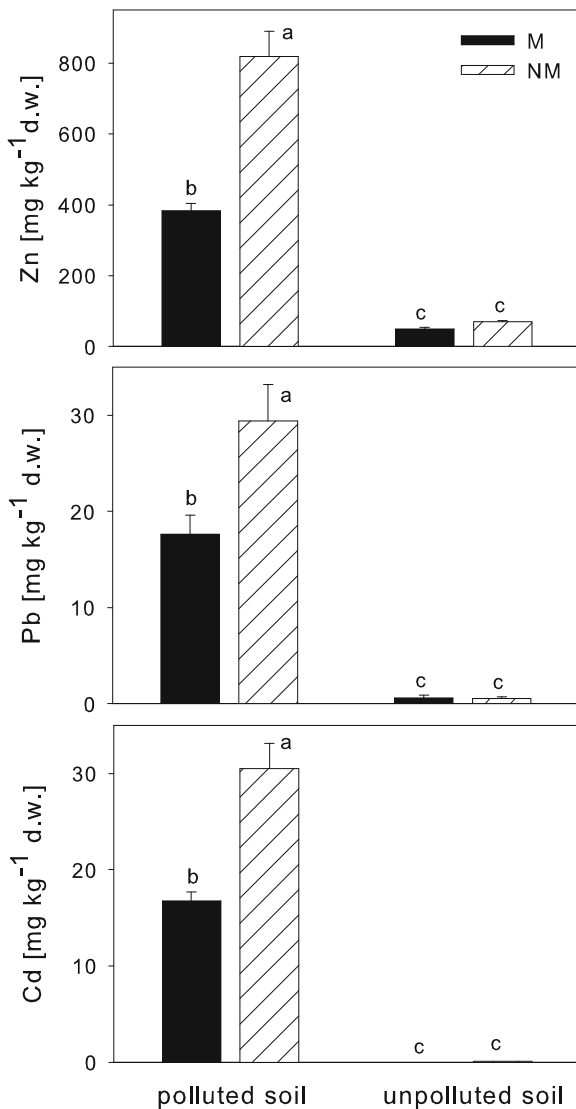
### Long-term soil experiment (chronic metal stress)

#### *Metal accumulation in plant shoots*

The two-way ANOVA revealed significant ( $p < 0.001$ ) differences in the accumulation of Zn, Pb, and Cd between the ecotypes, soils, as well as ecotype x soil interactions. After five weeks of growth on the waste heap substrate, higher accumulation of all the metals analysed (Zn, Pb, Cd) was found in the shoots of the NM ecotype (Fig. 1). The concentration of Zn was over 2-fold higher and those of Pb and Cd were 1.7 and 1.8 times higher, respectively, in comparison with the M ecotype. The accumulation of the metals from the uncontaminated soil was negligible in both ecotypes.

#### *Plant growth and accumulation of thiol peptides and organic acids*

The plants of both ecotypes grew well on the uncontaminated soil, while their growth was reduced on the metalliferous substrate (Fig. 2a, Suppl. Fig. 1). The fresh weight of shoots was lower by 41 and 51 % in the case of the M and NM plants, respectively,



**Fig. 1** Metal (Zn, Pb, Cd) concentrations in the shoots of metallicolous (M) and nonmetallicolous (NM) ecotypes of *D. carthusianorum* cultivated for 5 weeks on polluted (waste heap substrate) and unpolluted soils taken from the sites of origin of both ecotypes. Bars represent means  $\pm$  SE ( $n=5$ ). The values followed by the same letters are not significantly different at  $p<0.05$

cultivated on the metalliferous substrate in relation to plants from the unpolluted soil, although the differences between the ecotypes were not statistically significant ( $p=0.384$ ). Nevertheless, as shown in Suppl. Fig. 1, the NM plants were less tolerant to metal toxicity than the M plants. Besides the evident growth retardation, their youngest leaves became chlorotic and necrosis appeared along the edges of

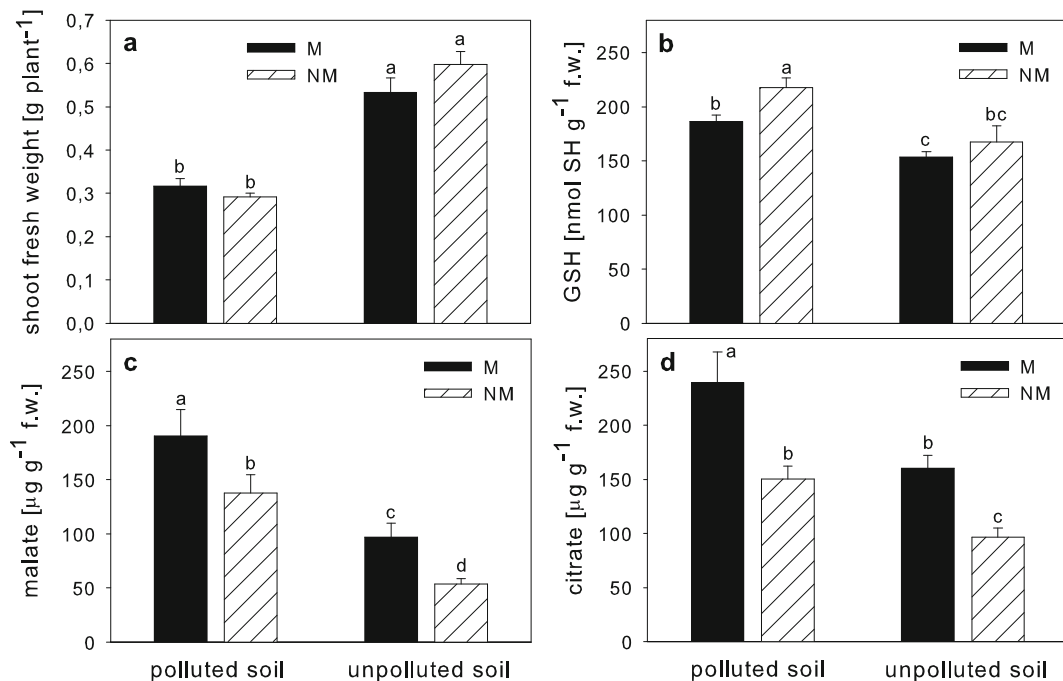
old leaves and on the tops of younger leaves, the symptoms becoming visible after 3–4 weeks of growth on the polluted substrate. Under the same conditions, the M plants were bigger and more vigorous.

No PC production was detected in the shoots of plants from the soil experiments. With respect of the accumulation of GSH and organic acids, significant differences between both ecotypes and different soil treatments were found (at the level of at least  $p<0.01$ ). In general, the concentrations of GSH, malate, and citrate were higher in the shoots of both ecotypes cultivated on the polluted soils (Fig. 2b–d). The concentrations of GSH were similar in the M and NM plants grown on the unpolluted soil; the presence of heavy metals in the growth medium increased the GSH concentrations by 20 and 30 % in the M and NM plants, respectively, resulting in a slightly higher GSH concentration in the plants of the NM ecotype cultivated on the waste heap substrate (Fig. 2b). In contrast to GSH, the concentrations of malate and citrate were significantly higher in the M plants, irrespective of the soil type (Fig. 2c, d). In comparison with the unpolluted soil, the metalliferous substrate induced higher malate (+97 % and +155 % in the M and NM plants, respectively) and citrate (+49 % and +56 % in the M and NM plants, respectively) accumulation.

#### Short-term hydroponic experiment (acute Zn stress)

##### *Zn accumulation in plants*

The zinc concentrations in the roots and shoots of both ecotypes increased with the increasing Zn concentration in the nutrient solution ( $p<0.001$ ) (Fig. 3). The metal concentrations in the shoots were much lower than those in the roots of both ecotypes (by factor of about 2 and 2.7–3.5 in the NM and M ecotypes, respectively). The shoot/root metal concentration ratio did not change significantly in an exposure-dependent manner. The root Zn concentrations were always higher in the M ecotype ( $p<0.001$ ); in the shoots, this was also the case at the 1,000  $\mu\text{M}$  Zn treatment. At lower Zn concentrations, the differences in the shoot metal concentration between the two ecotypes were not statistically significant. In both roots and shoots, statistically significant interactions were found between the ecotypes and Zn levels ( $p<0.001$  and  $p=0.002$ , respectively).



**Fig. 2** Shoot fresh weight (a) and the concentrations of GSH (b), malate (c), and citrate (d) in the shoots of metallicolous (M) and nonmetallicolous (NM) ecotypes of *D. carthusianorum* cultivated for five weeks on polluted (Zn-Pb waste heap substrate) or

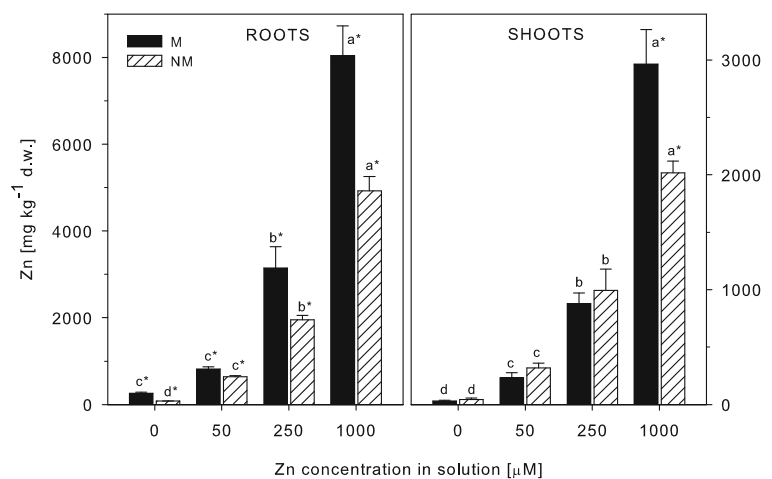
unpolluted soils taken from the sites of origin of both ecotypes. Bars represent means  $\pm$  SE ( $n=4-5$ ). The values followed by the same letters are not significantly different at  $p < 0.05$

#### Plant growth and accumulation of GSH and organic acids

Apart from the slightly reduced size of the plants cultivated hydroponically in the presence of 1,000  $\mu$ M Zn, no visual symptoms of Zn toxicity, such as chlorosis or necrosis, were found; the appearance of the plants was not different between the two ecotypes either (Suppl.

Fig. 2a). However, visualisation of root viability clearly indicated higher Zn resistance of the M plants, as demonstrated by the significantly lower amount of dead root cells in these plants (Suppl. Fig. 2b). Analysis of plant biomass provided further evidence for enhanced Zn tolerance of the M ecotype. The root and shoot fresh weight of the M plants was unaffected by any Zn treatment, whereas in the NM plants it was significantly

**Fig. 3** Zn concentrations in metallicolous (M) and nonmetallicolous (NM) ecotypes of *D. carthusianorum* cultivated in hydroponics for 14 days in the presence of Zn. Bars represent means  $\pm$  SE ( $n=3-4$ ). The values followed by the same letters (within an ecotype) are not significantly different at  $p < 0.05$ . Asterisks indicate significant differences between the ecotypes at the same Zn-treatments ( $p < 0.05$ )



reduced at 1,000  $\mu\text{M}$  Zn (by 41 % in roots and 35 % in shoots in relation to controls) (Fig. 4a). Comparison of net biomass of the two ecotypes following the Zn treatment may give confusing results due to constitutive differences in their growth rate. It should be noticed that the root fresh weight of the NM plants was higher than the M plants under 0–250  $\mu\text{M}$  Zn treatments but it became similar at 1,000  $\mu\text{M}$  Zn due to reduction of root biomass of the NM plants. The shoot fresh weight of both ecotypes was similar under the 0–250  $\mu\text{M}$  Zn treatments and it became significantly lower in the NM plants at 1,000  $\mu\text{M}$  Zn. Therefore, in order to avoid misinterpretation of the results, the percentage of growth inhibition should always be taken into account while comparing the responses to the metal in two contrasting ecotypes.

Higher Zn tolerance of the M ecotype was also confirmed by the lowest Zn concentration totally inhibiting root growth ( $\text{EC}_{100}$ ) - it was found to be 3,750  $\mu\text{M}$  in the M plants and 2,000  $\mu\text{M}$  in the case of the NM plants.

The glutathione concentration in the roots of both ecotypes did not change after any Zn treatment (Fig. 4b). In the shoots of the NM plants, the GSH concentration also remained unchanged, however it decreased in the shoots of the M plants exposed to 1,000  $\mu\text{M}$  Zn (by 37 % in relation to the control). There were no significant differences between the ecotypes in GSH accumulation ( $p=0.09$ ) (except for the roots of the control plants showing a higher GSH concentration in the M ecotype) or significant ecotype  $\times$  Zn interactions ( $p=0.336$ ).

As in the soil experiment, no PCs were detected in the root and shoot tissues of plants from both M and NM ecotypes at any Zn treatment in hydroponics.

With regard to organic acids, citrate was more abundant than malate, especially in the shoots of both ecotypes (Fig. 4c, d). Exposure to Zn did not affect the malate and citrate concentration in either the roots or shoots of both ecotypes. Although the content of both organic acids was similar in the roots of both ecotypes, significantly higher ( $p<0.001$ ) accumulation thereof, and especially malate, was found in the shoots of the M plants.

Multivariate comparison of chronic and acute stress responses

The compilation of all the data presented so far in this paper and concerning plant responses to the metal/s in

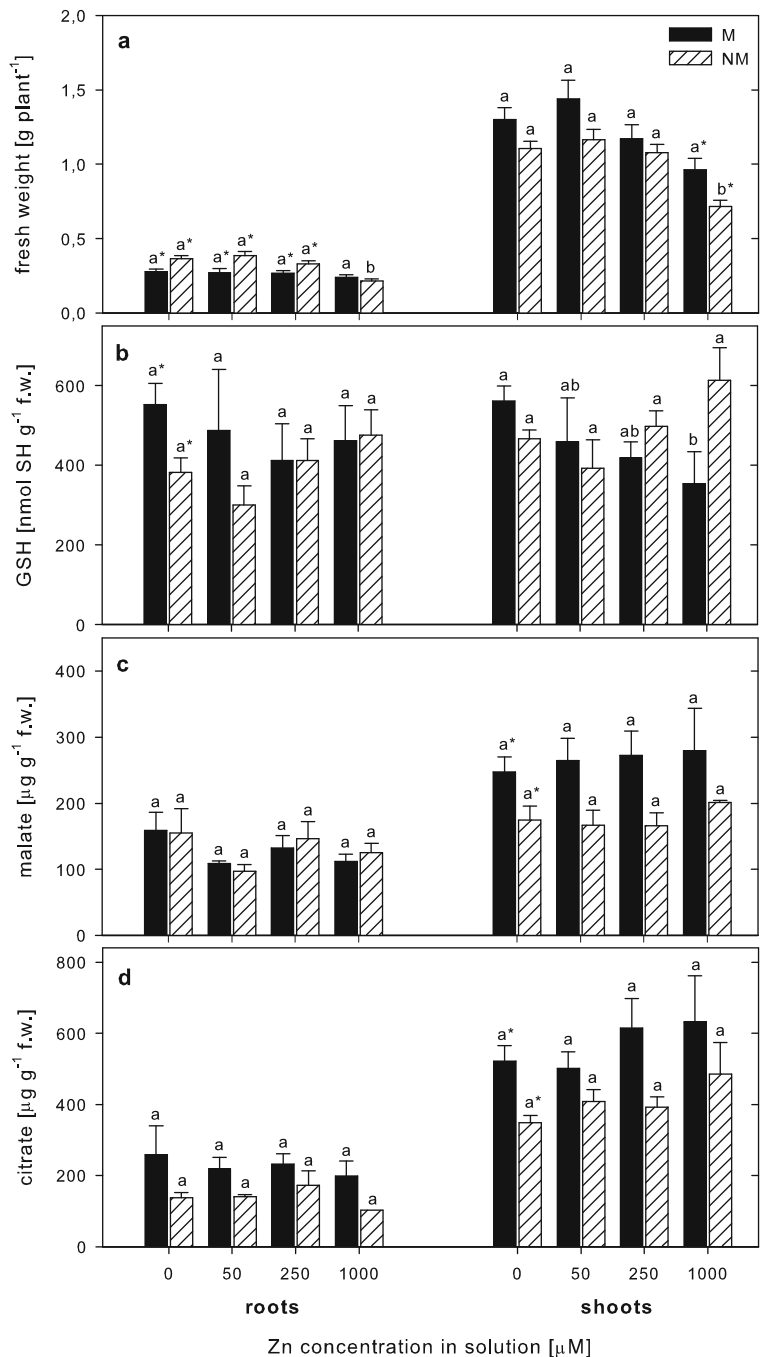
the soil and hydroponic experiments is presented in Fig. 5. Regarding chronic metal stress, the PCA separated the phenotypes into four groups according to their origin and growth conditions (Fig. 5a). Axis 1, representing 70.76 % of the total variation, was highly positively correlated with shoot fresh biomass and negatively correlated with the concentrations of all the metals (Zn, Pb, Cd) and GSH, and, to a lesser degree with malate. It clearly separated plants cultivated on the polluted (low biomass, high metal content) and uncontaminated (high biomass, low metal content) soils. Axis 2, representing 19.41 % of the total variation, was correlated with organic acids and it facilitated distinguishing between the M and NM ecotypes, especially those growing on the polluted soil. It was evident that the response of the M ecotype to the metalliferous growth medium was associated with the accumulation of organic acids, mainly malate. On the other hand, the NM ecotype was characterized by higher accumulation of all the metals, and interestingly, GSH in the shoots when exposed to chronic metal stress while growing on the waste heap substrate. A similar tendency towards accumulation of chelating ligands but not Zn was also indicated by the PCA analysis of the shoots of plants cultivated hydroponically (Fig. 5b). The variations between plant ecotypes under the control and Zn stress conditions were explained by the first two axes contributing with 42.41 and 26.11 % to the total variance, respectively. The first axis showed a positive correlation with organic acids, whose high concentration was characteristic for the M ecotype treated with Zn, and a negative correlation with GSH, whose higher concentration was found again in the NM ecotype, irrespective of the presence of Zn excess. However, in contrast to PCA of the long-term soil experiment, higher Zn accumulation was attributed to the M and not NM ecotype, as explained by the second PCA axis.

## Discussion

Constitutive or adaptive tolerance?

The results of the pot experiment clearly showed higher tolerance of the M ecotype than that of NM. This suggests that adaptive tolerance has developed in these plants through microevolutionary processes over at least the last 70 years since *D. carthusianorum* was first documented as occurring in the metal ore-bearing region

**Fig. 4** Fresh weight (**a**) and the content of GSH (**b**), malate (**c**), and citrate (**d**) in metalicolous (M) and nonmetalicolous (NM) ecotypes of *D. carthusianorum* cultivated in hydroponics for 14 days in the presence of Zn. Bars represent means  $\pm$  SE;  $n=5-8$  (**a**) or  $n=3-5$  (**b-d**). The values followed by the same letters (within an ecotype and plant organ) are not significantly different at  $p<0.05$ . Asterisks indicate significant differences between the ecotypes at the same Zn-treatments ( $p<0.05$ )

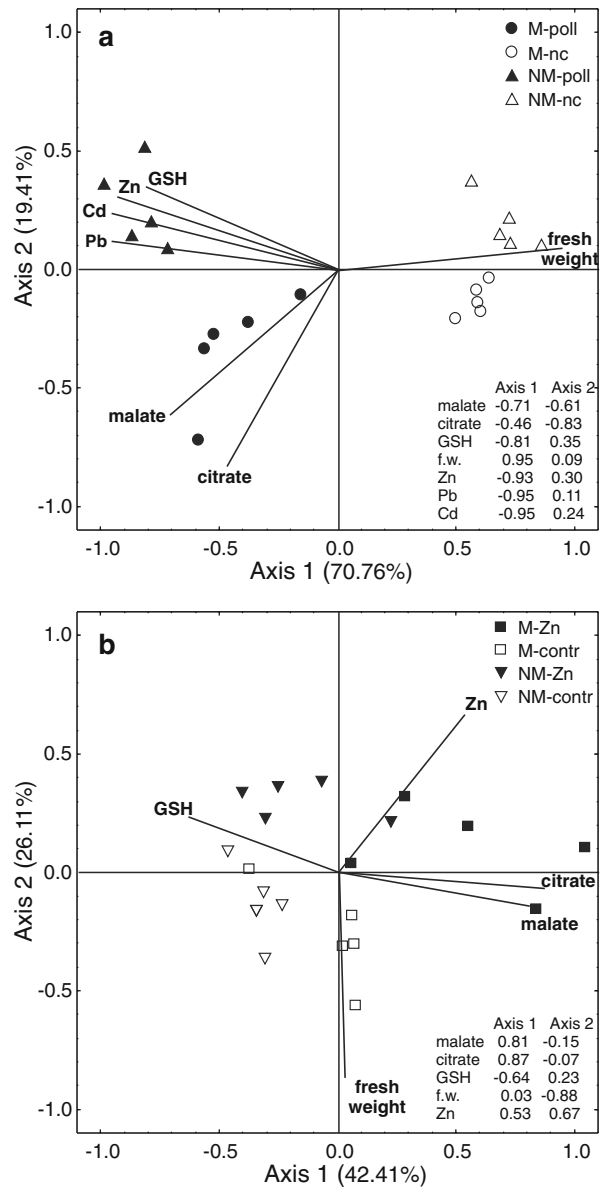


of Olkusz (Dobrzańska 1955). It is generally assumed that plant populations evolve tolerance specific to metals actually present in elevated concentrations in the soil (Remon et al. 2007; Colzi et al. 2014). To test this hypothesis further, the two ecotypes of *D. carthusianorum* (M and NM) were compared in a hydroponic experiment in respect of their tolerance to

Zn, the dominant pollutant of the waste heap. All toxicity tests applied (root elongation and viability, plant biomass) confirmed higher Zn tolerance of the M ecotype. Moreover, comparable differences between the two ecotypes have also been proven previously in the case of other metals present in the waste heap in toxic concentrations - Pb (Wójcik and Tukiendorf 2014) and



**Fig. 5** Principal component analysis (PCA) diagrams of phenotypic variables measured in the shoots the metallicolous (M) and nonmetallicolous (NM) ecotypes of *D. carthusianorum* cultivated for five weeks on soils taken from the sites of origin of the ecotypes (poll - polluted, nc - uncontaminated) (a) or for two weeks in hydroponics in the control conditions (contr) or in the presence of 1,000  $\mu\text{M}$  Zn (Zn) (b). The numbers in the parentheses at the axis names show the percent of explained variance. The data in the bottom right corners indicate contribution of particular traits to the principal components of variations



Cd (Wójcik et al. 2015), when applied individually in analogous hydroponic experiments. Taken together, the results demonstrate that the M ecotype of *D. carthusianorum* gained tolerance to all the metals present in excess in the substrate. Interestingly, not only elevated metal tolerance but also altered morphological traits and molecular marker signature were acquired in the M ecotype of *D. carthusianorum* during the adaptation process (Załęcka and Wierzbicka 2002; Wójcik et al. 2013). A similar phenomenon was observed in other species colonising this polluted area, e.g., in *Armeria maritima*, *Biscutella laevigata*, or *Silene*

*vulgaris* (Wierzbicka and Rostański 2002 and ref. therein).

It is noteworthy, however, that under acute Zn stress, the toxicity signs appeared in the NM plants only after two weeks of exposure to as much as 1,000  $\mu\text{M}$  Zn, i.e., when internal Zn concentrations reached 4,923 and 2,017  $\text{mg kg}^{-1}$  d.w. in roots and shoots, respectively. This is very remarkable, in view of the fact that toxic Zn levels in most plants usually range between 100 and 400  $\text{mg kg}^{-1}$  d.w. (Kabata-Pendias and Mukherjee 2007). There is no doubt, thus, that the basal level of Zn tolerance in this species is very high, suggesting the

existence of constitutive metal tolerance. In accordance with this, the previous experiments with Cd and Pb also showed increased metal sensitivity (on the fresh weight reduction basis) only at moderately high or high metal exposure levels (>15  $\mu\text{M}$  Pb; 15 and 50  $\mu\text{M}$  Cd for roots and shoots, respectively) (Wójcik and Tukiendorf 2014; Wójcik et al. 2015). Although the phenomenon of constitutive tolerance is still considered as a marginal feature in plants, it has previously been described in a number of species, including *Armeria maritima* (Köhl 1997), *Arabidopsis halleri* (Bert et al. 2000), *Thlaspi caerulescens* (Meerts and Van Isacker 1997), *Plantago arenaria* (Remon et al. 2007), *Calluna vulgaris* (Monni et al. 2000), or *Calamagrostis epigejos* (Lehmann and Rebele 2004). The ecological meaning of the constitutive metal tolerance in plants is not fully understood, but it was hypothesized to be correlated with other stress factors, e.g., water deficit (Bert et al. 2000; Remon et al. 2007). Indeed, the both ecotypes of *D. carthusianorum* studied, like many other constitutively tolerant species, originate from xerothermic environments. Although constitutive metal tolerance is not detrimental for growth on uncontaminated soil, it may enable the plant to colonise moderately polluted metalliferous habitats at early stages of succession, before adaptive tolerance develops through natural selection in some plant species.

The existence of constitutive tolerance was also revealed in our soil experiment - toxicity symptoms differentiating between the M and NM ecotypes appeared only after 3–4 weeks of growth on the waste heap substrate. The experiment was finished after 5 weeks when the differences between the ecotypes were clearly visible, but the NM plants were still vigorous. It is also worth mentioning that the experimental soil conditions were in fact harsher than the conditions in the field due to disturbances in the soil structure caused by homogenisation of the waste substrate for plant cultivation. Hence, the capability of the NM ecotype of *D. carthusianorum* to survive and grow on such a substrate is in itself evidence for the high level of basal tolerance and potential of the species to colonise adverse environments.

Is metal tolerance related to metal accumulation?

Our results showed that the answer to this question is different in the case of chronic and acute stress conditions. In the soil experiment, the visible toxicity

symptoms (Suppl. Fig. 1b) in the NM ecotype were connected with significantly higher accumulation of Zn, Pb, and Cd in the shoots. Therefore, the elevated tolerance of the M ecotype might be related to reduced uptake and/or translocation of the metals to the above ground parts, as observed previously in other tolerant plant populations subjected to chronic multi-metal stress (e.g., *Silene vulgaris* - Mohtadi et al. 2012 or *Thlaspi caerulescens* - Meerts and Van Isacker 1997). In fact, this seems to be a general tendency of the majority of non-hyperaccumulating metallophytes inhabiting metalliferous habitats to avoid metal presence in the shoots (Wójcik et al. 2014).

On the other hand, the relationship between metal tolerance and accumulation presented an opposite pattern under acute Zn stress in hydroponics. In this case, the M population accumulated much more Zn, both in the roots and shoots, but still showed higher Zn tolerance. A similar phenomenon was observed in an analogous experiment with *D. carthusianorum* exposed to Pb (Wójcik and Tukiendorf 2014), but no differences in Cd accumulation were found between the ecotypes in spite of the different Cd tolerance (Wójcik et al. 2015). It is difficult to compare the results obtained with the literature data concerning Zn accumulation and tolerance in metallicolous and nonmetallicolous ecotypes/populations due to very different experimental designs (e.g., different culture solutions, concentrations of metals, times of exposure, or different plant ages). That is why some reports are consistent (Thurman and Rankin 1982; Harmens et al. 1993; Schat et al. 2002; Sun et al. 2005; Deng et al. 2007) but some show an opposite tendency to the results of the present study (Bert et al. 2000; Colzi et al. 2014). Nonetheless, it seems that in a majority of cases, plant tolerance in hydroponic systems is not correlated with restricted metal uptake. Moreover, it may be concluded that short-term acute stress conditions are not appropriate to assess plant behaviour under long-term chronic metal stress, and especially the response of plants growing naturally in metalliferous habitats. In line with this conclusion, Köhl 1997 reported that under acute Zn stress a nonmetallicolous population of *Armeria maritima* was more Zn sensitive than the metallicolous one, whereas under chronic stress both populations exhibited a similar level of resistance. Although in the present study the differences in the metal tolerance between the M and NM ecotypes of *D. carthusianorum* were consistent in the soil and hydroponic experiments, the metal

accumulation patterns were different. This also implies the existence of different mechanisms of metal tolerance upon the two different kinds of stressful conditions.

Are thiol peptides and/or organic acids relevant in acute and chronic stress responses?

In the present study, acute Zn stress did not induce PC production, neither in the roots nor the shoots of the M and NM ecotypes of *D. carthusianorum*, in accordance with the results reported for *Sedum alfredii* (Sun et al. 2005) and *Armeria maritima* (Olko et al. 2008), indicating that other mechanisms must be crucial for Zn detoxification. Although an important role of PCs was suggested for Zn detoxification in *Arabidopsis thaliana* (Tennstedt et al. 2009; Sofo et al. 2013), comparative studies of Zn-tolerant and -sensitive lines of *Silene vulgaris* showed higher PC accumulation in the sensitive line, arguing at least against their role in enhanced Zn tolerance (Harmens et al. 1993; Schat et al. 2002). Similarly, more PCs were found in the NM ecotype of *D. carthusianorum* exposed to Cd and Pb, providing evidence that PC-based detoxification is not essential for enhanced tolerance to these metals either (Wójcik and Tukiendorf 2014; Wójcik et al. 2015).

No PC accumulation was found in the shoots of *D. carthusianorum* plants grown on the Zn-Pb waste heap substrate for five weeks. In fact, only very few reports have described the presence of PC in plants cultivated on metal-polluted soil and especially growing naturally in metalliferous habitats (Ernst et al. 2008; Machado-Estrada et al. 2013; Martínez-Alcalá et al. 2013). In accordance with the present results, leaves of metallicolous ecotypes of *Silene vulgaris* and *Thlaspi caerulescens* had no detectable amounts of PC when grown on the Zn-Pb spoil material from the site of their origin in Plombières, Belgium (Ernst et al. 2008; Wójcik et al. 2005). In contrast, in all these plants, PCs were induced by acute metal stress, particularly Cd stress, usually more efficiently in the NM ecotypes (Schat et al. 2002; Wójcik et al. 2005). Therefore, such response seems to be only relevant in acute metal stress and rather for basal metal tolerance.

Sun et al. (2005) proposed that another cellular thiol - GSH could be implicated in enhanced Zn tolerance. In their studies, following Zn exposure, GSH underwent a significant increase in all organs of plants from a mine population but only a slight increase in the stems of a control population of *Sedum alfredii*.

Similarly, the metallicolous population of *Armeria maritima* responded to Zn excess with a higher increase in the GSH level than the nonmetallicolous one (Olko et al. 2008). In contrast to these results, no significant variation in the GSH levels was observed in the roots of the Zn-treated *D. carthusianorum*, whereas a decrease in the GSH concentration was found in the shoots of the M ecotype at the highest Zn treatment. This, together with the similar level of GSH in both ecotypes, testifies against its role in Zn detoxification and enhanced tolerance of *D. carthusianorum* in hydroponic systems.

On the other hand, exposure of plants to chronic metal stress resulted in higher GSH accumulation in both ecotypes of *D. carthusianorum* in comparison with plants cultivated on uncontaminated soil, which could suggest its contribution to combating the metal toxicity. Since GSH was not utilized for PC synthesis, its enhanced accumulation must have constituted another advantage for plants, e.g., for direct metal chelation (metal inactivation) or for diminishing the deleterious effects of metals, such as oxidative stress. The latter seems more probable as GSH is regarded as a rather weak metal chelator (Satofuka et al. 2001) but a key player in the cell antioxidative system and in maintenance of cellular redox homeostasis (Jozefczak et al. 2012). Interestingly, both under the unpolluted and polluted soil conditions, higher GSH levels were found in the NM ecotype, supporting its role in basal metal tolerance but not in enhanced adaptive tolerance. This is also consistent with the hypothesis that investment of plants adapted to metalliferous habitats in increased thiols (GSH, PC) production seems to be unlikely due to too high costs of sulphur assimilation (Maestri et al. 2010). Therefore, this plant response was rather diminished in favour of other more effective ways of metal detoxification during (micro)evolution.

Unlike PCs, which are highly inducible stress peptides, and likewise GSH, organic acids are constitutively abundant plant compounds involved in a plethora of physiological functions (López-Bucio et al. 2000). Therefore, they are good candidates for detoxification of metals without any special additional costs of tolerance. Indeed, they were shown to be potent chelators for metals, especially Zn, in many plants species; moreover, their increased content was found in hyperaccumulators (Tolrá et al. 1996; Sarret et al. 2002). In the present study, irrespective of the growth conditions, considerably higher accumulation of malate and citrate was detected in the shoots of the M ecotype of

*D. carthusianorum*, implying their role in adaptive metal tolerance. This is fully consistent with the assumption of Mathys (1977), who considered malate as a major factor in the evolution of Zn resistance. Furthermore, in both ecotypes exposed to chronic metal stress, the level of organic acids was higher than in plants cultivated on unpolluted soil, thus confirming their importance in long-term exposure to sublethal metal concentrations. However, there again seems to be a discrepancy between the results of the soil and hydroponic experiments, since no alterations in the malate or citrate concentrations in either the roots or shoots of both ecotypes were observed with the increasing Zn concentrations in plants exposed to acute Zn stress. In accordance with the present results, Zn did not affect the malate and citrate concentrations in tolerant and sensitive ecotypes of *Silene vulgaris* (Harmens et al. 1994) and in the shoots of *Armeria maritima*, whereas in the roots of the latter it induced a decrease in these acids (Olko et al. 2008). There were no Pb- or Cd-induced changes in the amounts of malate and citrate in the plants of *D. carthusianorum* either (Wójcik and Tukiendorf 2014; Wójcik et al. 2015). It can be therefore concluded that these results exclude the role of organic acids in the tolerance of this species to Zn, Pb, and Cd under acute stress conditions, unless, as suggested previously (Wójcik and Tukiendorf 2014), the constitutively high content of malate, and especially citrate, is sufficient for effective chelation of the increasing tissue concentrations of the metals. However, the question arises then about the reason and relevance of enhanced production of organic acids under chronic metal stress, where tissue metal concentrations are by far lower than in hydroponics. Further research is necessary to support or contradict the role of organic acids in metal tolerance.

## Conclusions

Both soil and hydroponic experiments showed high constitutive metal tolerance of *D. carthusianorum*; however, the strong selective pressure occurring on the Zn-Pb waste deposit resulted in evolution of enhanced metal (Zn, Pb, Cd) tolerance (adaptive tolerance) in the M ecotype. The mechanisms of metal tolerance under acute and chronic metal stress were different, likewise the mechanisms of constitutive and adaptive tolerance. Under chronic metal stress, the enhanced tolerance of the M ecotype resulted from restricted metal uptake to

the shoots and possibly from high foliar accumulation of organic acids (malate and citrate). In contrast, under acute Zn stress, the M ecotype accumulated higher concentrations of Zn; however, its elevated tolerance was not dependent on organic acids neither on thiol peptides (GSH, PC). GSH-related mechanisms seem to be more important for basal metal tolerance, whereas organic acids may play a role in acquired metal tolerance. Understanding the differences between the physiological mechanisms of constitutive and adaptive metal tolerance is crucial for improving (by natural selection or genetic modifications) the desired characteristics of plants potentially useful for phytoremediation of contaminated areas. To understand these complex mechanisms fully, not only hydroponic experiments, but first of all soil experiments reflecting natural growth conditions or field trials should be performed.

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