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Nitrate Deficiency Reduces Cadmium and Nickel Accumulation in Chamomile Plants

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ABSTRACT: The effect of nitrogen (nitrate) deficiency (−N) on the accumulation of cadmium (Cd) and nickel (Ni) in chamomile (*Matricaria chamomilla*) plants was studied. Elimination of N from the culture medium led to decreases in N-based compounds (free amino acids and soluble proteins) and increases in C-based compounds (reducing sugars, soluble phenols, coumarins, phenolic acids, and partially flavonoids and lignin), being considerably affected by the metal presence. Proline, a known stress-protective amino acid, decreased in all −N variants. The activity of phenylalanine ammonia-lyase was stimulated only in −N control plants, whereas the activities of polyphenol oxidase and guaiacol peroxidase were never reduced in −N variants in comparison with respective +N counterparts. Among detected phenolic acids, chlorogenic acid strongly accumulated in all N-deficient variants in the free fraction and caffeic acid in the cell wall-bound fraction. Mineral nutrients were rather affected by a given metal than by N deficiency. Shoot and total root Cd and Ni amounts decreased in −N variants. On the contrary, ammonium-fed plants exposed to N deficiency did not show similar changes in Cd and Ni contents. The present findings are discussed with respect to the role of phenols and mineral nutrition in metal uptake.

KEYWORDS: amino acids, coumarin, phenolic metabolism, reactive oxygen species

INTRODUCTION

Nitrogen (N) is a crucial plant macronutrient because it is essential for the biosynthesis of amino acids, proteins, and enzymes.[1] Its deficiency has therefore an extensive impact on overall metabolism, and a shift from N-based to C-based compounds has been observed in different plant species[2−4] including *Matricaria chamomilla* L. (chamomile).[5−7] Apart from changes in sugar accumulation, enhanced accumulation of phenolic metabolites is a typical sign of N deficiency. For example, elevated amounts of phenolic acids, flavonoids, and coumarins have been observed.[2−7] This phenomenon was mainly attributed to the enhanced/maintained activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which is considered to be a pivotal step in the initial phase of the phenolic metabolism.[4,6]

Recent advances in industry and agriculture stimulate increased deposition of metals in the environment, and this is potentially toxic to human health either directly (food plants) or indirectly (food chain). Among other metals, cadmium (Cd) has no known biological function in organisms,[8] whereas nickel (Ni) is an essential "ultramicronutrient".[9] Despite the fact that both Cd and Ni are divalent and are unable to catalyze the production of reactive oxygen species (ROS) via Fenton−Haber−Weiss reactions, Cd shows more toxic effects, in comparison to Ni, when applied in equal doses.[10] This is also apparent from our previous works with chamomile, for example, at the level of chlorophylls, although growth was not damaged.[11,12] It is assumed that the main reason for such different toxic effects is higher endogenous Cd accumulation, as observed in several plants.[10−12]

Metal uptake is influenced by various factors including accumulation/exudation of organic compounds and mineral nutrition. Mainly quantitative changes of organic acids and amino acids in relation to Ni and Cd accumulation in hyperaccumulator plants have been studied.[13,14] Research in our laboratories is therefore focused on the role of phenolic metabolites in this process not only because of their abundance in plant tissue but also because they are potent antioxidants in the human diet. Manipulation of phenolic compound contents by exogenous factors is a suitable tool for this line of investigation. Our previous studies showed the active role of phenols in shoot Cd uptake,[15,16] but their role in Ni uptake is either negative[17] or nonactive.[16] Mineral nutrition may also affect the uptake of metals in both food and hyperaccumulator plants; for example, nitrate-fed rice and *Thlaspi caerulescens* contained more Cd than those ammonium-supplied.[18,19]

Chamomile is a widely used medicinal plant and, from the facts mentioned above, it is a good subject for the present study because it accumulates considerable amounts of Cd and Ni in the shoot[11,12] and, at the same time, its metabolism is strongly shifted toward the accumulation of phenolic metabolites under the conditions of N limitation.[5−7] Despite the recent progress of knowledge about the role of different N forms in the uptake of metals,[8] the effect of N deficiency on this process has not yet been studied. We therefore studied the impact of nitrogen/nitrate deficiency on metal uptake by chamomile plants, and the levels of both N-rich metabolites (free amino acids and proteins) and C-rich metabolites (phenolic acids, coumarins, lignin, flavonoids, soluble phenols, and reducing sugars) were investigated in detail. For comparison, the uptake of Cd and Ni was also assessed in

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ammonium-fed plants that were exposed to N limitation using the same N-deficient solution as for nitrate-fed plants.

MATERIALS AND METHODS

Plant Culture and Experimental Design. Twenty-one-day-old seedlings of *M. chamomilla* L. (tetraploid 'Lutea', Asteraceae) germinated in sand (with 3−4 first true leaves) were placed in Hoagland solution (containing 205 mg N L^{-1} = 14.6 mM) routinely used in our laboratory.[5−7,15−17] From this 14.6 mM, 14 mM was applied in the form of NO_3^- [$Ca(NO_3)_2 \cdot 4H_2O$ and KNO_3] and 0.6 mM as NH_4^+(NH4H2PO4). Despite this low ammonium concentration, this solution is considered to be a nitrate solution for the purpose of the present study (allowing comparison with our previous studies). In a parallel experiment, seedlings were cultured in ammonium solution containing macronutrients $CaCl_2 \cdot 2H_2O$, $NH_4H_2PO_4$, K_2SO_4, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ (with total N content = 14.6 mM) and micronutrients as for the nitrate solution. Uniform plants were cultivated in dark plastic boxes with 5 L of continually aerated solution (25 plants per box). The experiment was performed in a growth chamber under controlled conditions: 12 h day (6.00 a.m. to 6.00 p.m.); photon flux density ∼ 210 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes TLD 36W/33 (Philips, France); 25/20 °C day/night temperature; and relative humidity ∼ 60%. Under these conditions, plants form basal leaf rosettes only, and whole rosettes were analyzed for all parameters (= shoot in tables/figures). Solutions were renewed weekly to prevent nutrient depletion. Plants that had been cultivated hydroponically for 5 weeks were used in the experiment and further cultured in this N-sufficient or N-deficient medium [with macronutrients $CaCl_2 \cdot 2H_2O$, KH_2PO_4, K_2SO_4, $MgSO_4 \cdot 7H_2O$[5−7]] with or without the addition of 60 μM Cd or Ni (added in the form of $NiCl_2 \cdot 6H_2O$ and $CdCl_2 \cdot {}^{21}/_2H_2O$, Lachema Brno, Czech Republic) over 7 days. Concentrations of metals were selected on the basis of our previous studies, and pH was checked to be 6.0 ± 0.1 in all variants. Fresh and dry masses were estimated to determine the plant water content [100 − (dry mass × 100/fresh mass)], allowing recalculation of parameters measured in fresh samples. These dried samples were analyzed for free amino acids, phenolic acids, coumarins, lignin, reducing sugars, and mineral nutrients including Ni and Cd. Plants for fresh mass-requiring parameters were powdered using liquid N_2 and extracted as described below. For enzymes, selected supernatants were boiled to destroy enzyme activity and to check that the observed reaction was enzymatic. Spectrophotometry was carried out with an Uvi Light XTD 2 (Secomam, ALES Cedex, France).

Assay of Nitrogenous Compounds. Soluble proteins were quantified according to the Bradford method[20] using 20 μL of supernatants from homogenates for either PAL or polyphenol oxidase (PPO) activity (see below), and nonsignificant differences were observed; bovine serum albumin was used as standard, and data were expressed as mg g^{-1} DW. Free amino acids were extracted with 80% aqueous ethanol, and analyses were performed on an HP 1100 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) with fluorometric detector FLD HP 1100 and using precolumn derivatization with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate.[7]

Measurement of Carbonaceous Compounds and Phenolic Enzymes. Total soluble phenols were extracted with 80% methanol from fresh tissue and measured using the Folin−Ciocalteu method with gallic acid as standard; flavonoids were estimated in the same supernatant using the $AlCl_3$ procedure and quercetin as standard with modifications.[6,16] We note that in this assay for flavonoids, only flavonols were found to react with $AlCl_3$ reagent.[21] The selected cinnamic and benzoic acid derivatives were measured in 80% methanol extracts (free acids) and after alkaline hydrolysis of methanol-insoluble root residue (cell wall-bound compounds). Extraction was done using ether's method according to earlier work,[22] and quantification was done using the HPLC system with a mass selective HP MSD quadrupole detector (G1946A, Hewlett-Packard, Palo Alto, CA) as described previously.[5,23] Amounts of coumarin-related compounds [(*Z*)- and (*E*)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs) and herniarin] were estimated by gradient HPLC. Dried shoots were homogenized with sea sand and extracted with 80% methanol. Herniarin (Extrasynthese, Genay, France) was used for the quantification, and herniarin precursors (*Z*)- and (*E*)-GMCAs were prepared as described in the previous study.[24] Root lignin content was estimated by the thioglycolic acid reaction after purification of homogenized tissue with 1% Triton, 1 M NaCl, and acetone and expressed as mg g^{-1} DW.[23] Reducing sugars were extracted from dried material by homogenization with inert sand using a mortar and pestle (dilution ratio = 10 mg/4 mL of deionized water to achieve a final absorbance of ca. 0.3 in control samples) and determined colorimetrically using reaction with arsenomolybdate according to Somogyi-Nelson's method.[25] Briefly, supernatants (0.6 mL) were mixed with 1 mL of Somogyi's solution, heated for 20 min at 90 °C, and mixed with 0.6 mL of Nelson's solution and deionized water to a final volume of 7 mL. Samples were carefully shaken to remove bubbles, and absorbance was measured at 710 nm. Quantification was based on standard curve prepared with different concentrations of glucose (0.57−2.85 mg/L) according to the same procedure as for samples and expressed as mg g^{-1} DW. The activity of PAL (EC 4.3.1.5) was determined as the production of (*E*)-cinnamic acid from phenylalanine using the HPLC method[26] with a slightly modified protocol in homogenates prepared using sodium borate buffer (pH 8.7), and PAL activity was expressed as nmol (*E*)-cinnamic acid formed min^{-1} and mg^{-1} protein.[6,7] The activity of PPO (EC 1.10.3.2) was assayed in homogenates (1 g FW/5 mL) prepared with 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM polyvinyl polypyrrolidone as the oxidation of catechol (420 nm) monitored over 2 min. The activity was expressed as units of activity (UA); one unit was defined as the change in absorbance (ΔA = 1) min^{-1} g^{-1} FW and recalculated with the known protein content (UA mg^{-1} protein).[27]

Assay of ROS and Peroxidase Activity. Accumulations of hydrogen peroxide (using $TiCl_4$ method) and superoxide (using hydroxylamine method) were measured in potassium phosphate homogenates and expressed as μmol g^{-1} DW and μg g^{-1} DW, respectively.[6,27] The activity of guaiacol peroxidase (GPX, EC 1.11.1.7) was measured in the potassium phosphate homogenates as the oxidation of guaiacol (470 nm) and expressed as μmol min^{-1} mg^{-1} protein using an extinction coefficient of 25.5 mM^{-1} cm^{-1}.[26]

Quantification of Ni, Cd, and Selected Mineral Nutrients. Samples for the quantification of metals were prepared as described elsewhere:[15−17] dry material was kept overnight in a HNO_3 and H_2O_2 mixture (10 mL + 10 mL, Suprapur, Merck) at laboratory temperature and the next day evaporated to dryness at 90 °C in a water bath (5−6 h). Dry residue was dissolved in 5% HNO_3 and diluted to a final volume of 10 mL. All measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd., Mulgrave, Australia) and an air−acetylene flame. Samples for quantification of "intra-root" Ni and Cd were washed in 10 mM $CaCl_2$ (one root system in 300 mL) at 4 °C for 30 min to remove metals adsorbed at the root surface, and all other mineral nutrients were quantified in these samples. For quantification of total root Ni/Cd, samples were washed with deionized water only (and Ca content was determined in these samples). Nitrogen content was estimated according to the Kjeldahl method.[6] Briefly, dry material was mineralized using concentrated H_2SO_4 (dilution ratio 10 mg DW/1 mL) with selenium catalyst in Kjeldahl flasks heated by a sand bath. Clear solutions were then neutralized in a Parnass−Wagner distilling apparatus with 40% NaOH, and released ammonium was absorbed in 3% H_3BO_3. Nitrogen content was calculated after titration with 0.01 M H_2SO_4. The reproducibility of the apparatus was verified using a standard solution with a known amount of N (1 M $(NH_4)_2SO_4$) and found to be ca. 97%. We note that this type of Kjeldahl method detects only N in ammonium form, and it was used to prevent interferences caused by inorganic N.

Table 1. Selected Physiological and Biochemical Parameters in *Matricaria chamomilla* Plants after 7 Days of Exposure to 60 μM Cd or Ni in Nitrogen-Sufficient (+N) or Nitrogen-Deficient (−N) Solution[a]

	+N control	Cd	Ni	−N control	Cd	Ni
shoots						
tissue water content	92.26 ± 0.17 a	92.34 ± 0.32 a	92.32 ± 0.42 a	91.36 ± 0.08 b	90.97 ± 0.48 bc	89.86 ± 0.51 c
soluble proteins	103.9 ± 8.11 a	93.7 ± 4.55 a	98.4 ± 4.91 a	52.4 ± 4.56 c	78.3 ± 3.53 b	73.3 ± 5.64 b
hydrogen peroxide	6.51 ± 0.59 d	6.96 ± 0.62 d	6.12 ± 0.61 d	9.93 ± 0.97 c	20.03 ± 0.98 a	13.35 ± 1.33 b
superoxide radical	18.2 ± 1.59 b	17.5 ± 2.41 b	15.5 ± 2.09 b	11.2 ± 1.05 c	26.7 ± 2.23 a	17.6 ± 1.23 b
guaiacol peroxidase	0.15 ± 0.023 d	0.26 ± 0.019 c	0.17 ± 0.026 d	0.26 ± 0.034 c	0.34 ± 0.033 b	0.43 ± 0.027 a
polyphenol oxidase	1.35 ± 0.16 cd	1.66 ± 0.17 bc	1.31 ± 0.09 d	2.86 ± 0.27 a	1.83 ± 0.16 b	1.49 ± 0.13 cd
reducing sugars	21.6 ± 1.12 d	27.6 ± 1.42 c	16.8 ± 1.17 e	33.2 ± 1.08 b	41.1 ± 0.86 a	29.4 ± 1.56 c
roots						
tissue water content	93.82 ± 0.32 a	93.55 ± 0.40 ab	94.08 ± 0.21 a	93.75 ± 0.35 ab	92.65 ± 0.36 c	93.28 ± 0.24 bc
soluble proteins	37.2 ± 3.7 a	23.4 ± 2.9 b	38.9 ± 4.7 a	15.7 ± 2.3 c	14.9 ± 3.1 c	16.3 ± 1.54 c
hydrogen peroxide	8.63 ± 0.82 c	18.98 ± 0.88 a	9.81 ± 0.78 bc	6.29 ± 0.77 d	12.32 ± 1.64 b	12.06 ± 1.87 b
superoxide radical	28.5 ± 2.12 c	42.8 ± 2.67 a	42.1 ± 2.54 a	34.8 ± 1.76 b	44.3 ± 3.05 a	42.7 ± 1.28 a
guaiacol peroxidase	1.61 ± 0.16 c	2.48 ± 0.13 b	1.52 ± 0.28 c	2.59 ± 0.14 b	6.48 ± 0.32 a	1.27 ± 0.12 c
polyphenol oxidase	0.72 ± 0.06 d	1.28 ± 0.09 c	0.71 ± 0.09 d	1.32 ± 0.08 c	2.33 ± 0.10 a	1.74 ± 0.14 b
lignin	20.9 ± 1.24 c	27.4 ± 1.07 b	22.3 ± 2.20 c	25.7 ± 1.23 b	36.5 ± 2.19 a	21.5 ± 2.11 c

[a] N means nitrate in all tables. Data are the mean ± SD (n = 10 for tissue water content and n = 4 for all other parameters). Values within rows followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$). For the lucidity of table, units of individual parameters are mentioned under Materials and Methods. Note that shoots (leaf rosettes) and roots were evaluated separately.

Statistical Analyses. One box containing 25 plants was used for each treatment; thus, the whole experiment included 14 boxes. Two independent repetitions of the whole experiment were performed to check reproducibility, and nonsignificant differences were observed. Data were evaluated using two-way ANOVA followed by a Tukey's test (Minitab release 11, Minitab Inc., State College, PA) at $P < 0.05$. The number of replications (n) in tables/figures denotes individual plants measured for each parameter.

RESULTS

Growth Responses and Basic Physiological Parameters. The growth of plants was affected neither by N deficiency nor by metal excess after the given exposure period (data not shown). Leaf rosettes were free of necrosis or chlorosis under tested experimental conditions. Cd-exposed roots were visibly brown at the end of the experiment, whereas Ni-exposed plants did not show similar symptoms; these observations are in accordance with quantitative changes of lignin accumulation being expressed the most in Cd variants (+31 and +42% in Cd+N and Cd−N variants, respectively; Table 1). Tissue water content decreased in almost all shoots and roots of N-deficient plants in comparison with +N ones, and Cd and Ni variants did not differ (Table 1). Tissue water content in −N control roots was not reduced, but lignin content was elevated (+23%) when compared to +N control roots.

Quantitative Changes of Nitrogenous Compounds. Soluble proteins in +N plants were only reduced by Cd excess in the roots (−37%). N deficiency evoked a similar decrease in the root's proteins in all variants, whereas in the shoots both metals showed less pronounced effects than the N-deficient control alone (Table 1).

Among 17 detected amino acids, contents of aspartic acid, glycine, arginine, and valine decreased in both the shoots and roots of N-deficient plants, whereas 6 of them decreased only in shoots (Glu, His, Met, Ile, Leu, and Lys) and 4 only in the roots (Ser, Cys, Pro, and Ala; Table 2). Application of metals (either Cd or Ni) into the N-deficient medium further enhanced decrease in Asp and Phe in the shoots, and the same was visible (mainly in Ni−N) for Asp, Glu, Ser, Ala, Val, Met, Leu, and Lys (Table 2). On the other hand, accumulation of individual amino acids was enhanced in the +N variant with either Cd or Ni in both shoots (Glu, Thr, Arg, Ala, Tyr, Cys, Phe, Ile, and Lys) and roots (His, Cys, Phe, and Pro). Owing to these facts, the decrease in the sum of shoot's amino acids was more expressive in −N+metals (−48 and −43% in Cd and Ni, respectively) than in −N control (−17%) in comparison with respective +N counterparts. In the roots, the sum of amino acids was reduced by the same extent in control and Cd variant in response to N deficiency (32−40%), whereas Ni excess caused the most pronounced depletion (−64%) when compared to respective +N variants (Table 2).

Quantitative Changes of Carbonaceous Compounds and Phenolic Enzymes. Accumulation of reducing sugars was elevated by 53, 48, and 75% in control, Cd, and Ni N-deficient shoots in comparison with +N variants (Table 1).

The activity of PAL was stimulated by N deficiency in control shoots and roots (+63 and 54%, respectively), but it was reduced in N-deficient variants with metals in comparison with respective +N treatment (Figure 1). However, at the root level, Cd excess evoked the highest PAL activity in both +N and −N variants (Figure 1). In +N variants, Cd stimulated an increase in PPO activity in the roots, whereas Ni had no effect (Table 1). In terms of N deficiency, PPO activity in the shoots was significantly affected only in the control (increase by 112% in comparison with +N control; Table 1). In the roots, PPO activity was elevated in all N-deficient variants in comparison with respective +N treatments (+83, 82, and 145% in control, Cd, and Ni, respectively).

Accumulation of total soluble phenols was enhanced in both shoots and roots of all N-deprived variants (Figure 2), being expressed the most in Ni−N plants (+91 and 124% in shoots and roots, respectively). On the other hand, flavonoids were the most accumulated in Cd−N plants (+120 and 41% in shoots and roots, respectively; Figure 3).

Table 2. Accumulation of Free Amino Acids (μmol g^{-1} DW) in *Matricaria chamomilla* Plants after 7 Days of Exposure to 60 μM Cd or Ni in Nitrogen-Sufficient (+N) or Nitrogen-Deficient (−N) Solution[a]

	+N control	Cd	Ni	−N control	Cd	Ni
shoots						
aspartic acid	7.74 ± 0.25 a	8.05 ± 0.63 a	7.06 ± 0.20 a	5.84 ± 0.72 b	2.28 ± 0.26 c	2.33 ± 0.23 c
glutamic acid	1.19 ± 0.15 c	2.67 ± 0.34 b	4.21 ± 0.21 a	0.55 ± 0.33 d	0.48 ± 0.18 cd	0.79 ± 0.05 cd
serine	9.83 ± 1.96 a	11.8 ± 1.46 a	12.1 ± 1.47 a	11.2 ± 2.17 a	8.62 ± 0.60 a	8.50 ± 0.36 a
histidine	0.15 ± 0.02 a	0.14 ± 0.04 a	0.16 ± 0.02 a	0.08 ± 0.0082 b	0.08 ± 0.0021 b	0.06 ± 0.0051 b
glycine	1.20 ± 0.11 a	1.23 ± 0.21 a	1.34 ± 0.21 a	0.78 ± 0.02 b	0.64 ± 0.03 b	0.67 ± 0.05 b
threonine	1.47 ± 0.30 b	2.53 ± 0.21 a	2.63 ± 0.29 a	1.18 ± 0.07 b	1.73 ± 0.15 b	1.60 ± 0.28 b
arginine	0.95 ± 0.05 a	0.85 ± 0.13 b	0.85 ± 0.09 a	0.51 ± 0.06 c	0.46 ± 0.04 c	0.63 ± 0.06 c
alanine	11.7 ± 1.40 b	17.4 ± 2.19 a	13.1 ± 1.51 b	9.48 ± 0.42 bc	7.64 ± 0.70 c	9.71 ± 0.23 bc
tyrosine	0.43 ± 0.02 b	0.54 ± 0.03 a	0.41 ± 0.01 bc	0.35 ± 0.04 c	0.38 ± 0.03 bc	0.39 ± 0.03 bc
cysteine	0.50 ± 0.02 c	1.27 ± 0.10 a	1.02 ± 0.15 b	0.63 ± 0.03 c	0.62 ± 0.04 c	0.67 ± 0.01 c
valine	1.17 ± 0.20 a	1.29 ± 0.04 a	1.25 ± 0.07 a	0.62 ± 0.03 b	0.58 ± 0.03 b	0.46 ± 0.05 b
methionine	0.06 ± 0.005a	0.05 ± 0.004 a	0.05 ± 0.006 a	0.04 ± 0.002 b	0.04 ± 0.002 b	0.04 ± 0.003 b
phenylalanine	0.73 ± 0.03 b	0.96 ± 0.04 a	0.73 ± 0.06 b	0.67 ± 0.03 b	0.55 ± 0.04 c	0.48 ± 0.04 c
isoleucine	0.50 ± 0.03 c	0.88 ± 0.03 a	0.65 ± 0.03 b	0.38 ± 0.02 d	0.32 ± 0.02 d	0.36 ± 0.04 d
leucine	0.81 ± 0.02 a	0.79 ± 0.06 a	0.77 ± 0.02 a	0.57 ± 0.01 bc	0.53 ± 0.02 c	0.65 ± 0.04 b
lysine	0.42 ± 0.02 b	0.38 ± 0.04 bc	0.88 ± 0.06 a	0.30 ± 0.03 c	0.20 ± 0.02 d	0.34 ± 0.03 bc
proline	10.4 ± 1.42 c	14.2 ± 0.86 b	17.5 ± 2.16 a	7.82 ± 0.74 c	8.69 ± 0.43 c	8.96 ± 0.52 c
sum	49.2 ± 3.10 b	64.9 ± 2.91 a	64.8 ± 4.14 a	40.8 ± 2.35 c	33.8 ± 1.08 c	36.6 ± 2.45 c
roots						
aspartic acid	2.39 ± 0.21 a	2.21 ± 0.24 ab	2.59 ± 0.38 a	1.61 ± 0.35 bc	1.48 ± 0.12 c	0.47 ± 0.04 d
glutamic acid	1.56 ± 0.36 ab	1.52 ± 0.11 ab	1.56 ± 0.24 ab	0.97 ± 0.10 b	1.67 ± 0.38 a	0.26 ± 0.03 c
serine	4.14 ± 0.13 a	4.20 ± 0.39 a	3.30 ± 0.25 b	2.38 ± 0.32 c	2.61 ± 0.26 bc	1.27 ± 0.27 d
histidine	0.35 ± 0.02 cd	0.55 ± 0.04 b	0.90 ± 0.15 a	0.19 ± 0.04 d	0.41 ± 0.03 bc	0.24 ± 0.04 cd
glycine	2.43 ± 0.24 a	2.20 ± 0.25 a	1.86 ± 0.19 b	0.74 ± 0.04 cd	0.93 ± 0.05 c	0.38 ± 0.03 d
threonine	1.68 ± 0.20 a	1.59 ± 0.22 a	1.63 ± 0.11 a	1.24 ± 0.12 ab	1.52 ± 0.15 ab	1.13 ± 0.17 b
arginine	0.61 ± 0.03 b	0.82 ± 0.04 a	0.62 ± 0.02 b	0.47 ± 0.02 c	0.45 ± 0.03 c	0.48 ± 0.05 c
alanine	14.2 ± 1.78 a	13.1 ± 1.63 a	13.1 ± 1.74 a	8.73 ± 0.48 b	8.52 ± 0.40 b	3.86 ± 0.23 c
tyrosine	0.27 ± 0.07 a	0.34 ± 0.02 a	0.25 ± 0.04 a	0.28 ± 0.04 a	0.31 ± 0.03 a	0.26 ± 0.04 a
cysteine	1.56 ± 0.08 b	1.95 ± 0.19 a	1.31 ± 0.13 b	0.68 ± 0.02 cd	0.95 ± 0.10 c	0.54 ± 0.04 d
valine	1.81 ± 0.16 a	1.73 ± 0.10 a	1.82 ± 0.23 a	1.12 ± 0.15 b	1.07 ± 0.08 b	0.56 ± 0.03 c
methionine	0.15 ± 0.02 a	0.15 ± 0.02 a	0.16 ± 0.02 a	0.11 ± 0.01 b	0.12 ± 0.02 ab	0.06 ± 0.0042 c
phenylalanine	0.34 ± 0.03 b	0.43 ± 0.03 a	0.32 ± 0.04 b	0.39 ± 0.04 ab	0.20 ± 0.03 c	0.29 ± 0.02 b
isoleucine	1.08 ± 0.22 a	0.94 ± 0.22 a	1.08 ± 0.25 a	0.70 ± 0.03 ab	0.74 ± 0.03 ab	0.38 ± 0.02 b
leucine	0.98 ± 0.13 ab	1.10 ± 0.15 a	0.87 ± 0.13 ab	0.74 ± 0.05 b	0.75 ± 0.03 b	0.45 ± 0.01 c
lysine	0.39 ± 0.04 a	0.33 ± 0.04 ab	0.40 ± 0.06 a	0.26 ± 0.02 b	0.32 ± 0.04 ab	0.11 ± 0.01 c
proline	5.63 ± 0.23 b	5.26 ± 0.42 b	8.25 ± 0.42 a	3.38 ± 0.43 c	3.84 ± 0.27 c	3.62 ± 0.22 c
sum	39.9 ± 2.61 a	38.4 ± 3.06 a	40.0 ± 2.31 a	24.0 ± 1.42 b	25.9 ± 0.75 b	14.4 ± 0.69 c

[a] Data are the mean ± SD (n = 3). Other details are as in Table 1.

Among 14 detected free phenolic acids, the accumulation of gallic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzoic aldehyde, syringic acid, and vanillin was unaffected or only slightly affected by excess of metals or if +N and −N treatments were compared either in the shoots or in the roots (Table 3). The same was true for vanillic acid in the roots, whereas its shoot amounts were elevated in almost all N-deficient variants. Amounts of protocatechuic acid and its aldehyde were more affected in the shoots than in the roots by N deficiency. Among lignin precursors (*p*-coumaric, ferulic, and sinapic acids), the latter compound was affected the most in all N-deficient variants both in shoots and in roots (Table 3). The amount of salicylic acid was elevated by only Cd in +N plants, and the effect of N deficiency in combination with metals differed if Cd and Ni variants were compared. The accumulation of powerful antioxidative phenols, chlorogenic and caffeic acids, was elevated by N deficiency in all variants in both shoots and roots (in comparison with respective +N counterparts), and the former compound strongly increased in response to Cd application (Table 3). From the 14 detected phenolic acids in the free fraction, four of them were not detected after alkaline hydrolysis of methanol-insoluble root residue (Table 3). The quantity of the majority of detected compounds was lower in comparison with methanol extracts, with the exception of ferulic and caffeic acids, which strongly accumulated in this fraction (Table 3). The content of caffeic acid was extremely elevated by N deficiency in all variants (ca. 4−6 times over the respective control). The accumulation of ferulic acid was enhanced mainly by Cd excess in this fraction (Table 3).

At the level of coumarin-related compounds, both GMCA isomers increased more pronouncedly in response to Ni excess than to Cd excess in N-deficient variants in comparison with respective +N variants, thus leading to a higher sum of GMCAs in Ni−N (+148 and 65% in Ni and Cd, respectively). The amount of GMCAs was also almost doubled in −N versus +N control (Table 4). The accumulation of the GMCAs aglycone, herniarin, was elevated by Ni+N in comparison with +N control but decreased if Ni+N and Ni−N variants were compared.

ROS and Peroxidase Activity. In the shoots, the hydrogen peroxide level increased in all N-deficient variants (+52, 187, and 118% in control, Cd, and Ni, respectively), whereas in the roots it decreased in control and Cd-treated N-deficient plants in comparison with respective +N treatments (Table 1). Amounts of superoxide radical increased in the shoots of Cd−N variant, decreased in −N control, and remained unchanged in Ni−N variant. On the other hand, root superoxide was elevated in control only if +N and −N variants were compared (+22%, Table 1). The activity of guaiacol peroxidase was enhanced in

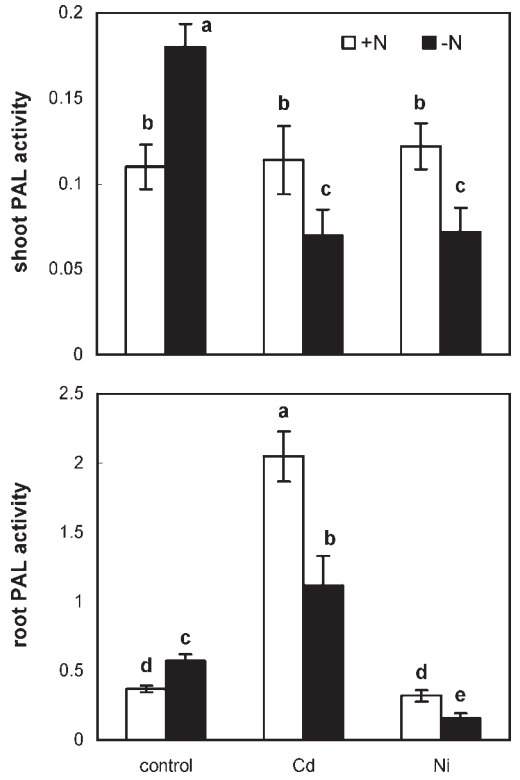

Figure 1. Activity of phenylalanine ammonia-lyase (PAL, nmol min^{-1} mg^{-1} protein) in *Matricaria chamomilla* plants after 7 days of exposure to 60 μM Cd or Ni in nitrogen-sufficient (+N) or nitrogen-deficient (−N) solution. Data are the mean ± SD (n = 4). Values within each graph followed by the same letter(s) are not significantly different according to Tukey's test (P < 0.05). N means nitrate in Figures 1−4.

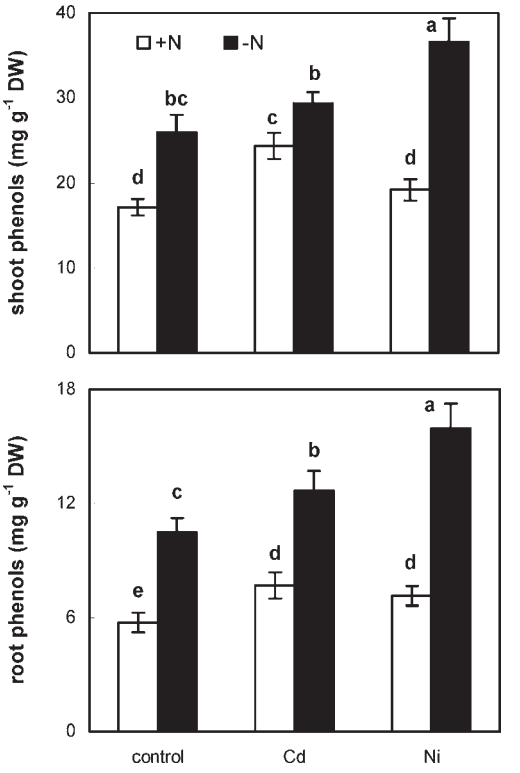

Figure 2. Accumulation of total soluble phenols in *Matricaria chamomilla* plants after 7 days of exposure to 60 μM Cd or Ni in nitrogen-sufficient (+N) or nitrogen-deficient (−N) solution. Data are the mean ± SD (n = 3). Other details are as in Figure 1.

all −N variants (in comparison with respective +N counterparts) with the exception of the root Ni variant (Table 1).

Effect on N Deficiency on the Uptake of Cd, Ni, and Selected Nutrients. The shoot amount of Cd and Ni decreased in response to N deficiency (by 46 and 33%, respectively; Figure 4). Total root metal content showed a similar trend in −N treatments (decrease by 21 and 19% in Cd and Ni, respectively), whereas intraroot amounts were not affected by N deficiency (Figure 4). Comparison of nitrate and ammonium solutions showed lower accumulation of Cd and Ni in the shoots (ca. −17% for both metals) and roots (−82%/−64% Cd/Ni) in +N solutions (Figure 5). N deficiency evoked decreases in both total Cd and total Ni in nitrate-fed chamomile plants as previously observed (Figure 4). On the other hand, elimination of N from ammonium-fed plants did not reveal similar quantitative changes: Ni accumulation was not significantly affected, and Cd content was even stimulated (+27 and 38% in the shoot and root, respectively; Figure 5).

Shoot N content was more reduced by application of metals than in control if +N and −N variants were compared (Table 5). Shoot amounts of Ca, Mg, Fe, and Cu were not significantly influenced if +N and −N variants were compared, and only K and Na accumulation showed slight changes in some variants (Table 5). At the root level, K and Mg contents were not affected by N deficiency in any variant, whereas Fe and Ca accumulation was depleted in all N-deficient variants (in comparison with respective +N counterparts). Na and Cu amounts decreased in response to metal application but were unaltered in control if +N and −N variants were compared (Table 5).

DISCUSSION

Concentrations of metals, exposure period to nitrogen/nitrate (N) deficiency, and age of plants have been selected on the basis of our earlier studies.[15−17] The main reason for this experimental design was to prevent damaging effects of high metal doses on growth and to avoid the trade-off between growth and metabolism in conditions of prolonged N deficiency.[6] It is undoubted that plants have some reserve of N after being exposed to N deficiency, but decrease in shoot N and nitrogenous compounds (Tables 1, 2, and 5) is a sufficient evidence that N metabolism was inhibited after the given exposure period (7 days).

To our knowledge, this experimental approach has not yet been used to test the toxicity of metals during N starvation. It was interesting to find that metals partially ameliorated the decrease in shoot proteins in N-deficient variants (Table 1). This could indicate the synthesis of protective enzymes, such as those being involved in ROS detoxification, and the decrease in many amino acids in N-deficient variants fits well with this assumption. Because metals alone stimulated an increase in free amino acids in +N variants, the decrease in their sum was more pronounced in −N variants, thus contributing to the decrease in the shoot N content. We found no data related to combined −N+metal treatments, but N deficiency alone evoked different responses of genes in a short-term experiment if leaves and roots of rice were analyzed; at the root level, expression of stress-related genes were both up- and down-regulated,[1] and we found enhanced activities of catalase and guaiacol peroxidase in a time course study.[6] We also note that calculation of enzymatic activities should be done with care in N-deficient plants just owing to the decrease in

proteins, and thus calculation per unit of proteins is physiologically more relevant in comparison with unit of biomass.[6] Less-pronounced decreases in shoot protein contents in −N variants with metal addition (in comparison with −N control) may also be covered by stimulation of guaiacol peroxidase activity owing to an increase in the H_2O_2 level (Table 1). Just soluble guaiacol peroxidase, which we measured, serves as an important scavenger of H_2O_2 in plants.[28] An almost overall decrease in root ROS level is another indication that below- and above-ground organs respond differently to metal excess and N deficiency.[6,7] It is probable that this difference is evoked by metal uptake being less visible in the roots, which are directly in contact with the polluted medium, thus leading to earlier responses of both antioxidative enzymes[29] and antioxidants.[23] Similar extensive inhibition of N metabolism has been observed in N-deficient *Arabidopsis* [3] and tobacco;[2] just nitrate deprivation evoked these changes as was confirmed by the readdition of nitrate and using nitrate reductase-deficient mutant.[2,3]

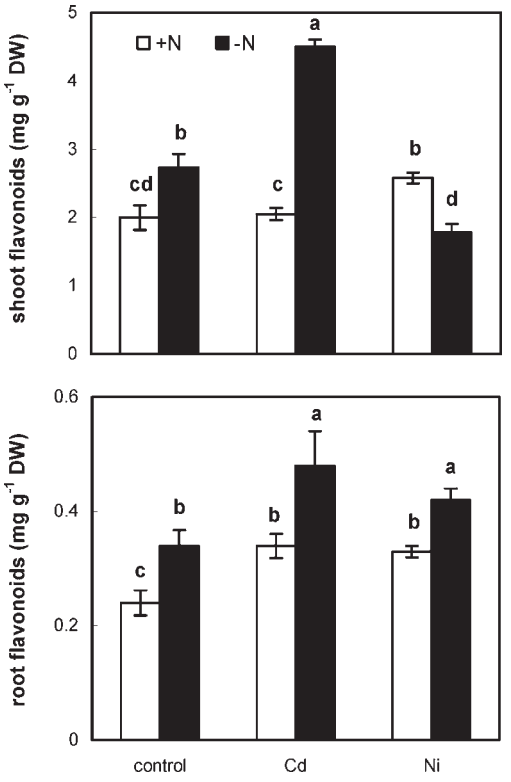

Figure 3. Amounts of flavonoids in *Matricaria chamomilla* plants after 7 days of exposure to 60 μM Cd or Ni in nitrogen-sufficient (+N) or nitrogen-deficient (−N) solution. Data are the mean ± SD (n = 3). Other details are as in Figure 1.

At the level of individual amino acids, some of them, such as histidine and cysteine, were suggested to be involved in Ni accumulation in hyperaccumulator plants (see ref 14 and the references cited therein). We did not observe extensive stimulation in +N variants, and their decrease in −N variants (with nonsignificant changes among all three treatments) is rather caused by N-deficient conditions (to maintain proteosynthesis as mentioned above) than by metal excess. Proline is another well-known protective amino acid in metal-exposed plants,[30] and it was surprising to find its decrease in both shoots and roots of N-deficient plants (Table 2). This protective role of proline may be compensated, at least partially, by enhanced accumulation of phenols (see below) as we found higher amounts of proline in chamomile with inhibited phenolic metabolism.[17,31] Phenylalanine accumulation was slightly affected in −N variants, and lower impact of Ni−N, in comparison with Cd−N in both shoots and roots, is well correlated with the most enhanced accumulation of total soluble phenols in Ni−N because Phe is a substrate for PAL and phenolic biosynthesis (Table 2 and Figure 2). Overall, the amino acid profile showed that N deficiency rather than excess of

Table 3. Accumulation of Free Phenolic Acids or Root Cell Wall-Bound Acids (μg g^{-1} DW) in *Matricaria chamomilla* Plants after 7 Days of Exposure to 60 μM Cd or Ni in Nitrogen-Sufficient (+N) or Nitrogen-Deficient (−N) Solution[a]

	Gal	Pro	Pro.a	pOHb	Chlor	Van	Caf	pOHb.a	Syr	vanillin	pCoum	Fer	Sin	Sal
shoots														
C+N	1.35 a	7.29 c	2.96 c	1.40 a	12.8 d	95.5 c	1.21 d	1.20 a	1.16 a	0.32 a	0.48 b	1.18 d	1.97 c	0.95 cd
Cd+N	1.12 a	6.67 c	2.89 c	1.34 a	21.7 c	107.3 bc	1.59 c	1.18 a	1.23 a	0.33 a	0.58 b	1.65 c	2.72 b	1.72 b
Ni+N	1.29 a	7.89 bc	2.83 c	1.37 a	11.3 d	81.7 c	1.42 c	1.18 a	1.22 a	0.27 ab	0.55 b	1.35 cd	2.54 c	1.03 c
C−N	1.23 a	7.59 bc	3.04 bc	1.42 a	24.2 c	126.4 ab	1.97 bc	1.24 a	1.19 a	0.28 ab	0.75 ab	2.14 b	2.65 b	1.16 c
Cd−N	1.21 a	10.4 a	3.44 a	1.44 a	64.5 a	113.9 b	2.93 a	1.21 a	1.26 a	0.24 ab	1.06 a	3.11 a	4.57 a	2.10 a
Ni−N	1.28 a	8.19 b	3.25 ab	1.36 a	51.7 b	131.2 a	2.34 ab	1.17 a	1.14 a	0.20 b	0.90 a	2.73 ab	3.93a	0.78 d
roots														
C+N	1.12 a	1.61 b	0.98 b	0.97 c	2.46 d	7.72 a	1.33 d	1.49 b	1.42 c	0.34 a	0.36 c	1.04 c	8.24 c	0.17 cd
Cd+N	1.15 a	1.94 ab	0.62 c	1.14 bc	7.41 c	10.3 a	1.97 c	1.71 b	2.15 a	0.26 ab	0.51 c	2.09 ab	19.5 b	0.38 a
Ni+N	1.07 a	1.58 b	0.53 c	1.28 b	2.15 d	8.48 a	1.82 c	1.76 b	1.81 ab	0.31 a	0.44 c	1.46 c	15.8 b	0.21 bc
C−N	1.18 a	2.21 a	1.64 a	1.62 a	12.9 b	9.88 a	4.29 b	2.13 a	1.72 b	0.24 ab	1.09 b	2.34 a	21.1 a	0.14 d
Cd−N	1.08 a	1.19 c	0.87 b	0.82 c	29.3 a	7.92 a	6.55 a	1.67 b	1.44 c	0.23 ab	1.92 a	1.65 bc	25.5 a	0.15 d
Ni−N	1.13 a	1.55 b	1.16 b	0.85 c	11.5 b	8.48 a	5.88 a	1.78 ab	1.24 c	0.19 b	1.37 b	1.71 b	26.3 a	0.37 a
alk.h														
C+N	nd	0.58 c	0.35 c	0.24 ab	nd	0.53 a	147.7 c	nd	0.25 a	nd	0.09 c	15.7 c	1.08 a	1.18 a
Cd+N	nd	0.74 ab	0.44 b	0.20 b	nd	0.48 a	106.1 d	nd	0.08 b	nd	0.11 c	23.5 b	0.97 a	1.43 a
Ni+N	nd	0.68 b	0.13 d	0.19 b	nd	0.51 a	115.8 d	nd	0.29 a	nd	0.08 c	19.4 bc	0.77 b	1.27 a
C−N	nd	0.66 b	0.12 d	0.27 a	nd	0.31 b	542.6 b	nd	0.31 a	nd	0.20 ab	33.1 a	0.84 ab	nd
Cd−N	nd	0.82 a	0.64 a	0.22 ab	nd	0.33 b	695.3 a	nd	0.26 a	nd	0.27 a	32.7 a	0.95 a	nd
Ni−N	nd	0.86 a	0.66 a	0.19 b	nd	0.27 b	465.5 b	nd	0.11 b	nd	0.18 b	22.9 b	0.64 b	nd

[a] Data are the mean (n = 3); for the lucidity of the table, SDs are not shown. Values within columns followed by the same letter(s) are not significantly different according to Tukey's test (P < 0.05). C, control; alk.h, alkaline hydrolysis (root cell wall-bound acids); Gal, gallic acid; Pro, protocatechuic acid, Pro.a, protocatechuic aldehyde; pOHb, *p*-hydroxybenzoic acid; Chlor, chlorogenic acid; Van, vanillic acid; Caf, caffeic acid; pOHb.a, *p*-hydroxybenzoic aldehyde; Syr, syringic acid; pCoum, *p*-coumaric acid; Fer, ferulic acid; Sin, sinapic acid; Sal, salicylic acid.

Table 4. Quantitative Changes of Coumarin-Related Compounds [(*Z*)- and (*E*)-2-β-D-Glucopyranosyloxy-4-methoxycinnamic Acids (GMCAs) and Herniarin] in *Matricaria chamomilla* Leaves after 7 Days of Exposure to 60 μM Cd or Ni in Nitrogen-Sufficient (+N) or Nitrogen-Deficient (−N) Solution[a]

	+N control	Cd	Ni	−N control	Cd	Ni
Z-GMCA	2.42 ± 0.29 c	2.51 ± 0.26 c	2.93 ± 0.38 c	4.02 ± 0.25 b	4.23 ± 0.17 b	5.36 ± 0.43 a
E-GMCA	5.42 ± 0.18 d	7.05 ± 0.53 c	7.19 ± 0.42 c	10.9 ± 0.45 b	11.5 ± 0.67 b	19.8 ± 0.32 a
GMCAs	7.84 ± 0.42 d	9.56 ± 0.46 c	10.1 ± 0.47 c	14.9 ± 0.69 b	15.8 ± 0.77 b	25.1 ± 0.57 a
herniarin	1.96 ± 0.17 b	1.81 ± 0.27 b	2.85 ± 0.31 a	1.99 ± 0.24 b	2.40 ± 0.43 ab	1.98 ± 0.12 b

[a] Data are the mean (mg g^{-1} DW) ± SD (n = 3). Other details are as in Table 1.

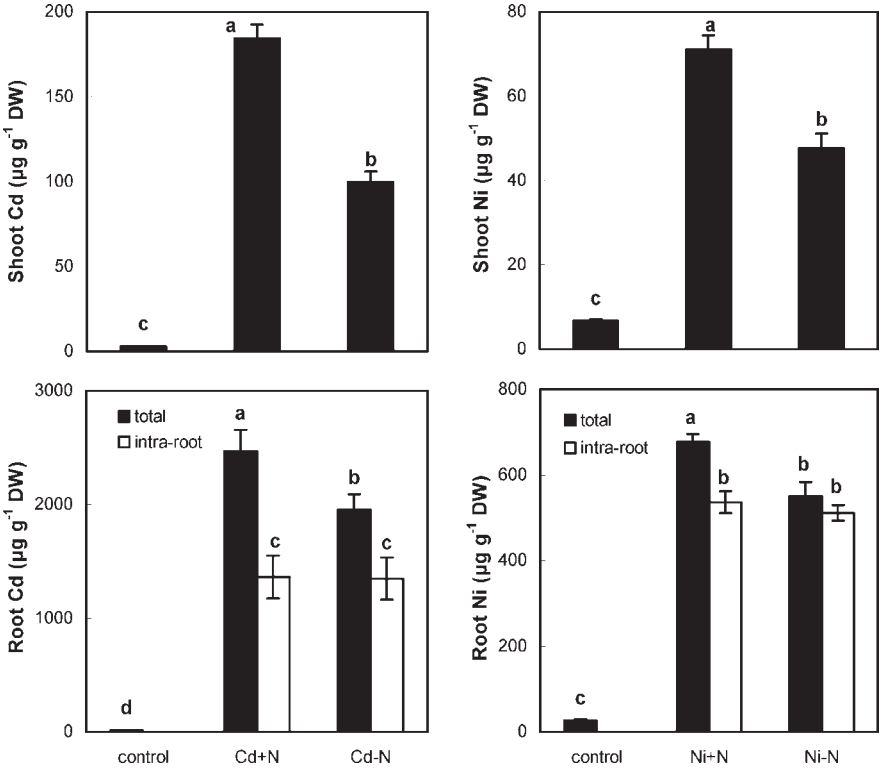

Figure 4. Uptake of cadmium and nickel by *Matricaria chamomilla* plants after 7 days of exposure to 60 μM Cd or Ni in nitrogen-sufficient (+N) or nitrogen-deficient (−N) solution. Data are the mean ± SD (n = 4). Other details are as in Figure 1.

metals regulates the soluble pool of amino acids in combined treatments, and these changes are correlated with the accumulation of soluble proteins to maintain essential enzymatic machinery, such as the peroxidase and phenolic enzyme activities we assayed.

Phenolic metabolites are both quantitatively and qualitatively important secondary metabolites, and their accumulation is elevated during both N deficiency[2−7] and metal excess.[15−17,23] We highlight that phenols in chamomile were suggested to be involved in shoot Cd uptake (positive correlation[15,16]), whereas the effect of phenols on Ni uptake is rather negative[17] or, in other words, they have no active role in shoot Ni accumulation.[16] Therefore, changes of phenolic metabolites cannot be evaluated without changes in metal uptake in the present study. A more pronounced impact of Ni, in comparison with Cd in N-deficient plants, on the accumulation of total soluble phenols is in contradiction with its low effect in +N medium (Figure 2), and the opposite was found in terms of flavonoid (flavonol) accumulation (Figure 3). These data again confirm the different impacts of Ni and Cd on phenolic metabolites, although both metals have similar physicochemical properties. The consequences of these observations in terms of metal uptake are discussed below.

Phenolic acids are simple phenols, which may serve as biosynthetic intermediates for more complex phenols (such as gallic acid for tannins, *p*-coumaric, ferulic, and sinapic acids for lignin), but they are also potent antioxidants, which accumulate under metal excess.[23] Among them, chlorogenic acid is a valuable compound,[28] and its amounts were considerably enhanced in all −N variants and mainly in Cd−N variants. Precursors of lignin monomers also increased in −N variants (Table 3), and lignin content was most elevated in Cd−N variants (Table 1). Just enhanced lignification may, for example, reduce apoplastic translocation of Cd,[23] which is a reason for reduced growth of roots in the early stages of ontogenesis.[32] N deficiency alone was also found to elevate lignification in tobacco plants,[2] and our quantitative data support this phenomenon in N-deficient chamomile roots (Table 1). The esterification of phenolic acids to the cell wall (revealed by alkaline hydrolysis of methanol-insoluble root residue) has been suggested to lead to the formation of lignin-like polymers by supplying lignin attachment sites to the matrix polysaccharides (see ref 23 and references

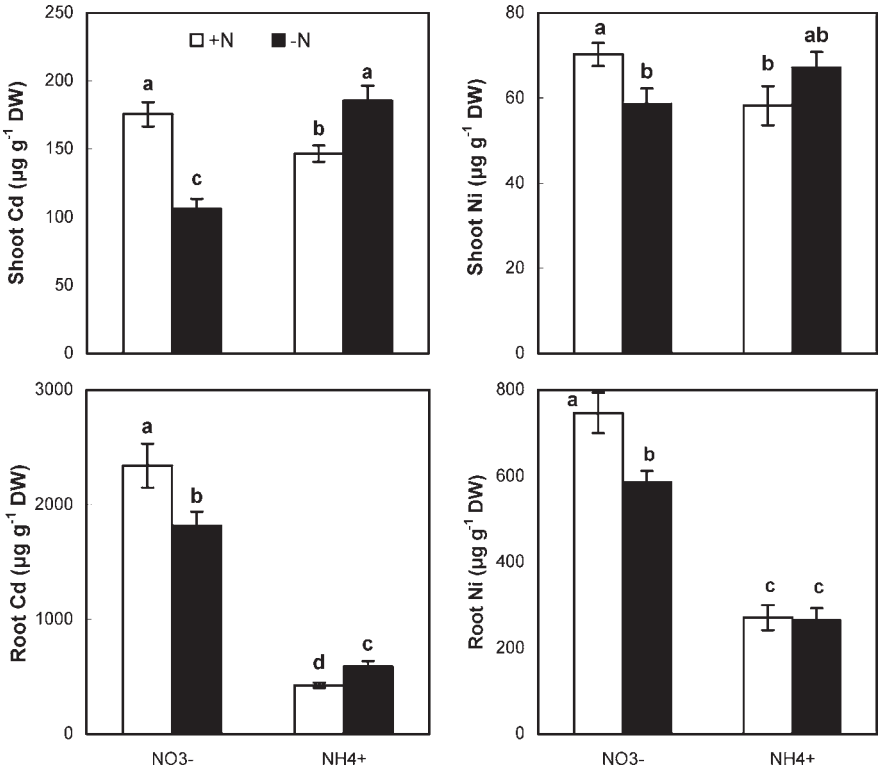

Figure 5. Effect of nitrogen deficiency (−N) on the uptake of cadmium and nickel by *Matricaria chamomilla* plants that had previously grown in either nitrate (NO_3^-) or ammonium (NH_4^+) solution. Data are the mean ± SD (n = 4). Values within each graph followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

Table 5. Amounts of Selected Mineral Nutrients in *Matricaria chamomilla* Plants after 7 Days of Exposure to 60 µM Cd or Ni in Nitrogen-Sufficient (+N) or Nitrogen-Deficient (−N) Solution[a]

	+N control	Cd	Ni	−N control	Cd	Ni
shoots						
N (mg g^{-1} DW)	56.7 ± 2.33 a	52.3 ± 2.81 a	56.4 ± 1.69 a	43.7 ± 0.86 b	38.9 ± 0.85 c	38.7 ± 1.41 c
K (mg g^{-1} DW)	99.9 ± 3.36 a	86.5 ± 3.63 bc	97.8 ± 4.18 a	93.7 ± 3.26 ab	78.6 ± 1.93 c	81.9 ± 4.10 c
Na (mg g^{-1} DW)	5.15 ± 0.08 c	5.85 ± 0.22 a	5.21 ± 0.25 bc	5.76 ± 0.07 ab	5.71 ± 0.37 abc	5.77 ± 0.16 ab
Ca (mg g^{-1} DW)	6.74 ± 0.32 a	5.62 ± 0.25 bc	6.15 ± 0.11 abc	6.28 ± 0.42 ab	5.51 ± 0.17 c	6.14 ± 0.08 abc
Mg (mg g^{-1} DW)	3.02 ± 0.11 ab	3.24 ± 0.05 a	2.94 ± 0.17 ab	3.07 ± 2.67 ab	3.01 ± 0.17 ab	2.74 ± 0.22 b
Fe (mg g^{-1} DW)	0.24 ± 0.03 a	0.21 ± 0.03 a	0.22 ± 0.04 a	0.21 ± 0.01 a	0.25 ± 0.03 a	0.23 ± 0.04 a
Cu (µg g^{-1} DW)	16.3 ± 2.12 a	10.7 ± 1.67 b	14.3 ± 2.00 ab	15.9 ± 1.36 a	10.5 ± 2.21 b	12.4 ± 0.80 ab
roots						
K (mg g^{-1} DW)	100 ± 3.31 a	80.9 ± 4.80 b	101 ± 6.34 a	107 ± 5.09 a	81.3 ± 4.56 b	102 ± 6.07 a
Na (mg g^{-1} DW)	4.88 ± 0.11 a	4.69 ± 0.59 a	4.50 ± 0.24 a	4.64 ± 0.20 a	2.60 ± 0.39 b	2.59 ± 0.18 b
Ca (mg g^{-1} DW)	8.70 ± 0.20 a	7.35 ± 0.29 b	7.55 ± 0.08 b	7.99 ± 0.38 b	5.12 ± 0.28 d	6.52 ± 0.12 c
Mg (mg g^{-1} DW)	1.57 ± 0.16 a	1.46 ± 0.25 a	1.47 ± 0.13 a	1.68 ± 0.16 a	1.36 ± 0.20 a	1.53 ± 0.11 a
Fe (mg g^{-1} DW)	8.18 ± 0.13 ab	8.32 ± 0.25 a	8.47 ± 0.38 a	6.21 ± 0.43 d	7.39 ± 0.21 bc	7.23 ± 0.30 c
Cu (µg g^{-1} DW)	40.6 ± 2.63 b	58.0 ± 4.20 a	55.0 ± 3.62 a	39.4 ± 4.25 b	19.7 ± 2.93 c	20.0 ± 5.66 c

[a] Data are the mean ± SD (n = 3). Other details are as in Table 1.

cited therein). This phenomenon was mainly visible in Cd−N variants, in accordance with the highest lignin deposition. Mainly caffeic acid was found in this fraction, an observation that was also made in Cu-exposed chamomile roots.[33] This indicates the essential role of caffeic acid in lignification, and a recent study has shown extensive effects of exogenous caffeic acid on lignin metabolism in soybean roots.[34] Increase in phenolic metabolites in N-deficient conditions is not only a passive mechanism owing to stimulation of respective enzymes, and their antioxidative (chlorogenic acid) or biosynthetic destiny (lignin monomers) is not the only role. For example, an increase in phenols was observed in P-deficient bean leaves, and exudation of phenolics into the culture medium was recorded in both P- and N-deficient conditions.[35] Released phenolics are thought to solubilize different nutrients from unavailable sources allowing their uptake and, for example, caffeic acid was found to be such a released compound.[36]

Coumarins are quantitatively important chamomile metabolites.[17] We note that herniarin and its precursors [(*Z*)- and (*E*)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs)] do not react with Folin−Ciocalteu reagent used for total soluble phenols, probably owing to the absence of a free hydroxyl group attached to the aromatic ring. It was shown that foliar Cu application led to a decrease

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in GMCAs and an increase in herniarin,[24] referred to as "stress-related" changes mediated by enzymatic glucose elimination from GMCA and spontaneous cyclization to herniarin. Further studies with hydroponical application of metals or inhibition of phenolic metabolism (see ref 17 and references cited therein) did not show a similar effect, and either partial or parallel increase or decrease in both GMCAs and herniarin has always been observed. These data strongly suggest that foliar Cu application simply directly disrupts biosynthesis owing to toxic concentration, and all other data indicate coordinated regulation of biosynthesis. Our present data are in accordance with this conclusion, but it was an unexpected finding that amounts of coumarin-related compounds stimulated the most in Ni−N variants (Table 4) because Ni accumulation was lower in comparison with Cd. The exact role of these metabolites in metal excess needs to be elucidated.

The activity of PAL after a given exposure period (7 days) was enhanced only in N-deficient plants (Figure 1). A similar observation was made in N-deficient *Achillea* leaves and roots even after 4 months of N deprivation.[4] Lower PAL activity in metal−N variants is in contradiction to the more expressive alteration of phenolic metabolites. This aspect may sufficiently be explained by the time dynamics of PAL activity (such as that found in +N Cd- and Cu-exposed chamomile[23]), and it could logically be expected that combined stress (N deficiency plus metal excess) will generate higher pressure to protective mechanisms, including phenolic metabolism at earlier exposure time points, which we did not quantify but was evidenced by increases in numerous phenolic metabolites. In contrast to PAL, the activity of PPO has never been reduced in −N variants, and it was mainly elevated in Cd−N roots. This is in accordance with the most visible brown coloration of roots in this variant because PPOs catalyze the oxygen-dependent oxidation of phenols to quinones and further melanin-like brown condensation polymers.[37] Therefore, both lignin accumulation and enhanced PPO activity contributed to observed color changes.

Sugars are other C-based compounds that usually increase in N-limited conditions.[2] Reducing sugars were stimulated by Cd but inhibited by Ni in +N variants, and they showed enhancement in −N variants, although Cd and Ni effects still differed (Table 1). This Cd effect has been previously found, for example, in Cd-exposed eggplant.[38] Although this different effect of Cd and Ni in N-limited conditions remains unclear, it is still another component of the mosaic showing different impacts of Cd and Ni excess on plant tissue.

An excess of metals usually reduces the accumulation of essential mineral nutrients also in chamomile.[15−17] It was therefore surprising that decreases in Cd and Ni amounts did not ameliorate, for example, K and Ca contents, although we observed this event in an earlier study.[17] Accumulation of Fe was even stimulated in metal−N variants in comparison with −N control, indicating the essentiality of this element under metal excess as it is a component of several ROS scavenging enzymes, including peroxidases. Overall, changes to mineral nutrients were influenced by metals rather than by N deficiency (Table 5).

The uptake of metals is affected by a wide range of factors, starting with plant species and ontogenetic stage. As mentioned above, phenols have a direct impact at least on Cd accumulation in chamomile. It is a new finding, which was yet not found in any plant species with sufficient N supply, that Ni had a more visible impact on phenols under N-deficient conditions in comparison with Cd. Higher accumulation of phenols and lower accumulation of Ni in Ni−N variant support our assumption that phenols serve as a barrier preventing Ni from reaching the above-ground biomass. This corresponds with the results from PAL-inhibited plants, in which a decrease in phenolic metabolites increased shoot Ni amount.[17] A decrease in shoot Cd amount in −N variants is in contradiction to our assumption about positive correlation between accumulation of phenols and Cd and suggests at least two possible explanations or their combination: (i) exudation of phenols (phloem translocation) reduces shoot Cd uptake, and such phloem retranslocation has been observed even in +N Cd-exposed *Arabidopsis*;[39] and (ii) elimination of nitrate (N deficiency) directly affects uptake. The latter reason has not yet been observed in −N conditions, but it may be judged from a recent paper by Jalloh et al.,[18] who found that nitrate-fed rice plants had the highest amount of Cd in all organs investigated. In terms of Ni uptake and N nutrition, it was found that sunflower plants contained ca. 3 times less Ni in a mixture of ammonium and nitrate in comparison with nitrate alone.[40] Comparison of metal uptake in nitrate- and ammonium-fed plants exposed to N deficiency also confirmed the assumption that nitrate is essential for metal uptake: NH_4^+-fed plants exposed to N deficiency did not show a decrease in Ni and even a stimulation of Cd uptake (Figure 5). Another aspect revealed by this comparison is the difference in shoot amounts of Cd and Ni in +N solutions; although NH_4^+-fed plants contained lower levels of metals than NO_3^--fed ones, this difference was relatively small in comparison with other plant species,[18,19,40] whereas differences in root metal contents were expressive (Figure 5). This indicates species-related differences and lower biomass of NH_4^+-fed chamomile plants (ca. 2−3 times, data not shown) could also contribute to the lower difference of shoot metal uptake in comparison with NO_3^--fed ones. However, these data indicate that accumulation of metals in N-deficient conditions is a more complex process owing to far-reaching consequences of N-limitation for overall metabolism. Strong accumulation of phenols has not yet been considered as a factor affecting metal uptake and/or retranslocation in any study published to date. We note that ammonium-fed plants exposed to N deficiency in combination with metal excess did not show enhanced accumulation of total soluble phenols (in comparison with +N variants, data not shown), suggesting strong differences between nitrate and ammonium nutrition, and further detailed studies will be conducted. The decrease in total root metal accumulation in −N variants (Figure 4) is well correlated with the decrease in shoot amount and again indicates retranslocation and exudation. Unaffected intraroot accumulation of metals is evidence of the impact of N deficiency on root-surface adsorbed fraction of metals and suggests that roots may actively affect the uptake of metals at the boundary root's surface rhizosphere.

In conclusion, nitrate deficiency evoked extensive changes in both primary and secondary metabolites. These changes were further enhanced or repressed by Cd and Ni excess, confirming different effects of these metals on plant tissue. These changes must therefore have either direct or indirect impact on metal translocation. Unexpected similar decreases in shoot and root accumulation of both Cd and Ni in N-deficient conditions indicate that strong accumulation of phenolic metabolites and nitrate elimination repressed Ni and Cd uptake. This conclusion was further confirmed by comparison with NH_4^+-fed chamomile plants exposed to N deficiency, for which decreases in metal uptake and/or enhancement of soluble phenols were not observed.

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