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ARTICLE

Copper Uptake Is Differentially Modulated by Phenylalanine Ammonia-lyase Inhibition in Diploid and Tetraploid Chamomile

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The effect of phenylalanine ammonia-lyase (PAL) inhibition by 2-aminoindane-2-phosphonic acid (AIP) in copper-exposed diploid and tetraploid chamomile (*Matricaria chamomilla*) roots has been studied in a short-term experiment (24 h). Cu evoked stronger induction of PAL activity and accumulation of soluble phenols, flavonols (quercetin and kaempferol), and lignin in diploid plants, whereas $AlCl_3$-reactive flavonoids and phenolic acids did not differ with respect to ploidy. Amounts of hydrogen peroxide and superoxide also preferentially increased in diploid. Surprisingly, PAL activity was restored in both +AIP cultivars, being inversely correlated with the accumulation of free phenylalanine. Notwithstanding this, total soluble phenols and flavonols were more depleted in Cu+AIP diploid roots. Soluble proteins decreased in response to Cu, and AIP had no effect. Among free amino acids, proline increased more visibly in Cu+AIP diploid, suggesting that this could be a protective mechanism in conditions with depleted content of phenols. Decrease in potassium content was ploidy-independent, calcium increased in all Cu variants, and Fe increased in Cu-exposed tetraploid. Shoot Cu content did not differ in Cu-exposed cultivars, but diploid roots contained more Cu. AIP decreased root Cu but increased shoot Cu amounts in diploid, whereas tetraploid plants did not exhibit similar responses. These data indicate that inhibition of root phenolic metabolism by AIP was effective enough, allowing Cu to accumulate in diploid shoots. The present findings are discussed in the context of available data about AIP effects and with respect to the role of phenols in metal uptake.

KEYWORDS: Heavy metals; phenols; reactive oxygen species

INTRODUCTION

Accumulation of metals by plants represents risk for human health if accumulated in crop plants (*1*). Copper (Cu) is an essential plant micronutrient, being a cofactor of many enzymes, but it can be phytotoxic at high concentrations (*2*). Owing to its wide use in chemicals for agricultural fungicides, it may easily enter the environment, leading to damage of plants when present in excess. It is a redox-active metal enhancing reactive oxygen species (ROS) formation by the Fenton−Haber−Weiss reactions (*3*), leading to higher toxicity in comparison with redox-inactive metals such as Cd (*4*, *5*).

Evolution of plants in changing environmental and aerobic conditions led to the development of an array of protective mechanisms such as antioxidative enzymes and low molecular weight antioxidants such as ascorbate, glutathione, and phenols. Especially phenolic metabolites are abundant compounds, forming a substantial part of the plant organic matter. Therefore, their responses to different stress effects including excess of metals are not surprising. For example, they scavenge ROS directly or through enzymatic reactions (*6*, *7*) and are able to chelate/precipitate metals (*8*, *9*). In the case of Cu excess, their exudation in the rhizosphere has been found (*10*), and strong quantitative changes in different plant species suggest an essential role in Cu detoxification (*7*, *11*, *12*).

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a pivotal step in the biosynthesis of phenols and is usually stimulated in metal-stressed plants, depending on the particular metal and its concentration (*13*). 2-Aminoindane-2-phosphonic acid (AIP) is the most effective known PAL inhibitor (*14*−*16*), providing a powerful tool for the manipulation of phenolic biosynthesis. Several studies have shown that PAL inhibition enhances the sensitivity of plants to stresses such as UV-B radiation (*17*) and low temperatures (*18*), but exact proof focused on the role of phenols in metal tolerance is still absent.

Polyploidization is a valuable tool to gain increased productivity of plants through higher organ size, but no corresponding increase in the content of active compounds has been observed in chamomile (*19*), although induction of several parameters differed depended on ploidy level (*20*). There exist only a limited number of studies about the role of ploidy in metal uptake focused mainly on Cd accumulation (*21*, *22*), but this phenomenon in

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Table 1. Selected Physiological and Stress-Related Parameters in the Roots of Diploid and Tetraploid *Matricaria chamomilla* Plants Exposed to Different Treatments for 24 h[a]

	diploid			tetraploid		
	control	Cu	Cu+AIP	control	Cu	Cu+AIP
tissue water content (%, $n = 10$)	93.68 ± 0.25 a	92.13 ± 0.27 b	92.59 ± 0.19 b	93.84 ± 0.34 a	93.82 ± 0.44 a	93.52 ± 0.23 a
soluble proteins (mg g^{-1} of DW; $n = 4$)	61.3 ± 4.62 a	14.1 ± 0.53 c	17.7 ± 0.80 bc	65.8 ± 3.13 a	20.8 ± 1.73 b	21.0 ± 1.61 b
hydrogen peroxide (μmol g^{-1} of DW; $n = 4$)	28.9 ± 3.61 d	66.5 ± 3.83 a	42.1 ± 1.44 c	29.2 ± 2.78 d	52.2 ± 4.51 b	55.3 ± 3.18 b
superoxide (μg g^{-1} of DW; $n = 4$)	43.1 ± 3.40 c	113.6 ± 10.5 a	102.4 ± 4.90 a	46.4 ± 1.87 c	75.5 ± 3.89 b	23.7 ± 2.03 d

[a] Cu was applied in 120 μM and AIP (PAL inhibitor) in 30 μM. Data are mean ± SD. Values within rows, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$).

terms of Cu accumulation has not been studied. Comparison of cultivars with different sensitivities to Cu did not show direct correlation between damage and Cu accumulation (*23*).

Chamomile (*Matricaria chamomilla* L.) is a widely used medicinal plant, and our previous studies using tetraploid plants have shown sensitivity to Cu (in comparison with Cd) and, at the same time, higher induction of specific phenolic metabolites in both roots (*13*) and above-ground biomass (*5*, *24*). Despite huge numbers of studies, information as to how phenols may affect uptake of metals is still absent. Fortunately, the existence of a specific inhibitor of PAL allows us to conduct deeper study by modulating this pathway. It was therefore the main aim of the present investigation to evaluate Cu uptake by two chamomile cultivars with different ploidy levels (diploid vs tetraploid) using AIP as a specific PAL inhibitor. We have studied phenolic metabolism-related parameters in the roots and the consequence for Cu distribution in the whole plants. Accumulations of free amino acids, ROS, and mineral nutrients were also measured. In addition, selected parameters in the shoots are also mentioned if appropriate for discussion.

MATERIALS AND METHODS

Cultivation of Plants and Experimental Design. Twenty-one-day-old seedlings of *Matricaria chamomilla* L. (diploid 'Novbona', $2n = 18$, and tetraploid 'Lutea', $2n = 36$ from our own seeds, identity verified by chromosome count and flow cytometry of nuclear DNA) germinated in sand (with three to four first true leaves) were placed in Hoagland solution routinely used in our laboratory (*5*, *13*, *20*, *24*−*28*). Uniform plants were cultivated in dark plastic 5 L boxes (25 plants per box) with continual aeration of the solutions. The experiment was performed in a growth chamber under controlled conditions: 12 h day (6:00 a.m. to 6:00 pm); 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%. In these conditions, plants form basal leaf rosettes only. Solutions were renewed weekly to prevent nutrient depletion. Plants that had been cultivated hydroponically for 7 weeks were used in the experiment and further cultured for 24 h in 120 μM Cu-enriched solutions (added in the form of $CuCl_2 \cdot 2H_2O$, Lachema Brno, Czech Republic) with or without addition of 30 μM AIP, and pH was corrected to 6.0 in all variants. AIP was synthesized according to earlier works (*14*−*16*). 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, lignin, and mineral nutrients including Cu. Plants for fresh mass-requiring parameters were powdered using liquid N_2 and extracted as described below. Spectrophotometry was carried out with an Uvi Light XTD 2 (Secomam, ALES Cedex, France).

Analyses of Phenolic Metabolism-Related Parameters. Total soluble phenols were extracted with 80% methanol from fresh tissue and measured using Folin−Ciocalteu's method with gallic acid as standard; flavonoids were estimated in the same supernatant using an $AlCl_3$ procedure and quercetin as standard (*20*, *24*). For quantification of selected flavonols (quercetin and kaempferol), 0.2 mL of 1 M HCl was added to 0.3 mL of methanol supernatants, and samples were heated for 1 h at 80 °C. Measurement has been realized using a HPLC system at 370 nm and calculation using a peak of quercetin and kaempferol standard compounds (*29*). The selected cinnamic and benzoic acid derivatives in the chamomile plants were measured in 80% methanol extracts. Extraction and repurification by solid phase extraction procedure at computer-controlled robot Aspec XL, Gilson (USA), HPLC conditions, and detection by a mass selective HP MSD quadrupole detector (G1946A, Hewlett-Packard, Palo Alto, CA) were done as described earlier (*5*, *13*, *26*). Root lignin content was estimated by the thioglycolic acid reaction (*13*).

Activity of PAL (EC 4.3.1.5) was determined as the production of (*E*)-cinnamic acid from phenylalanine using the HPLC method (*30*) with slightly modified protocol in homogenates prepared using sodium borate buffer, pH 8.7 (*13*).

Assay of Soluble Proteins, Reactive Oxygen Species, and Free Amino Acids. Samples for measurement of proteins and ROS were homogenized in potassium phosphate buffer, pH 7.0, using a cold mortar and pestle. Proteins were quantified according to the method of Bradford (*31*) using 20 μL of supernatants and bovine serum albumin as standard. Amounts of hydrogen peroxide (using $TiCl_4$ method) and superoxide (using hydroxylamine method) were calculated from standard curves prepared using known H_2O_2 and $NaNO_2$ concentrations, respectively (*5*, *27*). 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 (*26*, *28*).

Quantification of Cu and Selected Mineral Nutrients. Samples for quantification of metals were prepared as described elsewhere (*5*, *13*, *24*, *25*): dry material was kept overnight in 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.

Statistical Analyses. Data were evaluated using 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. One box containing 25 plants was used for each cultivar/treatment; thus, the whole experiment included six boxes. Two independent repetitions of the whole experiment were performed to check reproducibility.

RESULTS

Effect of Cu and AIP on Basic Growth Parameters and ROS Accumulation. A 24-h exposure had no effect on biomass accumulation (data not shown) as expected from short exposure time. The only visible symptom was brown coloration of the root tissue in Cu excess. Root tissue water content was reduced by Cu application in diploid plants, and AIP had no effect on this parameter. Similarly, AIP had no effect on Cu-induced depletion of soluble proteins (−77 and −71% in Cu and Cu+AIP in diploid and −68% in Cu and Cu+AIP in tetraploid ones; **Table 1**).

Amounts of H_2O_2 and superoxide were enhanced more expressively by Cu excess in diploid roots (2.3- and 2.6-multiple of control value, respectively), and AIP showed a different effect on these parameters (**Table 1**).

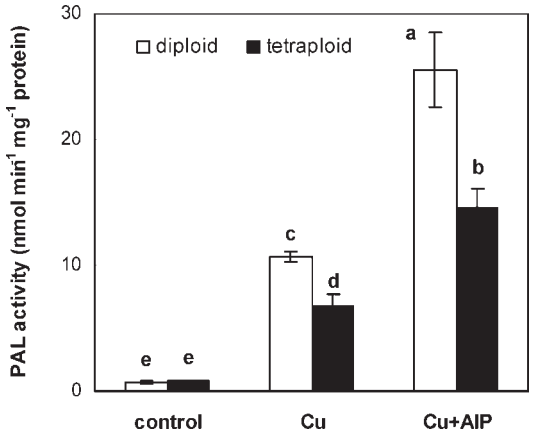

Figure 1. Activity of phenylalanine ammonia-lyase (PAL, $n = 4$) in the roots of diploid and tetraploid *Matricaria chamomilla* plants exposed to different treatments for 24 h. Cu was applied at 120 μM and AIP (PAL inhibitor) at 30 μM. Data are mean $\pm$ SD. Values within the graph, designated by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$).

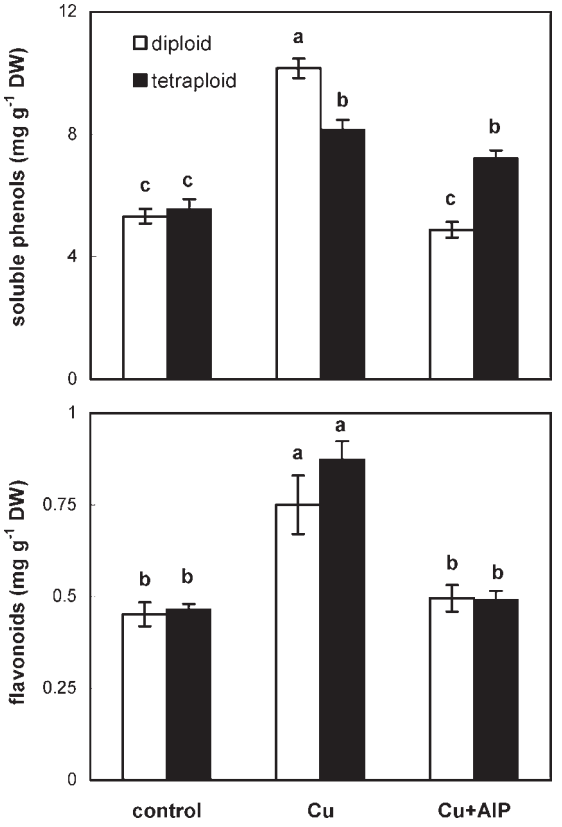

Figure 2. Accumulation of total soluble phenols and $AlCl_3$-reactive flavonoids in the roots of diploid and tetraploid *Matricaria chamomilla* plants exposed to different treatments for 24 h ($n = 4$). Other details are as in **Figure 1**.

Phenolic Metabolism-Related Parameters. The activity of PAL was strongly stimulated by Cu excess in both cultivars (16.5- and 8.7-fold over control in diploid and tetraploid, respectively) and further enhanced by AIP in combined treatment (2.4 and 2.1 times in comparison with respective Cu treatments, **Figure 1**). Accumulation of total soluble phenols was stimulated by Cu excess preferentially in diploid (+90%) and reduced by AIP to control level in this cultivar, whereas tetraploid showed a lower

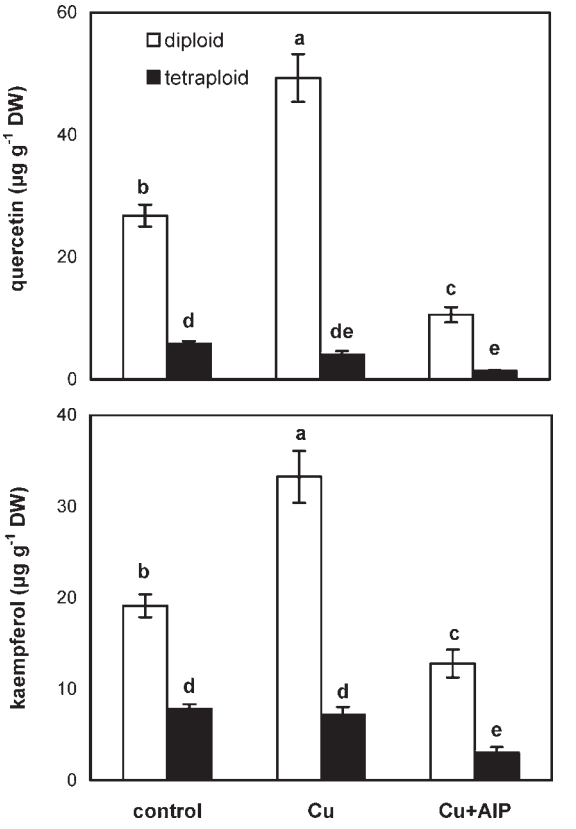

Figure 3. Amount of selected flavonols in the roots of diploid and tetraploid *Matricaria chamomilla* plants exposed to different treatments for 24 h ($n = 4$). Other details are as in **Figure 1**.

increase in Cu excess (+47%) but was unaffected by AIP application (**Figure 2**). Accumulation of flavonoids did not show a difference in terms of ploidy effect on Cu-induced changes (nonsignificant difference), and AIP reduced this parameter by the same extent in both cultivars (**Figure 2**).

Analyses of two flavonols (quercetin and kaempferol) showed their higher accumulation in diploid roots (**Figure 3**). Accumulation of both compounds strongly increased in diploid roots exposed to Cu (+83 and 73% for quercetin and kaempferol, respectively) and was significantly reduced by AIP application to even lower levels than respective controls (−60 and −33%).

Among 13 detected simple phenols (so-called phenolic acids), vanillin (+170 and 80%), chlorogenic (+117 and 149%), and ferulic (+100 and 70%) acids showed the most expressive quantitative increase in Cu excess in both cultivars (**Table 2**). These compounds mainly contributed to significantly nondifferent sum of phenolic acids if Cu-exposed cultivars are compared. AIP has reduced majority of detected compounds, leading to restoration of their sum to control level in both cultivars (**Table 2**).

Accumulation of lignin was more enhanced in diploid (+ 70%) in comparison with tetraploid roots (+39%), and AIP had no effect on Cu-induced lignification (+80 and 35% in diploid and tetraploid, respectively; **Table 2**).

Quantitative Changes of Free Amino Acids. Accumulation of free amino acids (sum) in Cu excess increased significantly only in tetraploid roots (**Table 3**). AIP caused further enhancement of the sum to similar levels in both cultivars. Within individual compounds, mainly serine, glycine, phenylalanine, and proline contributed to the mentioned increase in the sum of amino acids in AIP-exposed cultivars. This increase in serine/glycine/proline represented 1.78/4.12/2.8-multiple in diploid and 1.8/6.13/1.7-multiple in tetraploid in comparison with respective controls

Table 2. Accumulation of Selected Benzoic (Gallic−Salicylic) and Cinnamic Acid (Chlorogenic−*p*-Coumaric) Derivatives (Micrograms per Gram of Dry Weight) and Lignin (Milligrams per Gram of Dry Weight) in the Roots of Diploid and Tetraploid *Matricaria chamomilla* Plants Exposed to Different Treatments for 24 h ($n = 3$)[a]

	diploid			tetraploid		
	control	Cu	Cu+AIP	control	Cu	Cu+AIP
gallic acid	1.57 ± 0.14 a	1.01 ± 0.24 ab	0.43 ± 0.04 b	0.70 ± 0.25 b	0.69 ± 0.42 b	0.46 ± 0.13 b
protocatechuic acid	3.49 ± 0.05 b	6.57 ± 0.55 a	3.14 ± 0.12 b	1.65 ± 0.11 c	3.83 ± 0.26 b	1.93 ± 0.13 c
protocatechualdehyde	2.64 ± 0.34 bc	4.19 ± 0.21 a	2.82 ± 0.08 bc	1.61 ± 0.39 d	3.15 ± 0.23 b	2.34 ± 0.25 cd
p-hydroxybenzoic acid	1.70 ± 0.06 a	1.43 ± 0.22 ab	1.28 ± 0.16 ab	1.07 ± 0.22 b	1.26 ± 0.12 ab	1.11 ± 0.23 b
p-hydroxybenzoic aldehyde	3.14 ± 0.22 c	5.24 ± 0.19 b	7.10 ± 0.42 a	2.44 ± 0.30 c	5.06 ± 0.09 b	6.97 ± 0.59 a
vanillic acid	3.25 ± 0.09 b	3.31 ± 0.37 b	3.40 ± 0.19 ab	2.14 ± 0.28 c	3.98 ± 0.27 a	3.15 ± 0.14 b
vanillin	4.99 ± 0.26 c	13.56 ± 2.29 a	7.50 ± 0.28 b	7.40 ± 0.38 b	13.34 ± 1.19 a	8.14 ± 0.41 b
syringic acid	1.09 ± 0.11 b	1.25 ± 0.18 b	1.18 ± 0.21 b	1.11 ± 0.24 b	2.03 ± 0.02 a	1.84 ± 0.10 a
salicylic acid	0.61 ± 0.02 c	1.73 ± 0.19 a	1.21 ± 0.10 b	0.71 ± 0.03 c	1.26 ± 0.12 b	1.14 ± 0.29 b
chlorogenic acid	14.10 ± 2.39 b	30.62 ± 2.73 a	15.20 ± 2.08 b	13.49 ± 0.63 b	33.70 ± 3.12 a	4.23 ± 0.20 c
caffeic acid	1.96 ± 0.11 a	1.92 ± 0.21 a	1.26 ± 0.19 b	1.20 ± 0.16 b	1.02 ± 0.18 bc	0.72 ± 0.05 c
ferulic acid	5.23 ± 0.21 b	10.46 ± 1.45 a	5.01 ± 0.35 b	5.62 ± 0.04 b	9.67 ± 0.82 a	4.69 ± 0.32 b
p-coumaric acid	0.22 ± 0.02 ab	0.25 ± 0.02 a	0.15 ± 0.02 b	0.21 ± 0.03 ab	0.23 ± 0.03 a	0.19 ± 0.03 ab
sum	43.98 ± 2.15 cd	81.53 ± 2.59 a	49.67 ± 2.47 bc	39.36 ± 1.08 de	79.18 ± 3.44 a	36.93 ± 2.20 e
lignin	24.8 ± 2.29 c	42.5 ± 1.58 a	44.9 ± 4.59 a	25.5 ± 2.07 c	35.4 ± 0.75 b	34.6 ± 3.04 b

[a] Other details as in **Table 1**.

Table 3. Accumulation of Free Amino Acids (Micromoles per Gram of Dry Weight) in the Roots of Diploid and Tetraploid *Matricaria chamomilla* Plants Exposed to Different Treatments for 24 h ($n = 4$)[a]

	diploid			tetraploid		
	control	Cu	Cu+AIP	control	Cu	Cu+AIP
Asp	2.77 ± 0.23 a	0.84 ± 0.07 c	0.93 ± 0.04 c	2.58 ± 0.62 a	1.18 ± 0.16 bc	1.38 ± 0.09 b
Glu	1.32 ± 0.15 a	0.39 ± 0.04 c	0.47 ± 0.05 c	1.04 ± 0.12 ab	0.80 ± 0.076 b	0.99 ± 0.083 ab
Ser	4.10 ± 0.37 b	4.68 ± 0.62 b	7.33 ± 1.18 a	4.33 ± 0.51 b	4.51 ± 0.39 b	7.85 ± 0.88 a
His	0.35 ± 0.019 ab	0.27 ± 0.028 ab	0.40 ± 0.036 a	0.39 ± 0.022 a	0.21 ± 0.015 b	0.37 ± 0.034 a
Gly	1.48 ± 0.13 d	2.96 ± 0.21 c	6.11 ± 0.54 b	1.22 ± 0.09 d	3.58 ± 0.45 c	7.48 ± 0.67 a
Thr	1.19 ± 0.021 c	1.94 ± 0.35 b	2.32 ± 0.19 b	1.17 ± 0.019 c	2.25 ± 0.33 b	3.50 ± 0.41 a
Arg	0.77 ± 0.084 c	1.13 ± 0.14 bc	1.47 ± 0.31 ab	0.86 ± 0.093 c	0.98 ± 0.076 bc	1.95 ± 0.23 a
Ala	14.6 ± 2.37 a	15.8 ± 1.89 a	16.3 ± 2.06 a	12.9 ± 2.90 a	14.7 ± 1.51 a	16.5 ± 2.08 a
Tyr	0.41 ± 0.017 c	0.44 ± 0.015 bc	0.52 ± 0.033 ab	0.46 ± 0.051 bc	0.39 ± 0.023 c	0.64 ± 0.042 a
Cys	0.29 ± 0.022 ab	0.26 ± 0.014 b	0.30 ± 0.0.034 ab	0.27 ± 0.034 ab	0.23 ± 0.019 b	0.42 ± 0.031 a
Val	0.67 ± 0.042 c	0.81 ± 0.067 c	1.53 ± 0.19 b	0.74 ± 0.068 c	1.64 ± 0.09 b	2.78 ± 0.34 a
Met	0.041 ± 0.003 c	0.056 ± 0.004 bc	0.059 ± 0.005 ab	0.058 ± 0.007 ab	0.062 ± 0.006 ab	0.068 ± 0.005 a
Phe	0.28 ± 0.019 c	0.33 ± 0.029 c	4.49 ± 0.38 b	0.36 ± 0.041 c	0.31 ± 0.027 c	5.98 ± 0.63 a
Ile	0.69 ± 0.057 c	0.93 ± 0.11 c	1.67 ± 0.21 b	0.74 ± 0.083 c	1.52 ± 0.18 b	2.38 ± 0.29 a
Leu	1.07 ± 0.15 b	1.19 ± 0.24 b	2.16 ± 0.24 a	1.26 ± 0.19 b	1.33 ± 0.08 b	2.31 ± 0.18 a
Lys	0.11 ± 0.015 c	0.14 ± 0.022 c	0.35 ± 0.056 ab	0.17 ± 0.021 c	0.24 ± 0.027 b	0.46 ± 0.052 a
Pro	3.54 ± 0.29 d	4.93 ± 0.54 c	9.92 ± 1.02 a	4.61 ± 0.73 cd	6.85 ± 0.83 b	7.86 ± 0.41 b
sum	33.6 ± 2.59 c	37.2 ± 3.08 bc	58.8 ± 6.31 a	33.2 ± 1.84 c	40.8 ± 2.83 b	61.4 ± 4.22 a

[a] Other details as in **Table 1**.

(**Table 3**). Phenylalanine, owing to application of PAL inhibitor (AIP), has increased over 16 times in both cultivars. Tyrosine, another aromatic amino acid, also significantly increased in AIP treatments (+26 and +39% in diploid and tetraploid, respectively; **Table 3**).

Accumulation of Cu and Selected Mineral Nutrients. The amounts of Cu in the leaf rosettes and root were not different in control cultivars (**Figure 4**). Shoot Cu content was not affected by Cu excess either in diploid or in tetraploid plants, whereas application of AIP has elevated shoot Cu accumulation by about 2 times. At the root level, Cu-exposed diploid contained a higher amount of Cu in comparison with tetraploid (92 and 84 times over control, respectively). Application of AIP depleted root Cu content in diploid (78-fold of control) but had no effect on tetraploid ones (**Figure 4**).

Cu-induced decreased in potassium content was ploidy-independent (**Table 4**). Sodium content increased only in diploid (+54 and 50% in Cu and Cu+AIP, respectively). The amount of calcium was elevated by Cu excess in both cultivars (+38 and 30% in diploid and tetraploid, respectively), and AIP had no effect on this enhancement. Fe content was elevated in Cu (+51%) and Cu+AIP (+30%) variants in tetraploid roots, and accumulation of magnesium showed a similar trend (**Table 4**).

DISCUSSION

Although the Cu concentration that we used may be considered as environmentally nonrealistic, we used it to achieve possible visible changes of shoot Cu uptake after 24 h of exposure because Cu has low mobility in chamomile in terms of time dynamics (*25*). This was also reason why we studied parameters in the roots and consequences of root-induced changes for Cu uptake in whole plants. Similarly, the reason for short-time exposure to Cu or Cu+AIP was the fact that AIP is a reversible PAL inhibitor, and in terms of enzymatic kinetics this means that an increase in the enzyme's substrate (phenylalanine) will compete with inhibitor (AIP), thus leading to restoration of PAL activity. This was

observed in the present experiment as shown by higher PAL activity in both cultivars (Cu+AIP vs Cu, **Figure 1**) being inversely correlated with changes in free Phe accumulation in +AIP variants (lower amount of Phe reflects higher PAL activity, **Table 3**). This fact has not yet been published, although "superinduction" of PAL activity 24 h after application of AIP to chilling-treated soybean roots has been briefly mentioned (*18*). This could be explained, most probably, by an increase in PAL expression. Other PAL inhibitors induced accumulation of PAL enzyme and PAL mRNA, and the same is assumed for AIP (*32*). We note that AIP at 30 μM effectively reduced PAL activity in vitro in chamomile roots (ca. 95%, data not shown). Restoration of PAL activity, which we observed in vivo (**Figure 1**), has previously been judged from restored level of several groups of phenolic metabolites to control level several days after transfer from AIP-enriched to AIP-free medium (*33*). Our present study is direct evidence at the level of PAL activity that this restoration occurs in AIP-enriched medium. Additionally, increase in free Phe was observed in the shoots in +AIP variants (ca. 2.5 times, data not shown), indicating that AIP has already been partially translocated into the above-ground biomass.

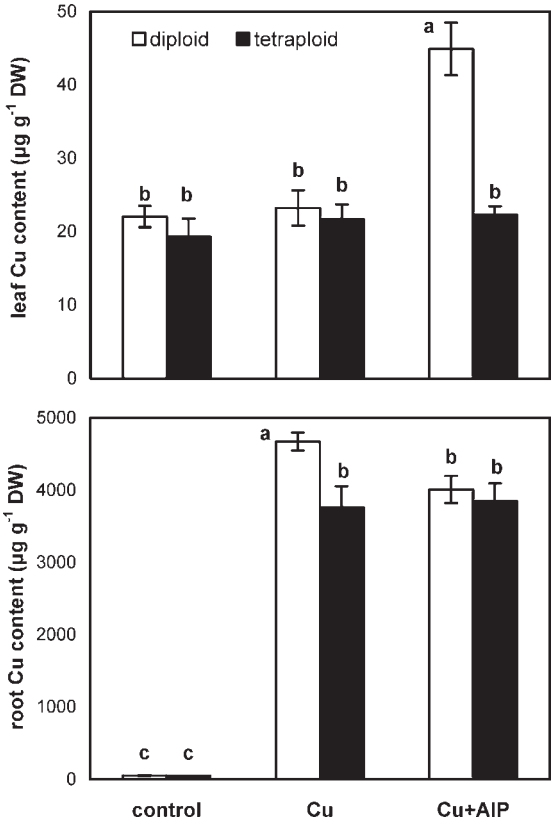

Figure 4. Accumulation of Cu in the leaf rosettes and roots of diploid and tetraploid *Matricaria chamomilla* plants exposed to different treatments for 24 h (n = 4). Other details are as in **Figure 1**.

The huge complexity of phenolic metabolism suggests that different responses of individual branches to AIP application may be expected. It was also pointed out that variable responses of different phenolics to AIP could emerge from different PAL isoforms (*34*). Within the compounds that we measured we did not find restoration in +AIP variants, and this can be related to short-term exposure (24 h), suggesting that despite restoration of PAL activity, accumulation of phenolic metabolites was yet effectively depleted. This is in accordance with a report on *Spirodela intermedia* cultured for 6 weeks in 10 μM AIP, where strong depletion of phenolics was observed (*17*). The fact that the depletion we found was not so fatal may be related to strong pro-oxidative properties of Cu leading to generation of ROS, which in turn affect biosynthesis of phenols (*35*). Study with chilling-treated soybean roots showed relatively low decrease in phenolic acids in chilling + AIP variant, where AIP was used in 100 μM concentration (*18*), but we found considerably higher depletion (**Table 2**). Similarly, 6-month-old plants of *Pinus banksiana* cultured in 10 μM AIP for 3 weeks showed only a small decrease in total soluble phenols in needles (measured by Folin−Ciocalteu's reagent), supporting the view about restoration of phenolic metabolism (*36*). To make these observations complex, chamomile plants cultured for 7 days in AIP-enriched solutions showed restored total soluble phenols in both control + AIP and nickel + AIP treatments (Kováčik and co-workers, unpublished results). At the level of specific compounds, phenolic acids are effective antioxidants, for example, against Cu-induced protein carbonylation (*37*). They also serve as storage compounds for the biosynthesis of more complex phenolics and were restored after transfer from AIP-enriched to AIP-free medium (*33*). Their lower level in the present study may therefore indicate use in the biosynthesis of other phenols at least in tetraploid roots (**Figure 2**). These data indicate that the effect of AIP may not be generalized and plant species, ontogenetic stage, AIP concentration, exposure period, and particular stress impact contribute to resulting responses within individual groups of phenols. In terms of regulation of PAL activity at the ploidy level, we found higher induction in Cu-exposed diploid roots being correlated with higher accumulation of soluble phenols, which is in accordance with our previous findings (*20*). Thus, it seems that although polyploidization increases plant height and organ size also in chamomile (*19*), this reduces the expression of selected genes, including phenolic metabolism-related parameters (*38*). This is also visible at the level of lignin accumulation, which was preferentially enhanced in Cu-exposed diploid roots (**Table 2**). In accordance, activities of cinnamyl alcohol dehydrogenase and polyphenol oxidase were more greatly induced in Cu-exposed diploid ones (Kováčik and co-workers, unpublished results).

Cu excess usually has a negative effect on plant nitrogen metabolism as observed in a sensitive population of *Silene vulgaris*, which exhibited inhibition of nitrate uptake and protein synthesis (*39*). This can be a reason for the increase in the sum of

Table 4. Accumulation of Selected Mineral Nutrients (Milligrams per Gram of Dry Weight) in the Roots of Diploid and Tetraploid *Matricaria chamomilla* Plants Exposed to Different Treatments for 24 h (n = 4)[a]

	diploid			tetraploid		
	control	Cu	Cu+AIP	control	Cu	Cu+AIP
K	85.82 ± 4.46 a	50.57 ± 4.03 bc	48.65 ± 3.12 c	90.28 ± 1.89 a	51.34 ± 2.54 bc	57.63 ± 2.06 b
Na	7.30 ± 0.35 b	11.29 ± 0.84 a	10.97 ± 1.54 a	7.38 ± 0.34 b	7.07 ± 0.36 b	7.16 ± 0.51 b
Ca	8.05 ± 0.36 b	11.17 ± 0.73 a	10.31 ± 0.40 a	7.89 ± 0.43 b	10.38 ± 0.44 a	11.26 ± 0.77 a
Fe	5.24 ± 0.26 b	5.27 ± 0.27 b	5.12 ± 0.35 b	5.03 ± 0.74 b	7.62 ± 0.26 a	6.56 ± 0.48 a
Mg	1.55 ± 0.13 c	1.90 ± 0.11 ab	2.12 ± 0.27 a	1.63 ± 0.08 bc	2.01 ± 0.08 a	2.17 ± 0.06 a

[a] Other details as in **Table 1**.

free amino acids and depletion of soluble proteins (cf. **Tables 1** and **3**). However, further enhancement of the sum of amino acids in +AIP variants was not correlated with further depletion of proteins, suggesting that AIP could affect the biosynthesis of amino acids without impact on proteosynthesis. A low, but significant, increase in free Tyr, which we observed (**Table 3**), was also found in *Betula pubescens*, and it has been suggested that AIP may affect other enzymes besides PAL, such as those in the shikimate−chorismate pathway leading to aromatic amino acids (*34*). Trp and Val also showed quantitative changes in AIP-treated plants (*18*, *34*). Besides Phe, also serine, glycine, and proline mainly contributed to the observed increase in the free amino acid pool (**Table 3**). We note that similar increases were not recorded in the shoots (data not shown) and were also absent in Cd- and Ni-exposed roots with the addition of AIP (Kováčik and co-workers, unpublished results). These facts confirm different impacts of metals on nitrogenous compounds and indicate that our present observations in the amino acid profile are Cu-dependent. Increase in free proline in metal-exposed plants is a well-known fact (*40*, *41*), and its ability to scavenge ROS is also known (*42*). Its increase in +AIP variants could be considered as one of the indirect impacts of AIP and, most probably, is related to the depletion of phenolic metabolites we recorded to maintain regulation of ROS level. This can also be judged from higher depletion of phenols and higher induction of Pro in the Cu+AIP diploid variant. It can only be speculated if changes in serine and glycine are related to synthesis of specific proteins, and further studies are needed. However, serine alone also increased in Cu-exposed *S. vulgaris* plants, and this could be a Cu-specific response (*28*). We note that cysteine increased in Cu+AIP diploid but not in tetraploid shoots (data not shown) as also shoot Cu content did (**Figure 4**). A study using exogenous application of amino acids to maize seedling has shown that just Cys application elevated shoot Cu accumulation (*43*).

ROS are unavoidable byproducts of aerobic metabolism, and specific levels are known to regulate different metabolic pathways including phenolic metabolism (*26*). Thus, more expressive increase in H_2O_2 and superoxide in Cu-exposed diploid roots may contribute to higher PAL activity and accumulation of soluble phenols in comparison with tetraploid ones (**Table 1**; **Figures 1** and **2**). In fact, superoxide generation was found to be essential for the induction of PAL activity (*44*). Increase in root Fe was observed in both Cu-exposed tetraploid variants, and this phenomenon has also been described in the roots of Cu-treated *Vitis vinifera* (*45*). We assume that this observation may be related to lower amounts of ROS in tetraploid cultivar, and recent study has shown that Fe supply to Cu-treated maize enhanced ROS-scavenging enzymes (*46*). Decrease in potassium content is a typical response to metal excess also in chamomile, being less visible in Ni- than in Cd-exposed plants (*20*). Cu caused a more visible decrease in K^+ amount, which was not ploidy-dependent, agreeing with our previous findings (*20*). The significance of the increase in Ca content remains unclear, but recent study has shown that exogenous application of Ca prevented ROS overproduction caused by Cd-induced depletion in endogenous Ca (*47*). A reduction in Ca supply may also affect wood formation and chemical modifications of lignin (*48*). Increase in Ca accumulation in Cu+AIP diploid but not in tetraploid shoots was observed (data not shown), as also shoot Cu content did (**Figure 4**).

Unchanged shoot Cu accumulation in Cu-exposed plants is in accordance with our previous time-dynamics study (*25*) and confirms the low mobility of Cu within plants even at high exogenous concentration (120 μM). The higher amount of Cu in Cu-exposed diploid roots is in accordance with a similar observation in Cd-exposed chamomile (*20*). With respect to higher induction of phenolic metabolites in diploid plants, their role, at least partially, as a "barrier" against root-to-shoot metal translocation may be assumed. This was also previously confirmed by salicylic acid-induced changes to accumulation of phenols in Cd- and Ni-exposed chamomile (*27*). Decrease in root Cu amount in diploid after application of AIP could be related to depletion of cell wall-bound phenolics, which are abundant in chamomile roots (*13*), thus reducing potential binding sites for Cu ions. AIP was found to cause depletion of cell wall-bound phenols in other plant objects (*18*). Another interesting observation is increase in shoot Cu content in Cu+AIP diploid plants. Because soluble phenols are mainly localized in the vacuoles (see ref *7* and the references therein) and these organelles are important for maintenance of metal ions away from the cytoplasm, a more pronounced decrease of phenols just in Cu+AIP diploid roots may therefore be an important factor for elevated shoot Cu accumulation.

In conclusion, our present data have shown that Cu-induced enhancement of different phenols is differentially affected by Cu excess and application of AIP, being more visible in diploid plants. These results suggest regulation of these processes at the level of gene expression because diploid plants showed higher PAL activity in both Cu and Cu+AIP treatment in comparison with tetraploid ones. The AIP-induced increase in shoot Cu accumulation in diploid plants seems to be mediated by the reduction in the accumulation of phenols, but the role of other compounds such as cysteine may also be expected. This is in accordance with our assumption about the "barrier" role of phenols during Cu excess in chamomile roots. Enhanced accumulation of proline in AIP treatments may be a protective mechanism in conditions of reduced accumulation of phenols.

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