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Chromium Uptake and Consequences for Metabolism and Oxidative Stress in Chamomile Plants

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ABSTRACT: Chromium Cr(III) toxicity toward chamomile metabolism and oxidative stress-related parameters after 7 days of exposure was studied. Cr preferentially accumulated in the roots and evoked extensive both dose-dependent and dose-independent increase in fluorescence signals of ROS, NO and thiols. Superoxide increased mainly at the highest Cr dose, whereas H_2O_2 accumulation revealed a discontinuous trend in relation to external Cr supply, and this could be owing to variation in activities of peroxidases. Glutathione and ascorbate quantification, using LC-MS/MS equipment, revealed strong stimulation despite low shoot Cr amounts. Phenolic enzyme activities, except for PAL, were depressed by Cr presence, whereas phenolic metabolites were stimulated, indicating various time dynamics. Among free amino acids, their sum and even proline decreased in the roots, whereas soluble proteins increased. Mineral nutrients showed negligible responses with only Zn and Cu being depleted in both shoots and roots. Cr staining using Cr(III)-specific (naphthalimide-rhodamine) and metal nonspecific (Phen Green) dyes indicated that the former correlated well with AAS quantification of Cr amount. Use of Phen Green is also discussed. These data indicate that Cr-induced oxidative stress is not simply a function of exposure time and applied concentration. Microscopic observations in terms of oxidative stress and chromium uptake are presented here for the first time.

KEYWORDS: *fluorescence microscopy, glutathione, mass spectrometry, nitric oxide, phenolic metabolism*

INTRODUCTION

Chromium (Cr) excess is an important environmental problem because it not only naturally occurs in soils but is also produced by anthropogenic activities.[1] The stable forms of Cr are trivalent Cr(III) and hexavalent Cr(VI) species.[1] Cr(III) is the most stable Cr form in soil,[2] but oxidation/reduction of Cr(III)/Cr(VI) can occur simultaneously in aerobic soils.[3] Notwithstanding toxicity if present in excess, low levels of Cr may have a positive effect on plant metabolism.[4,5]

Toxicity of Cr inside plant tissues occurs at several levels depending on the Cr concentration and oxidation state, exposure time, and ontogenetic stage.[1,6] Besides, individual Cr oxidation states have various stabilities and toxicities; for example, Cr(VI) shows a more toxic impact than Cr(III).[6] Cr(V) was identified as a mobile reduction product of Cr(VI) that was further reduced to Cr(III) in garlic roots.[2]

Excess of Cr, as in the case of many other metals, may stimulate reactive oxygen species (ROS) formation and usually elevates activities of antioxidative enzymes.[4,7] In terms of nonenzymatic antioxidants, glutathione (reduced form, GSH, and oxidized form, GSSG), ascorbic acid (AsA), and phenolic metabolites are the most important. Cr excess was found to elevate GSH and AsA contents,[7] whereas phenols were rather depleted.[8] These observations were found in response to Cr(VI), whereas Cr(III) impact has not been extensively studied. Besides, these metabolites were often assayed using simple spectrophotometric methods, which are prone to overestimation. In addition to ROS, nitric oxide (NO) was also found to modulate metabolism of plants exposed to Cr.[9]

Chamomile is a widely used medicinal plant accumulating considerable amounts of some metals (such as Cd and Ni[10]) in the shoot, which may represent a health risk. Other metals, such as Cu and Al, are only slightly accumulated.[11,12] It was therefore one of the aims to study Cr(III) uptake because previous data revealed the presence of Cr in chamomile flowers cultured under natural field conditions.[13]

Despite numerous studies focused on Cr toxicity in plants, usually Cr uptake and only basic physiological parameters were assayed, limiting more complex views on Cr action. It was also shown that numerous crop plants reduced applied Cr(VI) to Cr(III) already in roots,[14] urging for further study of Cr(III) toxicity. We used both classical (spectrophotometry) and modern (fluorescence microscopy, LC-MS/MS) methods to compare oxidative stress-related parameters and selected metabolites, and data are explanative compared with the available literature. Low and high Cr(III) concentrations were tested, aimed to study concentration-dependent toxicity. Comparison with previously tested metals is also possible[10−12] owing to identical concentrations and culture conditions.

MATERIALS AND METHODS

Cultivation, Experimental Design, and Statistics. Twenty-one-day-old seedlings of *Matricaria chamomilla* L. (tetraploid 'Lutea',

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Asteraceae) germinated in sand were placed into Hoagland solution.[10−12] Uniform plants were cultivated in dark plastic boxes with 5 L of continually aerated solutions (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, ∼270 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes L36W/840 (Lumilux, Osram, Germany) with a 25/20 °C day/night temperature and relative humidity of ∼60%. In these conditions, plants form basal leaf rosettes only. Solutions were renewed weekly to prevent nutrient depletion. Plants that had been cultivated hydroponically during 4 weeks were used in the experiment and further cultured in the mentioned Hoagland solution with the addition of Cr(III) concentrations of 3, 60, and 120 μM (added in the form of $Cr(NO_3)_3{\cdot}9H_2O$) over 7 days. Control did not contain Cr, and pH was checked to be 6.0 in all variants. Data were evaluated using ANOVA followed by a Tukey's test (MINITAB release 11, Minitab Inc.; State College, PA, USA) 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 treatment; thus, the whole experiment included four boxes. Two independent repetitions of the whole experiment were performed to check reproducibility. A subsequent experiment was focused on comparison of shoot Cr(III) uptake from either nitrate or chloride salt [$Cr(NO_3)_3{\cdot}9H_2O$ or $CrCl_3{\cdot}6H_2O$] using 1 L boxes. Another subsequent experiment studied Cr(III) uptake by chamomile seedlings. Seeds (100) were sown directly on filter paper placed on glass balls within Petri dishes containing identical Cr concentrations of $Cr(NO_3)_3{\cdot}9H_2O$ as mentioned above (but prepared using deionized water only). Seeds germinated within 48 h, and they were harvested 3 days later; whole exposure time was 5 days. Final Cr concentration of solutions was measured to verify that Cr was available through filter paper, and shoot Cr amounts were quantified (only germinated stems with cotyledons were carefully harvested).

Spectrophotometry has been carried out with Uvi Light XTD 2 (Secomam, ALES Cedex, France). Fluorescence microscopy was done with an Axioscop 40 (Carl Zeiss, Germany) microscope equipped with an appropriate set of excitation/emission filters.

For assay of metabolites and enzymes, individual plants were powdered using liquid N_2, and fresh material was extracted as described below. In parallel, fresh and dry masses (dried at 75 °C to constant weight) were measured to determine 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, coumarin-related compounds, lignin, reducing sugars, and mineral nutrients including Cr. Preparation of samples from both fresh and dry material (except for minerals) involved homogenization with inert sand using a mortar and pestle to achieve complete tissue disruption.

Measurement of Cr, Mineral Nutrients Content, and Cr Staining. Samples were prepared as described elsewhere: dry material was kept overnight in an HNO_3 and H_2O_2 mixture (10 + 10 mL, Suprapur, Merck) at laboratory temperature and 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 "intraroot" Cr were washed in 10 mM $CaCl_2$ (30 min, 4 °C) before drying.[15] Measurements were done as described previously.[10−13] The mineralization process was verified using microwave oven-assisted decomposition of selected samples and quantification by external addition of known Cr concentrations.

Cr was stained using nonspecific fluorescence metal indicator Phen Green SK, Diacetate ($492_{ex}/517_{em}$, Life Technologies Corp.). Although Phen Green is not metal-specific and is not indicated to visualize Cr ions (see Results and Discussion), we tested it to compare eventual similarities with specific Cr(III) reagent. Working solutions (10 μM) and observations were done according to the manufacturer's instructions and RNS/NO signal was transformed into pseudocolor green. For specific Cr(III) visualization, recently described fluorescence dye naphthalimide-rhodamine was used (kindly provided by Dr. Amitava Das, India); a working solution of 10 μM prepared in PBS (pH 7.4) and excitation/emission was done according to the first report of this reagent.[16] Primary roots were excised ca. 5 cm from leaf rosettes' base (ca. 3 cm below the surface of cultivation solutions) in the zone of lateral root formation. In the shoots, adult leaf's petioles of similar age from three individual plants were stained for Cr accumulation. For other microscopical parameters, roots were preferentially observed because of low shoot Cr uptake.

Physiological and Stress-Related Parameters. Reducing sugars were extracted with deionized water, determined using reaction with arsenomolybdate according to Somogyi−Nelson's method[17] with modifications and expressed as glucose equivalents.[13] ROS (hydrogen peroxide and superoxide content) were measured in homogenates prepared with potassium phosphate buffer containing 1% insoluble PVPP (pH 7.0) using $TiCl_4$ and the hydroxylamine method[18] with modifications, respectively.[11,19] ROS and RNS/NO were stained using CellROX Deep Red Reagent ($644_{ex}/665_{em}$, Life Technologies Corp.) and 2,3-diaminonaphthalene (Sigma-Aldrich) forming highly fluorescent 1*H*-naphthotriazole product ($365_{ex}/415_{em}$) in accordance with the manufacturer's instructions and RNS/NO signal was transformed into pseudocolor green. For NO microscopy, 4,5-diaminofluorescein diacetate ($495_{ex}/515_{em}$, Life Technologies Corp.) was also tested, and samples revealed a trend identical to that of 2,3-diaminonaphthalene (data not shown). Images were processed using NIS elements software (Nikon, Japan).

Reduced (GSH) and oxidized glutathione (GSSG) and ascorbic acid (AsA) were extracted with 0.1 M HCl (0.2 g FW/2 mL) and quantified using LC-MS/MS (Agilent 1200 series Rapid Resolution LC system coupled online to an Agilent 6460 triple-quadrupole detector with Agilent Jet Stream Technologies[20] at m/z values 308/76, 613/231,[21] and 177/95 in positive MRM mode, respectively. Separation was done using a Zorbax SB-C18 column, 50 × 2.1 mm, 1.8 μm particle size, and mobile phase consisting of 0.2% acetic acid and methanol (95:5). The flow rate was 0.6 mL/min, and the column temperature was set at 25 °C. Freshly prepared standards were used for calibration and quantification. Total thiols were stained with 5-(bromomethyl)fluorescein ($492_{ex}/515_{em}$, Life Technologies Corp.) in accordance with the manufacturer's instructions. Despite the fact that it may react with carboxylic acids and thiolated nucleotides/nucleosides, it forms highly stable thioether bonds and provides high intrinsic detectability.[22]

Peroxidase activities were measured in phosphate buffer homogenates prepared as mentioned above. Ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities were measured as the oxidation of ascorbate and guaiacol at 290 and 470 nm, respectively.[11,19]

Assay of Nitrogenous Metabolites. Soluble proteins were quantified according to the Bradford method[23] with bovine serum albumin as standard. 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.[24]

Phenolic Enzymes and Metabolites. The activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was determined as the production of (*E*)-cinnamic acid from phenylalanine using the HPLC method.[25] To determine the activities of shikimate dehydrogenase (SKDH, EC 1.1.1.25), cinnamylalcohol dehydrogenase (CAD, EC 1.1.1.195), and polyphenol oxidase (PPO, EC 1.10.3.2), samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% insoluble PVPP at 4 °C. Measurements and calculations were done as described earlier.[11] Soluble phenols and the sum of flavonols were extracted with 80% methanol and quantified using the Folin−Ciocalteu method with gallic acid as standard (detection at 750 nm)[26] and using the $AlCl_3$ method with quercetin as standard (detection at 420 nm).[27] Coumarin compounds [(*Z*)- and (*E*)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs) and herniarin] were estimated by gradient HPLC and quantified as described previously.[10] Root lignin content was measured by the thioglycolic acid reaction.[25]

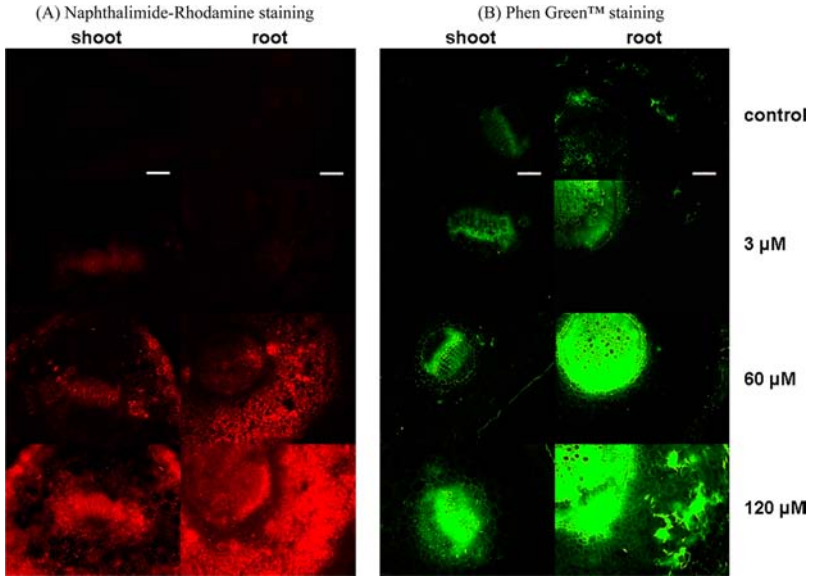

Figure 1. Qualitative visualization of Cr(III) uptake using fluorescent reagents: Cr(III)-specific naphthalimide-rhodamine (A) and metal-nonspecific Phen Green (B). Hand-cut cross sections of petioles (shoot) and primary roots (root) were done in triplicate, and representative photos are shown. Bars represent 200 μm. Note that exposure time for petioles stained with naphthalimide-rhodamine was 4 times higher than that for all other staining (600 vs 150 ms).

RESULTS AND DISCUSSION

Chromium Preferentially Accumulated in Chamomile Roots. It has been shown that Cr uptake is affected by its oxidation state, being more accumulated in shoots if Cr(VI) was applied.[6] This partially adds discredit to older observations where three crops reduced applied Cr(VI) to Cr(III) already in roots[14] but may be explained by mobile Cr(V) as one step of Cr(VI) reduction to Cr(III).[2] In fact, only Cr(III) was detected in Cr(VI)-exposed *Genipa americana* plants.[6] For this reason, we exactly mentioned the oxidation state of applied Cr for comparison with our data. It should be noted that Cr(III) in solution tends to form OH-containing complexes, leading to a decrease in pH and formation of hydroxides and oxides of insoluble Cr; precipitation of Cr also occurs in soil with pH >5.0.[6] However, we did not observe turbidity of treatment solutions or precipitates, and we maintained the pH (6.0) throughout the whole exposure period. Theoretical proof also did not reveal any turbidity in treatment solution with various pH values (4, 6 and 9) and using 120 μM Cr (data not shown). The present results confirmed that Cr(III) has low mobility in chamomile, and it was mainly retained in the roots (Figure 1). Preferential localization of Cr(III) in the roots known from various plants[1] was also microscopically confirmed in chamomile (Figure 1). It should be noted that the Phen Green fluorescent reagent we used is non-metal-specific, and therefore the signal observed in control variant is given by the presence of metallic micronutrients (Cu and Zn, for example). We therefore tested a recently described highly sensitive Cr(III) dye, naphthalimide-rhodamine (NR).[16] Its application showed a sharper concentration-dependent increase in fluorescence (Figure 1A) than that observed after Phen Green staining (Figure 1B). NR staining therefore correlated better with quantified Cr uptake (Figure 2). We also note that exposure time for NR-based shoot Cr visualization was 4 times higher than that in roots, supporting quantitative differences of AAS-measured Cr uptake. This was not observed in the case of Phen Green staining. However, Phen Green also showed

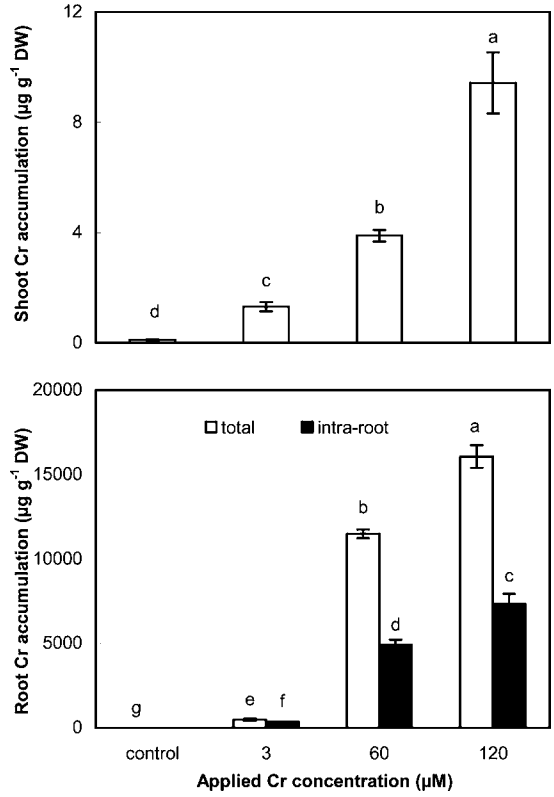

Figure 2. Quantitative uptake of Cr(III) by *Matricaria chamomilla* plants after 7 days of exposure to three concentrations measured by AAS. Data are means ± SDs (n = 4). Values within graphs followed by the same letter are not significantly different according to Tukey's test (P < 0.05).

concentration-dependent increase in fluorescence but mainly in the roots (Figure 1B). In contrast to many fluorescence probes, Phen Green upon binding to metal(s) should lose fluorescence[28] (thus yielding inverse correlation between metal content and fluorescence signal). Comparison of NR staining

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Table 1. Selected Physiological and Stress-Related Parameters in *Matricaria chamomilla* Plants after 7 Days of Exposure to Selected Cr(III) Concentrations[a]

	control	3 μM	60 μM	120 μM
shoots				
tissue water content (%)	90.82 ± 0.38 a	90.21 ± 0.87 a	90.84 ± 0.77 a	90.76 ± 0.66 a
soluble proteins (mg g^{-1} DW)	82.4 ± 4.16 a	82.3 ± 7.83 a	81.7 ± 3.20 a	68.6 ± 3.25 b
reducing sugars (mg g^{-1} DW)	23.0 ± 0.99 b	21.9 ± 2.07 b	23.1 ± 0.93 b	29.6 ± 1.55 a
hydrogen peroxide (μmol g^{-1} DW)	8.53 ± 0.46 bc	10.4 ± 1.18 ab	8.19 ± 0.47 c	11.2 ± 1.06 a
superoxide radical (μg g^{-1} DW)	11.9 ± 2.09 c	18.2 ± 2.05 bc	23.3 ± 3.11 ab	29.1 ± 2.36 a
guaiacol peroxidase (μmol min^{-1} mg^{-1} protein)	0.34 ± 0.03 b	0.42 ± 0.02 b	0.44 ± 0.05 b	0.75 ± 0.06 a
ascorbate peroxidase (nmol min^{-1} mg^{-1} protein)	40.9 ± 6.74 b	78.6 ± 7.29 a	87.2 ± 8.34 a	92.1 ± 10.3 a
roots				
tissue water content (%)	94.56 ± 0.57 a	94.40 ± 0.66 ab	94.22 ± 0.59 ab	93.55 ± 0.42 b
soluble proteins (mg g^{-1} DW)	33.0 ± 2.09 b	37.7 ± 2.50 ab	37.2 ± 2.48 ab	41.1 ± 3.02 a
reducing sugars (mg g^{-1} DW)	20.6 ± 0.65 c	23.0 ± 0.93 c	29.5 ± 1.72 b	58.1 ± 3.27 a
hydrogen peroxide (μmol g^{-1} DW)	4.71 ± 0.45 b	6.97 ± 0.47 a	6.15 ± 0.70 a	3.49 ± 0.25 b
superoxide radical (μg g^{-1} DW)	15.5 ± 3.34 b	16.9 ± 2.06 b	17.8 ± 2.71 b	27.0 ± 3.30 a
guaiacol peroxidase (μmol min^{-1} mg^{-1} protein)	0.96 ± 0.11 b	1.34 ± 0.09 b	2.08 ± 0.39 a	1.32 ± 0.27 b
ascorbate peroxidase (nmol min^{-1} mg^{-1} protein)	110.1 ± 9.28 a	70.8 ± 5.77 bc	56.4 ± 4.57 c	81.7 ± 4.10 b
shikimate dehydrogenase (nmol min^{-1} mg^{-1} protein)	91.0 ± 3.47 a	67.1 ± 5.19 b	61.7 ± 5.06 b	60.3 ± 3.66 b
cinnamylalcohol dehydrogenase (nmol min^{-1} mg^{-1} protein)	102.7 ± 12.8 a	103.3 ± 10.1 a	89.1 ± 7.94 a	55.8 ± 4.53 b
polyphenol oxidase (UA mg^{-1} protein)	1.04 ± 0.11 a	1.10 ± 0.10 a	0.58 ± 0.03 b	0.52 ± 0.07 b
lignin (mg g^{-1} DW)	18.8 ± 1.53 b	17.4 ± 1.92 b	18.3 ± 2.16 b	23.5 ± 1.25 a

[a]Data are means ± SDs (n = 20 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 ($P < 0.05$).

and Phen Green staining allowed us to assume (i) Phen Green fluorescence was not depleted, although Cr was unequivocally present in chamomile tissue; (ii) Phen Green therefore probably reacts with free metal ions because a green signal was mainly visible within and around vascular tissue through which Cr (and other metals) are transported; (iii) the absence of Cr staining by Phen Green in shoot (petioles) parenchyma and in almost all Cr treatments in cortex of roots suggests that Cr was bound by stronger bonds or inside organelles and thus is "invisible" for Phen Green. This was not observed in NR staining, and tissues revealed homogeneous fluorescence, indicating that NR may react with Cr distributed through various cell types. A similar increase in fluorescence was observed in NR-stained living human epidermoid A431 cells.[16] For complexity, we note that we found no paper related to PhenGreen staining on cross-cuts (transversal sections) of plant tissue, and therefore we are unable to discuss its specificity deeply. Other studies also showed parallel changes between uptake and fluorescence signal of Phen Green (unpublished works), and we strongly feel that responses of isolated organelles[28] or cell cultures differ from those of whole tissue preparations, in which it is more difficult to control the level of free metal ions across various types of tissue.

At the tissue level, cuts of chamomile petioles showed that Cr was mainly localized around and within vascular tissue, confirming Cr translocation through xylem.[1,29] At the root level, chromium in all concentrations was localized in all parts of the cortex after NR staining. This fact is closely similar to the transport of Cr(III) ions in *Gynura pseudochina*,[30] wherein symplastic chromium transport with subsequent redistribution into the cytoplasm of cortical cells was supposed. In the light of the role of transfer cells, they play an important role in chromium redistribution in the horizontal direction in both aerial and below-ground plant parts. Cell walls also provide the site of the Cr accumulation under its excess.[31] The content of Cr(III) in control plants probably arises from precultivation of seedlings in the sand. In fact, only a negligible signal was visible in roots of control plants using specific Cr(III) reagent (Figure 1A). The strong difference between shoot and root Cr accumulation in all Cr treatments analyzed here (Figure 2) is in agreement with studies on willow[4] or *Iris*[32] and indicates a similar accumulation pattern among various species.

We also compared Cr(III) applied in the form of nitrate or chloride, and results showed that shoot Cr uptake was slightly lower at 3 and 60 μM $CrCl_3$ but similar at 120 μM Cr(III) (10−20% less, data not shown). It is therefore assumed that the nitrate anion accompanying chromium could facilitate its uptake to some extent. This is in accordance with our previous results on chamomile, where the absence of nitrate in the cultivation solution evoked depletion of Cd and Ni accumulation.[10] Taken together, comparison of Cr uptake with previously tested metals in chamomile revealed that low shoot Cr accumulation is the most similar to low shoot Al uptake,[12] indicating similarities of these two trivalent cations. Among divalent cations, copper also revealed low shoot accumulation but evoked more expressive oxidative stress than Cr.[11]

Oxidative Parameters Were Mainly Stimulated in the Roots. Despite low accumulation of Cr in chamomile shoots, superoxide content increased with increasing external Cr supply and mainly at the highest Cr dose in both shoots and roots (Table 1). On the other hand, hydrogen peroxide accumulation revealed a discontinuous trend in relation to external Cr application, and this could be owing to variation in activities of peroxidases (Table 1). Besides, sensitivity of ascorbate peroxidase to Cr(VI) was reported in *Ocimum*.[33] Because fluorescent ROS reagent (CellROX Deep Red Reagent) is not specific to particular ROS, it rather provides information about "generalized oxidative stress" according to the manufacturer's Web site. This may explain the partial discrepancy between quantities of hydrogen peroxide and superoxide (Table 1) and ROS visualization (Figure 3) at the highest Cr concentration,

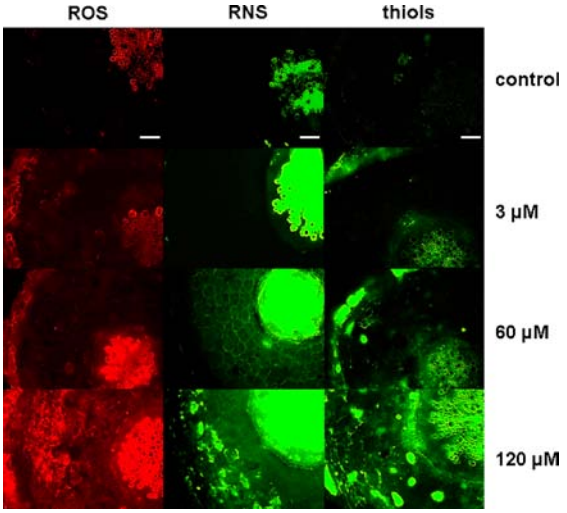

Figure 3. Qualitative visualization of reactive oxygen species (ROS), reactive nitrogenous species (RNS), and total thiols in the roots of *Matricaria chamomilla* after 7 days of exposure to three Cr(III) concentrations using respective fluorescent reagents. Hand-cut cross sections of primary roots were done in triplicate, and representative photos are shown. Bars represent 200 μm.

but quantitative and qualitative data fit well at 3 and 60 μM Cr. Involvement of other ROS in increased fluorescence at 120 μM is therefore assumed, and overall the most expressive changes in this Cr(III) concentration indicate the most-pronounced oxidative stress. At the tissue level, the ROS signal was mainly present in the outermost part of cortex and in 120 μM Cr treatment also in stele in parenchyma adjacent to xylem and especially in cortical cells. This fact may be evoked by redistribution of Cr (Figures 1 and 3). Besides, Cr concentration-independent ROS accumulation has been observed in rice roots exposed to hexavalent Cr.[7] In contrast to ROS, nitric oxide (NO) staining showed concentration-dependent changes. The fluorescence signal of NO was mainly visible in the stele in the lowest Cr concentration and in the cortex in two higher concentrations. It has been suggested that ROS−NO interaction may affect various metabolic processes including enzymatic activities.[24,34] In view of the strong increase, NO seems to be an important regulator of Cr toxicity. Accordingly, NO was found to be involved also in Cr(VI)-induced changes to protein with MAPK-like characteristics in *Zea mays* roots,[9] indicating impact on gene expression.

Glutathione and ascorbic acid are important general antioxidants. In terms of methodology, the highly sensitive LC-MS/MS system we used for chamomile gave GSH and GSSG control values similar to those found in *Capsicum* plants.[21] This is in strong contradiction to the value of ca. 1.2 mg g^{-1} FW in rice roots (~12 mg g^{-1} DW considering tissue water content 90%) and indicates overestimation in the

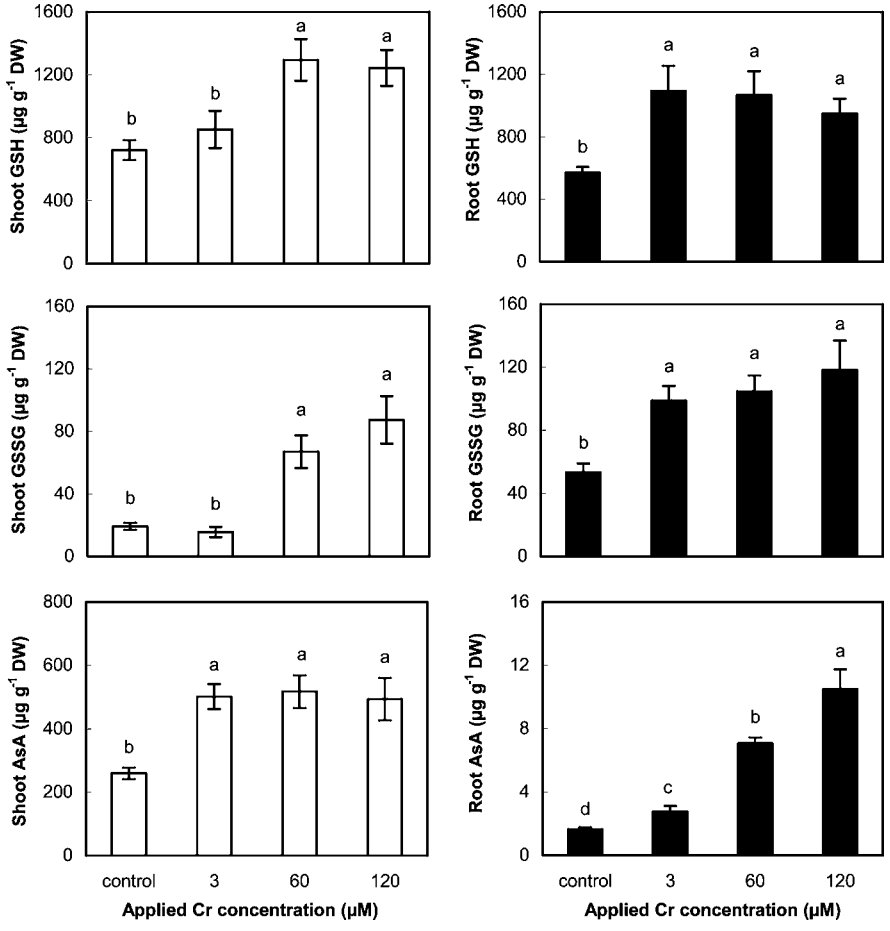

Figure 4. Quantitative changes of reduced and oxidized glutathione (GSH and GSSG, respectively) and ascorbic acid (AsA) in *Matricaria chamomilla* plants after 7 days of exposure to three Cr(III) concentrations (n = 3). Note strong quantitative difference of AsA between shoots and roots. Other details are as in the Figure 2.

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Table 2. Accumulation of Free Amino Acids (Micromoles per Gram Dry Weight) in *Matricaria chamomilla* Plants after 7 Days of Exposure to Selected Cr(III) Concentrations[a]

	control	3 μM	60 μM	120 μM
shoots				
aspartic acid	5.15 ± 1.07 a	5.19 ± 1.12 a	5.13 ± 0.83 a	5.98 ± 1.20 a
glutamic acid	1.35 ± 0.23 a	1.29 ± 0.31 ab	0.80 ± 0.09 bc	0.43 ± 0.09 c
serine	9.41 ± 0.59 a	8.00 ± 1.12 a	8.21 ± 0.87 a	7.46 ± 0.73 a
histidine	0.27 ± 0.03 a	0.27 ± 0.04 a	0.24 ± 0.03 ab	0.18 ± 0.02 b
glycine	1.24 ± 0.12 a	1.15 ± 0.09 a	1.02 ± 0.12 a	0.97 ± 0.16 a
threonine	1.66 ± 0.23 a	1.83 ± 0.19 a	1.96 ± 0.14 a	1.80 ± 0.07 a
arginine	1.53 ± 0.02 a	1.22 ± 0.17 a	1.44 ± 0.34 a	1.43 ± 0.20 a
alanine	10.7 ± 1.45 a	10.6 ± 0.63 a	10.6 ± 0.54 a	10.0 ± 0.87 a
tyrosine	0.98 ± 0.15 a	0.94 ± 0.03 a	0.94 ± 0.05 a	0.97 ± 0.12 a
cysteine	0.86 ± 0.03 b	1.05 ± 0.21 ab	1.17 ± 0.07 a	1.14 ± 0.01 ab
valine	1.48 ± 0.18 a	1.21 ± 0.18 ab	1.21 ± 0.17 ab	0.74 ± 0.21 b
methionine	0.16 ± 0.04 b	0.21 ± 0.01 ab	0.23 ± 0.04 ab	0.28 ± 0.04 a
phenylalanine	1.12 ± 0.15 a	1.08 ± 0.06 a	1.04 ± 0.08 a	0.98 ± 0.13 a
isoleucine	0.66 ± 0.06 a	0.67 ± 0.06 a	0.73 ± 0.04 a	0.68 ± 0.04 a
leucine	1.03 ± 0.08 a	1.05 ± 0.07 a	1.02 ± 0.09 a	0.95 ± 0.16 a
lysine	1.18 ± 0.20 b	4.48 ± 0.45 a	5.08 ± 0.78 a	4.70 ± 0.39 a
proline	12.3 ± 2.30 a	13.9 ± 1.11 a	14.1 ± 3.11 a	13.7 ± 3.71 a
sum	51.1 ± 3.71 a	54.1 ± 3.20 a	55.0 ± 2.77 a	52.5 ± 2.36 a
roots				
aspartic acid	2.21 ± 0.17 a	2.44 ± 0.40 a	2.41 ± 0.17 a	1.30 ± 0.26 b
glutamic acid	0.80 ± 0.08 c	1.85 ± 0.10 b	4.84 ± 0.32 a	1.00 ± 0.14 c
serine	5.25 ± 0.79 a	4.03 ± 0.40 a	2.37 ± 0.16 b	2.07 ± 0.25 b
histidine	0.42 ± 0.06 a	0.40 ± 0.06 a	0.44 ± 0.01 a	0.26 ± 0.04 b
glycine	2.45 ± 0.18 a	1.66 ± 0.17 b	0.83 ± 0.11 c	1.17 ± 0.11 c
threonine	1.68 ± 0.09 a	1.15 ± 0.03 b	0.99 ± 0.09 b	0.68 ± 0.01 c
arginine	0.46 ± 0.03 a	0.32 ± 0.03 b	0.19 ± 0.01 c	0.29 ± 0.02 b
alanine	9.35 ± 0.80 a	9.51 ± 0.73 a	7.14 ± 1.37 ab	5.37 ± 0.76 b
tyrosine	0.39 ± 0.02 a	0.48 ± 0.08 a	0.42 ± 0.05 a	0.42 ± 0.03 a
cysteine	1.68 ± 0.19 a	0.99 ± 0.06 b	0.49 ± 0.11 c	0.62 ± 0.02 c
valine	1.75 ± 0.09 a	1.45 ± 0.11 ab	1.31 ± 0.08 b	1.21 ± 0.18 b
methionine	0.60 ± 0.09 a	0.54 ± 0.02 ab	0.42 ± 0.02 bc	0.36 ± 0.04 c
phenylalanine	0.95 ± 0.13 a	0.70 ± 0.15 ab	0.66 ± 0.02 ab	0.56 ± 0.10 b
isoleucine	1.06 ± 0.17 b	1.44 ± 0.11 a	1.13 ± 0.02 b	1.00 ± 0.07 b
leucine	1.52 ± 0.12 ab	1.77 ± 0.11 a	1.38 ± 0.04 b	1.26 ± 0.16 b
lysine	0.64 ± 0.13 a	0.37 ± 0.02 b	0.31 ± 0.01 b	0.58 ± 0.09 ab
proline	9.74 ± 0.72 a	5.92 ± 0.61 b	5.42 ± 0.33 b	3.77 ± 0.43 c
sum	41.0 ± 2.28 a	35.0 ± 2.83 b	30.8 ± 2.28 b	21.9 ± 0.67 c

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

spectrophotometric assay; the same but with higher inaccuracy is true for ascorbic acid in the given rice roots[7] as we found only traces of AsA in roots and much higher content in shoots (Figure 4), not only in chamomile. We also note that 0.1 M HCl was found to be more suitable for extraction; deionized water gave very low signals of all compounds, and GSSG was almost undetectable. Notwithstanding these quantitative discrepancies, Cr(VI) applied to rice roots[7] evokes increases in both GSH and AsA as we observed in all Cr(III) treatments in chamomile (Figure 4). The elevated level of GSSG indicates oxidative stress but with low intensity considering GSSG quantity and considerable pool of GSH (Figure 4). The increase of root total thiols roughly correlates with the increase in GSH but at the highest Cr dose (Figure 3); accumulation of other thiols such as phytochelatins is assumed because Cr also stimulates synthesis of metallothioneins.[1] At the tissue level, thiols were mainly localized in the outermost part of the cortex that functionally replaces rhizodermis due to its decline during ontogenesis. This localization was well evident in all variants. In two higher Cr concentrations, thiols were localized also in the other cells of the cortex. The undamaged thiol-containing cells revealed significant green emission (Figure 3). On the basis of these observations, we can confirm that Cr is redistributed into the upper parts of roots (cortex) followed by generation of ROS and RNS and production of thiol-containing compounds.

The above-mentioned oxidative stress was also visible at the level of decreasing tissue water content in roots, although with relatively low intensity compared to other metals after 7 days of exposure.[19] The increase in reducing sugars (glucose equivalents) at high Cr doses (Table 1) indicates a shift of equilibrium between photosynthesis and respiration as previously observed in Cd but not in Ni excess.[10] Also, Al^{3+} revealed a similar impact in shoots and roots of chamomile.[12]

Nitrogenous Metabolites Were Differently Affected by Cr. Metal excess has usually a negative impact on protein synthesis and may therefore elevate individual amino acids.[34] A decrease in soluble proteins has also been observed under Cr excess, both Cr(III) and Cr(VI).[4,8] We observed a similar

negative impact of the highest Cr(III) dose in shoots, but roots revealed even a stimulatory effect (Table 1). The increase in soluble proteins in 120 μM Cr-treated roots could be a protective response through synthesis of specific proteins, and we previously observed such stimulation in Cd- and Ni-exposed chamomile subjected to N deficiency.[10] Proteomic analyses are required to identify such proteins. In accordance with elevated root proteins, depletion of amino acids was the most expressive in the highest Cr dose; however, individual amino acids such as glutamic acid revealed either decrease or increase in shoots or roots (Table 2). This suggests various regulation and probably rather inhibition of amino acid biosynthesis. In accordance, it was observed that higher Cr concentrations inhibit nitrate reductase activity[33] and glutamic acid-related enzymes (GS and GOGAT).[35]

Among individual amino acids, they were almost unaffected in shoots, and mainly glutamic acid, valine, and lysine revealed significant alterations: this also reflects low root-to-shoot Cr translocation. At the root level, depletion of almost all amino acids in all Cr treatments was visible (Table 2) and could be explained by impact on GS/GOGAT enzymes mentioned above.[35] Among others, decrease in phenylalanine/Phe fits well with depleted SKDH activity, an enzyme involved in Phe biosynthesis. However, the most interesting result was the decrease in proline, a known stress-induced amino acid.[36] This is in contradiction to Cr(VI)-exposed *Ocimum*, where strong elevation of Pro was found.[33] This indicates differences of Cr(VI) and Cr(III) ions and various impacts on general metabolism.[1] Besides, it should be noted that Pro values in the mentioned *Ocimum* were overestimated at first sight,[33] and thus the LC-DAD method we used is more precise. The content of alanine, a storage form of pyruvate, showed a tendency to decrease with significant depletion in 120 μM-exposed roots (Table 2), an observation that is not common under an excess of other metals.[36,37] This is another indication that Cr has specific impacts on nitrogenous metabolism, amino acid biosynthesis, and respiration, and further studies are needed.

Phenolic Metabolites Were Stimulated by Cr Uptake. Induction of phenolic metabolites in response to metal excess has been observed in various plants.[25,38,39] This was also found in the present experiment with Cr(III), and higher Cr doses evoked increases in total soluble phenols and the sum of flavonols (Figure 5). Including PAL, being the first enzyme in the general phenylpropanoid pathway, phenolic parameters were mainly stimulated in the roots as expected owing to preferential Cr accumulation in root tissue. In contrast to our data, Cr(VI) depleted soluble phenols in rice shoots and roots.[8] This partial discrepancy may be explained by various redox properties of Cr(VI) and Cr(III) as Cr(VI) may be reduced by caffeic acid at acidic pH.[40] Caffeic acid is an abundant compound in chamomile roots.[10]

Additional phenolic metabolism-related enzymes measured in root tissue revealed a common decrease in relation to elevated Cr (Table 1). Depletion of SKDH fits well with the decrease in free Phe as mentioned above. Depletion of CAD and PPO activities, enzymes that are involved in lignification and tissue browning, agrees with no intense change of root color and the relatively small increase in lignin amount (Table 1). Enhanced lignification is not important protection under Cr excess, although it plays a role under excess of other metals.[25]

Coumarins are quantitatively important shoot-localized chamomile metabolites.[10] 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 the total soluble phenols assay, probably owing to the absence of a free hydroxyl group attached to the aromatic ring. The sum of GMCAs was significantly elevated only by the highest Cr dose (8.25 ± 0.31 and 10.6 ± 0.57 mg g^{-1} DW in control and 120 μM Cr, respectively). This is in accordance with the overall trend of stimulation of phenols. Other metals such as Cd and Ni also stimulated an increase in GMCAs with similar intensity, although these metals were more shoot-accumulated.[10] On the other hand, Al^{3+} revealed a roughly similar increase in GMCAs, and low shoot Al uptake suggests similarities of these two metals.[12] The amount of herniarin, a product of GMCA hydrolysis, was not affected by Cr (mean value of 2.13 ± 0.27 mg g^{-1} DW from all treatments), indicating no impact on β-glucosidase mediating this reaction in damaged tissue.

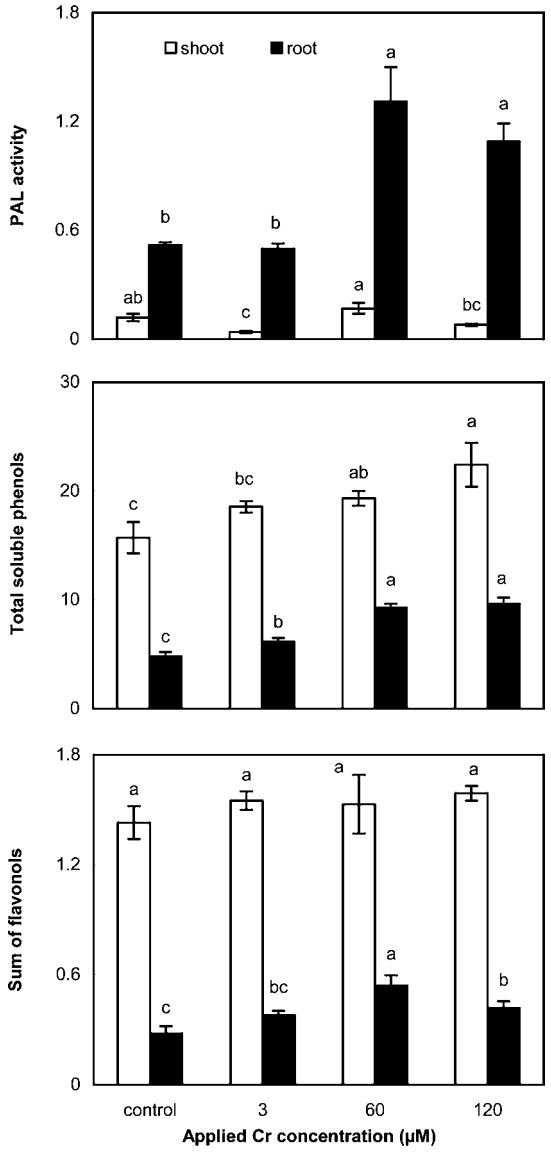

Figure 5. Activity of phenylalanine ammonia-lyase (PAL, nmol min^{-1} mg^{-1} protein), accumulation of total soluble phenols (mg g^{-1} DW), and sum of flavonols (mg g^{-1} DW) in *Matricaria chamomilla* plants after 7 days of exposure to three Cr(III) concentrations. Data are means ± SDs (n = 3). Values for shoot or root, followed by the same letter(s), are not significantly different according to Tukey's test (P < 0.05).

Table 3. Quantitative Changes of Selected Mineral Nutrients in *Matricaria chamomilla* Plants after 7 Days of Exposure to Selected Cr(III) Concentrations[a]

	control	3 μM	60 μM	120 μM
shoots				
K (mg g^{-1} DW)	99.0 ± 6.61 a	97.1 ± 5.72 a	91.8 ± 3.58 a	90.3 ± 4.63 a
Na (mg g^{-1} DW)	7.54 ± 0.59 a	7.76 ± 0.63 a	7.01 ± 0.46 a	7.84 ± 0.37 a
Ca (mg g^{-1} DW)	10.8 ± 1.16 a	11.2 ± 1.35 a	11.3 ± 1.44 a	10.9 ± 1.23 a
Mg (mg g^{-1} DW)	5.62 ± 0.31 a	5.74 ± 0.42 a	5.59 ± 0.43 a	5.85 ± 0.57 a
Fe (mg g^{-1} DW)	0.20 ± 0.01 a	0.25 ± 0.05 a	0.25 ± 0.03 a	0.24 ± 0.02 a
Zn (μg g^{-1} DW)	53.8 ± 3.15 a	46.6 ± 5.12 ab	43.8 ± 2.55 b	42.0 ± 2.80 b
Cu (μg g^{-1} DW)	19.1 ± 1.13 a	16.6 ± 1.46 ab	14.4 ± 0.70 bc	12.7 ± 0.75 c
roots				
K (mg g^{-1} DW)	94.9 ± 4.24 a	97.0 ± 5.39 a	88.6 ± 2.95 a	60.8 ± 5.83 b
Na (mg g^{-1} DW)	8.82 ± 0.46 a	9.63 ± 0.51 a	9.18 ± 0.67 a	8.85 ± 0.41 a
Ca (mg g^{-1} DW)	10.4 ± 0.87 a	10.7 ± 0.69 a	10.3 ± 0.71 a	10.4 ± 1.23 a
Mg (mg g^{-1} DW)	4.45 ± 0.39 a	4.67 ± 0.27 a	4.58 ± 0.33 a	4.43 ± 0.26 a
Fe (mg g^{-1} DW)	8.54 ± 0.64 a	8.29 ± 0.88 a	8.38 ± 0.72 a	6.96 ± 0.70 a
Zn (μg g^{-1} DW)	87.4 ± 4.80 a	85.5 ± 7.92 a	93.2 ± 6.90 a	69.3 ± 5.56 b
Cu (μg g^{-1} DW)	31.0 ± 3.40 a	26.8 ± 2.90 a	25.2 ± 4.39 a	15.6 ± 1.58 b

[a]Data are means ± SDs (n = 4). Other details are as in the Table 1.

Mineral Nutrients Showed Negligible Responses. Potassium was the only macroelement that decreased in roots (Table 3), and either limited uptake or its leakage is possible. Such leakage or decrease was observed in Cr(VI)-exposed *Ocimum* but with substantially higher intensity[33] and in Cr(III) and Cr(VI)-exposed *Genipa americana*.[6] Among micronutrients, both Cu and Zn decreased in shoots of plants exposed to 60 and 120 μM, whereas in the roots only after 120 μM treatment (Table 3). Similar responses were not observed in *Iris*[32] exposed to higher concentrations of $CrCl_3$, and interspecific differences are expected. However, even a decrease or no alteration of micronutrients confirms that staining of Cr using Phen Green (according to the manufacturer, it also reacts with Cu^{2+}, Cu^{+}, Fe^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, and Ni^{2+}) was not affected by changes of micronutrients; the visible signal observed in control petioles and roots may therefore be ascribed just to Cu and Zn presence (Figure 1B).

Impact of Cr on Chamomile Seedlings. To verify Cr uptake in early ontogenesis, chamomile seeds were sown directly on filter paper on Petri dishes supplemented with Cr(III) doses (Table 4) identical to those used for hydroponics (Figure 2). Analyses of final Cr concentrations in solution confirmed that a considerable part of Cr was available through filter paper to seedlings. Germination was not affected by any Cr doses. The amount of Cr in shoots of seedlings (exactly cotyledons with hypocotyls were harvested) confirmed low Cr amounts being only 2−3 times higher than those of hydroponically cultured plants (Figure 2). It is therefore visible that roots are the main site of Cr localization within chamomile tissues independent of developmental stage used for Cr(III) exposure. This proof (Cr applied to seedlings in water solution vs Cr applied to older plants through hydroponical solution) also indicates that low shoot Cr(III) accumulation is not evoked by the eventual formation of insoluble Cr(III) complexes in solution.[6]

Table 4. Effect of Cr(III) on Germination and Cr Amount in *Matricaria chamomilla* Seedlings Cultured on Petri Dishes over 5 Days[a]

initial Cr concn (μM)	final Cr concn (μM)	germination rate (%)	Cr content in shoots (μg g^{-1} DW)
0 (control)	0	97.0 a	nd
3	0.54 c	96.3 a	1.81 c
60	9.12 b	95.7 a	6.69 b
120	47.5 a	94.3 a	27.4 a

[a]Data are means (n = 3). Values within column followed by the same letter(s) are not significantly different according to Tukey's test (P < 0.05). nd, not detected.

Conclusions. To sum up, it was found that Cr(III) was mainly accumulated in chamomile roots as observed in other species. Notwithstanding this, Cr affected also shoot metabolism, although it was not accumulated in larger amounts. Phenolic metabolites showed stimulation, but selected enzymes in roots were rather depressed. ROS were not affected in a concentration-dependent manner, but RNS were, indicating the significance of NO in the amelioration of Cr toxicity. GSH and AsA seem to contribute to this amelioration, and the LC-MS/MS detection we used gives the most accurate data currently available. Depletion of proline is the most interesting finding, which had not yet been observed. Microscopy confirmed that Phen Green reagent is also suitable to visualize chromium uptake probably at the level of free ions, but specific Cr(III) dye naphthalimide-rhodamine correlated well with quantified Cr accumulation. Overall, low shoot Cr accumulation in chamomile suggests its safety in terms of trivalent chromium accumulation.

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Notes

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