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Hexavalent chromium damages chamomile plants by alteration of antioxidants and its uptake is prevented by calcium

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HIGHLIGHTS

- Cr mainly accumulated in the roots and reduction of Cr(VI) to Cr(III) was detected.
- 120 μM Cr(VI) depleted growth and antioxidants but increased ROS and NO signal.
- Amount of phenols and lignin increased strongly while thiols decreased under high Cr.
- In vitro assay confirmed oxidation of ascorbic acid in equimolar mixture with Cr(VI).
- Seedlings contained more Cr than older plants and Ca suppressed oxidative symptoms.

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ABSTRACT

Toxicity of low (3 μM) and high (60 and 120 μM) concentrations of hexavalent chromium/Cr(VI) in chamomile plants was studied. Fluorescence staining confirmed reduction of Cr(VI) to Cr(III). Cr was mainly accumulated in the roots with translocation factor <0.007. Notwithstanding this, both shoots and roots revealed increase in oxidative stress and depletion of glutathione, total thiols, ascorbic acid and activities of glutathione reductase and partially ascorbate peroxidase mainly at 120 μM Cr. Though some protective mechanisms were detected (elevation of nitric oxide, enhancement of GPX activity and increase in phenols and lignin), this was not sufficient to counteract the oxidative damage. Consequently, soluble proteins, tissue water content and biomass production were considerably depleted. Surprising increase in some mineral nutrients in roots (Ca, Fe, Zn and Cu) was also detected. Subsequent experiment confirmed that exogenous calcium suppressed oxidative symptoms and Cr uptake but growth of chamomile seedlings was not improved. Alteration of naturally present reductants could be a reason for Cr(III) signal detected using specific fluorescence reagent: in vitro assay confirmed disappearance of ascorbic acid in equimolar mixture with dichromate (>96% at pH 4 and 7) while such response of glutathione was substantially less visible.

1. Introduction

Chromium (Cr) excess is actual environmental problem because it is produced by numerous anthropogenic activities that include mainly production of hexavalent Cr [1]. The most stable oxidation states are Cr(III) and Cr(VI) with latter being actively taken up by plants [2,3]. Depending on the conditions, reduction of Cr(VI) to Cr(III) is often favored [1,4]. Toxicity of Cr in plant tissue depends on numerous factors including concentration, exposure time and plant ontogenetic stage. Cr(VI) seems to be more toxic probably owing to higher uptake if compared with Cr(III) [5].

Heavy metals typically stimulate reactive oxygen species (ROS) formation and usually elevate activities of antioxidative enzymes [6,7]. In terms of non-enzymatic antioxidants, glutathione (reduced form GSH and oxidized form GSSG), ascorbic acid (AsA) and phenolic metabolites are the most important. Cr(VI) excess was found to elevate GSH and AsA content [7] while phenols were rather depleted [8]. In addition to ROS, nitric oxide (NO) was also found to modulate metabolism of plants exposed to metals [9] including hexavalent [10] and trivalent Cr [11].

Chamomile is a widely-used medicinal plant accumulating considerable amount of some metals such as Cd and Ni in the shoot which may represent health risk [12]. Other metals including Cr(III)

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are only slightly accumulated [11]. It was therefore one of the aims of the present study to verify Cr(VI) uptake because previous data revealed presence of Cr in chamomile flowers cultured under natural field conditions [13].

Despite numerous studies focused on Cr toxicity in plants, relatively little attention was paid to Cr(VI) toxicity in terms of eventual reduction within plant tissue [5,14]. Reduction of Cr(VI) to Cr(III) could be assumed (unlike animals) owing to the presence of various reductants such as phenols, ascorbic acid or glutathione. For this purpose, Cr(VI) was applied in a range of concentrations (low vs. high) over sufficiently long period (7 days). We have also used both classical (spectrophotometry) and modern (fluorescence and confocal microscopy, LC–MS/MS) methods to compare oxidative stress-related parameters and mainly to achieve precise detection of antioxidants. Recently developed specific Cr(III) fluorescence reagent [15] was tested to verify its formation in Cr(VI)-exposed plants. Because calcium is known to ameliorate oxidative damage evoked by excess of metals in various plants including chamomile [16], we tested its impact on Cr(VI) excess using chamomile seedlings. In vitro assay of interaction of some antioxidants with Cr(VI) is presented here for the first time.

2. Materials and methods

2.1. Cultivation, experimental design and statistics

Twenty-one days old seedlings of *Matricaria chamomilla* L. (tetraploid 'Lutea', Asteraceae) germinated in sand were placed to Hoagland solution [9,11,12]. 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 am to 6.00 pm), the photon flux density was ~270 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes L36 W/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 for 7 days in Cr(VI)-enriched solutions (3, 60 and 120 μM of Cr, added in the form of $K_2Cr_2O_7$, Lachema Brno, Czech Republic). Control did not contain any additional chemicals and pH was checked to be 6.0 in all variants. 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)]. These dried samples were analyzed for soluble phenols, lignin and mineral nutrients including Cr. Subsequent experiment was focused on the impact of calcium (supplemented as $CaCl_2 \cdot 2H_2O$) on Cr(VI) toxicity in seedlings germinated directly in treatment solutions (120 μM for each chemical). Seeds (100) were sown on filter paper in Petri dishes and harvested 7 days later as described in detail previously [17]. Preparation of samples from both fresh and dry material (except for minerals) involved homogenization with inert sand using mortar and pestle to achieve complete tissue disruption.

Data were evaluated using one-way ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania) at $P < 0.05$. 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. For seedlings, biomass within one Petri dish was pooled to achieve enough material for quantification of minerals, glutathione and ascorbic acid and to identify germination (three individual dishes were used for each treatment and parameter then $n = 3$). Two independent repetitions of the whole experiment were performed in order to check reproducibility. Spectrophotometry was carried out with Uvi Light XTD 2 (Secomam, ALES Cedex, France) and fluorescence microscopy using Axioscop 40 microscope (Carl Zeiss, Germany) equipped with appropriate set of excitation/emission filters.

2.2. Measurement of Cr and mineral nutrients and fluorescence staining of Cr

Samples were prepared by mineralization of dry material in the mixture of concentrated HNO_3 and water (5 + 5 ml) using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) at 200 °C over 1 h. Resulting clear solution was quantitatively placed to glass flasks and diluted to a final volume of 20 ml. All measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd.; Mulgrave, Australia) and the air-acetylene flame. Samples for quantification of "intra-root" Cr were washed with 5 mM Na_2-EDTA before drying. Measurements of mineral nutrients were done as described previously [11,18].

For fluorescence visualization of Cr, primary roots were excised ca. 5 cm from leaf rosettes' base (ca. 3 cm below surface of cultivation solutions) in the zone of lateral roots formation. In the shoots, adult leaf's petioles of similar age from three individual plants were stained for Cr accumulation. Cr(VI) was visualized by 1,5-diphenylcarbazide. Freshly prepared hand-made sections were washed three times with hydrochloric acid/potassium chloride buffer (0.1 M, pH 1.8) and immediately incubated in a working solution: stock 0.25% solution of 1,5-diphenylcarbazide was prepared by dissolving of 0.5 g of substance in 100 ml of acetone and diluting to the final volume of 200 ml by water of ACS quality and then diluted to 0.05% with hydrochloric acid/potassium chloride buffer (0.1 M, pH 1.8). Sections were incubated in the working solution for 30 min at room temperature. After incubation, they were washed three times with hydrochloric acid/potassium chloride buffer and observed. For specific Cr(III) visualization, recently described fluorescence dye 6-((anthracen-9-yl) methyleneamino)-2H-chromen-2-one was synthesized and used [15]. Stock solution was prepared in DMSO and diluted to 10 μM with Phosphate Buffered Saline (PBS, pH 6.8). After 30 min of incubation, samples were washed in PBS and observed.

2.3. Assay of physiological and biochemical parameters

Proteins were quantified according to Bradford method with BSA as standard at 595 nm [19]. Soluble phenols were extracted with 80% methanol (20 mg/2 ml) and quantified using Folin-Ciocalteu method with gallic acid as standard [20]. Root lignin content was measured by the thioglycolic acid reaction as described in detail earlier [21].

To detect enzymatic activities, fresh material (1 g/5 ml) was homogenized in potassium phosphate buffer containing 1% insoluble PVPP (pH 7.0). Ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities were measured as the oxidation of ascorbic acid and guaiacol at 290 and 470 nm, respectively [18,22]. Glutathione reductase (GR) activity was assayed as the reduction of GSSG at 412 nm [22].

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 on-line to a detector Agilent 6460 Triple quadrupole with Agilent Jet Stream Technologies at m/z values 308/76, 613/231 [23] and 177/95 in positive MRM mode, respectively. Separation was done using column Zorbax SB-C18 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 column temperature was set at 25 °C. Freshly prepared