Ultrastructure and subcellular distribution of Cr in *Iris pseudacorus* L. using TEM and X-ray microanalysis.

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Abstract

Chromium pollution of fresh water is hazardous for humans and other organisms, and places a limitation on the use of polluted water sources. Phytoremediation, the use of plants to remove pollutants from the environment, is a cost-effective, environmentally friendly approach for water decontamination. To improve the efficiency of the process, it is essential to increase the current knowledge about Cr accumulation in macrophytes. Plants of *Iris pseudacorus* L. were treated with Cr(III) at 0.75 mM for five weeks to investigate Cr localization by means of transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX). Chromium induced severe ultrastructural alterations in the rhizodermis (cell wall disorganization, thickening, plasmolysis, electron-dense inclusions) and rhizome parenchyma (reduced cell size, cell wall detachment, vacuolation, opaque granules).

The highest Cr contents were found in the cell walls of the cortex in the roots, and in the cytoplasm and intercellular spaces of the rhizome. The Cr concentration in root tissues was in the order cortex>rhizodermis>stele, whereas in the rhizome, Cr was evenly distributed. It is proposed that root and rhizome have distinct functions in the response of *I. pseudacorus* to Cr. The rhizodermis limits Cr uptake by means of Si deposition and cell wall thickening. The rhizome cortex generates vacuoles and granules where Cr co-occurs with S, indicating Cr sequestration by metal-binding proteins.

Abbreviations

EDS	Energy Dispersive Spectrometer
EDX	Energy Dispersive X-ray analysis
LM	Light Microscopy
PC	Phytochelatins
TEM	Transmission Electron Microscopy
USEPA	United States Environmental Protection Agency

Introduction

Fresh water pollution with heavy metals is one of the major global environmental concerns. Toxic metals are hazardous for living organisms, strongly persistent in the environment and living tissues, and easily transferred to the food chain. Chromium pollution of water mainly originates from industrial processes such as the production of stainless and refractory steel, drilling muds, electroplating cleaning agents, catalytic manufacturing, leather, pigments, porcelain and pottery, and chemicals (Shanker et al, 2005).

Chromium is non-essential to plants and toxic for most agronomic species above 0.5-5.0 μg ml^{-1} (Davies et al, 2002). The toxic effects of Cr include decreases in seed germination, biomass production, root and shoot elongation, enzymatic activity, protein content and photosynthesis (Vajpayee et al, 1999 and 2001; Peralta et al, 2001; Appenroth et al, 2001), together with unbalanced mineral nutrition and altered pigment synthesis (Barceló et al, 1985; Vajpayee et al, 1999 and 2001). Chromium toxicity depends on its oxidation state. Chromium is naturally found in every oxidation state between –2 and +6, but the trivalent and the hexavalent are predominant (Barnhart, 1997). Hexavalent Cr is very soluble and toxic to living organisms at very low doses,

64 especially for aquatic species (Muramoto et al, 1991). In comparison, the less harmful
65 trivalent form is highly insoluble, and even promotes the growth of some plant species
66 (Samantaray et al, 1998). Cr(III) tends to adsorb to particulate matter and sediments,
67 and can form organic and inorganic complexes difficult to take up by plants
68 (Rowbotham et al, 2000). Most reported studies have been focused on the effects of
69 hexavalent Cr, because of its higher toxicity and bioavailability. However, both forms
70 can interconvert in the environment under specific conditions of pH and oxygen
71 concentration, and in the presence of appropriate ligands or catalysts (Kotaś and
72 Stasicka, 2000). Cr(III) predominates under anoxic or suboxic conditions, and in the
73 wastewater of tannery, textile and decorative plating industries. Moreover, Cr(VI) is
74 reduced to Cr(III) in plant tissues (Bluskov et al, 2005), and the mutagenicity of Cr(VI)
75 can be partially explained by the binding of Cr(III) to DNA (Zhitkovich, 2005). For all
76 these reasons, Cr (III) instead of Cr(VI) was selected to conduct the present research.
77 Current efforts to develop methods to clean up waters polluted with Cr have been
78 increasingly focussed on phytoremediation, which is the use of plants to remove
79 pollutants from the environment (Pilon-Smits, 2005). Macrophytes can accumulate high
80 amounts of Cr in their tissues, thus substantially contributing to successful removal of
81 Cr from water (Marchand et al, 2010). But this contribution can be insufficient or
82 seasonally dependent (Zhang et al, 2007; Paiva et al, 2009). Another limitation of the
83 phytoremediation technologies is the restricted tolerance of plants to high Cr levels
84 (Pilon-Smits, 2005). The typical concentration of Cr is of 0.5-100 nM in rivers and
85 lakes and of 0.1-16 nM in sea waters (Kotaś and Stasicka, 2000). But Cr concentrations
86 in polluted waters (Kumar and Riyazuddin, 2011), sediments (Roig et al, 2011) or
87 effluents (Vinodhini and Das, 2010; Yılmaz et al, 2010; Rehman, 2011) can be one to
88 four orders of magnitude higher. Under this scenario, it is critical to increase our

89 understanding of the mechanisms of Cr accumulation in aquatic plants at high Cr levels,
90 so that the efficiency of Cr removal can be improved.

91 Surprisingly, few studies deal with the localization of Cr in the cell compartments or
92 plant tissues. Only a small number of studies are devoted to aquatic plants. Liu and
93 colleagues (2009) examined the subcellular distribution of Cr in the marsh plant,
94 *Leersia hexandra* Swartz, and found that most of the metal was bound to the cell walls
95 of roots and the vacuoles of leaves. Other authors investigated Cr localization in crops
96 such as radish, maize, onion, tomato, *Brassica oleracea* L. and *Brassica juncea* L.
97 (Sanità di Toppi et al, 2002; Liu and Kottke, 2003; Bluskov et al, 2005; Mangabeira et
98 al, 2006; Lahouti et al, 2008). Most of these studies focused on the root, which plays a
99 key role in Cr detoxification and accumulates the highest amount of Cr in
100 non-hyperaccumulators (Salt et al, 1995). To the best of our knowledge, none of these
101 studies investigated Cr localization in the rhizomes. The existing literature about the
102 contribution of the rhizome to Cr accumulation is contradictory. Duman et al. (2007)
103 and Yang et al. (2008) analysed the Cr content in roots, rhizomes, stems and leaves of
104 *Phragmites australis* L. and *Schoenoplectus lacustris* and reported that rhizomes had an
105 accumulation capacity similar to stems, and much lower than roots. By contrast,
106 Calheiros et al. (2008) found much higher accumulation in the rhizome than in the
107 shoots and leaves of *P. australis* (4825, 883, and 627 mg Kg^{-1} respectively). Also
108 previous results in *I. pseudacorus* showed that rhizomes were able to accumulate Cr up
109 to 0.15% of dry weight (our unpublished observations). *I. pseudacorus* is useful for
110 water treatment purposes due to its high biomass production, tolerance to polluted
111 environments and metal extraction capacity. This plant has a strong stress-tolerance
112 response including low lipid peroxidation, increased proline and malondialdehyde
113 concentration, and increased peroxidase, catalase, superoxide dismutase, and ascorbate

peroxidase activity (Zhang et al, 2007; Qiu et al, 2008; Zhou et al, 2010). Compared with *Acorus gramineus*, *Acorus orientale*, *Acorus calamus*, *Lythrum salicaria* and *Reineckea carnea*, *I. pseudacorus* showed the best performance in reducing total nitrogen and phosphorus, chemical and biological oxygen demand, and heavy metals (Cr, Pb, Cd, Fe, Cu, and Mn) from sewage (Zhang et al, 2007).

Energy dispersive X-ray microanalysis (EDX) has been extensively utilized to analyse the elemental composition of tissues and cellular components. This technique allows for the detection of toxic metals, but also of metabolically relevant cations that might be involved in detoxification mechanisms. Sulphur is found in the thiol groups of metal-binding proteins involved in metal sequestration (Cobbett and Goldsbrough, 2002), whereas P and Si interact directly with metals and co-precipitate with them in the cell walls or vacuoles (Turnau et al, 2007; Van Bellenghem et al, 2007). Transmission electron microscopy (TEM) and EDX were conducted to assess the localization of Cr in both the subcellular and tissue levels, its relationship to the distribution of other elements, and the contribution of the rhizome to Cr accumulation and detoxification.

Considering all the existing evidence we addressed the hypotheses that (a) Cr is accumulated preferably in some tissues of the root or rhizome, and in metabolically-insensitive cellular compartments, (b) Cr co-localizes with S, Si or P in the cell walls and/or the vacuoles, and (c) there are significant differences in the accumulation patterns and co-localization with other elements between roots and rhizomes.

Materials and methods

Plant material and treatments

Plants of *Iris pseudacorus* L. were purchased from a local nursery (Bioriza, Breda, Spain) in 300 ml multipot containers. Roots were washed in tap water to remove the original peat-perlite substrate. Plants were weighed and placed in the greenhouse in individual 4 l pots filled with nutritive solution. This solution comprised 130.25 mg l^{-1} NO^{3-}, 5.5 mg l^{-1} NH^{4+}, 28.5 mg l^{-1} PO_4^{2-}, 35.5 mg l^{-1} K^+, 24.5 mg l^{-1} Ca^{2+}, 4 mg l^{-1} Mg^{2+}, 14.25 mg l^{-1} SO_4^{2-}, 0.325 mg l^{-1} Fe, 0.240 mg l^{-1} Mn, 0.09 mg l^{-1} Zn, 0.030 mg l^{-1} B, 0.090 mg l^{-1} Cu, 0.028 mg l^{-1} Mo, and 0.005 mg l^{-1} Co. After an acclimation period of two weeks, 10 individual plants were selected within a small range of initial fresh weight (104.0 ± 5.2 g expressed as average ± standard error) and randomly assigned to the 'control' or 'treatment' groups. The nutritive solution of five of the plants was then amended with $CrCl_3 \cdot 6H_2O$ at 200 µg ml^{-1} (Sigma-Aldrich, St. Louis, U.S.A, >98.0% purity), containing 0.75 mM Cr(III). This concentration is sufficient to allow the detection of Cr in plant tissues by microanalysis, and to induce ultrastructural modifications (Liu et al, 2009; Lahouti et al, 2008; Mangabeira et al, 2006; Liu and Kottke, 2003). It is also similar to the Cr content of wastewater from electroplating industry (Park et al, 2006). The other five plants continued with the un-amended nutritive solution and served as controls. Plants were distributed at random and grown under glasshouse conditions for five weeks during June and July. The average temperature was 18-36 ºC, the relative humidity 31-59%, the maximum global solar irradiance 1353 W m^{-2}, and the transmission of the greenhouse covers 51%. Nutritive solution was renewed regularly.

Ultrastructural studies and microanalysis

Segments of leaf, rhizome and root were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4), washed in phosphate buffer, and stained with 1% Os tetroxide for 1h. Fixed samples were washed in distilled water and dehydrated in an acetone series of increasing concentration to achieve 100%. All the fixation steps were carried at 4ºC. Samples were then polymerised in epoxy Spurr resin for 48h at 60ºC. Ultra-thin 50 nm sections were cut with a Reichert-Jung Ultracut E ultramicrotome (C. Reichert AG, Vienna, Austria), and observed in a Jeol JEM 1010 (Tokyo, Japan) transmission electron microscope at 80 kV. Photographs were taken with a 792 Bioscan camera (Gatan, Pleasanton, USA), sited in the technical services of the University of Barcelona. For light microscopy, semi-thin 1 μm sections were stained with methylene blue and photographed with a light microscope (Olympus CX41, Tokyo, Japan) coupled with a digital camera (Olympus DP70), in the same institution. The size of the cells and organelles was measured manually on the printed micrographs. To assess metal localization in cell organelles, EDX was performed on 150 nm unstained sections of the same samples mounted on nickel grids and coated with carbon. The preparation of samples detailed above has been described as causing the loss and redistribution of diffusible elements such as Na and K, and weakly-bound non-diffusible elements. However, it is accurate to analyse the strongly-bound elements that are the subject of this study (Mangabeira et al, 2006). To eliminate the interference of the grid, carbon coating and resin, C, H, O, N and Ni peaks were deducted from the spectra. Analyses were conducted in the Microscopy Service of the Autonomous University of Barcelona using an Energy Dispersive Spectrometer (EDS) INCA (Oxford Instruments, Abingdon, UK), coupled with a JEOL JEM-2011 TEM.

Statistical Methods

Student's T-tests for comparison of means were performed on the basis of a one-factor (either "Treatment" or "Tissue") design. The non-parametric Kruskal-Wallis test was used instead when variances were not homogeneous. To assess the differences between groups, pair-wise Mann-Whitney U-tests were conducted. The α was corrected for multiple comparisons. Spearman's correlation was used to test whether there was a relationship between Cr content and the concentration of other elements. The SPSS (Statistical Package for the Social Sciences) 2005 v14.0 for Windows was used for statistical analyses. Sigma Plot software 2006 (v10.0) was used for graphic representations and linear regressions.

Results

Transmission Electron Microscopy (TEM) and Light Microscopy (LM)

The most significant changes induced by heavy metals were found in the rhizome parenchyma. The normal ultrastructure of *I. pseudacorus* rhizome cells is shown in Fig.1a. After Cr exposure, the plasma membranes were detached from cell walls (Fig.1b). Vacuoles were full-sized and filled with opaque granules of diameter 2.2 ± 0.1 μm, which were present only in the cortex (Fig. 1c). The cells showed a reduced size and large intercellular spaces (Fig. 2). Chromium decreased the cell wall thickness and the size of amyloplasts (Table 1).

Fig 1, Fig 2, Table 1

The rhizodermis also displayed manifest deleterious effects due to Cr treatment. The cell walls of a healthy rhizodermis are well defined, as seen in Fig. 3a. Chromium caused disorganization of the cell walls (Fig. 3b), which were irregular with wavy margins. The thickness of the outer surface (in contact with the growth medium) increased significantly (Student's t = -2.9, df = 9, sig. = 0.001), from 1.1±0.1 μm in

Fig 3

controls (mean ± standard deviation) to 1.9±0.5 in Cr+. There was no sign of plasmatic membrane or organelles, indicating that cells were dead (Fig. 4). No opaque granules or vacuoles were detected in the root cells.

Fig 4

As compared with the controls, the mesophyll ultrastructure of Cr-exposed leaves suffered little damage (Fig. 5). The cell walls of the sclerenchyma situated in the vascular bundles of the leaves showed discontinuities (Fig. 5c). Loss of turgor was observed at low magnification (Fig. 6).

Fig 5, Fig 6

X-Ray Microanalysis

Chromium localization in roots and rhizomes

X-Ray analyses were performed in rhizome and root samples to locate Cr and quantify its accumulation in different compartments. Chromium was detected in all the Cr+ samples, and not in controls. There were no significant differences between the Cr content of the rhizome and the root taken as a whole (Kruskal-Wallis $\chi^2 = 0.7$, sig. = 0.4). However, the rhizome had a higher Cr content in the cytoplasm (Mann–Whitney U = 29, bilateral significance = 0.02) and in the intercellular spaces (U = 3, sig. = 0.02) (Fig. 7) than the root. In the rhizome, the Cr content varied between the cellular compartments ($\chi^2 = 32.4$, sig. = <0.001). It was higher in the cytoplasm and intercellular spaces than in the cell walls, vacuoles and granules (Table 2). The amyloplasts contained very little Cr, with it being close to the detection limit. In the roots, the Cr content of the cell walls, intercellular spaces and cytoplasm were not significantly different from each other. This was due to the heterogeneity of the samples, as reported below.

Fig 7, Table 2

To investigate the accumulation pattern of Cr and the variability of the root samples seen in Fig. 7, Cr content was examined in the epidermis, cortex and stele from both

roots and rhizomes. In the root, the Cr contents of the cell walls and the cytoplasm of the rhizodermis (Table 3) were very low as compared with the cortex. Only very few intercellular spaces could be analysed in the roots because the cells were very close to each other. There were no differences in the Cr content of the intercellular spaces between the rhizodermis and the cortex. The same was true for the cell walls, intercellular spaces, and cytoplasm in the rhizome (Table 4). Chromium was under the detection limit in vascular tissues and leaf tissues.

Tables 3 and 4

Distribution of other elements in relation to Cr

The accumulation of other elements was studied on the same samples to find possible relationships with the distribution of Cr. In the roots, Cr induced an increase in the Si content and a decrease in Cl, whereas in the rhizomes only a slight increase in Cl was noted (Table 5). These results were then analysed per tissue. In the rhizodermis, the cell walls had a higher Si content and a lower Ca content than the cortex (Table 3). The same was true for the Si content of the cytoplasm, but Ca was always below the detection limit. Thus Ca co-localized with Cr, whereas the Si distribution was opposite to the Cr distribution. This was further confirmed in the cell walls by the strong negative correlation of Si versus Ca or Cr (Table 6), and the linear relationship between them (Fig. 8a). In the cytoplasm, there was also a negative correlation and a linear relationship between Si and Cr (Table 6, Fig. 8b). The elemental composition of the intercellular spaces was the same in the rhizodermis and the cortex. The same was true for the cell walls, intercellular spaces, and cytoplasm in the rhizome (Table 4). The composition of the electron-dense granules and vacuoles found in Cr+ rhizomes showed a significant proportion of S (Table 7). In all the other samples analysed in this experiment, S was below the detection limit.

Table 5

Table 6, Fig 8

Table 7

Discussion

It is widely accepted that metals are principally retained in the roots of plants (Salt et al, 1995; Clemens, 2001). Metal accumulation in the roots is considered a general exclusion response of tolerant plants that are faced with metal toxicity, and which is aimed to prevent subsequent transport to the shoots. However, the literature concerning Cr localization in the root tissues of plants exposed to toxic levels of Cr is scarce and contradictory. Mangabeira et al. (2006) analysed tomato roots by ion microscopy and found that Cr was preferably accumulated in the vascular tissues. By contrast, electron energy loss spectroscopy and spectroscopic imaging revealed that Cr in *Allium cepa* accumulated mostly in electron-dense deposits in the cell walls and vacuoles of the root cortex (Liu and Kottke, 2003). The same study reported that Cr increased from the rhizodermis to the cortex, and decreased from there to the stele, where it was hardly detectable. The gradation of Cr content across the root was very similar to our results, where Cr content was low in the rhizodermis, high in the cortex and below the detection limit in the vascular tissues. A low Cr signal in the vascular tissue was also reported by Bluskov et al. (2005) in *Brassica juncea*, which they attributed to the barrier of the endodermis.

Several authors describe the cell walls of the root as one of the most important sinks for metal accumulation, including Cr (Liu and Kottke, 2003; Liu et al., 2009). Cell walls can accumulate metals before they enter the protoplast, thus functioning as barriers to limit passive absorption. Also, the metals removed from the protoplast can be extruded and sequestered in the cell walls to reduce cytotoxicity (Krzesłowska, 2010). Plants can improve the cation-binding capacity of cell walls in response to metals by either increasing pectin levels (Wierzbicka et al, 2007) or thickening the cell walls (Probst et

al, 2009). Cell wall polymers are also responsible for the biosorption of metals to dead biomass (Elangovan et al, 2008; Saha and Orvig, 2010). Accordingly, the highest Cr concentrations in this study were measured in the cell walls of the root cortex. The exterior walls of the rhizodermis also showed thickenings and electron-dense inclusions. This strongly supports the interpretation of the rhizodermis acting as a barrier to limit the passive uptake of Cr. Trivalent Cr, as used in this experiment, is taken up passively, whereas hexavalent Cr requires the intervention of specific transporters (Skeffington et al, 1976). Although the Cr content was higher in the cell walls, the levels attained by the cytoplasm and intercellular spaces were also notable. In our opinion, this illustrates the failure of the avoidance mechanisms following exposure to the high Cr concentration used to treat the plants (0.75 mM), and the duration of the experiment. Similarly, the cytoplasm and intercellular spaces of the rhizome had a higher Cr content than the cell walls, vacuoles or granules, which can be attributed to the same conditions.

Silicon has been extensively reviewed to increase plant tolerance to biotic and abiotic stresses including pathogens, salinity, drought, and metal toxicity (Liang et al, 2007; Zargar et al, 2010). The mechanisms responsible for the protective effect in the face of metal toxicity can operate both *in* and *ex planta*. The external mechanisms are based on decreasing the metal availability in the growth medium. Within the plant, Si diminishes metal toxicity and uptake and as well as contact with sensitive cellular components by means of, co-precipitation, increased compartmentation in vacuoles and cell walls, inhibited root-shoot transport, and increased production of antioxidants (Liang et al, 2007). Studies on plants under metal stress show the co-localization of Si with Al and Fe (Turnau et al, 2007), and the precipitation of Al, Sn and Zn silicates in the cell walls (Bringezu et al, 1999; Britez et al, 2002; Neuman and zur Nieden, 2001). However, Si does not always co-locate with metals (Bringezu et al, 1999). Nickel increased the Si

content of *Grevillea exul* var. Exul roots, and this was noted especially in the rhizodermis, where the concentration of Ni was lowest (Rabier et al, 2008, Table 1). Similarly, the localization of Si in the roots reported here was mainly in the rhizodermis, and was thus opposite to Cr. Also there was an increase in the Si content of the roots accompanied by a negative correlation between Cr and Si. This indicates that the function of Si deposition in the cell walls of the rhizodermis is not a direct interaction with Cr. We propose that this function is the reduction of Cr uptake, which is passive in the case of trivalent Cr (Skeffington et al, 1976). The thickening of the exterior cell walls also points to the creation of a barrier against Cr influx into the root.

Vacuoles, the same as cell walls, are a major sink for metal accumulation in plants under metal stress. The compartmentation of Cr in vacuoles has been reported in the roots of tolerant plants (Sanità di Toppi et al, 2002; Liu and Kottke, 2003; Lahouti et al, 2008), and in the leaves of hyperaccumulators (Liu et al, 2009), and the same is true for several other metals (Clemens et al, 2001). Again there is little evidence in the literature of metal-sequestering vacuoles in rhizomes. Shan et al. (2003) described the accumulation of rare earth elements in the vacuoles of both xylem and phloem cells of the rhizome in the hyperaccumulator fern, *Dricopteris dichotoma* (Thunb.) Bernh. The Cr-induced vacuoles of *I. pseudacorus* were only found in the cortical parenchyma of the rhizome, not in the vascular tissues. They contained a significant proportion of Cr, and were never detected in the roots or leaves. In addition, in the cytoplasm and intercellular spaces of the rhizome cells the Cr concentration was higher than in the root cells. Further research is required to determine whether this distribution of Cr-sequestering vacuoles is common to other tolerant rhizomatous plants and metals.

X-Ray analyses revealed that in these vacuoles and granules, Cr co-occurred with S. The co-localization of Cr with S in electron-dense vacuoles and vacuolar inclusions has

been established in previous work with *Brassica oleracea* (Sanità di Toppi et al, 2002) and *Raphanus sativus* (Lahouti et al, 2008). This can be attributed to Cr being sequestered by S-enriched metal-binding proteins like phytochelatins (PC) or metallothioneins, which lowers the metal levels in the cytoplasm and preserves the most sensitive cellular components from direct interaction. Metallothioneins are cysteine-rich low molecular weight proteins found in plants, animals and fungi, which are involved in metal detoxification and homeostasis in plants (Cobbett and Goldsbrough, 2002). The expression of these gene products by plants is promoted by Cr and other metals (Labra et al, 2006; Rodríguez-Llorente et al, 2010), but their exact function is still unknown. Phytochelatins (PC) are glutathione oligomers synthesised in response to metals and they are able to form stable complexes in vivo with several metals (Leita et al, 1991; Gupta et al, 1995; Iglesia-Turiño et al, 2006). Cadmium complexes with PC are pumped into the vacuoles and immobilized there (Salt et al, 1995; Cobbett and Goldsbrough, 2002). PC have also been recently described to be induced by Cr (Diwan et al, 2010), and most probably they form PC-Cr complexes that are sequestered in the vacuoles. In our study, electron dense vacuoles and granules did not occur in the roots, suggesting that the vacuolar compartmentation of protein-Cr complexes was restricted to the rhizomes.

Conclusions

From the present results it can be concluded that both the roots and rhizomes make an important contribution to Cr detoxification in *Iris pseudacorus.* It was shown that Cr localization in the root and rhizome is different at the subcellular and tissue levels. Chromium in the root is accumulated preferably in the cortical parenchyma, whereas in the rhizome the distribution is homogeneous. The highest Cr contents are found in the

cell walls of the cortex in the roots, and in the cytoplasm and intercellular spaces of the rhizomes. The high Cr content of the cytoplasm and intercellular spaces in both rhizomes and roots is indicative of the collapse of tolerance mechanisms, which are unable to effectively remove Cr from sensitive compartments. Several ultrastructural alterations confirm the toxic effect of Cr in roots (cell wall disorganization, thickening, plasmolysis, electron-dense inclusions) and rhizomes (reduced size, cell wall detachment, vacuolation, opaque granules).

Silicon and Cr exclude each other in the root. It is proposed that the rhizodermis acts as a barrier to limit Cr uptake by means of Si deposition and cell wall thickening. The rhizome cortex develops an extensive vacuole and granule system where Cr is sequestered in co-occurrence with S. This is attributed to Cr binding with PC or metallothioneins.

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Figure captions

Figure 1. Transmission electron micrographs of rhizome cortical parenchyma. **(a)** Control plants, **(b)** and **(c)** 0.75 mM Cr(III) treated plants; am = amyloplast, g = granule, vac = vacuole. Magnification = 3,000X (a) and (b), and 4,500X (c).

Figure 2. Light microscopy images of cross semi-thin sections of the rhizome. (a) Control plants, **(b)** 0.75 mM Cr(III) treated plants; ep = epidermis, par = parenchyma, vas = vascular tissues. Magnification 200X.

Figure 3. Transmission electron micrographs of the rhizodermis. (a) Control plants, **(b)** 0.75 mM Cr(III) treated plants; cw = cell wall, cyt = cytoplasm, ext = exterior, lu = lumen. Magnification = 20,000X.

Figure 4. Light microscopy images of cross semi-thin sections of the rhizodermis. (a) Control plants, **(b)** 0.75 mM Cr(III) treated plants; par = parenchyma, rd = rhizodermis. Magnification 200X.

Figure 5. Transmission electron micrographs of leaf mesophyll and sclerenchyma. **(a)** Control plants, **(b)** and **(c)** 0.75 mM Cr(III) treated plants; chl = chloroplast, cw = cell wall, n = nucleus. Magnification = 3,000X (a) and (b), and 15,000X (c).

Figure 6. Light microscopy images of cross semi-thin sections of leaf mesophyll and vascular bundles. (a) Control plants, **(b)** 0.75 mM Cr(III) treated plants; ep = epidermis, pl = palisade layer, sc = sclerenchyma, sp = spongy layer, vas = vascular tissues. Magnification 200X.

Figure 7. Chromium content in various subcellular compartments of rhizomes and roots of Cr+ plants. Values are means ± standard deviations, n ≥ 10 except for the intercellular spaces of roots (n = 4), which were sparse. Plants were treated with 0.75mM Cr(III). (*) indicates significant differences between rhizomes and roots, according to the Mann-Whitney U-test (pvalue < 0.05).

Figure 8. Linear regressions of Si with respect to Cr and Ca in the cell wall (a), and with respect to Cr in the cytoplasm (b) of Cr+ roots. Values are individual measurements ± standard deviations corresponding to the analytical error, n = 18 (cell wall) or 10 (cytoplasm). Plants were treated with 0.75mM Cr(III).

Table 1. Size of the cell wall and amyloplasts of the rhizome.[†]

	Control	Cr+	t-value	df	Significance
Cell wall	1.3±0.7	0.6±0.2	4.1	27.9	<0.001
Amyloplast	4.2±1.0	2.5±0.4	5.7	14.2	<0.001

[†]Values are means ± standard deviation, in µm. Cr+ plants were treated with 0.75mM Cr(III). T-value = Student-T test for equal means, df = degrees of freedom, n ranged from 10 to 23.

Table 2. Pairwise comparisons of the cellular compartments of the Cr+ rhizomes. [†]

	U-value	Significance
Cell wall vs Cytoplasm	8.0	<0.001
Cell wall vs Intercellular space	7.0	<0.001
Cell wall vs Vacuole+Granules	79.0	0.41
Cytoplasm vs Vacuole+Granules	5.0	<0.001
Cytoplasm vs Intercellular space	60.0	0.85
Intercellular space vs Vacuole+Granules	2.0	<0.001

[†]Dependent variable: mean Cr atomic %. Plants were treated with 0.75mM Cr(III). Significance is bilateral, U-value = Mann-Whitney U-test for equal medians, n ranged from 10 to 23.

Table 3. Element content of the rhizodermis and the cortex of Cr+ roots. [†]

Compartment	Element	Rhizodermis	Cortex	χ^2	Significance
Cell wall	Si	89.8±10.4	40.1±22.5	11.5	<0.001
	Cl	4.3±5.3	14.6±7.2	8.7	0.003
	Ca	0.0±0.0	13.7±11.7	11.0	<0.001
	Cr	5.9±5.2	31.5±18.9	9.8	0.002
Cytoplasm	Si	63.7±12.3	45.4±9.5	3.2	0.076
	Cl	18.3±9.0	28.7±7.7	2.5	0.117
	Cr	18.1±4.0	26.0±5.1	4.8	0.028

[†]Values are means ± standard deviation, in atomic %. Plants were treated with 0.75mM Cr(III). χ^2 = Kruskal-Wallis test for equal medians, n = 18 (cell wall) or 10 (cytoplasm).

Table 4. Element content of the epidermis and the cortex of Cr+ rhizomes. †

Compartment	Element	Epidermis	Cortex	χ^2	Significance
Cell wall	Si	25.2±3.2	29.3±7.8	1.4	0.239
	Cl	31.4±3.6	32.3±3.7	0.1	0.906
	Ca	15.2±3.3	12.0±6.8	0.7	0.409
	Cr	16.3±2.9	14.6±6.4	2.0	0.157
Cytoplasm	Si	46.5±9.6	54.4±15.5	0.3	0.606
	Cl	21.1±10.4	19.5±13.3	0.1	0.796
	Ca	1.73±4.1	0.0±0.0	1.3	0.248
	Cr	30.7±4.7	26.1±5.2	1.4	0.245
Intercellular space	Si	46.5±8.7	53.1±10.4	1.7	0.197
	Cl	20.5±12.6	21.3±7.5	0.7	0.796
	Cr	33.0±7.6	25.7±4.1	1.7	0.197

†Values are means ± standard deviation, in atomic %. Plants were treated with 0.75mM Cr(III). χ^2 = Kruskal-Wallis test for equal medians, n = 15 (cell wall), 14 (cytoplasm), or 9 (intercellular space).

Table 5. Effect of Cr on the element content of roots and rhizomes. †

Element	Control	Cr+	χ^2	Significance
Roots				
Si	28.3±17.2	57.7±26.0	5.3	0.021
Cl	43.5±24.3	15.2±10.6	4.6	0.033
Ca	18.6±25.8	5.4±9.9	1.9	0.172
Rhizomes				
Si	34.9±17.4	38.5±15.8	0.03	0.865
Cl	38.0±15.9	43.8±17.9	5.3	0.021
Ca	26.2±10.2	28.9±13.0	0.1	0.735

†Values are means ± standard deviation, in atomic %. Plants were treated with 0.75mM Cr(III). χ^2 = Kruskal-Wallis test for equal medians, n = 28 (Cr+ roots), 29 (Cr+ rhizomes) or 5 (Controls).

Table 6. Spearman's correlation of Si versus Ca and Cr in Cr+ roots. †

	Ca	Cr
Cell wall		
Coefficient	-0.688	-0.953
Sig.	0.002	<0.001
n	18	18
Cytoplasm		
Coefficient		-0.794
Sig.		0.006
n		10

†Significance is bilateral. Plants were treated with 0.75mM Cr(III). Ca was below the detection limit in the cytoplasm.

Table 7. Element content of electron dense granules and vacuoles of Cr+ rhizomes. †

Element	Atomic %
Si	26.8±17.5
S	19.3±15.0
Cl	32.1±15.2
Ca	4.9±11.9

†Values are means ± standard deviation, n=12. Plants were treated with 0.75mM Cr(III).