

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

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13 microanalysis.

14

15 **Abstract**

16 Chromium pollution of fresh water is hazardous for humans and other organisms,
17 and places a limitation on the use of polluted water sources. Phytoremediation, the use
18 of plants to remove pollutants from the environment, is a cost-effective,
19 environmentally friendly approach for water decontamination. To improve the
20 efficiency of the process, it is essential to increase the current knowledge about Cr
21 accumulation in macrophytes. Plants of *Iris pseudacorus* L. were treated with Cr(III) at
22 0.75 mM for five weeks to investigate Cr localization by means of transmission electron
23 microscopy (TEM) and energy dispersive X-ray analysis (EDX). Chromium induced
24 severe ultrastructural alterations in the rhizodermis (cell wall disorganization,
25 thickening, plasmolysis, electron-dense inclusions) and rhizome parenchyma (reduced
26 cell size, cell wall detachment, vacuolation, opaque granules).

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

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39 **Abbreviations**

40	EDS	Energy Dispersive Spectrometer
41	EDX	Energy Dispersive X-ray analysis
42	LM	Light Microscopy
43	PC	Phytochelatins
44	TEM	Transmission Electron Microscopy
45	USEPA	United States Environmental Protection Agency

46

47 **Introduction**

48 Fresh water pollution with heavy metals is one of the major global environmental
49 concerns. Toxic metals are hazardous for living organisms, strongly persistent in the
50 environment and living tissues, and easily transferred to the food chain. Chromium
51 pollution of water mainly originates from industrial processes such as the production of
52 stainless and refractory steel, drilling muds, electroplating cleaning agents, catalytic
53 manufacturing, leather, pigments, porcelain and pottery, and chemicals (Shanker et al,
54 2005).

55 Chromium is non-essential to plants and toxic for most agronomic species above
56 $0.5\text{-}5.0\ \mu\text{g ml}^{-1}$ (Davies et al, 2002). The toxic effects of Cr include decreases in seed
57 germination, biomass production, root and shoot elongation, enzymatic activity, protein
58 content and photosynthesis (Vajpayee et al, 1999 and 2001; Peralta et al, 2001;
59 Appenroth et al, 2001), together with unbalanced mineral nutrition and altered pigment
60 synthesis (Barceló et al, 1985; Vajpayee et al, 1999 and 2001). Chromium toxicity
61 depends on its oxidation state. Chromium is naturally found in every oxidation state
62 between -2 and $+6$, but the trivalent and the hexavalent are predominant (Barnhart,
63 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

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

119 Energy dispersive X-ray microanalysis (EDX) has been extensively utilized to
120 analyse the elemental composition of tissues and cellular components. This technique
121 allows for the detection of toxic metals, but also of metabolically relevant cations that
122 might be involved in detoxification mechanisms. Sulphur is found in the thiol groups of
123 metal-binding proteins involved in metal sequestration (Cobbett and Goldsbrough,
124 2002), whereas P and Si interact directly with metals and co-precipitate with them in the
125 cell walls or vacuoles (Turnau et al, 2007; Van Bellinghem et al, 2007). Transmission
126 electron microscopy (TEM) and EDX were conducted to assess the localization of Cr in
127 both the subcellular and tissue levels, its relationship to the distribution of other
128 elements, and the contribution of the rhizome to Cr accumulation and detoxification.

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

135

136 **Materials and methods**

137 *Plant material and treatments*

138 Plants of *Iris pseudacorus* L. were purchased from a local nursery (Bioriza, Breda,
139 Spain) in 300 ml multipot containers. Roots were washed in tap water to remove the
140 original peat-perlite substrate. Plants were weighed and placed in the greenhouse in
141 individual 4 l pots filled with nutritive solution. This solution comprised 130.25 mg l⁻¹
142 NO³⁻, 5.5 mg l⁻¹ NH⁴⁺, 28.5 mg l⁻¹ PO₄²⁻, 35.5 mg l⁻¹ K⁺, 24.5 mg l⁻¹ Ca²⁺, 4 mg l⁻¹ Mg²⁺,
143 14.25 mg l⁻¹ SO₄²⁻, 0.325 mg l⁻¹ Fe, 0.240 mg l⁻¹ Mn, 0.09 mg l⁻¹ Zn, 0.030 mg l⁻¹ B,
144 0.090 mg l⁻¹ Cu, 0.028 mg l⁻¹ Mo, and 0.005 mg l⁻¹ Co. After an acclimation period of
145 two weeks, 10 individual plants were selected within a small range of initial fresh
146 weight (104.0 ± 5.2 g expressed as average ± standard error) and randomly assigned to
147 the 'control' or 'treatment' groups. The nutritive solution of five of the plants was then
148 amended with CrCl₃·6H₂O at 200 µg ml⁻¹ (Sigma-Aldrich, St. Louis, U.S.A, >98.0%
149 purity), containing 0.75 mM Cr(III). This concentration is sufficient to allow the
150 detection of Cr in plant tissues by microanalysis, and to induce ultrastructural
151 modifications (Liu et al, 2009; Lahouti et al, 2008; Mangabeira et al, 2006; Liu and
152 Kottke, 2003). It is also similar to the Cr content of wastewater from electroplating
153 industry (Park et al, 2006). The other five plants continued with the un-amended
154 nutritive solution and served as controls. Plants were distributed at random and grown
155 under glasshouse conditions for five weeks during June and July. The average
156 temperature was 18-36 °C, the relative humidity 31-59%, the maximum global solar
157 irradiance 1353 W m⁻², and the transmission of the greenhouse covers 51%. Nutritive
158 solution was renewed regularly.

159

160 *Ultrastructural studies and microanalysis*

161 Segments of leaf, rhizome and root were fixed in a mixture of 2.5% glutaraldehyde
162 and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4), washed in phosphate
163 buffer, and stained with 1% Os tetroxide for 1h. Fixed samples were washed in distilled
164 water and dehydrated in an acetone series of increasing concentration to achieve 100%.
165 All the fixation steps were carried at 4°C. Samples were then polymerised in epoxy
166 Spurr resin for 48h at 60°C. Ultra-thin 50 nm sections were cut with a Reichert-Jung
167 Ultracut E ultramicrotome (C. Reichert AG, Vienna, Austria), and observed in a Jeol
168 JEM 1010 (Tokyo, Japan) transmission electron microscope at 80 kV. Photographs
169 were taken with a 792 Bioscan camera (Gatan, Pleasanton, USA), sited in the technical
170 services of the University of Barcelona. For light microscopy, semi-thin 1 µm sections
171 were stained with methylene blue and photographed with a light microscope (Olympus
172 CX41, Tokyo, Japan) coupled with a digital camera (Olympus DP70), in the same
173 institution. The size of the cells and organelles was measured manually on the printed
174 micrographs. To assess metal localization in cell organelles, EDX was performed on
175 150 nm unstained sections of the same samples mounted on nickel grids and coated
176 with carbon. The preparation of samples detailed above has been described as causing
177 the loss and redistribution of diffusible elements such as Na and K, and weakly-bound
178 non-diffusible elements. However, it is accurate to analyse the strongly-bound elements
179 that are the subject of this study (Mangabeira et al, 2006). To eliminate the interference
180 of the grid, carbon coating and resin, C, H, O, N and Ni peaks were deducted from the
181 spectra. Analyses were conducted in the Microscopy Service of the Autonomous
182 University of Barcelona using an Energy Dispersive Spectrometer (EDS) INCA
183 (Oxford Instruments, Abingdon, UK), coupled with a JEOL JEM-2011 TEM.

184

185 **Statistical Methods**

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

195

196 **Results**

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

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

Fig 1,
Fig 2,
Table 1

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

Fig 3

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

Fig 4

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

Fig 5,
Fig 6

217

218 **X-Ray Microanalysis**

219 *Chromium localization in roots and rhizomes*

220 X-Ray analyses were performed in rhizome and root samples to locate Cr and
221 quantify its accumulation in different compartments. Chromium was detected in all the
222 Cr⁺ samples, and not in controls. There were no significant differences between the Cr
223 content of the rhizome and the root taken as a whole (Kruskal-Wallis $\chi^2 = 0.7$, sig. =
224 0.4). However, the rhizome had a higher Cr content in the cytoplasm (Mann-Whitney U
225 = 29, bilateral significance = 0.02) and in the intercellular spaces (U = 3, sig. = 0.02)
226 (Fig. 7) than the root. In the rhizome, the Cr content varied between the cellular
227 compartments ($\chi^2 = 32.4$, sig. = <0.001). It was higher in the cytoplasm and intercellular
228 spaces than in the cell walls, vacuoles and granules (Table 2). The amyloplasts
229 contained very little Cr, with it being close to the detection limit. In the roots, the Cr
230 content of the cell walls, intercellular spaces and cytoplasm were not significantly
231 different from each other. This was due to the heterogeneity of the samples, as reported
232 below.

Fig 7,
Table 2

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

Tables
3 and 4

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

242

243 *Distribution of other elements in relation to Cr*

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

Table 5

Table 6,
Fig 8

Table 7

260

261 **Discussion**

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

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

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

298 Silicon has been extensively reviewed to increase plant tolerance to biotic and abiotic
299 stresses including pathogens, salinity, drought, and metal toxicity (Liang et al, 2007;
300 Zargar et al, 2010). The mechanisms responsible for the protective effect in the face of
301 metal toxicity can operate both *in* and *ex planta*. The external mechanisms are based on
302 decreasing the metal availability in the growth medium. Within the plant, Si diminishes
303 metal toxicity and uptake and as well as contact with sensitive cellular components by
304 means of, co-precipitation, increased compartmentation in vacuoles and cell walls,
305 inhibited root-shoot transport, and increased production of antioxidants (Liang et al,
306 2007). Studies on plants under metal stress show the co-localization of Si with Al and
307 Fe (Turnau et al, 2007), and the precipitation of Al, Sn and Zn silicates in the cell walls
308 (Bringezu et al, 1999; Britez et al, 2002; Neuman and zur Nieden, 2001). However, Si
309 does not always co-locate with metals (Bringezu et al, 1999). Nickel increased the Si

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

319 Vacuoles, the same as cell walls, are a major sink for metal accumulation in plants
320 under metal stress. The compartmentation of Cr in vacuoles has been reported in the
321 roots of tolerant plants (Sanità di Toppi et al, 2002; Liu and Kottke, 2003; Lahouti et al,
322 2008), and in the leaves of hyperaccumulators (Liu et al, 2009), and the same is true for
323 several other metals (Clemens et al, 2001). Again there is little evidence in the literature
324 of metal-sequestering vacuoles in rhizomes. Shan et al. (2003) described the
325 accumulation of rare earth elements in the vacuoles of both xylem and phloem cells of
326 the rhizome in the hyperaccumulator fern, *Dricopteris dichotoma* (Thunb.) Bernh. The
327 Cr-induced vacuoles of *I. pseudacorus* were only found in the cortical parenchyma of
328 the rhizome, not in the vascular tissues. They contained a significant proportion of Cr,
329 and were never detected in the roots or leaves. In addition, in the cytoplasm and
330 intercellular spaces of the rhizome cells the Cr concentration was higher than in the root
331 cells. Further research is required to determine whether this distribution of Cr-
332 sequestering vacuoles is common to other tolerant rhizomatous plants and metals.

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

335 been established in previous work with *Brassica oleracea* (Sanità di Toppi et al, 2002)
336 and *Raphanus sativus* (Lahouti et al, 2008). This can be attributed to Cr being
337 sequestered by S-enriched metal-binding proteins like phytochelatins (PC) or
338 metallothioneins, which lowers the metal levels in the cytoplasm and preserves the most
339 sensitive cellular components from direct interaction. Metallothioneins are cysteine-rich
340 low molecular weight proteins found in plants, animals and fungi, which are involved in
341 metal detoxification and homeostasis in plants (Cobbett and Goldsbrough, 2002). The
342 expression of these gene products by plants is promoted by Cr and other metals (Labra
343 et al, 2006; Rodríguez-Llorente et al, 2010), but their exact function is still unknown.
344 Phytochelatins (PC) are glutathione oligomers synthesised in response to metals and
345 they are able to form stable complexes in vivo with several metals (Leita et al, 1991;
346 Gupta et al, 1995; Iglesia-Turiño et al, 2006). Cadmium complexes with PC are pumped
347 into the vacuoles and immobilized there (Salt et al, 1995; Cobbett and Goldsbrough,
348 2002). PC have also been recently described to be induced by Cr (Diwan et al, 2010),
349 and most probably they form PC-Cr complexes that are sequestered in the vacuoles. In
350 our study, electron dense vacuoles and granules did not occur in the roots, suggesting
351 that the vacuolar compartmentation of protein-Cr complexes was restricted to the
352 rhizomes.

353

354 **Conclusions**

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

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

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

372

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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 \pm standard deviations, $n \geq 10$ 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 \pm 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).