Study of the spatial distribution of mercury in roots of vetiver grass (*Chrysopogon zizanioides*) by micro-PIXE spectrometry

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Abstract

Localization of Hg in root tissues of vetiver grass (*Chrysopogon zizanioides*) was investigated by micro-Proton Induced X-ray Emission (PIXE) spectrometry to gain a better understanding of Hg uptake and its translocation to the aerial plant parts.

Tillers of *C. zizanioides* were grown in a hydroponic culture for 3 weeks under controlled conditions and then exposed to Hg for 10 days with or without the addition of the chelators $(\text{NH}_4)_2\text{S}_2\text{O}_3$ or KI. These treatments were used to study the effects of these chelators on localization of Hg in the root tissues to allow better understanding of Hg uptake during its assisted-phytoextraction.

Qualitative elemental micro-PIXE analysis revealed that Hg was mainly localized in the root epidermis and exodermis, tissues containing suberin in all Hg treatments. Hg at trace levels was localized in the vascular bundle when plants were treated with a mercury solution only. However, higher Hg concentrations were found when the solution also contained $(\text{NH}_4)_2\text{S}_2\text{O}_3$ or KI. This finding is consistent with the observed increase in Hg translocation to the aerial parts of the plants in the case of chemically induced Hg phytoextraction.

Keywords: mercury, *Chrysopogon zizanioides*, micro-PIXE spectrometry, assisted-phytoextraction.

1. Introduction

Over the centuries, human activities have contaminated large areas in both developed and developing countries and heavy metals are a major factor of this pollution (Evangelou et al. 2007). Heavy metals and metalloids are of particular concern as they are frequently present at elevated concentrations in polluted areas. Their high toxicity and potential mobility can result in surface and groundwater contamination (Evangelou et al. 2007). In addition, heavy metals do not degrade in the environment as do organic contaminants. They can be transformed biotically and chemically, translocated into plants and further transferred into animal and human food chains (Lomonte et al. 2010, Sloan et al. 2001). Among the heavy metals frequently present in contaminated areas, mercury (Hg) is of the highest environmental and public health concern. This is due to its extremely high toxicity in both organic and inorganic forms and to its ability to bioaccumulate, thus further increasing the risks to exposure even at trace levels (Oliver et al. 2005). Combining anthropogenic inputs and natural sources, a total of approximately 8.06×10^8 kg Hg has been released into the soil, 1.18×10^8 kg into water, and 7.41×10^8 kg into the atmosphere (Kelly et al. 2006).

Since Hg is one of the most toxic heavy metals, mitigation of its effects is required. Recently, phytoremediation of Hg has been investigated more actively as an environmentally-friendly technology for the remediation of heavy metal contaminated sites and as an alternative to disruptive techniques (Lomonte et al. 2010, Issaro et al. 2009, Morena et al. a & b, Sierra et al. 2008). However, Hg has a very limited solubility in soil and biosolids, low availability for plant uptake and does not have any known biological function (Shiyab et al. 2009). In addition, there is a tendency for Hg to accumulate in roots (Beauford et al. 1977). Roots possess a significant cation-exchange capacity due largely to the presence of carboxyl groups. These may be responsible for moving ions through the outer part of the root to the plasmalemma where active absorption takes place (Patra and Sharma 2000). For this reason plant uptake of mobile metal ions present in the substrate solution, especially in the case of metal ions that can be strongly absorbed on the roots surface as Hg, depends significantly on the quantity of roots produced (Du et al. 2005). Translocation is also

relatively lower for Hg than for other metals (Wild 1988). Consequently, novel approaches are needed to improve phytoextraction technology. To enhance plant tolerance and metal uptake capacity conventional breeding approaches and genetic engineering have been suggested. However, both techniques are costly (Lindberg et al. 1979). To improve the metal accumulation capacities and uptake speed of non-hyperaccumulating plants, the development of low cost technologies with the addition of chelating agents has been proposed (Evangelou et al. 2007). An approach involving the use of thiol containing ligands to induce Hg accumulation in *Phaseolus vulgaris* (L.), *Brassica juncea* (L.), *Vicia villosa* Roth. (Moreno et al. 2005a &b), in the saltbush *Atriplex canescens* (Pursh) Nutt. and in the Ni hyperaccumulator *Berkheya coddii* (Roessler) (Moreno et al. 2004) has been proposed as a potential strategy for the removal of Hg from contaminated sites and for increasing the translocation of this metal to above-ground plant tissues. In *P. vulgaris*, *Br. juncea* and *V. villosa* thiosulphate salts (ammonium and sodium) mobilized Hg in the substrates and caused an increase in the Hg concentration in roots and shoots. With the same aim, iodide has also been utilized in hydroponic cultures to increase the phytoextraction of Hg by *Salix* spp. (Wang and Greger 2006).

Other studies on the phytoremediation of Hg in contaminated areas have been reported using different plant species such as *Rumex induratus* (Boiss. & Reut.) and *Marrubium vulgare* (L.) (Moreno-Jiménez et al. 2006), spruce (Godbold and Hutterman 1988), lupin (Moreno et al. 2004, Ximenez-Embun et al. 2001), wheat (Cavallini et al. 1999), pea (Patra and Sharma 2000), mustard (Shiyab et al. 2009), sorghum (Wong 2003), vetiver grass (Lomonte et al. 2010), wallaby and kangaroo grasses (Lomonte et al. 2010), rice (Du et al. 2005), tomato (Shanker et al. 1996), rosemary (Barghigiani and Ristori 1995), and tobacco (Suszeysky and Shann 1995). Among those plant species, vetiver grass (*Chrysopogon zizanioides* (L.) Robery) is known for its exceptional properties (Lomonte et al. 2011, Greenfield 1995). Unique morphology, physiology and symbiotic association render vetiver grass capable of tolerating environmental stresses (Srivastava et al. 2008). Vetiver grass is a high biomass plant with a long (3 - 4 m), massive, aerenchymatous and complex

root system, which can easily penetrate into the deeper layers of soil. It is capable of withstanding extremely harsh environmental conditions and climatic variations, such as prolonged drought, flood, submergence, fire, frost and extreme temperature (-20 to 60 °C) (Truong 2000). It is also tolerant to a wide range of soil acidity, alkalinity, salinity, sodicity, and elevated levels of metallic contaminants including Hg (Truong 2000, Wong 2003). The effectiveness of this grass in soil and sediment erosion control is due to its morphological and physiological distinctiveness (Srivastava et al. 2008). As with the other plant species during phytoextraction, Hg is mainly accumulated in the roots of vetiver grass and a small concentration of the metal is translocated to the above-ground tissues (1-5% of the total Hg taken up) (Greenfield 2002). To understand the mechanisms that allow this Hg-accumulating plant to tolerate and accumulate high concentrations of Hg in the roots and translocate it to shoots in a small scale, the spatial localization of the metal accumulation has to be known (Truong 2000). Micro-PIXE spectrometry has been used to study the localization of trace metals in metal indicator and hyperaccumulating plant species (Siegele 2008). To date, localization studies on hyperaccumulating plants have been predominantly conducted on herbaceous hyperaccumulators, mostly of Ni and in some cases of Zn, Cd, As, and Mn (Siegele 2008) but not many studies have been carried out on Hg accumulating plants species.

The aim of the research outlined in this paper was to investigate the distribution of Hg in root cross-sections of *C. zizanioides* by micro-PIXE spectrometry to permit a better understanding of its Hg uptake during phytoextraction. As the use of Hg chelators induced a larger uptake and translocation of the metal in plants, the localization of Hg in root tissues with or without chelators was also compared.

2. Materials and methods

2.1 Plant material

Tillers of *C. zizanioides* were grown in hydroponic culture with continuous aeration under glasshouse conditions with an ambient temperature of 24/20°C day/night and 14 h photoperiod.

Two 20 cm tall tillers of *C. zizanioides* were transferred to 3 L polypropylene containers filled with the following basal nutrient solution (pH 6): 1.5 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM NH_4NO_3 , 1 mM KNO_3 , 0.45 mM MgSO_4 , 0.05 mM MgCl_2 , 0.05 mM KH_2PO_4 , 0.7 μM MnSO_4 , 0.5 μM ZnSO_4 , 0.1 μM CuSO_4 , 0.1 μM NiCl_2 , 1 μM H_3BO_3 , 0.1 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 1 mM MES (2-(N-morpholino)ethanesulfonic acid), 0.5 mM NaOH, 0.1 mM FeNa-EDDHA (EDDHA = ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)). After 3 weeks of growth the nutrient solution (which was renewed every 7 days) was spiked with 0.8 mg L⁻¹ Hg (HgCl_2), and 3.2 mg L⁻¹ $\text{S}_2\text{O}_3^{2-}$ [$(\text{NH}_4)_2\text{S}_2\text{O}_3$] or 0.8 mg L⁻¹ Hg (HgCl_2), and 4.8 mg L⁻¹ I⁻ (KI). As a control, some plants were grown in the nutrient solution only. The use of $\text{S}_2\text{O}_3^{2-}$ and I⁻ as Hg chelators was chosen to gain a better understanding of the Hg uptake in the case of assisted-phytoextraction. The concentration of Hg spike was chosen to induce a high accumulation of Hg in plant roots in order to maximize the detection of the metal by micro-PIXE. The excess chelators guaranteed the complete complexation of Hg as $\text{Hg}(\text{S}_2\text{O}_3)$ and $[\text{HgI}_4]^{2-}$. The nutrient solutions of Hg treatment with or without chelator addition were renewed every 2 days. Plants were harvested after 10 days of treatment. At harvest, roots from the same treatment were washed with ultrapure Milli-Q water and were separated into two batches: one for total Hg determination by Atomic Fluorescence Spectrometry (AFS) and the other one for micro-PIXE analysis.

2.2 Micro-PIXE analysis

In micro-analysis of biological tissues, sample preparation is one of the most important steps, which is also the case for micro-PIXE measurements. For plant material exposed to metals, it has to be ensured that sample preparation does not result in the redistribution of the metal and that the cell structure is well preserved. Consequently, a cryofixation technique was employed: root fragments (about 1 cm) were immersed in liquid-nitrogen cooled propane and freeze-dried (Labconco Lyph.Loch[®] 6) for 72 h. The freeze-dried roots were thereafter hand cross-sectioned with a stainless steel razor blade under a dissection microscope. With the assumption that the cross-sections were

perfectly circular, root samples of about 2 mm in diameter were selected for analysis. Sections with an even thickness of about 50 μm were mounted on carbon tabs (PST-AI023) and then placed on a standard sample holder used for the micro-PIXE facility in ANSTO, Sydney. All samples were stored in a desiccator prior to analysis.

Plant samples were analyzed by the micro-PIXE spectrometry using the ANSTO High Energy Heavy Ion Microprobe (HIMP) (Siegele et al. 1999). Ion beams with an ME/q^2 of up to 100 can be focused with the HIMP within spot sizes down to 3 μm , thus providing sufficient current for conducting of various ion beam analytical techniques. The samples were analyzed using a 3 MeV proton beam with a typical spot size of 5 μm . At this spot size beam currents between 0.1 and 0.5 nA can be achieved, which is sufficient for PIXE analysis. The X-rays were measured by a high-purity Ge detector. A 100 μm Mylar foil was used to reduce low energy X-rays and thus pile-up in the micro-PIXE spectrum. The elemental maps and the analysis of micro-PIXE spectra were performed using GeoPIXE II software package (Chris Ryan, CSIRO).

2.3 Determination of Hg concentrations in roots

Mercury concentrations were measured by AFS (Millennium Merlin, PSA, England) in roots of *C. zizanioides* for all treatments. Concentrated nitric acid (5 mL) was used in the digestion of the corresponding plant material (0.2 g) (Cavallini et al. 1999). Prior to digestion plant material was dried at 60°C for 48 h. Hg concentrations are reported on a dry weight basis. It has been demonstrated experimentally that the loss of mercury at this temperature as a result of volatilization is insignificant (Lomonte et. al., 2011, Moreno et al., 2005b; Han et al., 2006). The significance of differences among treatment means was determined by one-way ANOVA. Comparisons among means were performed using Duncan's test ($P \leq 0.05$).

2.4 Microscopy

Prior to micro-PIXE analysis, light dissection microscopy was employed and photographs were taken to check the quality of samples loaded on the carbon tab. In order to examine the detailed cell

structure, cross-sections of fresh roots were examined and photographed under a microscope by using bright field or ultraviolet (UV) light. Toluidine blue (0.02%, 1 min) was used to stain the sections to achieve better contrast of cell structure and Nile Red (40 mg L⁻¹, 1h) was used to highlight red lipidic substances in plant cell walls.

3. Results and Discussion

3.1 Mercury accumulation

Table 1 confirms that the concentrations of Hg accumulated in roots of *C. zizanioides* were significantly different for the control and the three Hg treatments. The chelators added increased the Hg uptake into plant roots, which has already been demonstrated by other authors (Moreno et al. 2005 a & b, Moreno et al. 2004, Lomonte et al. 2011). In addition, KI induced a slightly greater Hg uptake (20% higher) than (NH₄)₂S₂O₃ when added to the Hg treatment. No symptoms of reduced growth or necrosis caused by Hg were observed during the period of the present study.

3.2 Light microscopy

The bright field light microscopy photograph of a *C. zizanioides* root cross-section (Fig. 1a) grown in nutrient solution with Hg and stained with toluidine blue identified the internal arrangement of the root tissues. This image displayed a schizogenous aerenchymatous cortex and well-developed xylem. In addition, the UV microscopy photograph (Fig. 1b) of a quarter of root cross-section stained with Nile Red highlighted in red the lipidic substances in the root tissues, such as suberin and essential oils. Fig. 1b showed that these lipidic substances were contained in the exodermis, cortical fibre and endodermis and also in the phloem and sclerenchyma fibre of the vascular cylinder. *C. zizanioides* roots contain high concentrations of the essential oil vetiverin (Wong 2003, Greenfield 2002, Nix et al. 2006).

3.3 Spatial distribution of Hg in roots

The *in situ* micro-PIXE distribution maps of Hg (HgL), K and Ca in the roots of *C. zizanioides*

without Hg treatment are shown in Fig 2. As expected, Hg was not present in the root cross-section of the control, the micro-PIXE images of the macro elements K and Ca were chosen to indicate the structure of the root cross-section. The presence of K and Ca in all samples is an indication that cell content has been retained at least to the cellular level, and that the sample preparation methodology is effective for the purposes of this investigation.

Considering the light micrographs in Fig. 1 as a reference for the root tissues arrangement, Hg appeared to be mainly localized in the outside zone of the *C. zizanioides* roots, particularly in the epidermis and the exodermis, as a result of the Hg treatment (Fig. 3 b, e and f). The epidermis and exodermis contain suberin, a waxy substance (Hose et al. 2001), and cortical cells which are known to contain essential oils, definitely present in the roots of *C. zizanioides* (Nix et al. 2006). The concentration of Hg in these root tissues might be explained with the fact that Hg binds lipidic substances (Beauford et al. 1977, Girault et al. 1995), therefore preventing significant translocation of the metal to the aerial parts of the plants. In addition, Hg acts as an efficient blocker of most aquaporins (water channel proteins) binding to the thiol group (-SH) of cysteine residues located in the vicinity of the aqueous pores, thereby blocking water permeation (Javot and Maurel 2002). Hg, as other metals, could be translocated to the above-ground tissues following the same pathway as that of nutrients present in solution. Therefore, it was suggested that Hg translocation was reduced because of the strong bond between this metal and cysteine thiol groups in roots, which resulted in blocking the transport of nutrients into the vascular tissue and consequently the supply to the aerial parts of the plants. This also might explain why Hg was not found to be concentrated in the root vascular bundle.

Figure 3 also shows that the Ca distribution map of *C. zizanioides* roots resembles qualitatively the corresponding Hg distribution (b, d, f and h). In fact, both Ca and Hg were found mainly in the outer tissues of the root suggesting a possible correlation between the transport of Ca and Hg from the nutrient solution to the internal parts of the roots. In support of this, it has already been hypothesized that Ca channels could partly contribute to Hg uptake as previously observed for Cd

(Esteban et al. 2008). In addition, the Ca and K maps of the controls appeared to be similar to the corresponding maps of the Hg-treated roots, suggesting that the Hg treatment did not produce any major effects on the uptake of these macro-nutrients.

Figures 4 and 5 show the effects of $(\text{NH}_4)_2\text{S}_2\text{O}_3$ and KI on the Hg distribution in the root cross-sections studied. In both cases Hg was mainly localized in the outside zone of the roots, particularly in the epidermis and the exodermis, which was similar to the Hg treatment without chelators. However, chelator treatments increased Hg localization in the vascular bundles; significant peaks of Hg were detected in the vascular bundle of the roots (Figs 3-5, b and f). A possible explanation for these results might be found in the different transport mechanisms of Hg alone and of its $\text{S}_2\text{O}_3^{2-}$ or I^- complexes. As previously suggested, Hg alone might strongly bind cysteine residues in the root epidermis and exodermis thus making its further movement into the vascular bundle and the consequent translocation to the above-ground tissues less likely. However, when Hg is complexed with $\text{S}_2\text{O}_3^{2-}$ or I^- , it was less likely to bind cysteine residues which could impede its transport from the root outside zone into the vascular tissues. This might explain the results of previous studies where the use of ammonium thiosulphate facilitated the translocation of Hg to the above-ground tissues of *Phaseolus vulgaris* (bush bean), *Br. juncea* (Indian mustard) and *V. villosa* (hairy vetch) (Moreno et al. 2005 a & b) and KI increased phytoextraction of the metal in *Salix* spp. (Wang and Greger 2006). Furthermore, the results shown in Figs 3-5 indicate higher concentrations of total Hg in the roots of *C. zizanioides* when treated with both chelators (Table 1) in comparison with the corresponding concentrations in roots treated with Hg only.

In order to have a better understanding of what might affect the Hg distribution in roots when $(\text{NH}_4)_2\text{S}_2\text{O}_3$ or KI were used, the distribution profiles of two macro-nutrients, K and Ca, obtained from the micro-PIXE analysis across a transverse area were considered (Figs 4 and 5). The profiles of both nutrients derived from treatment with Hg or with Hg + ammonium thiosulphate were similar, suggesting that transport of these nutrients was not affected by the presence of $(\text{NH}_4)_2\text{S}_2\text{O}_3$ (Figs 3b and 5b) which increased the Hg translocation into the vascular tissue. In comparison, the

distribution profiles of both K and Ca observed with the KI treatment (Figs 3b and 4b) showed significant differences. In this case most of the K was transported and concentrated in the vascular cylinder of the roots and it was almost absent in the cortex. In comparison with the other two treatments Ca was distributed more uniformly in all cortex and a very small portion of it was also found in the vascular tissue. In the other two treatments Ca was present mainly in the cortex. This suggests a different pathway of these macro-nutrients when KI was added as Hg chelator to the hydroponic solution.

4. Conclusions

Our study has demonstrated the capabilities of micro-PIXE in the localization of Hg in root tissues of a Hg-accumulating species. The micro-PIXE results have shown that Hg accumulated by *C. zizanioides* via root uptake is mainly present in the root epidermis and exodermis and its translocation to the aerial parts is insignificant. The finding that chelators ((NH₄)₂S₂O₃ and KI) additions increase the Hg distribution into the vascular bundles supports and likely explains the increase in Hg translocation when Hg phytoextraction is chemically induced. Further detailed studies on *C. zizanioides* are needed to fully understand the pathway of Hg uptake and its translocation processes, which will involve investigating the sub-cellular localization and biochemical transformation during metal accumulation.

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Fig. 1. Bright field light (a) and UV (b) microscopy photographs of fresh root cross-section of *C. zizanioides* grown in Hg nutrient solution and stained with (a) toluidine blue to show the internal tissue arrangement and (b) Nile Red to highlight red the lipidic substances in the root tissues (1 epidermis, 2 exodermis, 3 cortical fiber, 4 endodermis, 5 phloem, 6 xylem, 7 sclerenchyma fiber, 8 pith, 9 cortex and 10 vascular bundle). (See the online edition for a color version of this figure.)

Fig. 2: Dissection microscopy photograph (M) and micro-PIXE maps of Hg (HgL), K and Ca of freeze-dried root cross-sections of *C. zizanioides* without Hg treatment. (See the online edition for a color version of this figure.)

Fig. 3: A light microscopic image (a), micro-PIXE maps of Hg, K and Ca (b - d), a 2-element (Hg-K) micro-PIXE map (e) and qualitative Hg, K and Ca distribution profiles (f - h) of a cross-section of cryofixed and freeze-dried roots of *C. zizanioides* treated with Hg for 10 days. Qualitative distribution profiles were derived from the micro-PIXE analysis across the typical transverse area outlined in green on the corresponding maps. (See the online edition for a color version of this figure.)

Fig. 4: A light microscopic image (a), micro-PIXE maps of Hg, K and Ca (b - d), a 2-element (Hg-K) micro-PIXE map (e) and qualitative Hg, K and Ca distribution profiles (f - h) of a cross-section of cryofixed and freeze-dried roots of *C. zizanioides* treated with Hg and KI for 10 days. Qualitative distribution profiles were derived from the micro-PIXE analysis across the typical transverse area outlined in green on the corresponding maps. (See the online edition for a color version of this figure.)

Fig. 5: A light microscopic image (a), micro-PIXE maps of Hg, K and Ca (b - d), a 2-element (Hg-K) micro-PIXE map (e) and qualitative Hg, K and Ca distribution profiles (f - h) of a cross-section of cryofixed and freeze-dried roots of *C. zizanioides* treated with Hg and $(\text{NH}_4)_2\text{S}_2\text{O}_3$ for 10 days. Qualitative distribution profiles were derived from the micro-PIXE analysis across the typical transverse area outlined in green on the corresponding maps. (See the online edition for a color version of this figure.)

Table 1: Concentration of Hg in roots of *C. zizanioides* with or without a 10-day Hg treatment (n=3, $\pm$ SD, Different letters denote significant difference at the 0.05 probability level).

	Treatment			
	Control	Hg	Hg + (NH ₄) ₂ S ₂ O ₃	Hg + KI
Hg concentration (mg g⁻¹ dry wt.)	(2.16 $\pm$ 0.88) $\times 10^{-5}$ ^d	0.67 $\pm$ 0.21 ^c	1.09 $\pm$ 0.08 ^b	1.31 $\pm$ 0.13 ^a