Rhizostabilization of a mine tailing highly contaminated: Previous study of Cd localization and speciation in *Anthyllis vulneraria*

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**Abstract.** The plant *Anthyllis vulneraria* has been identified in mine tailings and the aim of this study is to determine the mechanisms developed by the plant and its symbiotic association *Mesorhizobium metallidurans* to tolerate Cd. We particulary intend to determine the distribution and speciation of Cd in plant using a combination of μXRF (X-ray fluorescence) and Cd XANES and μXANES (X-ray Absorption Near Edge Structure).

**Key words:** Rhizostabilisation, *Anthyllis vulneraria*, Cadmium (Cd), Localization, Speciation

**Introduction**

Mine tailings are highly metal contaminated areas, which are strongly affected by intensive rain and wind erosion and thus represent a source of environmental and health hazard. Due to their large surface and high level of contamination, conventional remediation techniques are not appropriate, and phytostabilization has emerged as an alternative technique during the last decade (Arthur et al., 2005). In this technique, metal tolerant plants are used to reduce the metal mobility and to prevent the migration of contaminants to groundwater or air, and their entry in the food chain. In this context, it is necessary to find plants that tolerate metals, and in a longer term would serve as a direct or indirect source of nutrients in low organic mining soils, thus facilitating the installation of other species. This latter condition can be satisfied by legumes, which are associated in root to rhizobium nodules able to fix atmospheric nitrogen, and thus act as pioneer species. The legume *Anthyllis vulneraria* (subsp carpatica) was identified in a highly contaminated mine tailing from South of France (les Avinières mine) contaminated with 161 000 ppm Zn and 1382 ppm Cd (Frérot et al., 2006). Interestingly, field experiments conducted in 2002 on the site showed that *A. vulneraria* progressively improved the biomass of grass species (Festuca) growing in its vicinity, thus promoting the revegetalization of the site (Frérot et al., 2006). Our general project focuses on the mechanisms developed by the symbiotic association *Anthyllis vulneraria-Mesorhizobium metallidurans* to tolerate Cd. A compartmentalization of the metal in specific organs/tissues as well as a binding with specific ligands may be a mechanism involved in metal tolerance. Hence, the specific objective of this present work is to determine Cd distribution and the ligands binding Cd in the leaves, roots of *A. vulneraria* as well as in its rhizobium nodules.

Micro X-ray absorption spectroscopy (μXAS) is a synchrotron based technique that has demonstrated great potential for the study of the chemical form of toxic trace metals in biological samples. Indeed, it is chemically selective, and the limits of detection are about ten mg kg⁻¹ of metal. In this work, XANES (X-ray Absorption Near Edge Structure) and μXANES were applied to probe chemical and structural environment of Cd at the bulk and micrometer scales. Finally μX-ray Fluorescence (μXRF) was used to determine elemental distributions and associations.

**Materials and Methods**

*Anthyllis vulneraria* originated from Les Avinières were germinated and grown in hydroponics. *Anthyllis
population was well described by Frérot et al. (2006) and Mahieu et al. (2011). Plants were inoculated with *Mesorhizobium metallidurans*. After nodulation, plants were exposed during four weeks to Cd present in two kind of substrate: (i) hydroponic solutions containing 10 or 70 µM Cd, and (ii) soil from Les Avinières (50%) mixed by attapulgite (50%) and 70 µM Cd. The addition of attapulgite was done in order to promote the nodulation. For both culture, aerial parts of plants were harvested and rinsed, roots and nodules were rinsed with a solution of CaCl₂ (5mM) and MilliQ Water and then separated.

**Cd concentrations**

For both experiments, one part of leaves and one parts of roots were oven-dried at 50°C, ground and homogenised. Aliquots were digested in 1:1 volume H₂O₂ and HNO₃ and filtered before analysis by ICP-MS (inductively coupled plasma – mass spectrometry). Quality control for plant samples was based on the use of certified standard samples (spinach leaves : SRM 1570a).

**Cd localization and speciation**

For both experiments, the other parts of leaves, and roots of *Anthyllis vulneraria* were frozen, ground in liquid nitrogen and prepared as frozen pressed pellets. Then, leaves and roots were analyzed by XANES as bulk samples. The other part of vegetal material frozen in liquid nitrogen was prepared as cross-sections using a cryomicrotome to investigate specific tissues of leaves (epidermis, mesophyll, veins), rhizobium nodules, and roots (xylem/phloem, endoderm, cortex, epidermis). Cd, S, and P distribution were studied in these cross-sections using μXRF. Then, Cd ligands were determined by Cd LIII-edge μXANES recorded on various tissues of storage evidenced by μXRF. The XANES and μXANES spectra were then compared to spectra of model-compounds including Cd minerals, Cd-sorbed minerals and Cd-complexed organic, already collected (Isaure et al., 2006, 2010), and fitted by linear combinations of these reference spectra. Chemical mapping and spectroscopy were performed on the beamline LUCIA (Soleil, Saclay, France) in cryogenic conditions to limit artefact from element redistribution and speciation (Soleil, Saclay, France) in cryogenic conditions to limit spectroscopy were performed on the beamline LUCIA (Soleil, Saclay, France) in cryogenic conditions to limit artefact from element redistribution and speciation (Soleil, Saclay, France) in cryogenic conditions to limit artefact from element redistribution and speciation.

**Results and Discussion**

**Cd concentrations**

Cd concentrations in roots and leaves of *Anthyllis* grown on hydroponics were presented in table 1. As expected, in hydroponics, Cd concentration in roots and shoots increased with Cd exposure (around 1200 mg Cd kg⁻¹ dry weight (DW) and 580 mg kg⁻¹ DW in roots for 70 and 10 µM Cd, respectively, and 270 and 30 mg Cd kg⁻¹ DW in shoots, respectively) but interestingly, no acute toxicity sign in the plant physiology was observed. Cd concentrations measured in leaves and roots of plants grown on the soil from Les Avinières mixed with attapulgite and 70 µM of Cd were intermediate and plants were healthy too. By comparison, Mahieu et al. (2011) measured 30.6 ± 5.5 mg Cd kg⁻¹ DW in aerial parts of *Anthyllis vulneraria* inoculated by the same *Mesorhizobium metallidurans* grown in pot filled with soil from Les Avinières. Although the soil from Les Avinières was very contaminated (around 1200 and 165 000 ppm for Cd and Zn respectively), Cd and Zn are less phytoavailable than in hydroponics.

<table>
<thead>
<tr>
<th>Type of substrate and content in Cd</th>
<th>Cd concentrations (mg kg⁻¹ DW) in leaves</th>
<th>Cd concentrations (mg kg⁻¹ DW) in roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydroponic solution with :</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM Cd</td>
<td>30 ± 2</td>
<td>580 ± 26</td>
</tr>
<tr>
<td>70 µM Cd</td>
<td>278 ± 26</td>
<td>1236 ± 79</td>
</tr>
<tr>
<td>soil Les Avinières + attapulgite + 70µM Cd</td>
<td>108</td>
<td>688</td>
</tr>
</tbody>
</table>

**Cd speciation and localization in *Anthyllis vulneraria***

Figure 1 shows the XANES spectra recorded on plant samples and on two Cd references: Cd-malate, as a representative of Cd-COOH/OH group with Cd-O/N bonds, and Cd-cysteine as a representative of Cd-thiols composed of Cd-S bonds. On Cd-malate spectrum, the first peak is typical of Cd-O ligands. Cd LIII-edge XANES measurements performed on bulk leaves and roots for the 10 µM Cd treatment showed this peak. Thus, by comparison with reference spectra, Cd was mainly bound to O/N ligands in these samples. On the contrary, S ligands seem dominant for plant samples exposed to the 70 µM Cd (Fig. 1). Cd exposure seemed to play a role in Cd speciation in leaves and roots samples. Verbruggen et al. (2009) reported that most of the hyperaccumulated metals are bound to ligands, such as organic acids, amino acids, peptides and proteins. Ligands depend on the metal, the function and the age of the plant tissue (Küpper et al., 2004). In mature and senescent leaves of *Thlaspi caerulescens*, oxygen ligands dominated and a fraction of the foliar Cd was bound to sulphur ligands. The detoxification of Cd by chelation in plants could involve thiol ligands such as glutathione, phytochelatines and metallothioneins (Verbruggen et al., 2009). Our results could highlight a probable toxicity in *Anthyllis vulneraria* exposed to 70 µM Cd and suggest a modification of the Cd sequestration form.

At the tissue scale, Cd was mainly bound to S in vascular bundles and in the nodules for the 10 µM treatment (Fig. 1). In leaf, Cd seems to be transported as Cd-S ligand, and possibly stored as Cd-O ligand in the
Fig. 1. Cd LIII edge XANES spectra collected on bulk leaves and roots from A. vulneraria inoculated by rhizobium grown on 10 or 70 µM Cd and Cd LIII-edge μXANES spectra collected in Cd enriched area of nodules and of vascular system of leaf of plant grown on 10 µM Cd (cf Fig. 2), compared to Cd references (Cd-malate and Cd-cysteine).

mesophyll. Will it be the same for 70 µM treatment? To answer, work is in progress.

At leaf scale, in the central vein, Cd was colocalized with P and S (Fig. 2) and Cd was mainly in vascular bound regardless the culture substrate (hydroponics or soil mixed with attapulgite and with 70 µM Cd by hydroponic – maps not shown). In addition, Cd was found in trichomes and cells of epiderm (maps not shown). To our knowledge, there is no studies that report Cd localization at leaf scale in Anthyllis vulneraria, but there are data for other plants. In A. halleri, Cd is mostly accumulated in mesophyll cells (Kupper et al., 2000; Zhao et al., 2000). In T. praecox (Vogel-Mikus et al., 2008), Cd is more concentrated in the epidermis, but the mesophyll is still the major storage compartment due to its larger volume (Vogel-Mikus et al., 2008). Enrichments in Cd were observed for A. halleri ssp. gemmifera (Hokura et al., 2006; Fukuda et al., 2008) and A. thaliana (Isaure et al., 2006). In leaf, Cd storage sites seem depend on plant species.

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References


relation to other elements in the hyperaccumulator 


