ESRF	**Experiment title:** Cd sequestration by the plant *Anthyllis vulneraria* from the field to lab cultures.	**Experiment number**: EV-28
Beamline: ID21	**Date of experiment**: from: 04/04/2013 to: 09/04/2013	**Date of report**: 30/08/2013
Shifts: 15	**Local contact(s)**: Hiram Castillo-Michel	*Received at ESRF:*

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Objective and expected results

The legume plant *Anthyllis vulneraria* has been revealed as a pionner plant to revegetalize mining sites, and the aim of our general project is to understand the mechanisms developped by the plant to cope with Zn and Cd toxicity in a context of phytostabilization. The aim of this experiment was to determine the mechanisms developped by the symbiotic association *Anthyllis vulneraria – Mezorhizobium metallidurans* to tolerate Cd and particularly to compare plants grown in controlled conditions and on the field.

Chemical analyses showed that the inoculation of *A. vulneraria* with metallicolous (MET) and non-metallicolous (N-MET) rhizobium strains decreased the metal concentration in leaves compared to the non-inoculated plant. Our objectives were to determine if specific tissues concentrated Cd depending on the inoculation. Particularly, we intented to specify if the storage of Cd in the rhizobium nodules varied with the strain and if the rhizobium strain impacted the distribution of Cd in nodules and roots but also in leaves, as well as the mechanisms of Cd binding. During our last experiment (EC-1050) we investigated roots and nodules of *A. vulneraria* grown in various conditions of bacterial inoculation (MET and N-MET rhizobium strains). We found that Cd was located in the middle of the rhizobium nodules, in cells infested by the bacteria, and that this distibution was similar for both rhizobium strains. We also found that for the inoculated roots, Cd was mainly distributed in the endodermis and the cortex whereas it was less concentrated in the vascular bundles. This distribution was similar for roots inoculated with the MET and N-MET rhizobium. This experiment was a continuation of the experiment EC-1050, and we investigated roots from non-inoculated plant and leaves. We especially expected to highlight the role of inoculation for Cd distribution in roots. For that purpose, we used a combination of chemical mapping using X-ray Fluorescence (μXRF) and X-ray Absorption Near Edge Structure spectroscopy (XANES and μXANES) at Cd L_{III}-edge.

Results and and the conclusions of the study

Anthyllis vulneraria were grown in hydroponics with 10 μM Cd, with inoculation or not with rhizobium from MET and N-MET strains) during 4 weeks. *A. vulneraria* was collected from the field in a mine tailing from South of France. Roots and leaves were collected and prepared as cryo cross-sections using a cryo-

microtome. The beam was focused with KB mirrors and the beamsize on the sample was 1 µm (H) x 0.6 µm (V). Cd distribution was studied by µXRF at 3.57 KeV collecting the fluorescence with a SDD detector. The Cd species were determined by Cd LIII-edge µXANES recorded on points of interest from the maps using the same lateral resolution. All measurements were performed at -170°C using a cryostat. µXRF data were treated using PYMCA software and µXANES spectra using Athena software. The µXANES spectra were then compared to spectra of Cd model-compounds and fitted by linear combinations of these reference spectra.

In roots of non-inoculated plants, Cd was mainly in vascular bundles and more concentrated in epidermis (Figure 1). Cd distribution was investigated in several roots of non-inoculated plant and there was no difference in this distribution. In the experiment EC-1050 focused on roots of inoculated plants, we found that Cd was mainly in the endodermis and cortex. Thus, Cd distribution differs in roots depending on the inoculation, which is an important result.

In Cd-enriched areas of root of non-inoculated plant, Cd was mainly bound to S ligands (Figure 2). There was no significant difference of S ligand proportion for all the investigated compartments of root *i.e.* epidermis and vascular bundles. However, Cd speciation in vascular bundles of non-inoculated seems to slightly differ from inoculated plant since the S ligand proportion was higher for inoculated plants.

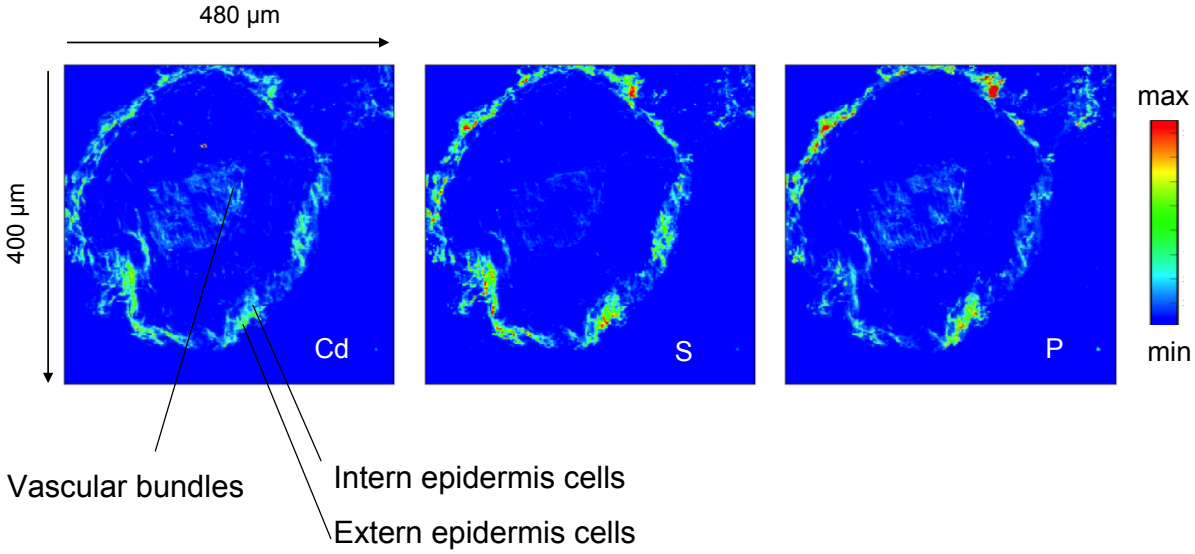

Figure 1: Elemental mapping of a root cross-section *A. vulneraria* non inoculated recorded by µXRF for Cd, P, S at 3.57 KeV. The step-size of elemental maps was 2µm and the counting time was 0.3s/pixel.

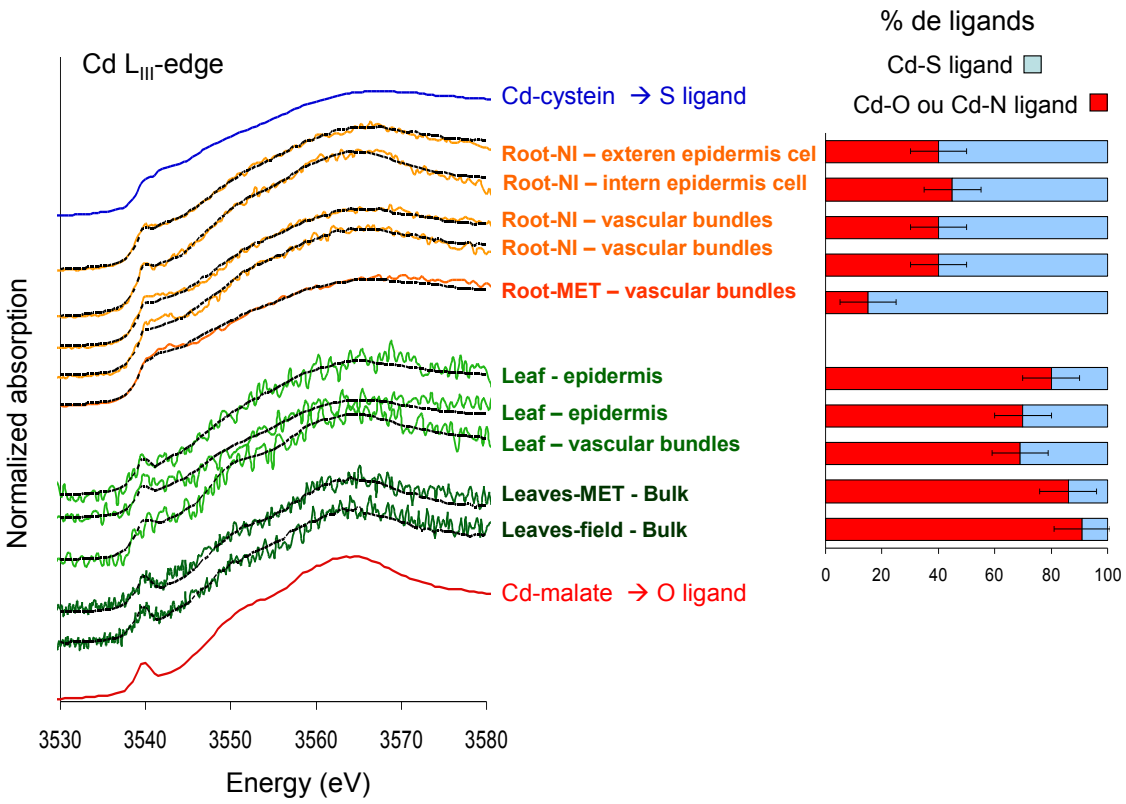

Figure 2: Cd L_{III} –edge XANES spectra collected on bulk leaves from *A. vulneraria* from the field or from hydroponics culture (inoculated with MET rhizobium strains and exposed to 10 µM Cd) and Cd L_{III}-edge µXANES spectra collected in various regions of interest from roots and leaves for non-inoculated (NI) and inoculated plants. Experimental spectra were compared to Cd references: Cd-malate as representative of Cd-COOH/OH group with Cd-O/N bonds and Cd-cysteine as representative of Cd-thiols composed of Cd-S ligands. Each spectrum (colored lines) is shown with its linear combination fit (black lines). Distribution of Cd species are presented for the samples after normalization of the percentages to 100%. The uncertainty is estimated to ±10%.

In leaves of *A. vulneraria* inoculated with MET rhizobium and exposed to 10 µM Cd, µXRF mapping showed that Cd was preferentially located in the vascular system and in the epidermis whilst it was less concentrated in the mesophyll (Figure 3). This distribution was the same for S while it was more homogeneous for P. For plants from the field, a similar distribution was evidenced (not shown). The Cd ligands were also similar in bulk leaves from the field and from the hydroponic culture: the metal was predominantly associated to O/N atoms while the thiol groups were very minor. When focusing the beam on the most Cd-enriched tissues of the leaves (epidermis and veins), µXANES spectra were very noisy especially in vascular bundles (Figure 3). Although the O/N ligands seemed to be dominant in epidermis it was unclear for the vascular bundles.

As a conclusion, thanks to this experiment, we showed that the inoculation with rhizobium impacted the Cd distribution in *A. vulneraria* roots. We also found that the metal distribution in leaves was similar in hydroponics and in the field. Concerning the Cd speciation, it was found that Cd was preferentially associated to thiol groups in the various tissues from roots, whereas O/N atoms were the main ligands in leaves. In parallel, we collected EXAFS data on bulk organs (roots, leaves and nodules) and data treatment is in progress.

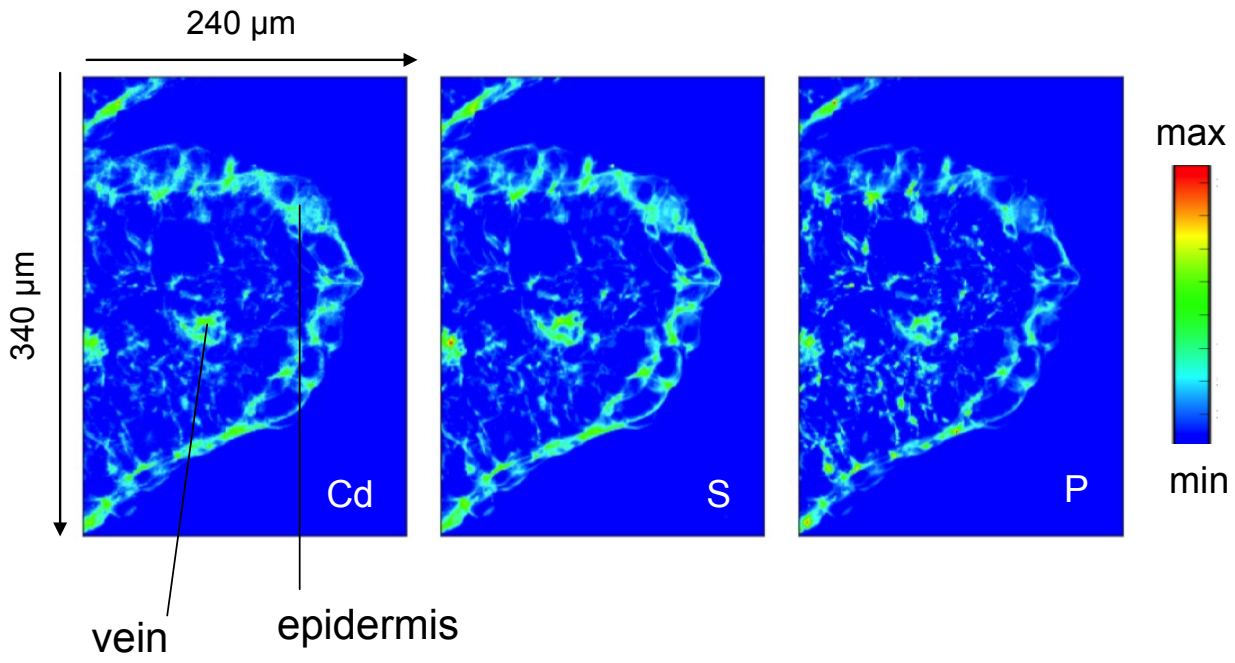

Figure 3: Elemental maps recorded by µXRF for Cd, S and P of a leaf cross-section of *A. vulneraria* inoculated with MET rhizobium strain grown on 10µM Cd. The step-size was 1µm and the counting time was 0.25s/pixel.

Justification and comments about the use of beam time (5 lines max) :
One shift was used for beam aligment and chamber cooling. 9 shifts were dedicated to XRF mapping on roots and leaves and 5 shifts were used for µXANES and bulk XANES.

Publications :
Huguet S., Soussou S., Clayet-Marel J.C., Proux O., Castillo-Michel H., Trcera N. and Isaure M.P. **Poster** at the 4th International Symposiulm of Metallomics® – Oviedo (Spain) – july 2103.