ESRF	**Experiment title:** Symbiotic association *Anthyllis vulneraria/Mesorhizobium metallidurans* for phytostabilization : resistance to Cd.	**Experiment number**: EC-1050
Beamline: ID21	**Date of experiment**: from: 24/01/2013 to: 29/01/2013	**Date of report**: 29/08/2013
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 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 our experiment was to determine the mechanisms developped by the symbiotic association *Anthyllis vulneraria – Mezorhizobium metallidurans* to tolerate Cd and specially to clarify the role of *rhizobium* in the storage of metals.

Recently, we have found 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. The aim of this proposal was to specify if the storage of Cd in the rhizobium nodules varied depending on the rhizobium strain and if this rhizobium strain impacted the distribution of Cd in nodules and plant organs, as well as the mechanisms of Cd binding. 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) using Cd L_{III}-edge. During this experiment, we investigated roots and nodules of *A. vulneraria* grown in various conditions of bacterial inoculation (metallicolous (MET) ou non-metallicolous (N-MET) strains).

Results and and the conclusions of the study

Anthyllis vulneraria were grown in hydroponics with 10 µM Cd during 4 weeks. Nodules and roots were collected and prepared as thin-sections using a cryo-microtome. Cd distribution was studied by µXRF at 3.57 KeV, K and Ca distribution at 4.1 KeV and chemical ligands were determined by Cd LIII-edge µXANES recorded on points of interest. The beam size on the sample was 0.6 µm (H) x 0.2 µm (V). 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 model-compounds and fitted by linear combinations of these reference spectra.

In nodules from MET strain, Cd is mainly in the interior of nodules, more precisely in cells infected with bacteria (Figure 1). Infected cells are known to be N-fixing cells. Cd distribution was also determined in nodules from N-MET strain. There was no significant difference of Cd distribution in nodules between MET and N-MET inoculated plant.

Figure 2 shows the XANES spectra recorded on nodule 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. This peak was observed on bulk nodule spectra. Bulk analyses showed that Cd was mainly bound to O ligands in nodules and there was no significant difference between Cd speciation in nodules between MET and N-MET inoculated plant (Figure 2). However, some micro-analyses performed on the most Cd-enriched areas of nodules showed that Cd was mainly bound to S ligand (figure 2), suggesting that the Cd chemical species are multiple in nodules. To our knowledge, it's the first study that reports metal distribution and speciation in symbiotic legumes, but bulk results should be confirmed by EXAFS.

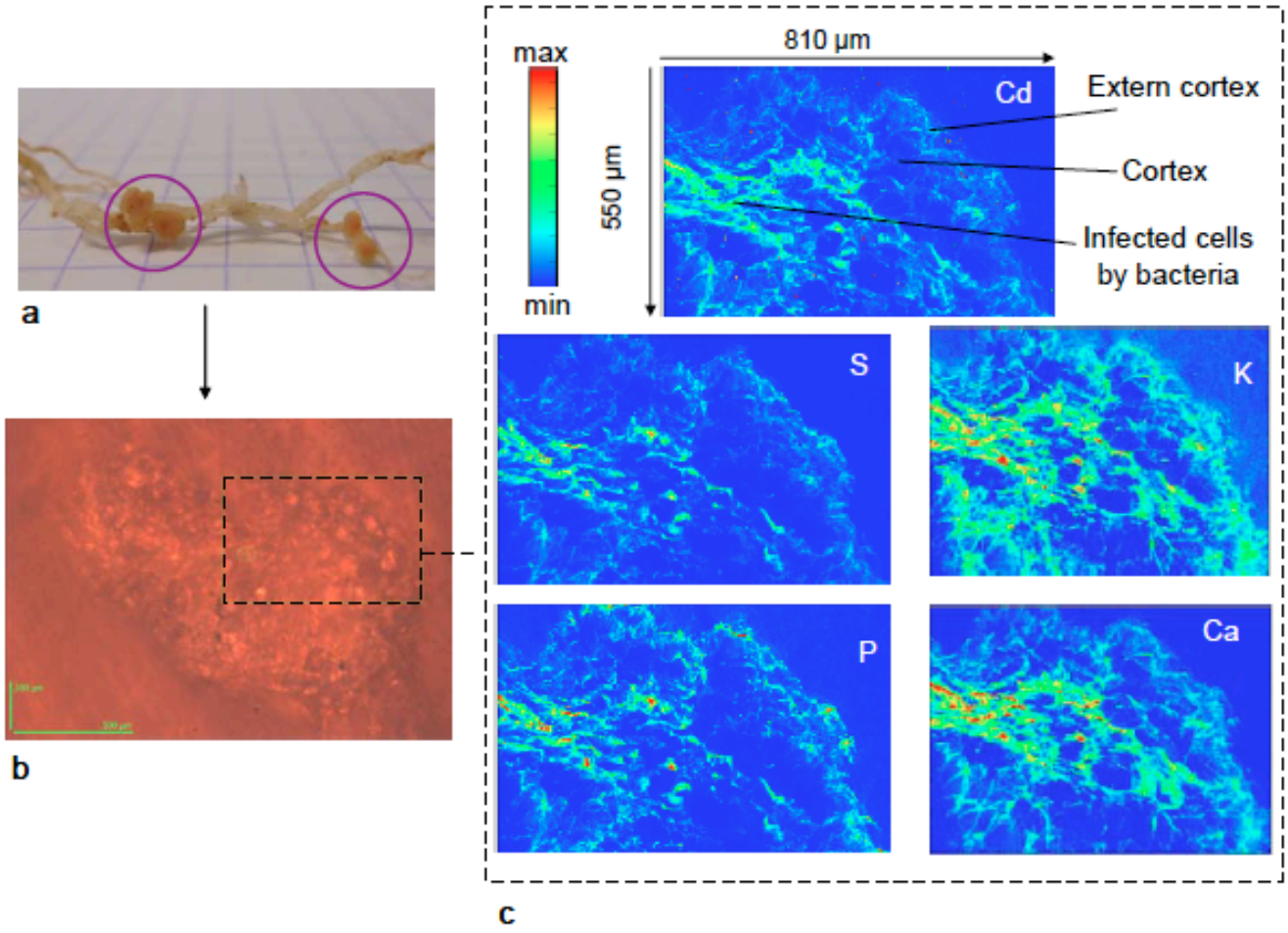

Figure 1: Photo of nodules of *A. vulneraria* inoculated with MET strain grown on 10µM Cd (a), and nodule cross-section observed by optical microscopy (b) and elemental mapping of this section recorded by µXRF for Cd, P, S K and Ca (c). The step-size of elemental maps was 3µm and the counting time was 0.5s/pixel.

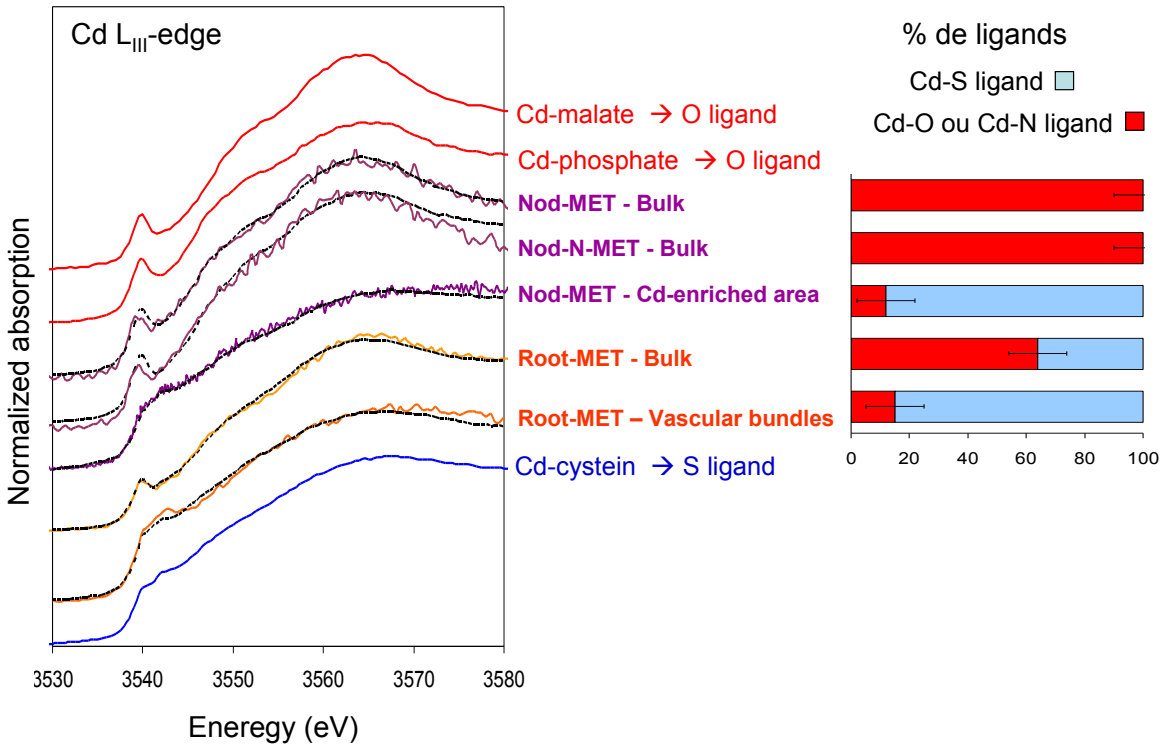

Figure 2 : Cd L_{III} –edge XANES spectra collected on bulk nodules from *A. vulneraria* inoculated by rhizobium with MET N-MET strains grown on 10 µM Cd and Cd L_{III}-edge µXANES spectra collected in Cd-enriched area of nodules and of vascular system of root, compared to Cd references : Cd-malate as 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 roots of MET inoculated plant, Cd is mainly in cortex, bundles and more concentrated in the endodermis (Figure 3). Cd distribution was investigated in several roots of N-MET inoculated plant and there was no significant difference of Cd distribution. In Cd-enriched areas of endodermis and vascular bundles, Cd was mainly bound to S ligands. The same observation was done by Salt et al. (1995) in roots of *Brassica Juncea* and they suggested that Cd could be transported as Cd-S ligand. It could be the same case in *A. vulneraria.*

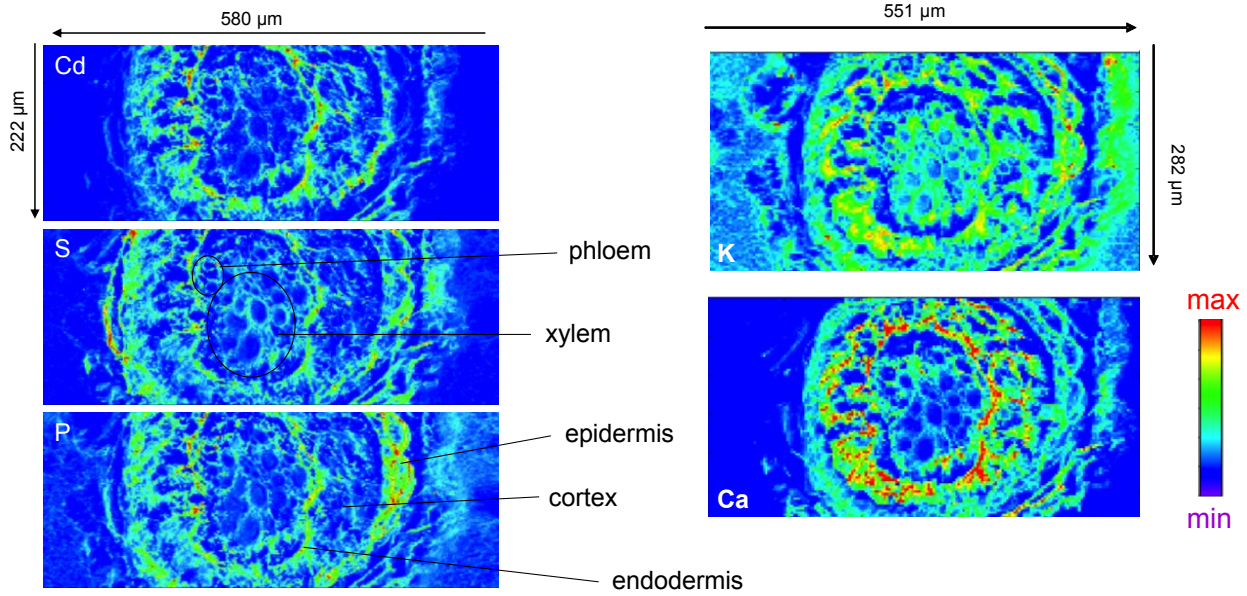

Figure 3 : Elemental maps recorded by µXRF for Cd, P, S K and Ca of a root cross-section of *A. vulneraria* inoculated with MET strain grown on 10µM Cd. The step-size was 15µm and the counting time was 1s/pixel.

Justification and comments about the use of beam time (5 lines max) :

One shift was used for beam alignment and cooling. Two shifts were dedicated to XANES on Cd-references and bulk samples. Eight shifts were used for the mapping of nodules and roots and four shifts were used for μXANES.

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) – 8-11 july 2013.