ESRF	**Experiment title:** Localization and chemical forms of Cd in the hyperaccumulating plant Arabidopsis halleri	**Experiment number**: ME 1262
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Report:

Introduction

Using metal hyperaccumulating higher plants to extract metals accumulated in soils (phytoextraction) might represent a low cost remediation strategy, but a better understanding of the mechanisms responsible for metal tolerance and hyperaccumulation is needed to develop the efficiency of this green technology. This experiment was part of a National Research Project funded by the CNRS (ECCO, 2004-2007), aiming at better understanding the mechanisms responsible for Cd tolerance and accumulation in the hyperaccumulating species *Arabidopsis halleri.* The originality of this project is to study two species presenting contrasting properties concerning metal tolerance and accumulation : *A. halleri*, the hyperaccumulating and tolerant plant, and *A. lyrata*, the non hyperaccumulating and non tolerant plant. A crossing between the two species gives a first progeny (F1), which is tolerant but not hyperaccumulating, and a second crossing between F1 and *A. lyrata* gives several back-crosses with different degrees of tolerance and accumulation. The study of both parents and progenies brings new insight on the relation between the mechanisms of Cd storage (localization and speciation) and the ability of the plant to tolerate and accumulate the metal.

The purpose of this experiment was to localize Cd in leaves (epidermis, mesophyll, veins, trichomes) and roots of both parents *A. halleri*, and *A. lyrata*, and to determine its chemical forms using Cd L_{III}-edge XANES spectroscopy. In parallel, we investigated the global speciation of Cd in the two parents and progenies in bulk samples using Cd K-edge EXAFS and XANES on FAME beamline.

Materials and Methods

A. halleri and *A. lyrata* were grown on 10 µM Cd contaminated medium in controlled conditions. Leaves and roots were collected and immersed in N_2 liquid. Others were embedded in a cryogenic protective compound (O.C.T.), immersed in N_2 liquid and prepared as 30 µm-thick thin sections using a cryomicrotome. Thin sections and frozen samples were analyzed under vacuum at -160°C using a N_2 cryostat available on ID21 beamline.

Elemental maps were collected for P, S, Cd, K, and Ca by recording the X-ray fluorescence with a Ge solid state detector (PGT). Because most of the samples contain a large amount of potassium, the K K_α (3.31 keV) overlaps the most intense Cd emission line, Cd $L_{\alpha 1}$ (3.13 keV) and two beam energies were required to map on one hand, P, S, and Cd (3.55 keV), and on the other hand K and Ca (4.10 keV). Samples were scanned with a beam size on the sample of H=0.7 µm x V=0.3 µm. Cd L_{III}-edge µ-XANES spectra were collected on points-of-interest selected from the elemental maps. Measurements were performed in fluorescence-yield

detection mode using the same Ge-detector. Sample spectra were compared to Cd- model compound spectra and treated using linear combination fitting, as presented in Isaure *et al.*, 2006.

Results

Microfluorescence maps showed that in young leaves of *A. halleri*, Cd was distributed in veins, and to a lesser extent in the mesophyll (Figure 1A). Epidermis was found not to be a Cd accumulating tissue. High concentrations of Cd were evidenced in localized rings of trichomes, as for *A. thaliana* (Isaure *et al*, 2006), possibly co-located with phosphorus (Figure 1B). Cd concentration in roots was higher than in leaves, suggesting that the translocation of the metal from roots to leaves was not very efficient. Cd was likely preferentially localized in the cortex of the roots (Figure 1C). In *A. lyrata*, Cd was also found preferentially in the veins of leaves, but not in the mesophyll nor in the trichomes contrary to *A. halleri* (Figure 2A). In roots, the metal seems homogeneously distributed, possibly in association with sulfur (Figure 2B).

Cd L_{III}-edge XANES spectroscopy enables a clear distinction between O and S ligands since O/N ligands induce a peak at about 3539 eV, as shown by the references Cd-malate, Cd-pectin, and Cd-phosphate in opposition to Cd-cysteine, which contains S-ligands (Figure 3). µXANES spectra collected on three points of interest of *A. halleri* trichomes indicate a high heterogeneity of Cd species at the micron scaled level. In the ring (points 2 and 3 in Figure 1B), Cd was preferentially associated to O ligands. However, Cd spectrum was mainly simulated by 65% Cd-pectin and 30% Cd-cysteine in point 2, whereas in point 3, Cd was found as a more mineral form, constituted of 65% Cd-phosphate and 30% of other O-ligands containing compound. Out of the ring (point 1 in Figure 1B), Cd was rather bound to S ligands (70% S ligand + 25% O ligands), in agreement with Cd and S maps displayed in Figure 1B. In the mesophyll and veins of *A. halleri*, Cd seems equivalently distributed between S and O ligands (40% Cd-pectin and 55% Cd-cysteine for mesophyll, 50% Cd-pectin and 42% Cd-cysteine for veins). On the contrary, for veins in *A. lyrata*, XANES indicated that the main part of the metal (~ 70%) is bound to S ligands and that a minor part (~ 30%) is associated to oxygen atoms. In roots of both species, 50% of Cd species was encountered in association with S ligands while the other part was associated to O groups-containing species.

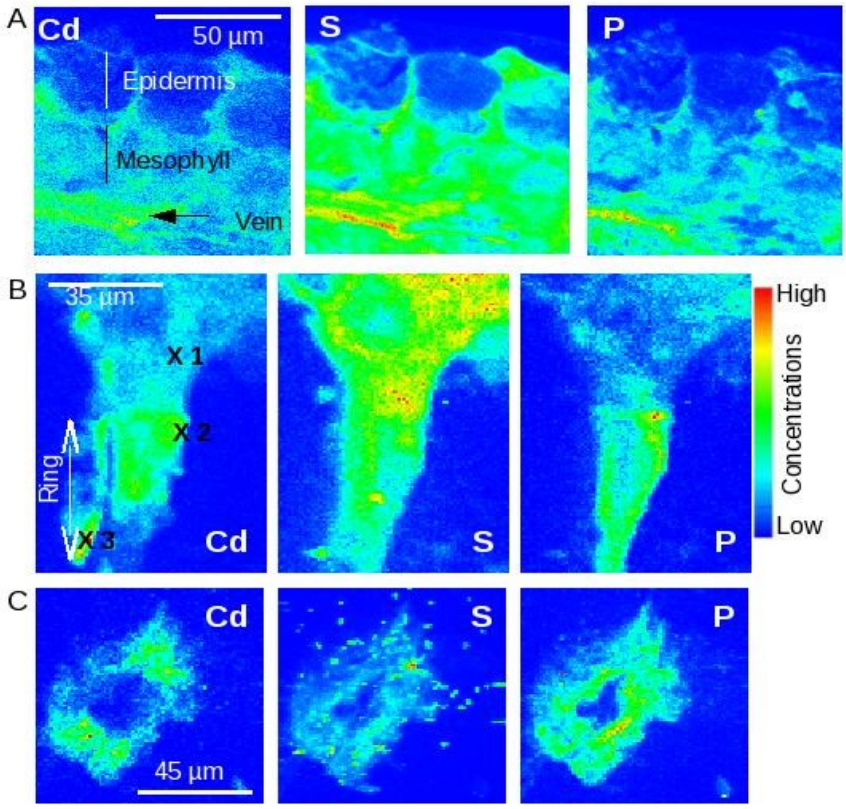
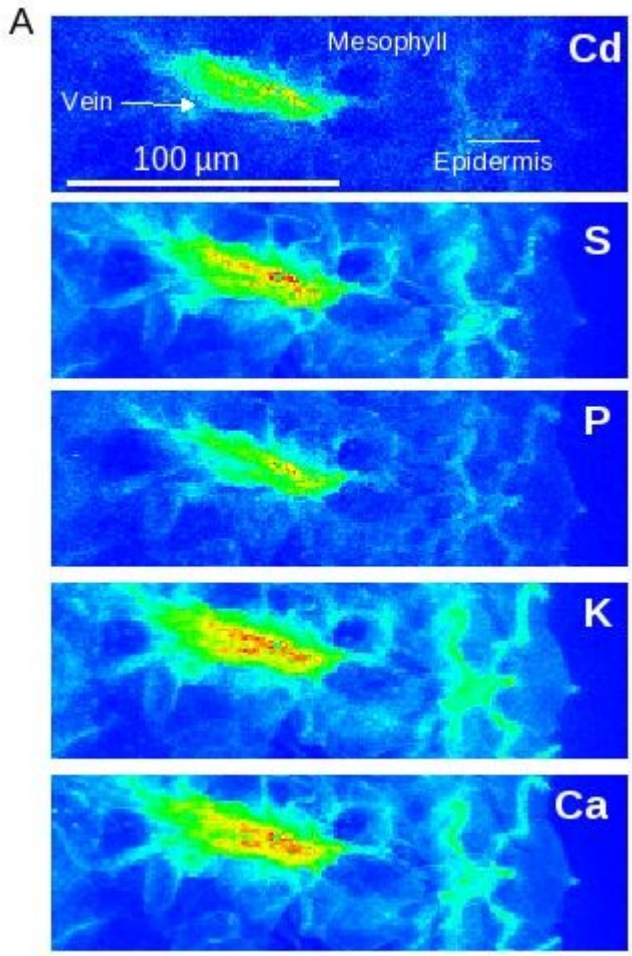


Figure 1 : False-color µ-XRF Cd, S, and P maps recorded at 3550 eV on young leaf (A), trichome (B), and root (C) of *A. halleri*.
Beam-size : H= 0.7 µm * V = 0.3 µm.
For A, step size = 0.5 µm, dwell time = 600 ms,
For B, step size = 0.8 µm, dwell time = 400 ms,
For C, step size = 1 µm, dwell time = 500 ms.



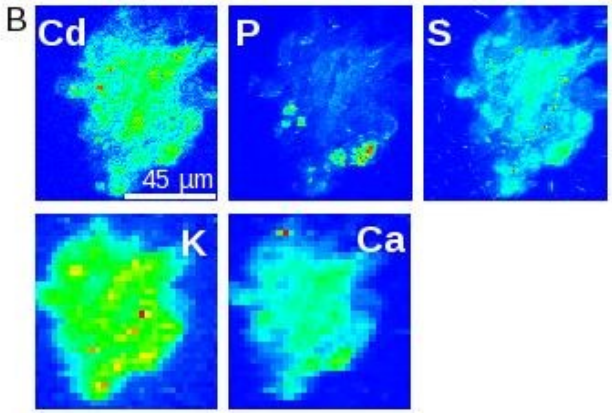


Figure 2 : False-color µ-XRF Cd, S, P, K, and Ca maps recorded on leaf (A), and root (B) of *A. lyrata*. Cd, S, and P maps were recorded at 3550 eV, and K and Ca maps were recorded at 4100 eV.
For A, for Cd, S and P, step size = 1 µm, dwell time = 500 ms, and for K, and Ca, step size = 1 µm, dwell time = 300 ms.
For B, for Cd, S and P, step size = 1 µm, dwell time = 500 ms, and for K, and Ca, step size = 3 µm, dwell time = 100 ms.

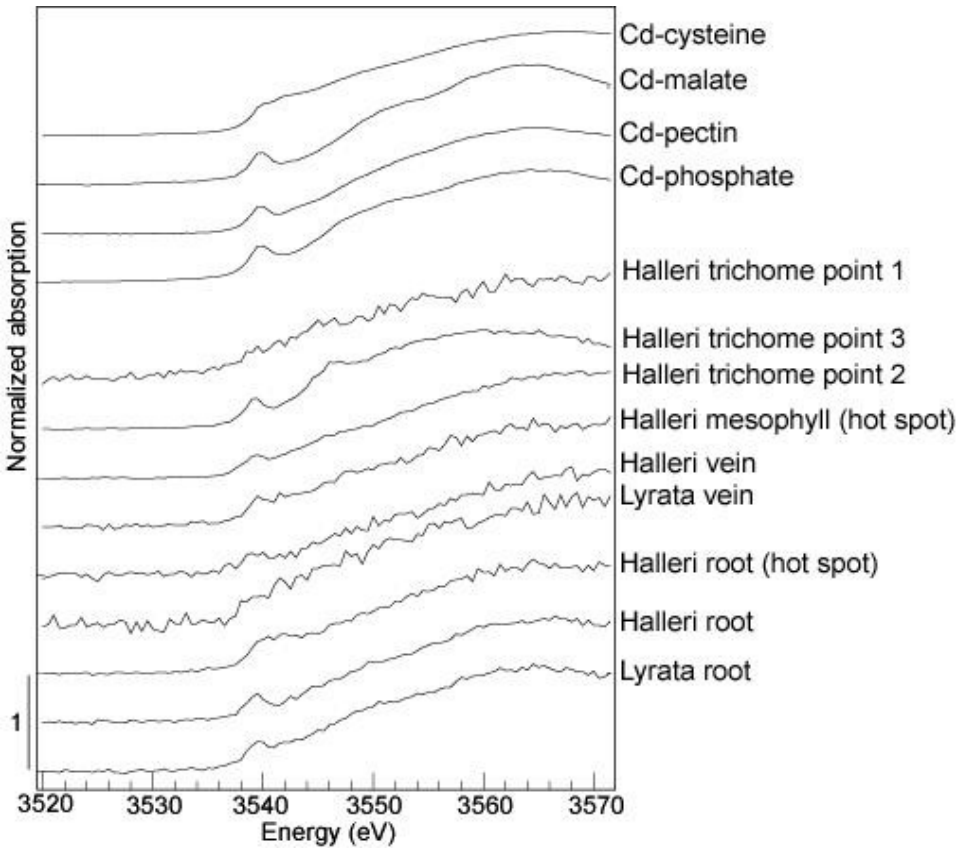


Figure 3 : Cd LIII-edge XANES spectra of four Cd model compounds (Cd-cysteine, Cd-malate, Cd-pectin, and Cd-phosphate), and of points of interest of leaves and roots from *A. halleri* and *A. lyrata*.

Results obtained on bulk samples using Cd K-edge XANES and EXAFS spectroscopy on FAME beamline (Report n°30 02 760) showed that in mature leaves of *A. halleri*, Cd was only bound to sulfur-containing groups, while a contribution of oxygen binding (~ 30%) was observed for young leaves. This is in agreement with results obtained in this experiment for young leaves, and suggest that the trichomes, in which Cd is associated to O ligands, constitute a minor compartment of Cd storage in leaves. Interestingly, we showed in this project that the mechanism of Cd storage in *A. halleri* is different from Cd storage in *Thlaspi caerulecens*, a more Cd accumulating species, where Cd is bound to O/N groups (Kupper et al., 2004). In *A. lyrata* we showed that Cd was essentially associated to S-containing groups, corroborating µXANES results obtained on veins. S-ligands were generally detected as the dominant ligands in F1, and tolerant and sensible progenies.

Conclusions

Combination of synchrotron techniques using focused beam (ID21) and not focused beam (FAME) showed that Cd was essentially bound to S ligands in the leaves of *A. halleri*, *A. lyrata* and progenies with different Cd tolerance and accumulation. These results suggest that Cd speciation would not be related to the character of tolerance and/or accumulation in these plants. Results obtained at the micron scaled level on ID21, showed that Cd is bound to O-containing groups in trichomes, but that this compartment is minor. We also showed that veins, and to a lesser extent, mesophyll represent the main Cd accumulating compartment for *A. halleri*. For *A. lyrata* leaves, Cd was only accumulated in veins. These results are new findings compared to the Cd hyperaccumulator *Thlaspi caerulescens*, where only O/N ligands were identified. They suggest that *A. halleri* would not be a real Cd hyperaccumulator.

During this experiment and previous ones on ID21, we also investigated Cd, Zn-containing grains excreted by trichomes of tobacco plants. This excretion was shown to represent a novel mechanism for plants to resist metal toxicity**.** A paper was published on this topic in 2007 (See reference and abstract below). Another one is in preparation.

Sarret G, Isaure MP, Marcus MA, Harada E, Choi YE, Pairis S, Fakra S, Manceau A. 2007. Chemical forms of calcium in Ca, Zn- and Ca, Cd-containing grains excreted by tobacco trichomes. *The Canadian Journal of Chemistry, 85, 738-746.*

Abstract : Tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants exposed to toxic levels of zinc and cadmium excrete metals through their leaf trichomes (epidermal hairs) as Zn,Ca- and Cd,Ca-containing grains. Little is known about the nature and formation mechanism of these precipitates. The chemical, crystalline, and non-crystalline compositions of individual grains produced by tobacco were studied by scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM-EDX), micro-X-ray diffraction (µXRD) and calcium K-edge micro X-ray absorption near edge structure (µXANES) spectroscopy. Zinc is predominantly incorporated in calcite, and cadmium in calcite and vaterite. Aragonite, which occurs occasionally, does not seem to contain trace metals. In addition to being precipitated in its three possible polymorphic forms, calcite, aragonite and vaterite, calcium also is speciated as amorphous CaCO3 and possibly organic Ca in some grains. Most often, a particular grain consists of two or more crystalline and non-crystalline phases. The observed variability of intra- and inter-grain elemental and phase composition suggests that this biomineralization process is not constrained by biological factors, but instead results from thermodynamically and kinetically controlled reactions. This study illustrates the potential of laterally resolved X-ray synchrotron radiation techniques (µXRD and µXANES) to study biomineralization and metal immobilization processes in plants.

Key words : biomineralization, detoxification, micro-XANES, micro-XRD.

References

Isaure MP, Fayard B, Sarret G, Pairis S, Bourguignon J. 2006. Localization and chemical forms of cadmium in plant samples by combining analytical electron microscopy and X-ray spectromicroscopy. *Spectrochimica Acta- Part B Atomic Spectroscopy*, 61, 1242-1252.

Küpper H, Mijovilovich A, Meyer-Klaucke W, and Kroneck PMH. 2004. Tissue- and age- dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy. *Plant Physiology* 134(2), 748-757.