# ESRF

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## Report:

**Introduction**

Certain plant species have the ability to survive and reproduce on soils containing high concentrations of metals, and to store large amounts of metals in their aerial parts (more than 10000 mg kg$^{-1}$ dry weight for Zn). These plants present a great interest for phytoremediation, a soft method in which plants are used for the cleanup of metal-polluted soils. The project aimed at better understanding the molecular mechanisms underlying the immobilization of zinc as non-toxic forms in the Zn and Cd hyperaccumulator *Arabidopsis halleri*. This species is a good model since its genome is simple and very close to *A. thaliana*, the well known genetic model for higher plants, so the molecular tools developed for *A. thaliana* (genetic map, gene ships, …) can be transferred to *A. halleri*. In a previous experiment, we used Zn K-edge EXAFS spectroscopy to identify the major form of zinc in *A. halleri* and in the non-tolerant and non- hyperaccumulating species *A. lyrata* (Sarret *et al.*, 2002). We concluded that Zn occurred as Zn malate in *A. halleri*, and as Zn phosphate in *A. lyrata*. There was no influence of the plant origin and of the concentration of Zn in the nutrient solution. However, the EXAFS spectra were recorded on freeze-dried leaves. This treatment may induce some artifacts, therefore these analyses had to be repeated on frozen hydrated samples.

More recently, we showed that the base of the trichomes (epidermal hairs) of both species was highly enriched in Zn and other metals. However, this compartment accounted for a minor proportion of total Zn present in the leaves, especially for *A. halleri* (Sarret *et al.*, in preparation)

Zn tolerance and Zn hyperaccumulation traits are genetically independent. Therefore, an interspecific crossing between these two species yielded a first generation of non accumulating and slightly tolerant plants (F1 progeny), and second generation of plants presenting new phenotypes, such as tolerant and non-hyperaccumulator, or moderately tolerant and hyperaccumulator (F2 progeny). This original material allows the study of the two traits, independently.

The objectives of this study were to (1) check that previous results obtained on freeze-dried samples of *A. halleri* and *A. lyrata* were valid, (2) determine the chemical form of Zn in the trichomes, and (3) evaluate the relationship between the genetic traits and the mode of Zn sequestration by studying plants of the F2 progeny.

## Materials and Methods

Plants were grown for three weeks on Zn-contaminated compost (treatment 1), and *A. halleri* plants were collected on a smelter-impacted soil containing about 2% weight Zn (treatment 1). Plant leaves (young and mature) were ground and pressed as pellets in liquid nitrogen, and transferred into a Helium cryostat. Trichomes could not be isolated from hydrated leaves, therefore they were collected on freeze-dried leaves, ground and pressed as pellets. Zn reference compounds included various solutions containing Zn nitrate and an organic acid, amino-acid or protein, or a mixture of these ligands were prepared. All spectra were recorded at 20°K, using a Canberra 30-element detector.

## Results

First, we analyzed Zn reference compounds in solution in order to test the sensitivity of EXAFS for these complexes (Figure 1). Zn oxalate has a characteristic spectrum. This complex in solution has the same structure as the solid-state complex, with two oxalate chelating the metal in the equatorial plane, and two axial water molecules. Zn histidine also has a characteristic spectrum owing to multiple scattering in the imidazole ring (Krämer et al., 1996).
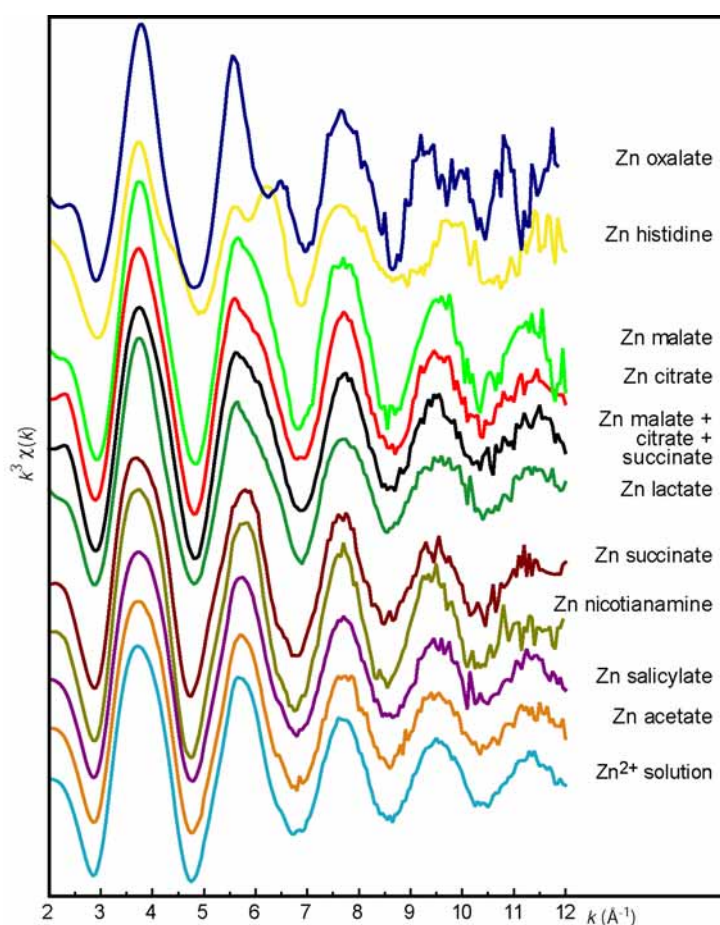


Figure 1: Zn K-edge EXAFS spectra of reference compounds in solution. The pH of all solutions is 5.5. The Zn/ligand ratio is 1/10 for all references except Zn acetate (1/2), Zn nicotianamine and Zn oxalate (1/4), and Zn salicylate (1/5).

The other spectra can be divided in two groups: Zn malate to Zn lactate spectra (group 1) and Zn succinate to Zn acetate spectra (group 2). The great spectral similarity within each group suggests that in the absence of complementary information, it may be difficult to distinguish these ligands in unknown samples. Some of the

spectra of group 2 are close to $Zn^{2+}$ spectrum, which suggests a weak interaction bteween Zn and the ligand. To compensate the relatively weak sensitivity of EXAFS for Zn complexes in solution, the concentration of organic acids was measured in the plant samples by ionic chromatography.
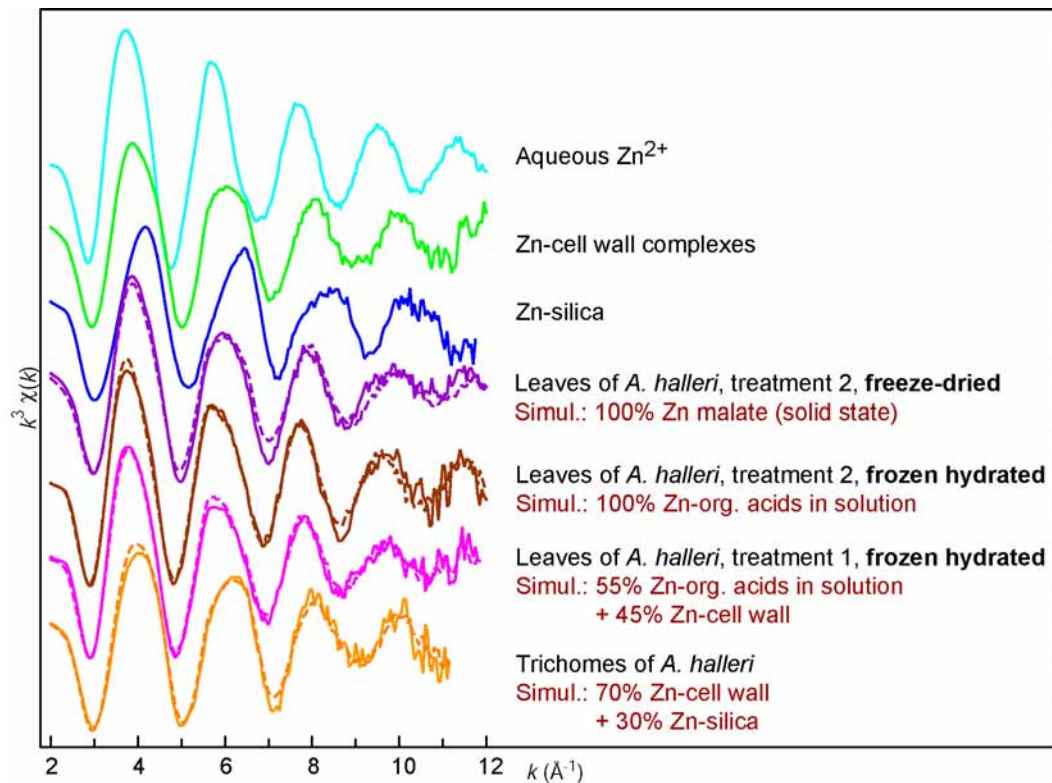


Figure 2: Zn K-edge EXAFS spectra for some reference compounds, plant samples (plain lines) and their simulations using linear combinations of reference spectra (dashed lines). Treatment 1: Plants grown for 3 weeks in a compost contaminated with $ZnSO_4$, and contained 9000 mg kg$^{-1}$ d. w. Zn in their shoots. Treatment 2: Plants were collected in a smelter-impacted soil, and the contaminated, and contained 15 400 mg kg$^{-1}$ d. w. Zn in their shoots.

Figure 2 shows significant differences between the spectra for freeze-dried and frozen hydrated leaves of *A. halleri* from the contaminated soil (treatment 2). There was no difference between young and mature leaves. The best simulation for the frozen hydrated sample was obtained with a mixture of Zn-organic acids in solution including malate, citrate and succinate. Chemical analyses showed that malate was the most concentrated organic acid (Zn/malate molar ratio > 1), and that citrate was present in minor amount (other ligands were not measured). This is consistent with a sequestration of the metal in the vacuolar compartment, which is rich in simple organic acids. Based on these concentrations and on the complexation constants, and supposing that the species are all present in the vacuoles at a pH of 5.5, we calculated Zn speciation in solution using *PhreeqC* software. Second to this calculation, Zn is present as 55% Zn citrate, 20% $Zn^{2+}$ and 25% Zn malate.

Figure 2 shows significant differences between *A. halleri* collected in a contaminated soil (treatment 2) and grown on Zn-amended compost (treatment 1). For the latter sample, the best simulation was obtained with a combination of Zn-organic acid complexes in solution and Zn complexed with isolated cell walls. This suggests the presence of Zn in the vacuoles and in the apoplasm. TEM-EDX observations are planned to confirm this hypothesis.

This difference in the mode of sequestration of Zn may arise form the differences in the duration of exposure to Zn, from the interference of other metallic contaminants for the treatment 2, or from genetic differences between the plants. In order to test the first hypothesis, which seems the most plausible, we plan to compare plants at various durations of exposure.

The spectrum for frozen hydrated leaves of *A. lyrata* and of the F2 plants differed from *A. halleri* spectra (not shown). Data treatment and simulations are under way for these samples.
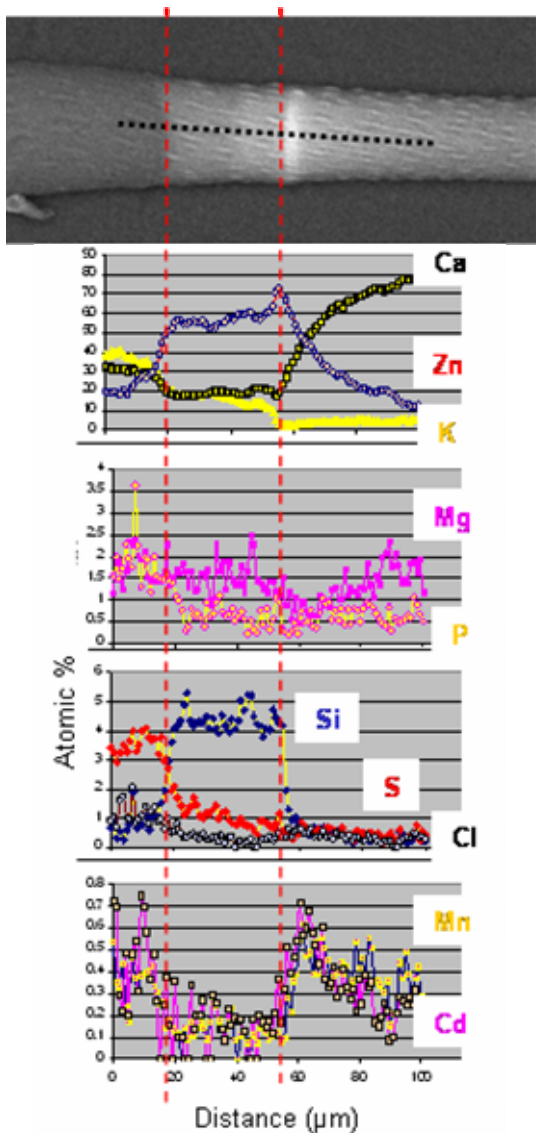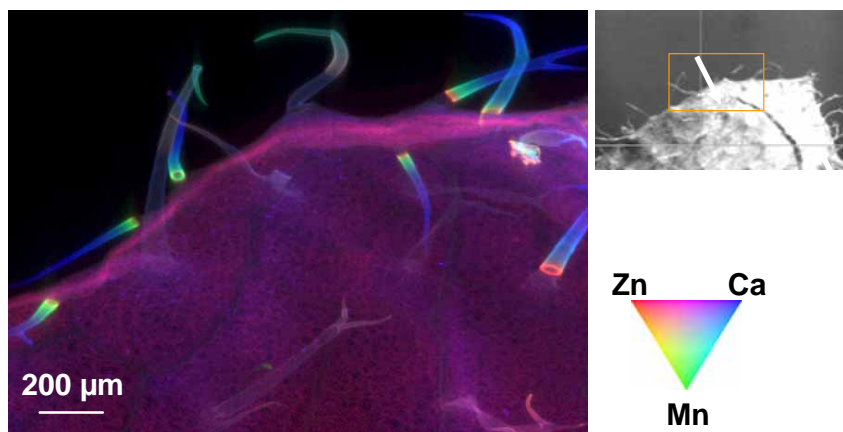
Figure 4: Micro X-ray fluorescence map of a portion of leaf of *A. halleri*. Some of the trichomes present localized Zn enrichments in the form of a ring, which suggests an association with the cell wall and cuticle.

Concerning the trichomes of *A. halleri*, two simulations of equivalent quality were obtained, one with Zn phosphate and one with Zn-cell wall and Zn-silica. The first one was rejected since phosphorus was not detected by SEM-EDX (Fig. 3). The second one is consistent with the localization of Zn in the external part of the trichomes observed by micro-X-ray fluroescence (Fig. 4).

Similar metal accumulations were observed in the trichomes of the two species, so this phenomenon is not related to the tolerance and hyperaccumulation.



Figure 3: Profile of elements near the Zn-richest region of the trichome. Phosphorus is almost absent.

## Conclusions

Working on frozen hydrated samples is essential to limit artifacts on these types of samples. The combination of bulk EXAFS spectroscopy and micro-imaging techniques including micro-X-ray fluroescence and SEM-EDX revealed very powerfull to study the mechanisms responsible for Zn tolerance and hyperaccumulation in plants.

## References

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Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A, Marcus MAM, Manceau A (2002). Plant Physiol. 130: 1815-1826

## Scientific production related to this experiment

**Sarret** G., Willems, G., Manceau A., Marcus M.A., Saumitou-Laprade P., 2005, Relationships between zinc tolerance and hyperaccumulation and zinc localization and speciation in *Arabidopsis halleri, 8th International Conference on the Biogeochemistry of Trace Elements (ICOBTE)*, 3-7 April 2005, Adelaïde, Australia

**Sarret** G., Isaure M.P., Marcus M.A.M., Saumitou-Laprade P., and Willems G., Relationships between Zn chemical form and Zn tolerance and accumulation traits: an EXAFS study of *Arabidospis halleri* and *Arabidopsis lyrata* interspecific crosses. *In preparation.*