Do Hyperaccumulators develop Specific Chelates for Nickel Transport and Storage? The cases of Senecio coronatus and Berkheya coddii.

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Abstract
In order to unravel nickel speciation within shoots of two hyperaccumulating plants *Senecio coronatus* and *Berkheya coddii*, spatially resolved X-ray absorption experiments were carried out at the Ni K-edge, using a micro-focused beam. Citrate and malate appeared as the main complexing ligands for nickel in the different investigated regions i.e. mesophyll, epidermis, conductive vessels (xylem and phloem).

Introduction
Hyperaccumulation of heavy metals in plants is intriguing biologically and extremely rare (exhibited by <0.2% of angiosperms). It has been reported for more than 450 species, mainly Ni accumulating species (70%), encountered on all continents, both in temperate and tropical environments. Such metal-tolerant plants absorb metals through their roots and translocate them to aerial parts, stems and leaves, displaying high metal contents, e.g. up to 7.6 % of nickel in leaves (Mesjasz-Przybylowicz et al., 2004). The uptake mechanism of metals is still not well understood, despite increasing number of investigations concentrating on different aspects of hyperaccumulation (e.g. Chardot et al. 2005, Broadhurst et al., 2009). One aspect concerns nickel chemical status and the existence of specific chelatants involved not only in the transport from the rhizosphere to storage area but also in the storage process itself. In recent work focused on European species including *Alyssum murale* and *Thlaspi caerulescens*, Ni was evidenced to be predominantly complexed by citrate and malate organic ligands, on the basis of X-ray absorption spectroscopy (XAS) data (Montargès-Pelletier et al., 2008). From these data collected in bulk mode, it was not possible to distinguish Ni speciation between storage and transport tissues. Spatially resolved experiments with a focused incident X-ray beam are needed to study properly the specificity of transport mechanism in such plants. Moreover, most results reported so far concern model plants like *Thlaspi sp.* and *Alyssum sp.* (e.g. Broadhurst et al., 2009, McNear et al., 2010), whereas the diversity within hyperaccumulating plant species suggests that several mechanisms might be responsible for this phenomenon. A distinctly different picture of hyperaccumulators emerges from sites in the tropical zone, where hyperaccumulators are never from the Brassicaceae family but belong mainly to Asteraceae, Euphorbiaceae, Rubiaceae, Sapotaceae and other families. In addition, previous cited work referred to bulk measurements and did not provide distinct information concerning the transport and storage of nickel. The following paragraphs present spatially resolved XAS measurements at the Ni K-edge for two Asteraceae hyperaccumulating plants from ultramafic outcrops in Barberton area, South Africa (Mesjasz-Przybylowicz et al., 2004, 2007).

Methods
Cross-sections of leaves from *Berkheya coddii* Roessler and *Senecio coronatus* (Thunb.) Harv. collected from their native habitat on ultramafic soils were cryofixed in liquid propane and freeze-dried.
Several solutions of Ni-complexes were prepared as model compounds for XAS experiments. Solutions were prepared with metal:ligand ratio equal to 1:10 to enhance multidentate complex formation. Five different ligands were selected: malate, citrate, methionine, nicotianamine and histidine. XAS spectra were collected for plants and reference solutions in fluorescence mode on the SAMBA beamline at the SOLEIL synchrotron, Saint-Aubin, France (Belin et al., 2005). X-ray beam was reduced in size with a pinhole to ca. 150 micrometers along x-axis, and ca. 350 micrometers along z-axis.

Results and discussion
X-ray Absorption Near Edge Spectroscopy (XANES) provides relevant information about oxidation state and first coordination shell of the element concerned. Extended X-ray Absorption Fine Structure (EXAFS) oscillations are sensitive to the nature and distribution of neighboring atoms of first and further coordination shells. In the case of Ni(II)-carboxylate complexes, XANES spectra are very similar to the XANES spectrum of hydrated $Ni^{2+}(H_2O)_6$ (Figure 1, left plot), and EXAFS curves are dominated by the signal of first neighboring oxygen atoms. Ni(II)-histidine complex is easily distinguished by the broadening of the main absorption peak in the XANES region and by a marked asymmetry of the second EXAFS oscillation centered at $6Å^{-1}$, due to oxygen and carbon atoms from n+1 shells (Montargès-Pelletier et al., 2008). (Figure 1).

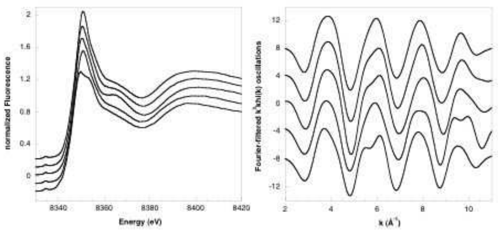

Figure 1: XANES spectra (left) and Fourier-filtered EXAFS oscillations (right) from Ni(II)-organic solutions; from top to bottom: Ni-methionine, Ni-malate, Ni-citrate, Ni-nicotianamine, Ni-histidine.

XAS data from different points of interest (POI) on *S. coronatus* leaf (Figure 2) revealed the complexation of Ni by a mixture of malate and citrate. The second maximum of EXAFS oscillations suggests the predominance of citrate ligands for mesophyll and epidermis POIs. The chemical status of nickel within vascular bundles is slightly different, involving weaker complexing ligands.

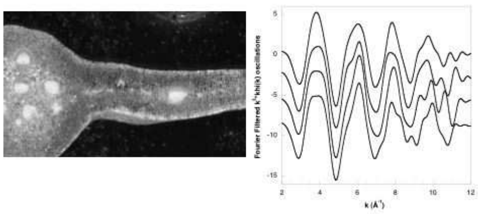

Figure 2: Light microscope picture of leaf cross-section of Senecio coronatus. Fourier-filtered EXAFS oscillations (right) - from top to bottom: vascular bundle, mesophyll, lower epidermis, upper epidermis.

Very similar results were obtained for *B. coddii* (Figure 3).

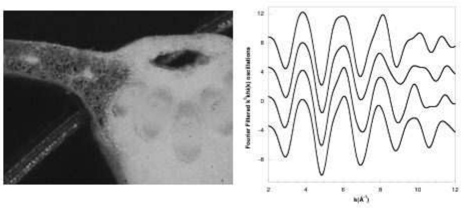

Figure 3: Light microscope pictures of leaf cross-section of Berkheya coddii. Fourier filtered EXAFS oscillations - from top to bottom: xylem, phloem, mesophyll, epidermis.

XAS data show very tiny differences between plants and tissues. However, different nickel status could be evidenced from transport and storage tissues. Due to the similarity of signals, and to the predominance of first coordination shell within the oscillations (6 oxygen atoms), linear combination was not an appropriate method to unravel univocally the nature of complexing ligands. However, the detailed study of scattering paths other than that of the first shell coordination could reveal the predominance of malate and citrate molecules as major organic ligands of nickel.

Conclusions

Histidine could be excluded from major organic ligands responsible for nickel transport and storage in the hyperaccumulators *S. coronatus* and *B. coddii*, growing in their natural environment. This study confirmed that citrate and malate, already reported in earlier studies about hyperaccumulation (Lee et al., 1977), play a major role in metal tolerance.

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