The hyperaccumulator *Alyssum murale* uses complexation with nitrogen and oxygen donor ligands for Ni transport and storage

David H. McNear Jr. a,*, Rufus L. Chaney b, Donald L. Sparks c

a Rhizosphere Science Laboratory, Department of Plant and Soil Sciences, University of Kentucky, N1225 Agricultural Sciences North Building, 1100 Nicholasville Road, Lexington, KY 40546-0091, USA

b USDA-ARS, Environmental Management and Byproducts Laboratory, Beltsville, MD 20705, USA

c Environmental Soil Chemistry Research Group, Department of Plant and Soil Sciences, University of Delaware, 152 Townsend Hall, Newark, DE 19717-1303, USA

Abstract

The Kotodesh genotype of the nickel (Ni) hyperaccumulator *Alyssum murale* was examined to determine the compartmentalization and internal speciation of Ni, and other elements, in an effort to ascertain the mechanism used by this plant to tolerate extremely high shoot (stem and leaf) Ni concentrations. Plants were grown either hydroponically or in Ni enriched soils from an area surrounding an historic Ni refinery in Port Colborne, Ontario, Canada. Electron probe micro-analysis (EPMA) and synchrotron based micro X-ray fluorescence (μ-SXRF) spectroscopy were used to determine the metal distribution and co-localization and synchrotron X-ray and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopies were used to determine the Ni speciation in plant parts and extracted sap. Nickel is concentrated in the dermal leaf and stem tissues of *A. murale* bound primarily to malate along with other low molecular weight organic acids or counter-ions (e.g., sulfate). Ni is present in the plant sap and vascular bound to histidine, malate and other low molecular weight compounds. The data presented herein supports a model in which Ni is transported from the roots to the shoots complexed with histidine and stored within the plant leaf dermal tissues complexed with malate, and other low molecular weight organic acids or counter-ions.

1. Introduction

There is much to be learned about the biochemical or physiological mechanisms responsible for excess metal uptake and storage in hyperaccumulating plants. Understanding these mechanisms would be beneficial for increasing the efficiency of current hyperaccumulating cultivars or transferring this quality to higher biomass, faster growing and climatically adapted plant species for use in either phytoremediation or phytomining of metal-enriched soils across a variety of landscapes. Improving our understanding of metal physiology in hyperaccumulating plants will also assist in efforts to fortify staple agronomic food crops with these mechanisms which established that a plant’s ability to hyperaccumulate metals can depend on the plant species, the chemical properties of the element being detoxified (Assuncao et al., 2003) or, in some cases, tissue type and age (Kupper et al., 2004). For Ni (and other transition metals), most studies have implicated oxygen or nitrogen donor ligands such as citrate, malate, malonate or histidine as the compounds responsible for transport and storage of the metal. Histidine, in the Ni hyperaccumulator *Alyssum lesbiacum*, is the only amino acid shown to increase in xylem sap in response to increased metal concentrations in the growth media (Kramer et al., 1996). There is no evidence to support that endogenous overproduction of either organic or amino acids are a specific response resulting from elevated metal exposure. Instead, selected hyperaccumulators have been found to have a constant and unusually high organic and/or amino acid concentration which implies that excess metal accumulation in some species may be a constitutive property (Boyd and Martens, 1998; Ostergren et al., 1999). In this case, tolerance to internally high heavy metal concentrations, perhaps as an evolutionary adaptation to growing either on metal rich soils or in response to selective pressures from herbivores, would be a sufficient explanation for increased metal uptake.

---

* Corresponding author. Tel.: +1 859 257 8627. E-mail address: dave.mcnear@uky.edu (D.H. McNear).
In some plants, the cysteine-containing metal binding ligands metallothioneins (MT) and phytochelatins (PC) have been shown to play a role in metal tolerance when accumulated metals are at phytotoxic levels, but less so in metal tolerant plants (Schat et al., 2002). Phytochelatins are comprised of cysteine, glycine and glutamate and are used in algae and plants to detoxify excess cellular metal. PCs are readily produced in metal stressed, non-hypertolerant organisms (plants, insects), but their production in hypertolerant species is only inducible at extremely high exogenous metal concentration (Schat et al., 2002; Ebbs et al., 2002). Therefore, it is unlikely that they have a direct role in transition metal tolerance in hyperaccumulating plants. Phytochelatins have been found to have a role in the detoxification of arsenic in Pteris vittata (Chinese Brake fern), which several researchers have shown to have inducible PC production (Zhang and Cai, 2003). Nickel hyperaccumulating plants have been the most studied of the hyperaccumulators, likely because over three quarters of the roughly 450 metal hyperaccumulators identified to date are hyperaccumulators of Ni. In the current paper, we investigated the biochemical mechanisms of Ni hyperaccumulation in Alyssum murale; one of the few hyperaccumulators to be developed as a commercial crop for the removal of metals from metal-enriched soils (Zhang et al., 2007). A. murale is native to Mediterranean serpentine soils and has the ability to hyperaccumulate Ni and Co (Broadhurst et al., 2004b), using different mechanisms to tolerate each.

Tappero et al. (2007), using a variety of techniques, examined the co-tolerance mechanisms in A. murale exposed to different combinations of Zn, Co and Ni. They showed that A. murale does not hyperaccumulate Zn and stores Co in the apoplasm of leaf mesophyll, in the leaf tips and possibly as extracellular precipitates on the leaf surface. They found that exposure of A. murale to either Zn or Co had no effect on the amount and localization of Ni within the plants, that Zn was not accumulated, nor did it interfere with Ni uptake, finally concluding that A. murale used different tolerance mechanisms for Co and Ni. Broadhurst et al. (2004b, 2009), using scanning electron microscopy with energy dispersive X-ray detection (SEM/EDX), also reported that A. murale preferentially stores Mn in the basal compartments of the Ca rich leaf trichomes. From these studies, it is apparent that different biochemical mechanisms govern the way in which A. murale tolerates each of these metals.

More is known about the chemical mechanisms used for Ni tolerance in A. murale compared to either Mn or Co. Nickel accumulates primarily in the cell vacuoles of epidermal stem and leaf tissues and trichome basal compartments (Broadhurst et al., 2004a; McNear et al., 2005), with palisade mesophyll cells becoming important only at higher soil Ni concentrations probably resulting from overflow of the primary cellular storage compartments (Broadhurst et al., 2004a). The transport and storage of Ni in several Alyssum species has been attributed to organic acids and possibly histidine. Little information has been reported, however, on the mechanism used by A. murale to tolerate such high Ni concentrations, let alone the chemical mechanism used to tolerate Co, Mn and Ca. Montargès-Pelletier et al. (2008) using unfocused (bulk) synchrotron X-ray spectroscopy found that A. murale stored Ni in its leaves as Ni–malate and in the stems as Ni–citrate complexes. The bulk X-ray methods used in their studies relied on dried and homogenized tissues which provided a good measure of the average Ni speciation present in the plant tissues. One drawback, as pointed out by Sarret et al. (2002), when analyzing ground and homogenized plant samples, is the possibility that contents of different cellular compartments can come into contact and thus change the metal distribution. In addition, in heterogeneous materials like plant tissues, where the metal speciation can vary over microns depending on the tissue it is found in, bulk-XEAF spectroscopy may not adequately ascertain the minor metal complexes which may have a major role in the plants mechanisms of detoxification, transport or storage. The authors themselves note this limitation and state that microbeam methods are necessary to "unravel the complete Ni pathway".

The objectives of this research were to perform a detailed characterization of the chemical mechanisms responsible for the transport and storage of Ni in the 'Kotodesh' genotype of the Ni hyperaccumulator Alyssum murale using a combination of synchrotron based microspectroscopic techniques, electron microscopy and Fourier transform infrared spectroscopy, respectively.

2. Results

2.1. Element distribution and compartmentalization

Fig. 1a is a false colored confocal micrograph showing the primary fluorescent generated from the different tissue types within a leaf from A. murale and is provided here to highlight the location of the epidermal and vascular tissues (green1) in relation to the mesophyll (red). Presented in Fig. 1b is the scanning electron microscope (SEM) image of an entire leaf cross-section with boxes highlighting examples of the regions analyzed in Fig. 1c and d. Fig. 1c and d presents the backscattered electron (BSE; large) and energy dispersive (EDS; small) micrographs of mid-leaf and mid-rib cross-sections. Ni is located in the dermal tissues, vascular bundles, mid-rib and leaf veins (Fig. 1c and d), and is absent from the mesophyll. Sulfur and potassium are concentrated in the mesophyll and calcium to the leaf surface, none of which are correlated with Ni. The distribution of Mn is less obvious from Fig. 1c, although Mn does appear more enriched in the dermal tissues than the mesophyll. Fig. 2a–f presents the synchrotron based X-ray fluorescence spectroscopy (SXRF) images simultaneously showing the distribution of Ni, Mn and Ca within several leaves from soil and hydroponically grown A. murale plants. Ni appears evenly distributed throughout all of the leaves, with enrichment in the vascular tissues (Fig. 2a, b and f), as well as an apparent enrichment in the leaf margins (Fig. 2b, e and c). Vascular enrichment was observed with SEM in this study and others (McNear et al., 2005; Tappero et al., 2007) and is not interpreted as Ni bound to vascular tissues. Instead, because the leaves were analyzed relatively fresh (i.e., removed from the plant just prior to analysis), Ni was likely trapped there upon freezing. The apparent enrichment of the leaf margins is likely an aberration of the 2D technique resulting because the X-ray beam samples a larger volume of dermal tissue (which was noted to contain the most Ni) at these locations compared to others. For example, the influence of sample volume on fluorescence response is apparent in Fig. 2a, where the leaf tip was folded over and the X-ray beam sampled two layers of leaf tissue resulting in higher relative fluorescence from this region. Similar to the study of Broadhurst et al. (2004), Mn (green) was found to be localized in the region of the Ca rich trichomes (blue) on the leaf surface of A. murale.

2.2. Spectroscopic characterization of standards

The ATR-FTIR spectra for oxalate, malonate, acetate, citrate, malate, tartrate, histidine and each with and without Ni, are shown in Fig. 3. For all IR spectra (Fig. 3), the region between 1000 and 1800 cm⁻¹ (i.e., near-IR region) was isolated because it provided the most information about the coordination environment of the metal complexes with the functional groups found in organic and amino acids. The pH of the aqueous standards was adjusted to match the pH found in typical plant xylem (i.e., pH 1 For interpretation of color in Figs. 1 and 2, the reader is referred to the web version of this article.
6.5–7.0). At these values, all of the carboxyl groups of the organic and amino acids are expected to be fully deprotonated in Ni free solutions. For the Ni-organic/amino acid complexes, the ligand was provided in considerable excess (4–10 \( \times \)) in order to assure that the standard represented the fully complexed Ni-organic/amino acid species.

In general, the IR features (Fig. 3) occurring between 1690–1750 and 1200–1300 cm\(^{-1}\) are attributed to carbonyl stretching (\( \nu(C=O) \)) and C–OH vibrations (\( \nu(C–OH) \)) of the protonated carboxylic acid anion (Cabaniss et al., 1998). Upon deprotonation, the \( \nu(C=O) \) stretching mode becomes more symmetric and shifts to higher energy and the asymmetric mode of the \( \nu(C–OH) \) to lower energy. Shifts in this region are most notable for oxalate followed by citrate and malate indicating the involvement of one or more carboxyl groups in the complexation of Ni by these stronger metal chelates. Conversely, the acacetin spectra changes very little with Ni addition indicating only a weak, outer sphere interaction between the two. Changes in the 1300–1500 cm\(^{-1}\) range of the malonate spectra, notably the peak at 1440 cm\(^{-1}\), are attributed to dipole moment changes resulting from straining of the central –CH\(_2\) group most likely from Ni complexation with the two malonate carboxyl groups forming a mononuclear, bidentate configuration (Dobson and McQuillan, 1999). For the organic acids malate, tartrate, citrate and the amino acid histidine (Fig. 3), absorption bands between 1000 and 1200 cm\(^{-1}\) were observed which may be attributed to the C–O stretching of the intermediary alcohol groups (\( \nu(C–OH) \)) (Cabaniss and McVey, 1995) or, for histidine, C–N stretching (\( \nu(C–N) \)). Also in the histidine IR spectra (Fig. 3) were absorption bands at 1602 and 1518 cm\(^{-1}\) associated with the imidazole ring motion and ring vibration, respectively.

The IR spectrum for cysteine (Fig. 3) contains absorption bands at around 1600 and 1400 cm\(^{-1}\) representing the \( \nu_{as}(\text{C–OH}) \) and \( \nu_{s}(\text{C–OH}) \) stretching modes, respectively, of the carboxyl group. The bands remain virtually unchanged after Ni addition indicating that the carboxyl group is not participating in Ni complexation. The absorption band at 1620 cm\(^{-1}\) result from interactions with the amine group (\( \delta(NH) \)) and is shifted to lower energy after complexation with Ni. The sharp peaks below the \( \nu_{as}(\text{C–OH}) \) band at

Fig. 1. Panels (a) and (b) show a laser scanning confocal micrograph and backscatter electron (BSE) image of an Alyssum murale leaf cross-section, respectively (bar = 250 \( \mu \)m). Panels (c) and (d) show a BSE close-up (large) and energy dispersive spectroscopy (EDS) elemental distribution maps (small) through the mid-leaf (c) and mid-rib regions (d) (bar = 20 \( \mu \)m), of an A. murale leaf cross-section (boxes in panels (a) and (b) indicate regions analyzed in panels (c) and (d)). The circle in panel (c) indicates the location of the vascular tissue and the arrows point in the direction of the leaf margin.
1400 cm\(^{-1}\), specifically bands at 1348 and 1302 cm\(^{-1}\), whose intensity increases upon complexation with Ni are likely due to either bending or twisting modes of the coordinating amine group (Pawlukojc et al., 2005; Ramos et al., 2008). From the IR data, it is difficult to assess the involvement of S in the complexation of Ni as the associated IR bands appear around 300 cm\(^{-1}\) and which is below the resolution of the instrument. No IR spectra were collected for either Ni–glutathione or Ni–glycine.

Fig. 4 shows the XANES, \(k^2\chi(k)\)-spectra and radial structure functions (RSF) for the organic reference phases, and Table S1 (Supplementary data), the results from non-linear least squares fitting. Notable features in these spectra, which indicate the formation of stronger metal ligand complexes, include a widening of the first primary absorption peak at 8.33 keV and a shoulder between 8.34 and 8.35 keV in the XANES spectra (arrows Fig. 4a) and the appearance of a pronounced second shell carbon contribution in the RSF (Fig. 4c). Strathmann and Myneni (2004), using both FTIR and synchrotron X-ray spectroscopy to characterize the binding environment of Ni with several organic acids, noted that longer chain organic acids, where the carboxylate functional groups are separated by one or more methylene groups form weak complexes with Ni and thus have spectra similar to that of the hydrated Ni species. However, those organic or amino acids with shorter chains or closely packed carboxyl (COOH) groups supported by adjacent alcohol \((\alpha(C-OH))\) donor groups or ring structures (e.g., the imidazole ring of histidine) form more rigid bonds (i.e., stronger complexes) leading to the diagnostic spectral features described previously. Supporting these observations (Table S1 Supplementary data), only the first coordination shell of oxygen could be fit \((CN_{Ni-O} \approx 6; R_{Ni-O} = 2.05 \text{ Å})\) for Ni–succinate, Ni–aconitate and the aqueous Ni standard while the remaining organic/amino acid standards (histidine, malate, malonate, citrate, oxalate, tartrate, cysteine and glycine) all had significant second shell carbon contributions \((CN_{Ni-C} \approx 1.9–5.5; R_{Ni-C} \approx 2.82–2.92)\) (see Supplemental data for further fitting details).
Additional diagnostic features include asymmetry in the oscillations between 3 and 7 Å⁻¹ in the $k^3\chi(k)$-spectra (arrows Fig. 4b). Montargès-Pelletier et al. (2008), using multiple Fourier back-filtering ranges of the RSF, demonstrated for the Ni–citrate standard that the asymmetry that occurs in the 3–7 Å⁻¹ region of the $k^3\chi(k)$-spectra can be attributed to C or O atoms beyond the second coordination shell. Extending the Fourier filtering range beyond 4 Å, while certainly contributing to the features in the overall $k^3\chi(k)$-spectra, had more of an influence at higher $k$ ranges (i.e., >10) in the $k^3\chi(k)$-spectra. Therefore, the features between 3 and
7 Å$^{-1}$ may prove more diagnostic than the region of the $k^3\chi(k)$-spectra >7 Å$^{-1}$ since the higher $k$ region of the spectra tends to be very noisy.

Unique among the XAFS standard spectra were those for the tripeptide glutathione and its components (along with glutamate) glycine and cysteine (Fig. 4). Most notably, cysteine had a significant pre-edge peak in the XANES spectra (arrow Fig. 4) indicating significant orbital mixing ($1s \rightarrow 3d$ or $1s \rightarrow 4pz$) because of the non-centrosymmetric structure formed when complexed with Ni. Best fits for the Ni cysteine complex were achieved by including both N and S donor ligands in the first shell (CNNi–S = 2 sulfur atoms at $R_{Ni–S} = 2.14 \text{ Å}$ and CNNi–N = 2 nitrogen atoms at $R_{Ni–N} = 1.96 \text{ Å}$) with C and the oxygen of the hydrating water in the second (CNNi–C = 4 carbon atoms at $R_{Ni–C} = 2.98 \text{ Å}$ and CNNi–O = 1.10 Å oxygen atoms at $R_{Ni–O} = 2.41 \text{ Å}$). Fits for the Ni–glycine complex indicates that Ni is bound to the amine group and terminal carboxyl groups (CNNi–N = 2 nitrogen atoms at $R_{Ni–O} = 2.04 \text{ Å}$ and CNNi–O = 2 oxygen atoms at $R_{Ni–O} = 2.13 \text{ Å}$) of two glycine molecules with Ni acting as the bridging atom forming a dimer.

2.3. Ni speciation in A. murale – evidence from X-ray and ATR-FTIR spectroscopy

The numbered spots in the µ-SXRF maps in Fig. 2 correspond to the XANES, $k^3\chi(k)$-spectra and RSF shown in Fig. 5. The dataset included a total of 19 spectra including bulk-EXAFS spectra from extracted sap (#15) and freeze-dried and ground stem (spectra #16 and 18) and leaf tissue (#17 and 19) from soil grown plants. While the spectra are very similar, there are subtleties in each that provide an indication of the Ni coordination. The arrows in Fig. 5a and b point to regions in the XANES and $k^3\chi(k)$-spectra possessing the most diagnostic features. As mentioned previously, the feature in the XANES spectra at about 8.338 keV results from $1s \rightarrow 3d$ or $1s \rightarrow 4pz$ electronic transitions (i.e., orbital mixing), and is indicative of non-centrosymmetric coordination resulting from either tetrahedral or square pyramidal complexes (Colpas et al., 1991; Feth et al., 2003; Strathmann and Myneni, 2004). In the sample spectra, this feature is small, likely arising from slight distortions in symmetry permitting some p–d orbital mixing which increases the probability of $1s \rightarrow 3d$ transitions. The fact that this feature is small and similar to those found in the Ni carboxylic acid and hydrated Ni standard spectra indicates that Ni in the samples has an octahedral geometry.

In some of the spectra (e.g., 1 and 2), there is obvious splitting in the peak around 5.8 Å$^{-1}$ in the $k^3\chi(k)$-spectra which, as was observed while fitting the standards, is indicative of a stronger Ni–ligand complex. The RSF of all spectra show a prominent first shell peak at ~1.6 Å (uncorrected for phase shift) and, in many, another smaller peak at ~2.3 Å (uncorrected for phase shift).

An initial assessment of the coordination environment of Ni in the plant tissues was conducted using conventional NLLSF. Table 1 shows the NLLSF results from fitting the sample spectra using phase
and amplitude functions generated from either Ni(sal)₂(H₂O)₂ (Stewart et al., 1961) for Ni–C paths or Ni–imidazole ((C₃H₄N₂)₆-Ni(NO₃)₂) for Ni–N paths (Santoro et al., 1969). The first coordination shell in all the spectra is occupied by approximately six oxygen/nitrogen atoms at \( R_{\text{Ni–O/N}} \approx 2.03–2.06 \text{ Å} \) indicating, as was suggested by the small pre-edge feature in the XANES spectra, that the Ni is in an octahedral coordination. Some spectra had a significant second shell contribution from carbon at \( R_{\text{Ni–C}} \approx 2.76–2.91 \text{ Å} \) with coordination numbers ranging from \( CN_{\text{Ni–C}} \approx 1.00 \) to 4.67. It should be noted that second shell coordination numbers less than 2 are outside the error associated with EXAFS spectroscopy second shell coordination number estimates (±40%). However, each of the fits was performed with carbon and without, and carbon was only included when it significantly improved the fit (based on a 20% or greater improvement in the % residual error; \( \chi^2\text{res} \)). The NLLSF results show that only oxygen or nitrogen donor ligands participate in the complexation of Ni in the tissues of \( A. \text{murale} \).

The species contributing to these fitting results were determined by principal component analysis (PCA) using all 19 sample spectra over the \( k \) range of 2–10 Å⁻¹. The weights of the first four

---

**Fig. 5.** (a) X-ray absorption near-edge spectra (XANES), (b) \( k^2\mu(k) \)-spectra and (c) radial structure functions from the points indicated in Fig. 2 throughout several \( Alyssum \) murale leaves. Note: spectra 15 was collected from pressure bomb extracted plant sap and 16–19 were collected from homogenized muck and loam grown, stem and leaf tissues, respectively, and thus are not represented in Fig. 2.
components were 140, 21, 18 and 13 with indicator values of 0.0286, 0.0280, 0.0275 and 0.0288, respectively. The total normalized sum square values (a “badness” of fit parameter) significantly improved when going from 2 to 3 components (from 0.0526 to 0.0376), but not four. Therefore, PCA indicates that, while the first component contributes significantly more to the overall fit (i.e., 0.0526 compared to that stored in the bulk of the leaf dermal tissues. Since points 1 and 15 were collected from a non-specific region within a plant leaf, point 15 was the bulk-EXAFS spectra. To exclude them from further consideration. Target transformation (TT) was done using our characterized references to determine the species represented by the abstract principal components. We initially used a reference dataset that included both inorganic and organic standards (see McNear et al. (2007) for entire data set); however, the spoil values for the inorganic standards were large enough (ranging from 6.2 to 25) to exclude them from further consideration. Target transformation indicated that the top three species most likely in our dataset (Table S2 Supplementary data; 3–7 Å) were Ni–malate and Ni(aq), Ni–succinate, Ni–aconitate, Ni(aq) and Ni–malate as the primary species (Table S2 Supplementary data; 3–7 Å). Unique to the fits over both spectral regions were Ni–malate and Ni(aq), while PCA over the 3–7 Å region left out Ni–tartrate, but included both Ni–succinate and Ni–aconitate. Including Ni–succinate and Ni–aconitate in the LLSF of the entire spectra resulted in an improvement of the original fits for most of the spectra with the exception of those collected at points 1, 12 and 15 (Fig. 2). Point 1 was collected from a vein of a leaf, point 15 was the bulk-EXAFS spectra collected from the extracted plant sap and point 12 was collected from a non-specific region within a plant leaf. Since points 1 and 15 were collected either from a vein or bulk plant sap, further efforts were made using ATR-FTIR to assess whether Ni in the vacuolation of the plant was present in a different chemical form compared to that stored in the bulk of the leaf dermal tissues. FTIR has been used to investigate a variety of constituents in plants including proteins (Surewicz et al., 1993) and cell wall materials (McCann et al., 1992). No studies, to these authors’ knowledge, have used ATR-FTIR methods to characterize plant sap extracts from specialized hyperaccumulating plants.

Table 1

<table>
<thead>
<tr>
<th>Spectra #</th>
<th>Atom</th>
<th>First shell</th>
<th>Atom</th>
<th>Second shell</th>
<th>( \Delta E_p ) (eV)</th>
<th>( \chi^2_{res} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/O</td>
<td>5.60 2.05 0.005</td>
<td>C</td>
<td>4.67 2.91 0.008</td>
<td>-2.19</td>
<td>3.57</td>
</tr>
<tr>
<td>2</td>
<td>N/O</td>
<td>6.30 2.04 0.006</td>
<td>C</td>
<td>3.00 2.89 0.004</td>
<td>-3.56</td>
<td>13.89</td>
</tr>
<tr>
<td>3</td>
<td>O</td>
<td>6.80 2.05 0.006</td>
<td>C</td>
<td>1.18 2.87 0.001</td>
<td>1.19</td>
<td>2.09</td>
</tr>
<tr>
<td>4</td>
<td>O</td>
<td>6.50 2.05 0.005</td>
<td>C</td>
<td>1.50 2.86 0.007</td>
<td>0.59</td>
<td>3.01</td>
</tr>
<tr>
<td>5</td>
<td>O</td>
<td>6.75 2.06 0.004</td>
<td>C</td>
<td>1.00 2.81 0.002</td>
<td>2.07</td>
<td>2.74</td>
</tr>
<tr>
<td>6</td>
<td>O</td>
<td>6.53 2.05 0.005</td>
<td>C</td>
<td>2.30 2.84 0.009</td>
<td>1.03</td>
<td>6.0S</td>
</tr>
<tr>
<td>7</td>
<td>O</td>
<td>6.50 2.05 0.005</td>
<td>C</td>
<td>1.64 2.78 0.005</td>
<td>1.30</td>
<td>4.53</td>
</tr>
<tr>
<td>8</td>
<td>O</td>
<td>6.07 2.05 0.005</td>
<td>C</td>
<td>-  -  -</td>
<td>1.77</td>
<td>4.98</td>
</tr>
<tr>
<td>9</td>
<td>O</td>
<td>6.32 2.04 0.004</td>
<td>C</td>
<td>2.30 2.88 0.004</td>
<td>0.44</td>
<td>4.22</td>
</tr>
<tr>
<td>10</td>
<td>O</td>
<td>6.00 2.05 0.005</td>
<td>C</td>
<td>2.70 2.88 0.008</td>
<td>2.38</td>
<td>9.02</td>
</tr>
<tr>
<td>11</td>
<td>O</td>
<td>5.21 2.03 0.005</td>
<td>C</td>
<td>2.58 2.78 0.003</td>
<td>-0.45</td>
<td>8.17</td>
</tr>
<tr>
<td>12</td>
<td>O</td>
<td>6.20 2.06 0.006</td>
<td>C</td>
<td>2.00 2.83 0.005</td>
<td>1.55</td>
<td>2.83</td>
</tr>
<tr>
<td>13</td>
<td>O</td>
<td>6.24 2.04 0.004</td>
<td>C</td>
<td>1.50 2.83 0.005</td>
<td>-0.25</td>
<td>5.00</td>
</tr>
<tr>
<td>14</td>
<td>O</td>
<td>6.39 2.04 0.005</td>
<td>C</td>
<td>1.93 2.88 0.007</td>
<td>0.64</td>
<td>4.10</td>
</tr>
<tr>
<td>15</td>
<td>O</td>
<td>4.50 2.03 0.005</td>
<td>C</td>
<td>3.00 2.76 0.005</td>
<td>-0.21</td>
<td>3.30</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>5.60 2.03 0.004</td>
<td>C</td>
<td>-  -  -</td>
<td>2.60°</td>
<td>10.19</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>6.00 2.05 0.004</td>
<td>C</td>
<td>2.00 2.84 0.006</td>
<td>-0.09</td>
<td>5.72</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>6.00 2.04 0.005</td>
<td>C</td>
<td>1.50 2.81 0.009</td>
<td>-0.11</td>
<td>9.27</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>6.00 2.04 0.004</td>
<td>C</td>
<td>1.30 2.86 0.004</td>
<td>-0.92</td>
<td>9.95</td>
</tr>
</tbody>
</table>

\( CN = \) coordination number (±20% (Scheidegger et al., 1997)).
\( R = \) inter-atomic distance (±0.02 Å for first shell and ±0.05 Å for second shell (Scheidegger et al., 1997)).
\( \Delta E_p = \) phase shift.
\( \chi^2_{res} = \) residual.
\( \text{NSS} = \frac{\sum [k^2_p(k_{\text{exp}}) - k^2_p(k_{\text{recon}})^2]}{\sum k^2_p(k_{\text{exp}})} \).
at 1300–1380 cm$^{-1}$ which was only found in the histidine spectra. Malate is included for comparison because, as was demonstrated earlier, it has consistently been identified using PCA as a component within our sample EXAFS spectral dataset. There are, however, few similarities between the Ni–malate IR standard and the plant saps, lending some support to the hypothesis that Ni is sequestered by a different combination of ligands during transport and for storage.

Based on these findings, all of the sample spectra were re-evaluated using the following criteria: (1) NLLSF parameters, (2) diagnostic features in the XANES spectra, (3) goodness (based on NSS) of the LLSF with particular attention paid to the diagnostic features between 3 and 7 Å$^1$ and (4) ATR-FTIR data. For example, fits with a high NSS value were inspected to determine where the data were inadequately fit, paying particular attention to the diagnostic region between 3 and 7 Å$^1$. In addition, the coordination number and atomic distances for the ligand identified by NLLSF, and the metal species identified by LLSF for the sample spectra were compared to determine if it was appropriate to include the identified ligand in the NLLSF. That is, if the NLLSF indicates that the EXAFS spectra are comprised primarily of histidine, there should be a significant second shell carbon contribution at a slightly longer bond distance in the LLSF of that spectra, as well as splitting in the $k^2\chi(k)$-spectra at $\sim$5.8 Å$^{-1}$ and broadening of the maximum absorption peak in the XANES spectra.

The LLSF results from using these revised fitting criteria can be found in Table 1. The average distribution of Ni species within all of the sample spectra was found to be 34% Ni–malate, 16% Ni(aq), 15% Ni–tartrate, 14% Ni–succinate, 12% Ni–aconitate and 5% Ni histidine. The inclusion of Ni–succinate and Ni–aconitate was found to significantly improve the fit for some of the spectra. Fits from the bulk-EXAFS spectra of leaves and stems from plants grown in Ni enriched soils (Fig. S1; Supplemental data) show a similar trend in that 30–67% of the Ni is complexed with tartrate and the balance with succinate and/or counter-ions.

3. Discussion

Determination of metal speciation in plant tissues is difficult due to the complex and dynamic matrix within which they reside. Identification of ligands responsible for metal detoxification in hyperaccumulating plants using solely chromatographic methods are limited by the typically low recovery of metals from the tissue extractions, the inability to specify which cellular compartments the complex comes from (e.g., vacuole vs vasculature), as well as potential mixing, degradation or transformation of the cellular components once extracted. Direct measures of metal speciation in plant tissues using spectroscopic methods may overcome some of these limitations, but they too have their drawbacks. Using solely hard X-ray spectroscopic methods to directly speciate metals in biological tissues is complicated because the ligands often associated with the metals are comprised of weak backscattering atoms (e.g., C) which provide very little structure to the EXAFS spectra. In this paper, we have attempted to overcome many of these concerns by combining multiple analytical techniques and statistical data fitting methods to ascertain the speciation and distribution of Ni within A. murale.

Using electron microscopy and µ-SXRF we found that Ni is evenly distributed throughout the epidermal tissues, entrained in xylem vesicles, and absent from the mesophyll of the leaf. There is no clear correlation of Ni with any of the other elements analyzed. Sulfur was found to be concentrated in the mesophyll where it has been shown to have a functional role in several metabolic processes. The majority of sulfur is stored in a plant as the inorganic anion sulfate with the remainder incorporated into organic forms such as proteins, chloroplast lipids, or secondary metabolites (Marschner, 1995). Sulfur is a key constituent, both structural and functional, in proteins, namely cysteine, and the strong antioxidant glutathione. Many of the functions requiring S take place in chloroplasts of photosynthetically active tissues of the plant and thus it is not surprising that the S content of these tissues would be elevated. Kupper et al. (2001) and Broadhurst et al. (2004) both found Ni to be associated with sulfur in the plant epidermal cell vacuoles in A. lesbiacum, A. bertolonii and A. murale, which is opposite to the pattern we observed. They concluded that the excess sulfur was probably sulfate acting as a counter-ion to Ni, and possibly an inorganic nickel sulfate species stored in the epidermal vacuoles. In the Kupper et al. (2001) study, the excess Ni treatments were supplied to the plants as NiSO$_4$ (up to 4000 mg/kg) which may have induced the observed Ni–S associations. Sulfate can be directly taken up

from the growth solution and, if not used for other plant functions, the excess stored in the cell vacuole (Lombi et al., 2002). However, Broadhurst et al. (2004) noted in their studies that regardless of the sulfate concentration and type of Ni supplied (i.e., NiSO\textsubscript{4} vs Ni-C\textsubscript{4}H\textsubscript{6}O\textsubscript{4}), S concentration in the leaves (on a total S basis) was still elevated. Tappero et al. (2007) also found elevated shoot S concentrations in \textit{A. murale} grown in mixed metal (Ni + Co, Ni + Zn and Ni + Co + Zn) hydroponic solutions noting that in the Co treated plants (Ni + Co, Ni + Co + Zn) the S content significantly increased compared to the Ni only treatment and, based on the SXRF maps, plants (Ni + Co, Ni + Co + Zn) hydroponic solutions noting that in the Co treated plants (Ni + Co, Ni + Co + Zn) the S content significantly increased compared to the Ni only treatment and, based on the SXRF maps, was correlated with Co. Their study suggests that the mechanisms used by \textit{A. murale} to detoxify Ni are different than those used for Co, for which S may play a role. Ingle et al. (2005) found that at Ni concentrations high enough to support Ni hyperaccumulation (i.e., 0.03 mM NiSO\textsubscript{4}) there were no S-metabolism-related genes upregulated; however, when moderately high Ni was added (0.3 mM NiSO\textsubscript{4}), genes responsible for S-metabolism were upregulated. This only highlights the point that even metal hyperaccumulating plants that have evolved the ability to tolerate high metal concentrations have an upper threshold beyond which inducible chemical stress responses are upregulated. In the current study, these solution concentrations were not sufficient enough to satisfy the necessary conditions after approximately 11 h of Ni exposure (see Fig. 1 Kerkeb and Kramer (2003)). This may indicate that while histidine is important in the delivery of Ni to the xylem from the roots, and some histidine may concurrently be released into the xylem, other ligands or counter-ions will be necessary to balance the molar excess of Ni. The results in this study are in agreement with this interpretation, and supports a model where transport in the xylem is facilitated by chelation of Ni in some part by histidine and other non-specific organic acids, after which it is delivered from the leaf veins to the vacuoles of the leaf epidermal cells where it is stored complexed with organic acids (most notably malate) or with less specific ligands (e.g., acconitate, succinate) or counter-ions (e.g., sulfate).

4. Conclusions

The findings contained herein contribute to the current understanding of metal tolerance and hyperaccumulation and provide an example of how coupling complementary techniques at multiple scales can provide a unique picture of metal sequestration and compartmentalization in biological tissues. While this study contributes to the growing evidence for the importance of organic/arnino acids and counter-ions in the transport and storage of metals, there are still many questions to be answered regarding metal uptake, delivery and storage in hyperaccumulating plants. Little is known about the physiological, biochemical and genetic mechanisms involved in metal uptake from the rhizosphere, transport through the vasculature and preferential offloading of metals into cellular compartments within leaf and stem tissues (e.g., epidermal vacuoles, trichome basal compartments). Understanding these mechanisms will not only satiate our curiosity as to the fundamental evolutionary and ecological processes that guided these plants to the hyperaccumulation phenotype, but they will also help in efforts to improve food crop production via, for example, biofortification or improved pathogen resistance. The results obtained and methods used in this study should assist in these efforts.

5. Experimental

5.1. \textit{A. murale} propagation

The 'Kotodesh' accession of the Ni hyperaccumulator \textit{A. murale} (Waldst. & Kitt.) was grown in a glass house in nickel enriched soils collected from an area adjacent to an historic Ni refinery in Port Colborne, Ontario, Canada. The speciation of Ni in these soils and the soil physicochemical characteristics have been described.
elsewhere (Kukier and Chaney, 2001; McNear et al., 2005). Soils were sieved wet, mixed thoroughly and 1.5 kg (dry weight) of loam soil or 0.8 kg (dry weight) of muck soil were placed in 1.5 l plastic pots. Based on Mehlich III soil test analyses, the total amounts of macro- and micro-nutrients applied to each pot were: 154 mg N (as calcium nitrate), 291 mg P (as calcium phosphate and potassium phosphate), 231 mg K (as potassium phosphate), 22 mg Mg (as magnesium sulfate) and 1 mg B (as boric acid) (Siebiedel et al., 2007). All fertilizers were added as solutions with the exception of calcium phosphate, which was added as a dry powder. A previous study has shown that Port Colborne soils can develop a Mn deficiency, particularly when limed to alleviate Ni phytotoxicity (Kukier and Chaney, 2001). Therefore, MnSO₄ was added at a rate of 200 kg Mn/h to each pot (Siebiedel and Chaney, 2006). The fertilizers were mixed with the soil in plastic bags and added to the pots. The pots were watered and incubated for 7 days after which the soils were mixed again and cultivated. Pots were watered as needed using reverse osmosis filtered H₂O and saucers were used to prevent loss of leachate. Control plants were grown in soils from the same region but without Ni enrichment. Some plants were grown in the greenhouse aeroponically in a General Hydroponics Clone Machine 20 (General Hydroponics, Sebastopol, CA) using modified 1/4 strength Hoagland’s with Fe(III)–HBED (N,N,N,N′-di(2-hydroxybenzoyl)-ethylenediamine,N,N′-diacetic acid) to avoid metal chelator interactions (Chaney, 1988). Control plants were grown in solutions containing 0.125 μM Ni while elevated treatments had 50 μM Ni (added as Ni(NO₃)₂). Both soil and aeroponically grown plants received light for 16 h each day using a combination of high pressure sodium vapor lamps and natural sunlight. Day/night temperatures were approximately 25 and 21 °C, respectively.

5.2. Electron probe micro-analysis (EPMA)

Plant samples were prepared by immersing whole sections of shoot in liq. N₂ after which they were dried under vacuum at −80 °C for 24 h to prevent ice crystal formation. Leaves were then fractured to expose the interior of the leaf and pieces were placed on a carbon stub. Prior to analysis the samples were sputter-coated with carbon and then examined on a JOEL JXA-8600 microprobe (John Hopkins University) with wavelength and energy dispersive detectors (WDS and EDS, respectively). Samples were first scanned manually from 50 to 300× using background corrected and normalized. Background subtraction was performed by fitting a linear polynomial to the pre-edge region between 150 and 500 eV above the Ni K-edge. The threshold energy (E₀) was determined by selecting the root of the second derivative through the absorption edge of the differentiated spectra, and used to convert the spectra from energy to k-space (photoelectron wave vector (Å⁻¹)). A cubic spline function with ≤7 knots was then used to remove the contribution to the spectrum resulting from atomic absorption in the absence of backscattering contributions. This step generated the EXAFS function (χ(k)), which was then weighted by k², to compensate for dampening of the EXAFS amplitude with increasing k. The k²χ(k)-spectra were then Fourier transformed using a Bessel window with a smoothing parameter of 3–4 to reduce artifacts due to the finite Fourier filtering range used. This step produced radial structural functions (RSF) which were not corrected for phase shift.

5.3. ATR-FTIR standard and plant sap characterization

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was employed to characterize the organic and amino acid standards used in EXAFS PCA-LLSF, as well as in characterization of extracted xylem sap from A. murale. Plant sap was collected from hydroponically grown A. murale plants by inserting cut sections of shoots into a pressure bomb apparatus (Soil Moisture Equip. Corp., Goleta, CA), adjusting the pressure to 500 atm and using reverse osmosis filtered H₂O and saucers under reverse pressure. Tapes were used to generate all standards. The pH was adjusted using trace metal grade nitric acid (HNO₃) or potassium hydroxide (KOH).

XAS data for standards were collected on beamline X-11A at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, Upton, NY (unless otherwise stated). The electron beam energy was 2.5–2.8 GeV with a maximum beam current of 300 mA. The monochromator consists of two parallel Si(1 1 1) crystals with a vertical entrance slit opening of 0.5 mm. The beam size on the sample was maintained at 2 × 10 mm for all samples and standards. Prior to data collection, the energy was calibrated to the first inflection point on the K absorption edge of a Ni metal foil standard (E₀ = 8.333 keV for Ni). Aqueous standards were loaded into individual acrylic sample holders and sealed with Kapton™ tape. A non-adhesive Kapton™ film was placed on the adhesive side of the tape where it covered the sample reservoir to avoid any interaction of the sample with the tape adhesive. The samples were then mounted 45° to the incident beam and data collected at the Ni K-edge over the energy range 8183–9082 eV in fluorescence mode using a N₂/Ar (95/5%) filled Lytle Cell. To optimize the Ni signal and remove elastically scattered radiation, the fluorescence signal was filtered using a Co foil, one to two sheets of aluminum foil and Soller slits. Harmonic rejection was achieved by detuning the monochromator 20% of k₀. Multiple scans (≥3) were collected for each sample to improve the signal-to-noise ratio. EXAFS spectroscopy data analyses were performed using WinXAS 3.1 (Ressler, 1997). Prior to averaging, the individual spectra were background corrected and normalized. Background subtraction was performed by fitting a linear polynomial to the pre-edge region between 150 and 500 eV below the Ni K-edge. The threshold energy (E₀) was determined by selecting the root of the second derivative through the absorption edge of the differentiated spectra, and used to convert the spectra from energy to k-space (photoelectron wave vector (Å⁻¹)). A cubic spline function with ≤7 knots was then used to remove the contribution to the spectrum resulting from atomic absorption in the absence of backscattering contributions. This step generated the EXAFS function (χ(k)), which was then weighted by k², to compensate for dampening of the EXAFS amplitude with increasing k. The k²χ(k)-spectra were then Fourier transformed using a Bessel window with a smoothing parameter of 3–4 to reduce artifacts due to the finite Fourier filtering range used. This step produced radial structural functions (RSF) which were not corrected for phase shift.

The first two major shells below 3.5 Å were individually selected, back-transformed, and fit using a non-linear least squares fitting (NLLSF) approach and theoretical scattering paths generated using ATOMS and FEFF 7.02 software packages (Zabinski et al., 2011).
5.5. μ-SXRF and μ-EXAFS spectroscopy's data collection and analysis

Micro-EXAFS and μ-synchrotron X-ray fluorescence (μ-SXRF) data were collected on beamline 10.3.2 (1.9 GeV and 300 mA) at the Advanced Light Source, Lawrence Berkeley National Lab (Berkeley, CA). Leaves and stems were removed from living plants and mounted directly to a Peltier cold stage using metal-free silicon vacuum grease and rapidly frozen to ~30 °C. The sample stage was then oriented 45° to the incident X-ray beam. Fluorescence signals were collected using a Ge solid-state multi-element detector. To assess the spatial distribution of Ni and other elements in the samples, fluorescence maps were collected over 1000 μm² (coarse map) and 200 μm² (fine map) with a beam size of 16 × 7 and 5 × 5 μm, respectively, using a step size of 20 and 5 μm and an integration time of 100 ms. For mapping, the beam energy was set to 11 keV to allow the detection of Ni, Fe, Ca, Co, Cu, Zn, and Mn. To determine the Ni speciation at regions of interest in the samples, μ-XAS spectra were collected up to 500 eV above the Ni K-edge. Prior to μ-XAS data collection, the beamline energy was calibrated to the first inflection point of the Ni metal foil standard (E₀ = 8.333 keV).

Analysis of the EXAFS spectra was done using both NLLSF as was described above for fitting the standards and principal component analysis linear least squares fitting (PCA-LLSF). Traditional shell fitting was used as an initial assessment of the coordination environment of Ni with particular focus on the second shell coordination number as an indication of the relative strength of the complex. PCA-LLSF was then performed using Labview™ based data analysis software developed by 10.3.2 beamline scientists (available for free download at their website: http://xrayweb.lbl.gov/uxas/Beamline/Software/Software.htm), to identify and quantify the Ni–ligand complexes contributing to the spectra. Both of these fitting procedures were chosen because analysis of EXAFS spectra collected from multicomponent systems (such as plants) cannot rely on traditional fitting procedures alone in which atomic shells are individually selected and fit. The difficulty arises because multiple species in the system may have overlapping atomic shells making it difficult if not impossible to separate each of them out. Therefore, to determine the species present within a mixed system, a dataset of spectra from multiple spots throughout a sample are analyzed statistically using principal component analysis (PCA) (Wasserman et al., 1999). The PCA technique determines if the data set can be described as weighted sums of a smaller number of components, which would be the case if each spot in the dataset is comprised of a smaller number of distinct compounds. Selection of the number of principal components was made where the empirical indicator (IND) and Eigenvalues were at their minimum (Malinowski, 1977). Target transformation (TT) was then used to identify the components by taking a spectrum of a known reference compound and mathematically removing from the spectrum anything that did not look like principal components identified by PCA. If minimal information is removed from the known reference spectrum, then it is most likely present in the sample. Reference spectra are evaluated for their “goodness of fit” by the SPOIL value (Malinowski, 1978). Generally, numbers <1.5 are considered excellent, 1.5–3 good, 3–4.5 fair, 4.5–6 poor and >6 unacceptable. After the contributing standard phases are identified, linear least squares fitting (LLSF) is used to determine the amount (%) of each standard within the individual sample spectra making up the dataset. The fit is optimized where the normalized sum squared (NSS = \[\sum (k^2 \chi(k) - \chi(k_{\text{ref}}))^2 \] ) value is at a minimum. A reference phase was included in the fit only if it decreased the NSS by 20% or more. The accuracy of this fitting approach is dependent upon the data quality, the completeness of the standards data set, and the range over which the data were fit (Manseau et al., 2002).

Acknowledgements

The authors would like to thank Dr. Ken Livi of the Department of Earth and Planetary Sciences at Johns Hopkins University for help with EMPA data collection, analysis and interpretation. We thank Dr. Kirk Czymek of the Delaware Biotechnology Institute for help collecting the confocal and SEM image of leaf cross-sections. We thank Dr. Ryan Tappero for assistance/instruction with plant propagation and Dr. Mike Borda for help with collecting ATR-FTIR spectra. We thank Dr. Timothy Stratham for providing EXAFS spectra for selected Ni-organic acid standards. We thank Matthew Marcus for assistance with μ-EXAFS data collection at the Advanced Light Source (ALS) beamline 10.3.2 and the staff of beamline X11A at the National Synchrotron Light Source (NSLS) for their assistance in the collection of EXAFS standards spectra. The Advanced Light Source is supported by the Office of Science, Basic Energy Sciences, Division of Materials Science of the US Department of Energy under Contract No. DE-AC02-05CH11231. Use of the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-98CH10886.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.10.023.

References


