Accumulation Forms of Zn and Pb in Phaseolus vulgaris in the Presence and Absence of EDTA

Geraldine Sarret, Jean-François Vangronsveld, Alain Mancau, Martine Mussot, Jan D'Haen, Jean-Jacques Menthonnex, and Jean-Louis Hazemann

Environmental Geochemistry Group, LGIT-IRIGM, University of Grenoble and CNRS, BP 53, 38041 Grenoble Cedex 9, France, Environmental Biology and Institute of Material Sciences, Limburgs Universitair Centrum, Universitaire Campus, building D, B-3590 Diepenbeek, Belgium, and Laboratoire de Cristallographie, CNRS, 25 avenue des Martyrs, BP 166, 38042 Grenoble Cedex 9, France

The internalized speciation of Zn and Pb in roots and leaves of Phaseolus vulgaris grown in zinc sulfate, zinc EDTA, lead nitrate, and lead EDTA solutions was studied by electron microscopy (Zn) and extended X-ray absorption fine structure (EXAFS) spectroscopy (Zn and Pb). Zn was predominantly present as Zn phosphate dihydrate in the roots and leaves of the plant regardless of its form in solution. Pb was predominantly found in the leaves as cerussite (lead carbonate) when the plant was grown in Pb nitrate solution and as a mixture of PbEDTA and an undetermined species in contact with PbEDTA solution. Therefore, Phaseolus vulgaris is able to dissociate totally (Zn) or partly (Pb) the two metal–EDTA complexes from the nutrient solution and to bind these metals in other forms.

Introduction

Phytoextraction, an emerging biotechnology that uses plants to transfer trace elements from polluted soils to plant shoots, is both technically and economically attractive in comparison to transfer trace elements from polluted soils to plant shoots, is both technically and economically attractive in comparison to soil manipulation. Initial phytoremediation studies focused on hyperaccumulating species such as Thlaspi caerulescens (1–3), Thlaspi rotundifolium (4), and Alyssum lesbiacum (5) which can grow in extremely polluted soils and can accumulate up to several percent metal in their shoots. However, a general characteristic of these species is their limited biomass production. Therefore, some researchers estimated that these species could not meet the requirements for the design of an economically realistic biotechnology, which are, in the case of Pb, 1% metal concentration in shoots and 20 t ha⁻¹ year⁻¹ shoot biomass productivity (6). For this reason, more recent research on phytoextraction focused on crop species such as Indian mustard, corn, oat, barley, pea, and ryegrass that display significant heavy metal tolerance combined with a high biomass productivity (6–13). To compensate for the relatively low metal accumulation capacities of these species, some researchers proposed to favor the soil/root transfer of metals by adding chelates such as EDTA, DTPA, CDTA, EGTA, or citric acid, which favor metal desorption from minerals (6, 8, 9, 11–14). Among these chelates, EDTA was shown to be the most efficient, as Pb shoot concentrations increased from less than 100 mg/Kg to 1.5% for Indian mustard grown in soils containing 600 mg/Kg Pb (8) and from less than 500 mg/Kg to 2% for corn grown in soils containing 2500 mg/Kg Pb (9).

EDTA has been shown not only to enhance Pb desorption from the soil components to the soil solution but also to increase its transport into the xylem and its transfer from the roots to the shoots (9, 14). Several studies on Pb accumulation in plants in the presence of ¹⁸⁷⁷C-labeled EDTA showed that both Pb and EDTA were present in the shoots, suggesting that the metal was absorbed and transferred as a PbEDTA complex (8, 14, 15). The physiological basis of the uptake of the complex and particularly the possibility for this negatively charged large molecule to cross the membrane is unknown. However, Vassil et al. (15) suggested that EDTA could damage the membrane of root cells by chelating Zn²⁺ and Ca²⁺ cations that stabilize this membrane, thus allowing free equilibration between the soil solution and the xylem sap. However, no evidence has been found that favors the hypothesis of an uptake of the PbEDTA⁻² complex or of Pb²⁺ and EDTA⁻⁴ separately.

To our knowledge, no studies have been conducted on Zn and EDTA phytoaccumulation, but given the high affinity of this metal for EDTA (logK = 16.44 for ZnEDTA⁻² compared to 17.88 for PbEDTA⁻² (16)), the chelate is expected to have a positive effect on Zn phytoextraction. Despite its effectiveness in phytoextraction, the use of EDTA in the environment is subject to discussion because of its low biodegradability.

Different mechanisms of Zn accumulation in the plant tissues have been proposed depending on the plant species studied (17). Brookes et al. (18) showed that zinc-resistant clones of Deschampsia caespitosa were able to actively pump zinc into the vacuoles of root cells, whereas zinc-sensitive clones had a much lower capacity to do so. Some Zn-containing granules have been identified by transmission electron microscopy and energy-dispersive X-ray analysis (TEM–EDX) in small vacuoles of root cells of various plants species (Deschampsia caespitosa and some crop species) grown in Zn solutions (19–22). Based on the presence of P, Mg, and K in these globular deposits and on the relatively constant Zn/P, Mg/P, and K/P elemental net count ratios (0.29–0.48, 0.32–0.41, and 0.39–0.43, respectively) the authors concluded that they contained Zn phytate, a myo-inositol kis-hexaphosphate. Phytate is a molecule present in the cells of grains, seeds, and plants, generally present as CaMg salt and associated with carbohydrates and proteins to form globular bodies, and whose main function is P, Mg, and K storage (23). Phytate presents a high affinity for Zn and Fe and could act as a metal-immobilizing molecule as well (24). Vazquez et al. (25) identified by TEM–EDX some Zn-containing globular crystals in vacuoles of Thlaspi caerulescens grown in Zn-containing nutrient solutions but with higher Zn/P elemental net count ratios (up to 6.9 in leaves and 8.8 in roots) and no Mg. They concluded that this Zn/P ratio was incompatible with Zn phosphate (Zn/P = 1.5) and Zn phytate composition (Zn/P = 0.33 for Zn₉₋₇-phytate and 0.17 for ZnNa₁₋₇-phytate). Other chemical forms of Zn were...
TABLE 1. Calculated Speciation of Zn and Pb in the Nutrient Solutions

<table>
<thead>
<tr>
<th>nutrient solution</th>
<th>composition*</th>
<th>metal speciation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnEDTA</td>
<td>Hoagland + 50 μM ZnSO₄ + 50 μM Na₂H₂EDTA</td>
<td>96% ZnEDTA, 4% Zn²⁺</td>
</tr>
<tr>
<td>Zn sulfate</td>
<td>Hoagland + 50 μM ZnSO₄</td>
<td>97% Zn²⁺, 3% ZnSO₄</td>
</tr>
<tr>
<td>PbEDTA</td>
<td>Hoagland + 125 μM Pb(NO₃)₂ + 125 μM Na₂H₂EDTA</td>
<td>100% PbEDTA²⁺</td>
</tr>
<tr>
<td>Pb nitrate</td>
<td>tap water + 1666 μM Pb(NO₃)₂</td>
<td>80% Pb²⁺, 14% PbSO₄PbO, 3% PbCl⁻, 3% PbNO₃⁻</td>
</tr>
</tbody>
</table>

*The pH was fixed at 5.5 for the four solutions.

identified by X-ray absorption spectroscopy in the same plant species also grown in Zn-containing nutrient solution. Salt et al. (26) found that Zn was mostly complexed to histidine in roots, transported as Zn²⁺ in the xylem sap, and complexed to organic acids in leaves.

In the case of Pb, some authors suggested that cell walls could play an important role in the accumulation of metals (17). This hypothesis has been verified by EXAFS spectroscopy in the case of the lichen Xanthoria parietina (27) and by TEM-EDX and EXAFS spectroscopy in the case of the heavy metal tolerant grass Agrostis capillaris (28). This latter species presented extracellular Pb-containing grains in the outermost layer of root cells predominantly composed of pyromorphite, a lead phosphate mineral.

The S-containing proteins, phytochelatins, have been often advocated to complex metals in plants, but this hypothesis has not been demonstrated yet for Zn and Pb.

In this paper, EXAFS spectroscopy and TEM-EDX were used to investigate the accumulation forms of Zn and Pb in bean (Phaseolus vulgaris) grown in metal-containing solutions, in the presence and absence of EDTA.

Materials and Methods

Materials. Bean seeds (Phaseolus vulgaris L. cv. Limburgse vroeg) received a cold treatment (+4 °C) for 3 days to break dormancy and to synchronize germination. They were transferred to a growth chamber to germinate between two layers of water-soaked rock wool for 4 days. Subsequently, seed coats were removed, and seedlings with a root length of approximately 1.5 cm were grown in 3-mm thick polystyrene squares by fixing the roots through 5-mm holes (9 of approximately 1.5 cm were grown in 3-mm thick polyethylene seed coats were removed, and seedlings with a root length transferred to a growth chamber to germinate between two layers of root cells predominantly composed of pyromorphite, a lead phosphate mineral.

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calculated ab initio from the structure of hopeite (Zn$_3$(PO$_4$)$_2$·4H$_2$O) (30) using FEFF7 code (31).

Results and Discussion

Metal Concentration. Measured Zn concentrations were 1468 (74 and 254 (20 mg/Kg dry weight in roots and leaves of the plants grown in Zn sulfate solution, respectively, and 503 (86 and 600 (39 mg/Kg in roots and leaves of the plants grown in ZnEDTA solution. These values suggest that Zn transfer from roots to shoots is increased in the presence of EDTA, which is in agreement with observations made on Pb accumulation (9, 14). Pb concentration in roots was not determined. Measured Pb concentrations in the leaves were 272 (25 mg/Kg for the plants grown in Pb nitrate solution and 1047 (40 mg/Kg for the plants grown in PbEDTA solution.

Transmission Electron Microscopy and X-ray Microanalysis. TEM observation of leaf cells for P. vulgaris exposed to 50 μM Zn solution revealed the presence of small particles (<0.1 μm) in the cytoplasm and in some chloroplasts (arrows in Figure 1A). EDX analysis of these precipitates showed that they contain high amounts of Zn and P, a small amount of Ca, but no K (Figure 1B). The Zn/P net count ratio was relatively constant (0.5 ± 0.1). This value lies between values reported in the literature for other plant species (see Introduction). For the control plants, no granules were observed, and no Zn was detected. These results suggest that Zn is accumulated in the leaves of P. vulgaris as a phosphate compound, but one cannot conclude on the nature of the P-containing ligand. To obtain structural information on the Zn chemical form and to compare Zn and Pb speciation in P. vulgaris, leaves and roots samples were analyzed by EXAFS spectroscopy.

EXAFS. Zn K-Edge. Four samples, including the roots and the leaves of P. vulgaris grown in ZnEDTA (samples A and B) and Zn sulfate media (samples C and D), were examined at the Zn K-edge. The spectra were compared to a database of mineral and organic Zn compounds (32), a selection of which is shown in Figure 2. The spectra for samples A and B are clearly different from the ZnEDTA model compound (FM = 2.42 × 10$^{-3}$ and 1.43 × 10$^{-3}$, respectively). In the nutrient solution containing Zn and EDTA, the calculated Zn speciation is 96% ZnEDTA$^{2-}$ and 4% Zn$^{2+}$ (compared to 97% Zn$^{2+}$ and 3% ZnSO$_4$ in the nutrient solution containing Zn sulfate). Thus, as suggested by TEM-EDX, EXAFS shows that the Zn chemical form has changed from the ZnEDTA solution to the plant. For those two samples, the closest spectra were Zn phosphate dihydrate (FM = 36 to 133). Sample C spectrum presents a great similarity with Zn phytate spectrum, particularly the asymmetric shape of the second oscillation and the presence of two shoulders on its left tail and the occurrence of a shoulder on the third oscillation.
Phaseolus vulgaris

The three references have the Zn first atom in common, which is composed of four oxygen atoms (Table 2, (33)). The second shell is composed of phosphorus atoms in Zn phosphate dihydrate and Zn phytate, respectively. The position of the imaginary function in the four Fourier transform spectra, which is shifted to the left for C atoms, is typical of tetrahedral coordination (1.96 Å for RO, 0.01 Å for R2, 0.004 Å for N, and 0.003 Å for α2). Thus, it is possible to distinguish P and C nearest neighbors and of carbon in Zn benzoate. As previously described (34), it is possible to distinguish P and C nearest neighbors by comparing the imaginary part of the second peak of the Fourier transform spectra, which is shifted to the left for C atoms. The position of the imaginary function in the four plant spectra matched that in the Zn phosphate dihydrate and the Zn phytate (Inset in Figure 3). Moreover, the amplitude of the second RSF peak was higher for the plant samples than for Zn phytate. These observations tend to suggest that in the four plant samples, Zn is bound to inorganic phosphate groups (e.g. Zn phosphate) rather than organic (e.g. phytate).

The structural parameters for the Zn phosphate dihydrate and phytate references (whose crystallographic structures are not known) and the four plant samples were determined by quantitative analysis of the EXAFS spectra (Table 2). For both references, the first shell was simulated by a tetrahedral oxygen shell containing 4 ± 0.7 P atoms at 1.97 ± 0.01 Å and 1.98 ± 0.01 Å for Zn phosphate dihydrate and Zn phytate, respectively. The second shell was simulated by two subshells containing 4 ± 0.7 P atoms in Zn phosphate dihydrate and only 1.5 ± 0.5 P atoms in Zn phytate, which indicates that Zn is bound on average to less than four phosphate groups in this latter compound (Figure 4). The plant spectra were simulated by a first shell composed of oxygen with distances typical of tetrahedral coordination (1.96 ± 0.01 Å for the roots and 1.99 ± 0.01 Å for the leaves) and by a second shell composed of two P subshells containing 1.8 to 2.4 ± 0.7 P atoms (Table 2). The number of next nearest P atoms is higher than for Zn phytate, as expected from the comparison of the second RSF peaks (Figure 3). If Zn was present as Zn phytate in the plant samples, the number of atomic neighbors could be lower than in the reference because of lower crystallinity. Thus, it is concluded that Zn phosphate dihydrate is the major form of Zn in the four plant samples, whatever the form of Zn (Zn²⁺, ZnEDTA⁻) in the growing solution.

Pb L_{III}-edge. The fingerprint approach is particularly adapted to Pb L_{III}-edge spectra because this metal exhibits a large variety of local environments, thus important differences in frequency and amplitude are observed between Pb reference spectra (35, 36). Pb L_{III}-edge EXAFS spectra of P. vulgaris leaves were compared to a database of mineral and organic Pb compound spectra (32, 36). For the leaves of the plant grown in the absence of EDTA (sample E), a very good spectral agreement was obtained with cerussite (anhydrous lead carbonate) (FM = 0.0165, Figure 5). Particularly, the shoulders on the third and fifth oscillations at 4.7 and 7.5 Å⁻¹, that constitute good fingerprints for PbCO³, are observed on the “leaves” spectrum. However, this latter spectrum presents a slightly lower amplitude, which likely results from the contribution of minor Pb species. In an attempt to identify the minor species, the spectrum was simulated by a linear combination of two reference spectra. A combination of 85% cerussite and 15% pyromorphite (Figure 5) and a combination of 83% cerussite and 17% Pb benzoate (not shown) provided the same quality of fit (FM = 0.0043). Thus, it is concluded that the major form of Pb in leaves of P. vulgaris grown in Pb nitrate-containing solution is cerussite, but the nature of the minor species, whose proportion is lower than 20%, remains uncertain. Cerussite was absent from the nutrient solution, as shown by the calculated speciation of Pb in the tap water (80% Pb²⁺, 14% larnakite (PbSO4PbO), 3% PbCl⁺, and 3% PbNO³⁻, Table 1). Thus, cerussite likely precipitated in the plant itself.

TABLE 2. Structural Parameters Determined by Simulating EXAFS Spectra for Zn in Phaseolus vulgaris Roots and Leaves

<table>
<thead>
<tr>
<th>Sample</th>
<th>First O-shell</th>
<th>Second P-shell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R0 (Å)</td>
<td>N₀</td>
</tr>
<tr>
<td>Zn phosphate</td>
<td>1.97</td>
<td>4.0</td>
</tr>
<tr>
<td>Zn phytate</td>
<td>1.98</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Pb L_{III}-edge EXAFS spectra of P. vulgaris grown in ZnEDTA- (A: roots, B: leaves) and Zn sulfate-containing nutrient solution (C: roots, D: leaves), and for Zn benzoate, Zn phytate and Zn phosphate dihydrate. Inset: Zoom on the imaginary parts in the 2.3–3.3 Å range.
For the plant grown in the presence of PbEDTA (sample F), MINTEQA2 calculation showed that 100% of Pb is complexed to EDTA in the solution (Table 1). However, the spectra for sample F and for PbEDTA$^{2-}$ are clearly distinct. Also, spectrum F differs from spectrum E (Figure 5). The amplitude of spectrum F is particularly low, which is indicative of a distribution of species. Accordingly, no good agreement was obtained with one reference spectrum. The best statistical solution was obtained for a combination of 51% pyromorphite, 27% PbEDTA$^{2-}$, and 22% Pb salicylate (FM $= 4.4 \times 10^{-3}$, Figure 5), but satisfying solutions were also obtained with mixtures of cerussite, PbEDTA$^{2-}$, and various Pb-organic acid complexes. In all cases, PbEDTA$^{2-}$ systematically showed up, and, therefore, its presence is very likely. When PbEDTA$^{2-}$ was excluded from the fit, FM values increased to $7.0 \times 10^{-3}$. Thus, Pb is probably present as a mixture of PbEDTA$^{2-}$ and other species that are difficult to identify because of the low amplitude of Pb-organic compounds EXAFS spectra. Thus, it can be concluded from these results that both Pb and EDTA can be absorbed by the plant and that one part of Pb present in the leaves is complexed to EDTA. The mechanism of PbEDTA$^{2-}$ or Pb$^{2+}$ and EDTA$^{4-}$ absorption and transport through the membrane is still to be elucidated.

This study shows that the mechanism of metal accumulation in P. vulgaris depends on the nature of the metal studied. For Zn, no difference was observed between plants grown in ZnEDTA and Zn sulfate solution. In contrast, cerussite was the predominant Pb species in the absence of EDTA, but in the presence of EDTA, a mixture of PbEDTA$^{2-}$ and unidentified Pb species was the result. Thus, highly stable metal–EDTA complexes present in soil solutions can be totally (Zn), or partly (Pb), dissociated when absorbed by the plant.

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Literature Cited