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To cite this version:
Anne Straczek, Geraldine Sarret, Alain Manceau, Philippe Hinsinger, Nicolas Geoffroy, et al.. Zinc distribution and speciation in roots of various genotypes of tobacco exposed to Zn. Environmental and Experimental Botany, Elsevier, 2008, 63, pp.80-90. hal-00311796

HAL Id: hal-00311796
https://hal.archives-ouvertes.fr/hal-00311796
Submitted on 21 Aug 2008

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**Full title:** Zinc distribution and speciation in roots of various genotypes of tobacco exposed to Zn

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**Abstract**

Cell walls of roots have a great reactivity towards metals, and may act as a barrier limiting the entry of metals, especially in non hyperaccumulating species. The aim of this study was to determine the localization and speciation of Zn in roots of tobacco (*Nicotiana tabacum*) grown in Zn-contaminated substrates. Chemical extractions and EXAFS spectroscopy were applied on whole roots and on isolated cell walls of roots. Our results show that cell walls of roots exhibited a distribution of Zn affinity sites, from water-soluble to non-exchangeable Zn. In whole roots, Zn was bound with oxalate and other COOH/OH groups: the first species was probably intracellular while the second was attributed to Zn bound to the cell walls and, to a lesser extent, to intracellular organic acids. Moreover, Zn phosphate was also identified, and this species was CuSO₄-extractable. It probably resulted from chemical precipitation in the apoplast, and explained the steady increase in exchangeable root Zn observed in root of
tobacco during the culture. This study shows the strength of combining EXAFS and chemical extractions for studying localization and speciation of metals in plants.

**Keywords**

Cation Exchange Capacity of Roots (CECR), Cell walls, chemical extractions, EXAFS, pectin, cellulose

**Abbreviations**

A1, control genotype of tobacco (wild-type genotype neutrally transformed with a CaMV 35S promoter-GUS construct);

CaMV, cauliflower mosaic virus;

CECR, cation exchange capacity of roots;

C5, genotype of tobacco genetically transformed to over-accumulate the Fe storage protein ferritin in the cytoplasm;

EDTA, ethylene-diamine-tetra-acetic acid;

ESRF, European Synchrotron Radiation Facility;

EXAFS, extended X-ray absorption fine structure;

Fe-EDTA, ethylene-diamine-tetra-acetic acid iron(III) sodium salt;

GUS, beta-D-glucuronidase (EC 3.2.1.31);

NSS, normalized sum-squares;

TEM-EDX, Transmission electron microscopy and Energy dispersive X ray;
Introduction

Numerous authors (e.g. Haynes, 1980; Sattelmacher, 2001) have shown that the cell walls of plant roots are involved in the acquisition of mineral elements. This compartment also plays a role in metal tolerance by acting as a barrier for some elements (Ernst et al., 1992). Main components of plant cell walls are cellulose, hemicellulose, pectin and glycoproteins. The cation exchange capacity of roots (CECR) arises mostly from carboxyl and hydroxyl groups, and to a minor extent from phenolic and amine groups (Meychik et al., 2001). The structure and the composition of the cell walls (and consequently the CECR) vary as a function of the plant species, of its nutrition and of the age of the plant tissues. Particularly, the development of secondary cell walls in older tissues induces a decrease in CECR because of the lower pectin and higher lignin content of this structure. The CECR ranges between 10 and 20 cmol$_c$ kg$^{-1}$ (or meq 100 g$^{-1}$) for monocot species and between 20 and 50 cmol$_c$ kg$^{-1}$ for dicot species (Dufey et al., 2001). The affinity of cations for exchangeable sites on root cell walls decreases in the order H > Cu > Ca > Zn according to Nishizono et al. (1987), and H > Cu > Zn > Ca according to Ernst et al. (1992). A similar order of affinity was found for pectin (Franco et al., 2002). Based on the high affinity of Cu for the cell walls, Dufey and Braun (1986) showed that saturating the cell walls with Cu, and then extracting it using HCl, was an easy and reliable way to measure the CECR because they obtained comparable CECR values by this method and by acid-base titration of roots.

An overview of the literature shows a great variability in Zn localization and exchangeability in plants roots: exchangeable Zn represented 10% of total root Zn in Silene vulgaris (Harmens et al., 1993), 16% in wheat (Triticum aestivum) and 46% in soybean (Glycine max) (Steveninck et al., 1993), 27% in barley (Hordeum vulgare) (Wu et al., 2005), 60% in the hyperaccumulator Thlaspi caerulescens (Lasat et al., 1998), and 67 to 87% in the Zn-tolerant fern Athyrium yokoscense (Nishizono et al., 1987). This wide range of responses...
may result from actual differences between species, but also from the techniques used for measuring the so called "exchangeable" Zn (isolation of cell walls, chemical extractions, isotopic exchange, transmission electron microscopy coupled with energy dispersive X-ray microanalysis, etc…). Other possible sources of differences include the duration and intensity of Zn exposure (Vasquez et al., 1994), and the age of the plants.

Although zinc has a high affinity for cell walls, there is no consensus on the stability of Zn-root cell wall complexes. Nishizono et al. (1987) showed that Zn associated to isolated root cell walls of *Athyrium yokoscence* was totally exchangeable. Lasat et al. (1998) found that exchangeable Zn represented the majority (but not all) of apoplasmic Zn in the roots of *Thlaspi caerulescens*. Similarly, Hart et al. (1998) found a small proportion of strongly bound Zn (*i.e.*, non exchangeable) on cell walls of wheat roots.

Extended X-ray absorption fine structure (EXAFS) spectroscopy is well adapted for the study of metal speciation in plant samples because it is an element-specific probe sensitive to the short-range order (Salt et al., 2002). The main limitation of bulk EXAFS is that it provides averaged information. For instance, the spectrum for whole roots would contain averaged contribution of the different cell compartments (apoplasm, symplasm, etc…), and it may be difficult to isolate them and to obtain structural information on each one. Combining this spectroscopic method with chemical extractions could be a way to overcome this limitation.

In this study, the distribution and the speciation of Zn in roots of tobacco was studied by a purely chemical approach and a purely (Zn K-edge EXAFS) spectroscopic approach which was conducted on whole roots and on isolated cell walls of roots, and a combination of chemical approach and EXAFS spectroscopic approach on whole roots.

**Material and methods**

*Plant material and preculture of tobacco*
The plant materials were two genotypes of tobacco (*Nicotiana tabacum cv SR1*). A control genotype (A1) was a wild-type genotype transformed with a CAMV 35S promoter-GUS construct without any gene insert. The other genotype (C5) was genetically transformed to over-accumulate ferritin in the cytosol (van Wuytswinkel et al., 1999). Ferritin is an iron storage protein naturally present in plants. Animal ferritins are known to bind Zn (Briat and Lebrun, 1999), whereas this has not been demonstrated for plant ferritins.

Seeds were surface sterilised with NaOCl for 25 min, then carefully washed with sterile water. Plants were cultivated in a cropping device designed to easily separate the roots from the growing soil at harvest (Niebes et al., 1993). The plant container was made of a PVC cylinder (inner diameter 40 mm) closed at the bottom by a fine polyamide mesh (30 μm pore diameter, Sefar Nytel/Fyltis). For the preculture, plant containers were placed on a nutrient gel in sterile and capped cropping boxes (150×150×135 mm, MERCK eurolab, Polylabo).

The nutrient gel was prepared by adding 1.0 g L\(^{-1}\) gelrite (Sigma G1910) and 0.6 g L\(^{-1}\) phytagel (Sigma P8169) to a Hoagland solution containing 5 mM KNO\(_3\), 5 mM Ca(NO\(_3\))\(_2\), 2 mM MgSO\(_4\), 1 mM KH\(_2\)PO\(_4\), 50 μM H\(_3\)BO\(_3\), 50 μM MnSO\(_4\), 50 μM Fe-EDTA, 15 μM ZnSO\(_4\), 3 μM (NH\(_4\))MoO\(_4\), 2.5 μM KI, 50 nM CoCl\(_2\), and 50 nM CuSO\(_4\). Five seeds were put in each plant container, and each cropping box contained 9 containers. Boxes were placed in a growth chamber with a 16/8 h day/night cycle, light intensity of 250 μmol photons m\(^{-2}\) s\(^{-1}\), temperature of 23/20°C and 75/80% relative humidity. After two weeks, the cropping boxes were progressively opened for 3 days so that plants could adapt to ambient culture conditions.

The containers were then transferred in a nutrient solution containing 1 mM KNO\(_3\), 1 mM Ca(NO\(_3\))\(_2\), 0.5 mM MgSO\(_4\), 20 μM Fe-EDTA, 10 μM H\(_3\)BO\(_3\), 5 μM KH\(_2\)PO\(_4\), 2 μM MnCl\(_2\), 0.5 μM MoNaO\(_4\), 0.5 μM ZnSO\(_4\) and 0.2 μM CuCl\(_2\) (10 plant containers per 5 L bucket). The solution was renewed weekly. After two weeks, the plants were then 4-week old, and each
container presented a homogeneous root mat formed by the roots of 5 plants. The pH of the nutrient solution was between 5.5 and 6.1.

Culture of tobacco in hydroponics

All culture conditions are summarized in Table 1. For the measurement of the CECR (culture n°1), plant containers containing 4-week old plants (A1 and C5 genotype) were transferred in a nutrient solution devoid of Fe, and containing 100 µM ZnSO$_4$, 1 mM KNO$_3$, 1 mM Ca(NO$_3$)$_2$, 0.5 mM MgSO$_4$, 10 µM H$_3$BO$_3$, 5 µM KH$_2$PO$_4$, 2 µM MnCl$_2$, 0.5 µM MoNaO$_4$, and 0.2 µM CuCl$_2$. Note that P concentration was low (5 µM) to avoid precipitations with Zn, as predicted by the SOILCHEM speciation code (Sposito and Coves, 1988). Plants were grown for 2, 4, 7 and 14 days (8 plant containers per 5 L bucket). The pH of the solution was 5.5 at the beginning of culture.

For the comparison of the sequential extraction procedures, 4-week old plants (A1 genotype) were grown in the same conditions for 4 days (culture n°2). For the EXAFS analyses, 4-week old plants (C5 genotype) were grown in the same conditions except Zn concentration (200 µM instead of 100 µM ZnSO$_4$) for 4 days (culture n°3). No toxicity symptoms were observed in any culture, probably due to the presence of Ca in the nutrient medium which partially alleviates Zn toxicity in tobacco (Sarret et al., 2006).

Culture of tobacco on artificial substrates (culture n°4)

Artificial substrates were made of agarose nutrient gel containing various Zn-bearing minerals to provide a range of Zn availabilities with in spite of an identical total Zn content in the substrates. The nutrient solution contained 1 mM KNO$_3$, 0.625 mM Ca(NO$_3$)$_2$, 0.5 mM MgSO$_4$, 0.375 mM (NH$_4$)$_2$SO$_4$, 10 µM H$_3$BO$_3$, 5 µM KH$_2$PO$_4$, 2 µM MnCl$_2$H$_2$O, 0.5 µM MoNaO$_4$, 2H$_2$O, and 0.2 µM CuCl$_2$. Zn-bearing minerals included Zn-sorbed synthetic...
ferrihydrite and Zn-sorbed hectorite containing 0.2% dry weight Zn. Ferrihydrite is a poorly crystalline iron oxyhydroxide with a high sorption capacity, and hectorite (SHCa-1 from the Source Clay Repository of the Clay Minerals Society) is a magnesian smectite composed of an octahedral sheet of magnesium sandwiched between two tetrahedral sheets of silicon. The substrates contained 49 g L$^{-1}$ of Zn-sorbed ferrihydrite or hectorite, and 10 g L$^{-1}$ of agarose. A control culture substrate was made with agarose only, the nutrient solution being supplemented with 1500 µM ZnSO$_4$. Note that a fraction of added Zn is finally adsorbed on agarose gel (Calba et al., 1999). Four-week old plants (A1 and C5 genotypes) were grown for 4 days on Zn-ferrihydrite, Zn-hectorite and Zn-agarose substrates (Table 1). Table 2 shows that Zn root concentrations increased in the order hectorite < ferrihydrite < agarose. At the end of the culture, shoots and roots were harvested separately and stored for further chemical and EXAFS analyses.

Extraction of cell walls of roots of tobacco

Fresh roots of 4-week old A1 genotype tobacco were harvested and then immersed in a 1% v:v Triton X100 detergent solution with 1 mM CaCl$_2$ to dissolve the cell content (Calba et al., 1999). The detergent solution was renewed periodically for 28 days. The detergent was then removed by washing the material for 15 days with a 1 mM CaCl$_2$ solution. The entire treatment was carried out at 4°C. Zinc-cell wall complexes were conditioned prior to sequential extractions as follows. Three g of cell walls were placed in 1 L of nutrient solution (the same as the one used for the hydroponic culture without Fe-EDTA) containing 100 µM ZnSO$_4$, then shaken end over end for 24 hours. Other Zn-cell wall complexes were prepared for EXAFS analysis (see the EXAFS section).
Sequential extractions of whole roots and isolated cell walls of roots of tobacco

The CECR was determined by sequential extractions by adapting the procedure of Dufey and Braun (1986). Roots of A1 and C5 genotype tobacco plants from culture n°1 were harvested. Each root sample was made of the roots of 5 plants grown in the same container. An aliquot was oven dried at 105°C, digested and analyzed for total Zn. The remainder (25 mg ± 8 mg dry weight) was shaken end over end in 5 mL of 10 mM CuSO$_4$ during 30 min. The initial pH of the solution was 4.8. The suspension was then filtered, and Ca and Zn concentration in the filtrate were measured. Copper is supposed to displace all cations associated to the cell walls and to saturate the CECR. The roots were then briefly rinsed with a solution containing 0.1 mM CuSO$_4$ to reduce the excess Cu in the interstitial volume of roots before to be shaken end over end in 50 mL of 100 mM HCl during 20 min to extract Cu, the suspension was filtered, and Cu concentration in the filtrate was measured. The acidic extraction is supposed to desorb Cu from the cell walls. The CECR was thus estimated from the amount of desorbed Cu, by considering Cu as a divalent cation.

Sequential extractions were performed on whole roots of A1 tobacco from culture n°2 (Table 1) and on isolated cell walls. Samples (22 mg ± 7 mg dry weight for roots and 32 mg ± 5 mg dry weight for cell walls) were treated with 10 mM CuSO$_4$, then 100 mM HCl as described above. This procedure was realized at 25°C and at 4°C. Other extraction procedures were tested at both temperatures. The first one involved three successive extractions in 10 mM CuSO$_4$ during 30 min, and then an extraction in 50 mL of 100 mM HCl during 20 min. The second one involved an extraction in 5 mL ultra pure water during 2 hours, followed by an extraction in 5 mL of 10 mM CaCl$_2$ during 2 hours. The third one involved an extraction in 50 mL of 10 mM EDTA pH 7 during 2 hours. For all procedures, after each extraction, the root suspension was filtered over an ashless filter paper (Whatman 40), and elemental concentrations were determined in the filtrate and in the extracted roots.
Roots of C5 genotype tobacco from culture n°3 (Table 1) were treated at 25°C following the CuSO$_4$/HCl procedure, and aliquots of non-extracted, CuSO$_4$-extracted and CuSO$_4$/HCl-extracted roots were kept for EXAFS analyses.

Chemical analyses of plants and solutions

Samples (shoots, whole roots, isolated cell walls of roots and root residues after extractions depending on the experiment) were weighed, oven-dried at 105°C and digested in a 1:1 mixture of hot concentrated HNO$_3$ and HClO$_4$ (A.O.A.C., 1975). Ca, Zn and Cu concentrations were determined in the digests and in the filtrates of the chemical extractions by flame atomic absorption spectrometry (Varian SpectrAA-600, Australia). Malate, citrate and oxalate concentrations were measured in the roots of A1 genotype tobacco at the end of the preculture (4-week old plants). One g of fresh roots was put in 10 mL of HCl 100 mM at 60°C. After 20 min, solution was filtered over an ashless filter paper (Whatman 40) and the supernatant was analyzed by High Pressure Ionic Chromatography (Dionex 4,000) using an AS11 column. The elution was performed with a NaOH gradient, and the signal was detected by conductimetry, and analyzed with a integrator Chromjet (Spectra-Physics) integrator.

For each analysis, four replicates were prepared and analyzed. All results are expressed relative to dry weight. Statistical analysis was performed using the ANOVA procedure with the test of least significant difference (LSD, p=0.05) of the Statistica Software (Statsoft Inc.).

Zn K-edge EXAFS spectroscopy

Zn model compounds

A variety of Zn-model compounds were used for the EXAFS data analysis. Zn-oxalate dihydrate and Zn-citrate dihydrate were purchased from Alfa (Berkshire, UK). The preparation of Zn-malate and Zn-sorbed hydroxylapatite were described previously (Sarret et
al., 2002; Panfili et al., 2005). The Zn-cysteine spectrum was provided by S. Beauchemin (Beauchemin et al. 2004). The Zn-cell wall complexes containing 0.75, 1.4, 12.7 and 69.6 mmol kg\(^{-1}\) d.w. Zn were prepared by placing 100 mg (dry weight) of isolated cell walls of roots in 50 mL of 1.5, 6.1, 30.3 and 303 µM Zn(NO\(_3\))\(_2\) at pH 5.0, respectively, and shaking end over end for 24 hours. Final pH values were 5.0, 5.4, 5.4, and 5.4, respectively. The suspensions were then centrifuged, and the Zn loading was determined by difference between initial and supernatant Zn concentrations.

For the Zn-cellulose complexes, 200 mg of cellulose (Sigma-Aldrich) were suspended in 60 mL of water and the pH was adjusted to 5.0. Two samples were prepared: after addition of 1 and 2 mL of 1.53 mM Zn(NO\(_3\))\(_2\) at pH 5.0, respectively, the suspensions were stirred during 3 hours at fixed pH 5.0 by adding 0.5M NaOH or HNO\(_3\), then centrifuged. The Zn content in the Zn-cellulose complexes was calculated as the difference between the amount of Zn introduced and the amount of Zn measured in the supernatant: they were 1.27 and 3.82 mmol kg\(^{-1}\) d.w. Zn. For each Zn concentration, half of the Zn-cellulose samples was freeze-dried, and half was kept in wet state for EXAFS analysis. For the Zn-pectin complexes, 166 mg of pectin extracted from apples esterified at 70 to 75% (Fluka) were dissolved in 30 mL of water, and the pH was adjusted to 5.0. Two samples were prepared: after addition of 0.4 and 0.9 mL of 4.31 mM Zn(NO\(_3\))\(_2\) at pH 5.0, respectively, the suspensions were stirred during 3 hours at fixed pH 5.0 by adding 0.5M NaOH or HNO\(_3\). The Zn-pectin complexes were directly freeze-dried because they could not be concentrated by centrifugation. Zn concentrations were 15.29 and 7.65 mmol kg\(^{-1}\) d.w. of pectin, respectively.

EXAFS data acquisition and treatment

Zinc K-edge EXAFS analyses were performed on untreated whole roots of A1 and C5 genotype tobacco grown for 4 days on artificial substrates as described above (culture n°4),
and on whole roots of C5 genotype tobacco grown for 4 days in 200 μM Zn, untreated and treated by chemical extractions (culture n°3). After harvesting, root samples were freeze-dried, ground and pressed as pellets. EXAFS experiments were performed on beamlines BM32 and FAME at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) in transmission mode for the references, and in fluorescence mode using a 30-element solid-state Ge detector (Canberra) for the root samples. The great sensitivity of the spectrometer made it possible to study samples containing down to 0.76 mmol Zn kg$^{-1}$ dry weight. EXAFS data extraction was performed according to standard methods. Spectra were simulated by linear combination fits using a library of Zn reference compounds, including Zn complexed to simple organic acids and amino acids, cellulose, pectin, and isolated cell walls of roots, and mineral and organic Zn-phosphate compounds (Guiné et al., 2006). For the first shell simulation, EXAFS spectra were Fourier transformed, and the contribution of the first coordination shell was simulated in $k$ and $R$ space. Theoretical functions for the Zn-O and Zn-S pair were calculated by FEFF7 (Rehr et al., 1991) from the structure of Zn-malate dihydrate (Reed and Karipides, 1976) and sphalerite (Jumpertz, 1955) respectively.

Results

Accumulation of Zn in tobacco

In culture n°1, A1 and C5 genotypes of tobacco were cultivated in hydroponics without Fe and with 100 μM Zn. After 14 days of culture, total Zn uptake and Zn shoot content were comparable for both genotypes: total uptakes were 1.1 ± 0.2 and 1.3 ± 0.2 μmol Zn per A1 and C5 plants, and shoot contents were 15.1 ± 1.7 and 15.9 ± 1.6 μmol Zn per g, respectively. At the opposite, the Zn concentration of roots was larger for the ferritin overexpressor (98.8 ± 5.2 μmol g$^{-1}$) than for the wild type (73.5 ± 5.8 μmol g$^{-1}$). For both genotypes, the pH of the
nutrient solution increased from 5.5 at the beginning, to 6.0 ± 0.1 after two days and to 7.0 ± 0.1 after 14 days of culture. The calculation of Zn speciation with SOILCHEM (Sposito and Coves, 1988) suggests a minor precipitation at pH 7.0: At pH 5.5, calculated Zn species are 93% free Zn$^{2+}$ and 6% ZnSO$_4$. At pH 7.0, they are 86% free Zn$^{2+}$, 6% ZnSO$_4$, 1% ZnB(OH)$_4$ and 6% precipitated Zn-phosphate.

Changes in cation exchange capacity of roots (CECR) of tobacco

The cation exchange capacity of the roots (CECR) was determined at different times of the culture. It did not vary significantly between 0 and 14 days, and was comparable for the two genotypes (Figure 1a). The mean value for the two genotypes and all exposure durations was 32 ± 3 cmol$_c$ kg$^{-1}$. The ratio of Ca extractable by CuSO$_4$ to the CECR (“Ca:CECR”), which corresponds to the fraction of the CECR occupied by Ca, did not vary significantly (Fig. 1b). The concentration of Ca(NO$_3$)$_2$ being kept the same (1.02 ± 0.05 mM) in the preculture and culture solutions during the 14 days of culture, this steady-state was expected. Again, there was no significant difference between the two genotypes. Calcium accounted for 52 ± 10% of the CECR (mean value for the two genotypes and all exposure durations). On the contrary, an increase in the fraction of the CECR occupied by Zn (“Zn:CECR”) was expected because Zn concentration increased from 0.5 µM in the preculture to 100 µM in the culture solution.

Cations exchange between the solution and roots is supposed to reach equilibrium within 48h (Meychik et al., 2001). In our experiment, we observed a continuous increase in Zn:CECR during the 14 days of culture (Fig. 1b). At the end of the culture, the sum of Ca:CECR and Zn:CECR accounted for 90±5% of the CECR for A1, and 110±5% of the CECR for C5 tobacco.

Comparison of different sequential extractions of Zn accumulated in roots of tobacco
The accumulation compartments of Zn in A1 tobacco roots exposed to 100 µM Zn for 4 days (culture n°2) were studied by chemical extractions (Fig. 2). Various procedures were compared:

(i) 10 mM CuSO$_4$, then 100 mM HCl, (ii) three successive extractions with 10 mM CuSO$_4$, then 100 mM HCl, (iii) pure water, then 10 mM CaCl$_2$, and (iv) 10 mM EDTA.

Each procedure was done at 4°C and 25°C to evaluate the role of active Zn transport during the treatments. Chemical extractions on whole roots and isolated cell walls of roots were compared to distinguish the intra- and extra-cellular contributions.

On whole roots, water extracted about 20% of total Zn, and the CaCl$_2$ solution removed another 20%. The one-step extraction with CuSO$_4$ yielded similar results as the water + CaCl$_2$ extraction (40 ± 8 % and 39 ± 5 respectively). The three-step CuSO$_4$ and the EDTA extraction were slightly more efficient (52 ± 4 % and 55 ± 9% of total Zn, respectively). These data are consistent with the occurrence of a distribution of affinity sites. The results obtained at 4°C and 25°C were roughly similar except for the HCl treatment (extraction doubled at 25°C compared to 4°C). This suggests that Zn transport through the cell membranes during the water, CaCl$_2$, CuSO$_4$, and EDTA extractions was insignificant. This result also suggests that cation diffusion within the roots is not significantly different at 4°C and at 25°C. In contrast, HCl extractant is likely resulting in some damage of the integrity of cell membranes and thereby in the release of intracellular Zn, as suggested for Cu by Iwasaki et al. (1990).

Results obtained on the isolated cell walls also suggested a distribution of affinity sites. Zn extractability was higher for the isolated cell walls than for the whole roots (73 to 96% compared to 39 to 55%).

**Determination of Zn speciation in tobacco roots**
First, in order to give an idea of the sensitivity of EXAFS spectroscopy, Figure 3 shows the spectra for various Zn reference compounds of interest for this study. The spectrum for Zn-oxalate dihydrate presents a characteristic shoulder between 6.1 and 6.6 Å\(^{-1}\). It is due to the well ordered structure of this organic compound, Zn being bound to four carboxyl groups in a planar configuration (Fig. 3). Citrate, malate and pectin contain hydroxyl and carboxyl functional groups. The spectrum for Zn-citrate exhibits a weakly pronounced shoulder around 6.5 Å\(^{-1}\), and the spectrum for Zn-malate is even smoother. This reflects an increase in disorder from Zn-oxalate to Zn-citrate, and from Zn-citrate to Zn-malate. The spectra for Zn-pectin and for Zn-cell walls at various Zn concentrations present strong similarities with Zn-malate, which suggests a similar Zn local structure. Thus, in the cell walls and in Zn-pectin, the metal is probably bound to hydroxyl and carboxyl groups. The spectra for Zn-cellulose (recorded in freeze-dried and hydrated state) have a markedly higher frequency relative to Zn-pectin, and present some similarities with aqueous Zn\(^{2+}\). This suggests an outer-sphere configuration, i.e., Zn being fully hydrated and bound to cellulose through weak interactions. This is consistent with the fact that cellulose contains hydroxyl groups only, and that these groups are fully protonated at pH 5.0, and deprotonate in alkaline conditions (pH > 10) (Smith and Martell, 1982). The structural parameters for Zn first coordination shell in these compounds were determined. For Zn-cell walls and Zn-pectin, Zn-O distances were 1.99 and 2.00 Å, respectively (Table 2). Considering typical Zn-O distances for tetrahedral and octahedral coordination (1.95 to 2.0 and 2.0 to 2.2 Å, respectively, Sarret et al., 1998), this suggests that the metal occupies both types of coordination sites in these samples. For Zn-cellulose, a Zn-O distance of 2.07 Å was found, indicating an octahedral coordination. Figure 3 also shows the spectra for an inorganic and organic Zn-phosphate, Zn-sorbed hydroxylapatite and Zn-phytate, respectively. Zn is in tetrahedral coordination in both compounds (Table 2). The similarity between the two spectra suggests that it may be difficult to distinguish between
mineral and organic Zn-phosphate, especially in case of a mixture of Zn species. Finally, figure 3 shows the spectrum for Zn-sorbed ferrihydrite, which is used as a proxy for Zn in ferritin (Briat and Lebrun, 1999).

Figure 4 presents the spectra for the whole roots of A1 and C5 genotype tobacco grown on agarose, ferrihydrite and hectorite substrates (culture n°4). They present slight differences in frequency and shape of the oscillations. For instance, some of them exhibit a shoulder on the second oscillation similar to (but less pronounced than) Zn-oxalate dihydrate. This feature suggests that Zn-oxalate might be present as a minor species. Because of the limited number of spectra, principal component analysis could not be used, and spectra were simulated by linear combinations of reference spectra (Table 2). A combination of two to three components was sufficient to provide satisfactory fits, and four-component fits did not decrease normalized sum-squares (NSS, formula given in Table 2) significantly (< 5%). As anticipated, Zn-oxalate was identified, and represented up to 30 % of total Zn in some whole roots. For these samples, unsatisfactory fits were obtained if Zn-oxalate was removed from the set of references (NSS increased by more than 10%). For technical reasons, oxalate concentration was not measured in the roots studied by EXAFS, but in the whole roots of A1 genotype tobacco grown on a Zn-free medium (Table 3). This concentration (497 mmol kg\(^{-1}\)) was by far sufficient to explain the highest Zn-oxalate concentration determined by EXAFS (8.2 mmol kg\(^{-1}\) for the A1 genotype tobacco grown in the agarose medium, value obtained by multiplying the molar percentage of Zn oxalate determined by EXAFS with the total Zn concentration in the roots).

The second (and most represented) Zn species identified was Zn-pectin and/or Zn-malate. The similarity between the two EXAFS spectra prevents the positive identification of one or the other compound (see above). Whole roots do contain malate (Table 3). However, they are richer in oxalate and citrate (Table 3), and these two organic acids have a higher
affinity for Zn than malate (log K = 4.0 for Zn oxalate, and 4.9 for Zn citrate, compared to 2.9 for Zn malate, Smith and Martell, 1982). Therefore, the presence of Zn-malate as major Zn species is unlikely in these root samples. Zn-pectin which can be used as a proxy for Zn-cell walls (see above) is more likely. Because of the similarity between Zn-pectin, Zn-malate and Zn-citrate, this pool may contain a minor proportion of complexes of Zn with simple organic acids complexes: it is referred to as "Zn-COOH/OH" in Table 2.

The third species identified was Zn-phosphate. Depending on the samples, this pool was simulated by mineral (Zn-sorbed hydroxylapatite and parahopeite) or organic (Zn-phytate) references. Although this species was the least abundant in the whole roots, unsatisfactory fits were obtained if Zn-phosphate references were removed from the set of references (NSS increased by more than 10%). As explained above, it was not possible to conclude on the exact nature of Zn-phosphate. Finally, the Zn-sorbed ferrihydrite reference, used as a proxy for Zn incorporated in ferritin, did not show up it the simulations, suggesting that this species was insignificant.

Table 2 summarizes the results of EXAFS analyses and shows that the "Zn-COOH/OH" pool was the major species (43 to 80% of total Zn depending on the root sample), followed by Zn-oxalate (0 to 30%) and Zn-phosphate (0 to 16%).

In order to get some insights on the localization of these three Zn species, whole roots before and after extraction with CuSO₄ and CuSO₄ / HCl were studied by EXAFS spectroscopy (Fig. 4 and Table 2). For this purpose, whole roots of C5 genotype tobacco were grown in hydroponics containing 200 µM Zn were used (culture n° 3, chemical extraction experiment in Table 2). The speciation of Zn in this nutrient solution was similar to the one calculated for the 100 µM Zn solution, with 92% free Zn²⁺ and 6% ZnSO₄ at pH 5.5, and 88% free Zn²⁺, 7% ZnSO₄, 1% ZnB(OH)₄ and 3.5% of precipitated Zn-phosphate at pH 7.0. For
the untreated roots, the distribution of Zn species was comparable to what was found for
plants grown in solid substrates. Roots contained $81 \pm 4\%$ Zn-COOH/OH, $6 \pm 4\%$ Zn-oxalate, and $6 \pm 4\%$ Zn-phosphate. By multiplying these percentages with Zn total root concentration, one obtains the molar concentration of Zn for each species, i.e., $39.9 \pm 1.9, 3.0 \pm 2.0$ and $3.0 \pm 2.0$ mmol Zn kg$^{-1}$, respectively. In the CuSO$_4$-extracted roots, the proportion of Zn-COOH/OH species was decreased to $72 \pm 10\%$ ($23.3 \pm 3.2$ mmol Zn kg$^{-1}$). The Zn-phosphate species identified in the untreated roots was absent, and the change in Zn-oxalate concentration was within experimental error. These results indicate that the CuSO$_4$ extraction removed all of the Zn-phosphate pool, about half of the Zn-COOH/OH pool, but did not alter the Zn-oxalate pool.

The spectrum for the residues after the HCl extractions strongly differed from the other root spectra, and could not be simulated by the three reference spectra used before. Its frequency was intermediate between the untreated root spectrum and the Zn-cysteine, in which Zn is bound to sulfur atoms. The first shell simulation showed that Zn was bound to 4.5 sulfur atoms at 2.28 Å, and 1.3 oxygen atoms at 2.13 Å (Figure 5 and Table 2). The presence of sulfur atomic neighbours might suggest the binding of Zn by glutathione, phytochelatins or metallothioneins. However, considering Zn concentration in the HCl-extracted roots (14.00 mmol kg$^{-1}$) and the CuSO$_4$-extracted roots (32.31 mmol kg$^{-1}$), S neighbours should have been detected in the CuSO$_4$-extracted roots. As this was not the case, it is concluded that some redistribution of Zn occurred during the HCl extraction. Thus, this acidic treatment, which was supposed to desorb Cu from the cell walls, probably damaged a breakage of cellular membranes and induced the subsequent release of intracellular proteins, leading to the formation of Zn-S bonds. Note that this artefact was not observed for CuSO$_4$–extracted roots.

Discussion
Concentrations of Zn in shoots and roots of crop species exposed to high Zn concentration are highly variable (Table 4). In crop species grown in similar conditions of Zn exposure, Zn shoots concentration ranged between 1 and 19 mmol kg$^{-1}$ and Zn roots concentration ranged between 5 and 150 mmol kg$^{-1}$ (Fargasova et al., 2001; van Steveninck et al., 1993; Fontes and Cox, 1998; Brune et al., 1994). With a concentration of 15-16 mmol kg$^{-1}$ and 74-99 mmol kg$^{-1}$, tobacco appears as a crop species with high Zn uptake capacity. Similar Zn concentrations were observed in shoots and roots of the wild species Silene vulgaris (Harmens et al., 1993; Chardonnens et al., 1998) and of the hyperaccumulator Arabidopsis halleri (Zhao et al., 2000). Higher Zn concentrations were measured in the shoots of the hyperaccumulator Thlaspi caerulescens and of the non hyperaccumulator Thlaspi ochroleucum regardless of Zn exposure (Shen et al., 1997).

The roots of the C5 genotype of tobacco accumulated more Zn than those of the A1 genotype, as shown by Vansuyt et al. (2000) in a pot culture. In the C5 genotype, the overexpression of ferritin induced a decrease in physiologically available iron, and an activation of the mechanisms of iron uptake. Ferritin was thus a possible candidate for Zn storage in the C5 genotype plants. However, no Zn-ferrihydrite association was found in C5 roots.

Results of CECR showed a continuous increase in Zn:CECR during the culture, the sum of Ca:CECR and Zn:CECR being close to 100% of the CECR after 14 days of culture. Three interpretations can be proposed. Firstly, all cations originally occupying the CECR except Ca might be progressively replaced by Zn. This seems unlikely. Secondly, part of intracellular Zn might be released, either by active transport or by damage of the membrane during the CuSO$_4$ extraction. The temperature had no effect on Zn extraction by CuSO$_4$, thus invalidating the possible role of an active transport. The efficiency of the CuSO$_4$ extraction was comparable to the extraction with water and then CaCl$_2$, which are supposed to preserve the membrane.
integrity. Moreover, EXAFS did evidence a damage of the membranes for the HCl-treated roots, not for the CuSO$_4$-extracted ones. Therefore, the second hypothesis can be ruled out.

Thirdly, CuSO$_4$ may solubilize some Zn precipitated in the apoplasm. This hypothesis is supported by EXAFS results. In order to avoid (or at least to limit) this precipitation of Zn, next experiments were performed on roots after only 4 days of exposure to Zn (i.e., when Zn occupies about 10 % of the CECR).

Results of the chemical extractions on whole roots and on isolated cell walls evidenced a distribution of Zn affinity sites, from the least to the most strongly bound Zn: (i) soluble in water, (ii) extractable by CaCl$_2$ or by CuSO$_4$ in one step, (iii) extractable by EDTA or by CuSO$_4$ in three steps, and finally (iv) non-exchangeable Zn. In whole roots, this latter pool represented 45 to 48% of total root Zn and may include intracellular Zn, and extracellular Zn bound to high affinity sites or precipitated. In isolated cell walls, strongly bound Zn accounted for a small fraction (4 to 5%) of Zn, as observed for *Thlaspi caerulescens* (Lasat et al., 1998) and wheat (Hart et al., 1998).

The EXAFS analyses of tobacco roots showed that zinc was predominantly bound to COOH/OH groups (40 to 80% of total root Zn). Only half of this pool was CuSO$_4$-exchangeable. The second half might correspond to strong Zn-cell wall complexes and/or to intracellular complexes with organic acids such as citrate and malate. Zn oxalate was found as a minor component (0 to 30% of total Zn). It was not affected by the CuSO$_4$ treatment, which suggests an intracellular localization. Oxalate was observed as free anion and as Ca-oxalate crystals in the vacuoles of tobacco leaves (Wang et al., 1992, Bouropoulos et al., 2001). Ca-oxalate crystals are also excreted through the trichomes of tobacco (Sarret et al., 2006). The role of oxalate in the detoxification of Zn in fungi and lichens is well known (Dutton and Evans, 1996, Adamo and Violante, 2000). In higher plants, oxalate has been shown to
detoxify Al, Sr, Pb and Cu (Franceschi and Nakata, 2005). The present study suggests a role in Zn detoxification. Zn-phosphate was present as a minor component in whole roots (0 to 16% of total Zn). This species has been observed on the surface roots in hydroponics (Küpper et al., 2000, Sarret et al., 2002), and inside the root cells of several crop species (Van Steveninck et al., 1994). In the present case, Zn-phosphate was removed by CuSO₄, thus suggesting an extracellular location. No relationship could be drawn between the distribution of Zn species and Zn total concentration, plant genotype or to the composition of the growing medium. Moreover, a relatively large dispersion in the percentages of Zn species was observed between the samples. Further investigations are necessary to better interpret these observations.

Because of the low Zn concentration of the roots EXAFS spectra could not be recorded on hydrated samples, but on freeze-dried materials. The structure of the Zn-cell wall complexes and Zn-phosphate precipitates should not be affected by this dehydrating treatment (Guiné et al., 2006). At the opposite, Zn-organic acid complexes present in solution are likely to be precipitated by the freeze-drying treatment. Considering the one identified in this study (Zn-oxalate), results should not be affected though: indeed the spectra for solid Zn-oxalate dihydrate and Zn-oxalate in solution are similar (Sarret et al., 1998). The occurrence of free Zn²⁺ may be overlooked due to the freeze-drying treatment. However, Zn²⁺ is unlikely to be present in significant amount in the cell walls and inside the cells because of the high concentration of ligands in these compartments. Free Zn²⁺ was observed in the xylem sap of a hyperaccumulating plant (Salt et al., 1999). However, xylem sap certainly accounts for a minor fraction of total root Zn. Therefore, the freeze-drying treatment should not modify significantly the distribution of Zn species in the roots.

In conclusion, the combination of chemical extractions and EXAFS spectroscopy, generally used for the study of soils and sediments, proved as an interesting approach for
plants. It overcame the relatively low sensitivity of EXAFS for organic complexes and for mixed species, and enabled the identification of intracellular and extracellular Zn species. Among our findings, Zn-phosphate was found in the apoplasm of the tobacco roots although the P concentration in culture solution was 5 µM only, and intracellular Zn oxalate was identified in these roots.

Acknowledgments

This work was supported in part by the “Programme National de Recherche Sols et Erosion” funded by CNRS and INRA, France. We would like to thank the ESRF for the provision of beamtime. Zn cysteine reference spectrum was provided by S. Beauchemin. We thank also Nicole Balsera, Denis Loisel and Michaël Clairotte from the UMR Rhizosphère & Symbiose team, and Nicolas Geoffroy, Martine Lanson and Delphine Tisserand from the LGIT for their technical help.

References


Table 1: Culture conditions and investigations

<table>
<thead>
<tr>
<th>Culture number</th>
<th>Growing medium</th>
<th>Zn concentration in the medium</th>
<th>Duration of Zn exposure (days)</th>
<th>Genotype</th>
<th>Investigations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydroponic</td>
<td>100 µM ZnSO₄</td>
<td>2, 4, 7, 14</td>
<td>A1, C5</td>
<td>CECR, CuSO₄-extractable Ca, CuSO₄-extractable Zn</td>
</tr>
<tr>
<td>2</td>
<td>Hydroponic</td>
<td>200 µM ZnSO₄</td>
<td>4</td>
<td>A1</td>
<td>Chemical extractions</td>
</tr>
<tr>
<td>3</td>
<td>Solid substrates</td>
<td>49 g L⁻¹ of ferrihydrite containing 0.2% Zn</td>
<td>4</td>
<td>A1, C5</td>
<td>Zn K-edge EXAFS on non-extracted roots and residues after extraction</td>
</tr>
<tr>
<td>4</td>
<td>Agarose</td>
<td>1500 µM ZnSO₄</td>
<td>4</td>
<td>A1, C5</td>
<td>Zn K-edge EXAFS on non-extracted roots</td>
</tr>
</tbody>
</table>

Table 2: EXAFS results obtained for the tobacco roots and for Zn references

<table>
<thead>
<tr>
<th>Samples</th>
<th>Zn conc. (mmol kg⁻¹, d.w.)</th>
<th>Linear Combination Fits</th>
<th>First shell simulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn oxalate dihydrate</td>
<td></td>
<td>Distribution of Zn species (molar % of total Zn)</td>
<td>Structural parameters</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zn oxalate</td>
<td>Zn-COOH/ OH</td>
</tr>
<tr>
<td>Zn citrate dihydrate</td>
<td></td>
<td>O</td>
<td>2.07</td>
</tr>
<tr>
<td>Zn malate</td>
<td></td>
<td>O</td>
<td>2.03</td>
</tr>
<tr>
<td>Zn-pectin</td>
<td>7.65 and 15.29</td>
<td>O</td>
<td>2.01</td>
</tr>
<tr>
<td>Zn-isolated cell walls</td>
<td>0.76 to 69.58</td>
<td>O</td>
<td>2.00</td>
</tr>
<tr>
<td>Aqueous Zn⁺</td>
<td></td>
<td>O</td>
<td>1.99</td>
</tr>
<tr>
<td>Zn-cellulose</td>
<td>1.27 to 3.82</td>
<td>O</td>
<td>2.07</td>
</tr>
<tr>
<td>Zn-sorbed hydroxylapatite</td>
<td>152.9</td>
<td>O</td>
<td>2.07</td>
</tr>
<tr>
<td>Zn phytate</td>
<td></td>
<td>O</td>
<td>1.97</td>
</tr>
<tr>
<td>Zn cysteine</td>
<td></td>
<td>O</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>2.35</td>
</tr>
</tbody>
</table>

*Genotype and growth medium*
A1, agarose 35.70 23 ± 3 69 ± 4 0 4.1 O 2.04 5.7 0.011 0.1
C5, agarose 21.20 0 80 ± 2 16 ± 2 4.1 O 2.01 5.0 0.010 0.3
A1, ferrihydrite 7.95 31 ± 3 51 ± 11 6 ± 10 8.7 O 2.05 6.0 0.011 1.1
C5, ferrihydrite 5.21 30 ± 4 43 ± 8 13 ± 6 4.9 O 2.05 5.9 0.011 1.1
A1, hectorite 2.11 23 ± 3 68 ± 12 14 ± 19 3.0 O 2.02 5.3 0.010 0.3
C5, hectorite 2.05 17 ± 3 80 ± 8 12 ± 5 4.5 O 2.02 5.3 0.010 0.2

Chemical extraction experiment
C5, hydroponics 49.23 6 ± 4 81 ± 4 6 ± 4 2.4 O 2.01 4.2 0.010 0.7
Same root, CuSO₄-extracted 32.31 20 ± 6 72 ± 10 0 3.7 O 2.02 4.7 0.010 1.3
Same root, CuSO₄/HCl-extracted 14.00 { O 2.11 1.4 0.006 0.8

Values expressed as mean ± SD over the best fits, defined by a normalized sum-squares (NSS) value comprised between the value obtained for the best simulation (NSS_{best}) and 1.1 × NSS_{best}. b NSS = [\sum (\chi(k)_{exp} - \chi(k)_{fit})^2 / \sum \chi(k)_{exp}^2] * 100. c Interatomic distance (Å). d Coordination number. e Debye-Waller disorder factor (Å²).

No satisfactory linear combination fit was obtained with the three components for this spectrum.

Table 3: Concentration of malate, oxalate and citrate in root of A1 genotype of tobacco.

<table>
<thead>
<tr>
<th>Organic anion</th>
<th>Concentration in roots (mmol kg⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>154 ± 46</td>
</tr>
<tr>
<td>Oxalate</td>
<td>497 ± 56</td>
</tr>
<tr>
<td>Citrate</td>
<td>187 ± 37</td>
</tr>
</tbody>
</table>

Table 4: Concentration of Zn in shoot and root of other plant species grown in hydroponics

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Exposure duration, days</th>
<th>Zn Concentration in solution, µM</th>
<th>Zn Concentration in shoots, mmol kg⁻¹ DW</th>
<th>Zn Concentration in roots, mmol kg⁻¹ DW</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Mustard</td>
<td>8</td>
<td>105</td>
<td>0.8</td>
<td>5</td>
<td>from Fargosova, 2001</td>
</tr>
<tr>
<td>Wheat</td>
<td>8</td>
<td>100</td>
<td>4</td>
<td>59</td>
<td>from van Steveninck et al., 1993</td>
</tr>
<tr>
<td>Soybean</td>
<td>14</td>
<td>40</td>
<td>14</td>
<td>79</td>
<td>from Fontes and Cox, 1998</td>
</tr>
<tr>
<td>Rye grass</td>
<td>15</td>
<td>1000</td>
<td>22</td>
<td>132</td>
<td>from Monnet et al., 2001</td>
</tr>
<tr>
<td>Plant</td>
<td>Zn Concentration</td>
<td>Sensitivity 1</td>
<td>Sensitivity 2</td>
<td>Year</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>10</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>from Brune et al., 1994</td>
</tr>
<tr>
<td>Barley</td>
<td>10</td>
<td>400</td>
<td>19</td>
<td>145</td>
<td>from Brune et al., 1994</td>
</tr>
<tr>
<td><em>Silene vulgaris</em></td>
<td>7</td>
<td>100</td>
<td>19 / 13</td>
<td>150 / 120</td>
<td>after Harmens et al., 1993*</td>
</tr>
<tr>
<td><em>Silene vulgaris</em></td>
<td>14</td>
<td>150</td>
<td>23 / 10</td>
<td>-</td>
<td>after Chardonnens et al., 1999*</td>
</tr>
<tr>
<td><em>Thlaspi caerulescens</em></td>
<td>16</td>
<td>10</td>
<td>46</td>
<td>&lt;15</td>
<td>after Shen et al., 1997*</td>
</tr>
<tr>
<td><em>Thlaspi caerulescens</em></td>
<td>16</td>
<td>500</td>
<td>431</td>
<td>185</td>
<td>after Shen et al., 1997*</td>
</tr>
<tr>
<td><em>Thlaspi ochroleucum</em></td>
<td>16</td>
<td>10</td>
<td>31</td>
<td>46</td>
<td>after Shen et al., 1997*</td>
</tr>
<tr>
<td><em>Thlaspi ochroleucum</em></td>
<td>16</td>
<td>500</td>
<td>215</td>
<td>431</td>
<td>after Shen et al., 1997*</td>
</tr>
<tr>
<td><em>Arabidopsis halleri</em></td>
<td>28</td>
<td>100</td>
<td>31</td>
<td>77</td>
<td>after Zhao et al., 2000*</td>
</tr>
<tr>
<td>Tobacco A1 / C5</td>
<td>14</td>
<td>100</td>
<td>15 / 16</td>
<td>74 / 99</td>
<td>this study</td>
</tr>
</tbody>
</table>

* Values of Zn concentration were read on graphs
Figure 1a. Variation of the cation exchange capacity of roots (CECR) for A1 and C5 genotype tobaccos. The horizontal line corresponds to the average CECR for A1 and C5. b. Variation of the CuSO$_4$-extracted Ca : CECR ratio (white bars) and of the CuSO$_4$-extracted Zn : CECR ratio (hatched bars). For both graphs, tobacco were cultivated for 14 days in hydroponics with 100 µM ZnSO$_4$, and errors bars represent standard deviations.

Figure 2. Comparison of four types of sequential extractions at 4 and 25°C on roots of tobacco cultivated for 4 days in hydroponics with 100 µM ZnSO$_4$ (R), and at 25°C on isolated root cell walls of tobacco incubated for 24 hours in hydroponics with 100 µM ZnSO$_4$ (CW). Res: residual Zn. Values are normalized to Zn total content, which ranges between 45 and 69 mmol kg$^{-1}$ for the roots, and between 39 and 96 mmol kg$^{-1}$ for the cell walls. Errors bars represent standard deviations.

Figure 3. Zn K-edge EXAFS spectra for Zn reference compounds. Values in parentheses indicate the Zn content, in mmol kg$^{-1}$, dry weight.

Figure 4. Zn K-edge EXAFS spectra for some Zn reference compounds (Zn content, in mmol kg$^{-1}$, dry weight in parentheses), and for roots of A1 and C5 genotype tobacco grown on artificial substrates and on hydroponics, and for the residues after the CuSO$_4$ and HCl treatment. Dashed lines are linear combination fits using reference spectra (proportions of the species given in Table 2).

Figure 5. Fourier transformed EXAFS spectra for the untreated C5 root and for the residues after chemical extraction and their first shell simulation (dotted lines, structural parameters given in Table 2).
Figure 1a

Figure 1b
Figure 2

- Water, CaCl$_2$
- CuSO$_4$, HCl
- 3x CuSO$_4$, HCl
- EDTA

Zn extracted, mol mol$^{-1}$

- Water
- CaCl$_2$
- CuSO$_4$
- HCl
- 3x CuSO$_4$
- EDTA

Conditions:
- R, 4°C
- R, 25°C
- CW, 25°C

Zn extracted values:
- 0.0
- 0.2
- 0.4
- 0.6
- 0.8
- 1.0

Acids and salts used:
1. HCl
2. CuSO$_4$
3. EDTA

Temperature conditions:
- 4°C
- 25°C
Figure 4