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14 **088Abstract**

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

26 tobacco during the culture. This study shows the strength of combining EXAFS and chemical
27 extractions for studying localization and speciation of metals in plants.

28

29 **Keywords**

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

32

33 **Abbreviations**

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

36 CaMV, cauliflower mosaic virus;

37 CECR, cation exchange capacity of roots;

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

40 EDTA, ethylene-diamine-tetra-acetic acid;

41 ESRF, European Synchrotron Radiation Facility;

42 EXAFS, extended X-ray absorption fine structure;

43 Fe-EDTA, ethylene-diamine-tetra-acetic acid iron^{III} sodium salt;

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

45 NSS, normalized sum-squares;

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

47

48

49

50

51 **Introduction**

52 Numerous authors (e.g. Haynes, 1980; Sattelmacher, 2001) have shown that the cell
53 walls of plant roots are involved in the acquisition of mineral elements. This compartment
54 also plays a role in metal tolerance by acting as a barrier for some elements (Ernst et al.,
55 1992). Main components of plant cell walls are cellulose, hemicellulose, pectin and
56 glycoproteins. The cation exchange capacity of roots (CECR) arises mostly from carboxyl
57 and hydroxyl groups, and to a minor extent from phenolic and amine groups (Meychik et al.,
58 2001). The structure and the composition of the cell walls (and consequently the CECR) vary
59 as a function of the plant species, of its nutrition and of the age of the plant tissues.
60 Particularly, the development of secondary cell walls in older tissues induces a decrease in
61 CECR because of the lower pectin and higher lignin content of this structure. The CECR
62 ranges between 10 and 20 $\text{cmol}_c \text{ kg}^{-1}$ (or $\text{meq } 100 \text{ g}^{-1}$) for monocot species and between 20
63 and 50 $\text{cmol}_c \text{ kg}^{-1}$ for dicot species (Dufey et al., 2001). The affinity of cations for
64 exchangeable sites on root cell walls decreases in the order $\text{H} > \text{Cu} > \text{Ca} > \text{Zn}$ according to
65 Nishizono et al. (1987), and $\text{H} > \text{Cu} > \text{Zn} > \text{Ca}$ according to Ernst et al. (1992). A similar
66 order of affinity was found for pectin (Franco et al., 2002). Based on the high affinity of Cu
67 for the cell walls, Dufey and Braun (1986) showed that saturating the cell walls with Cu, and
68 then extracting it using HCl, was an easy and reliable way to measure the CECR because they
69 obtained comparable CECR values by this method and by acid-base titration of roots.

70 An overview of the literature shows a great variability in Zn localization and
71 exchangeability in plants roots: exchangeable Zn represented 10% of total root Zn in *Silene*
72 *vulgaris* (Harmens et al., 1993), 16% in wheat (*Triticum aestivum*) and 46% in soybean
73 (*Glycine max*) (Steveninck et al., 1993), 27% in barley (*Hordeum vulgare*) (Wu et al., 2005),
74 60% in the hyperaccumulator *Thlaspi caerulescens* (Lasat et al., 1998), and 67 to 87% in the
75 Zn-tolerant fern *Athyrium yokoscense* (Nishizono et al., 1987). This wide range of responses

76 may result from actual differences between species, but also from the techniques used for
77 measuring the so called "exchangeable" Zn (isolation of cell walls, chemical extractions,
78 isotopic exchange, transmission electron microscopy coupled with energy dispersive X-ray
79 microanalysis, etc...). Other possible sources of differences include the duration and intensity
80 of Zn exposure (Vasquez et al., 1994), and the age of the plants.

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

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

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

98

99 **Material and methods**

100 *Plant material and preculture of tobacco*

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

107 Seeds were surface sterilised with NaOCl for 25 min, then carefully washed with sterile
108 water. Plants were cultivated in a cropping device designed to easily separate the roots from
109 the growing soil at harvest (Niebes et al., 1993). The plant container was made of a PVC
110 cylinder (inner diameter 40 mm) closed at the bottom by a fine polyamide mesh (30 μm pore
111 diameter, Sefar Nynetel/Fyltis). For the preculture, plant containers were placed on a nutrient
112 gel in sterile and capped cropping boxes (150 \times 150 \times 135 mm, MERCK eurolab, Polylabo).
113 The nutrient gel was prepared by adding 1.0 g L⁻¹ gelrite (Sigma G1910) and 0.6 g L⁻¹
114 phytigel (Sigma P8169) to a Hoagland solution containing 5 mM KNO₃, 5 mM Ca(NO₃)₂, 2
115 mM MgSO₄, 1 mM KH₂PO₄, 50 μM H₃BO₃, 50 μM MnSO₄, 50 μM Fe-EDTA, 15 μM
116 ZnSO₄, 3 μM (NH₄)MoO₄, 2.5 μM KI, 50 nM CoCl₂, and 50 nM CuSO₄. Five seeds were put
117 in each plant container, and each cropping box contained 9 containers. Boxes were placed in a
118 growth chamber with a 16/8 h day/night cycle, light intensity of 250 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$,
119 temperature of 23/20°C and 75/80% relative humidity. After two weeks, the cropping boxes
120 were progressively opened for 3 days so that plants could adapt to ambient culture conditions.
121 The containers were then transferred in a nutrient solution containing 1 mM KNO₃, 1 mM
122 Ca(NO₃)₂, 0.5 mM MgSO₄, 20 μM Fe-EDTA, 10 μM H₃BO₃, 5 μM KH₂PO₄, 2 μM MnCl₂,
123 0.5 μM MoNaO₄, 0.5 μM ZnSO₄ and 0.2 μM CuCl₂ (10 plant containers per 5 L bucket). The
124 solution was renewed weekly. After two weeks, the plants were then 4-week old, and each

125 container presented a homogeneous root mat formed by the roots of 5 plants. The pH of the
126 nutrient solution was between 5.5 and 6.1.

127

128 *Culture of tobacco in hydroponics*

129 All culture conditions are summarized in Table 1. For the measurement of the CECR (culture
130 n°1), plant containers containing 4-week old plants (A1 and C5 genotype) were transferred in
131 a nutrient solution devoid of Fe, and containing 100 μM ZnSO_4 , 1 mM KNO_3 , 1 mM
132 $\text{Ca}(\text{NO}_3)_2$, 0.5 mM MgSO_4 , 10 μM H_3BO_3 , 5 μM KH_2PO_4 , 2 μM MnCl_2 , 0.5 μM MoNaO_4 ,
133 and 0.2 μM CuCl_2 . Note that P concentration was low (5 μM) to avoid precipitations with Zn,
134 as predicted by the SOILCHEM speciation code (Sposito and Coves, 1988). Plants were
135 grown for 2, 4, 7 and 14 days (8 plant containers per 5 L bucket). The pH of the solution was
136 5.5 at the beginning of culture.

137 For the comparison of the sequential extraction procedures, 4-week old plants (A1
138 genotype) were grown in the same conditions for 4 days (culture n°2). For the EXAFS
139 analyses, 4-week old plants (C5 genotype) were grown in the same conditions except Zn
140 concentration (200 μM instead of 100 μM ZnSO_4) for 4 days (culture n°3). No toxicity
141 symptoms were observed in any culture, probably due to the presence of Ca in the nutrient
142 medium which partially alleviates Zn toxicity in tobacco (Sarret et al., 2006).

143

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

145 Artificial substrates were made of agarose nutrient gel containing various Zn-bearing minerals
146 to provide a range of Zn availabilities with in spite of an identical total Zn content in the
147 substrates. The nutrient solution contained 1 mM KNO_3 , 0.625 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM
148 MgSO_4 , 0.375 mM $(\text{NH}_4)_2\text{SO}_4$, 10 μM H_3BO_3 , 5 μM KH_2PO_4 , 2 μM $\text{MnCl}_2 \cdot \text{H}_2\text{O}$, 0.5 μM
149 $\text{MoNaO}_4 \cdot 2\text{H}_2\text{O}$, and 0.2 μM CuCl_2 . Zn-bearing minerals included Zn-sorbed synthetic

150 ferrihydrite and Zn-sorbed hectorite containing 0.2% dry weight Zn. Ferrihydrite is a poorly
151 crystalline iron oxyhydroxide with a high sorption capacity, and hectorite (SHCa-1 from the
152 Source Clay Repository of the Clay Minerals Society) is a magnesian smectite composed of
153 an octahedral sheet of magnesium sandwiched between two tetrahedral sheets of silicon. The
154 substrates contained 49 g L^{-1} of Zn-sorbed ferrihydrite or hectorite, and 10 g L^{-1} of agarose.
155 A control culture substrate was made with agarose only, the nutrient solution being
156 supplemented with $1500 \text{ }\mu\text{M ZnSO}_4$. Note that a fraction of added Zn is finally adsorbed on
157 agarose gel (Calba et al, 1999). Four-week old plants (A1 and C5 genotypes) were grown for
158 4 days on Zn-ferrihydrite, Zn-hectorite and Zn-agarose substrates (Table 1). Table 2 shows
159 that Zn root concentrations increased in the order hectorite < ferrihydrite < agarose. At the
160 end of the culture, shoots and roots were harvested separately and stored for further chemical
161 and EXAFS analyses.

162

163 *Extraction of cell walls of roots of tobacco*

164 Fresh roots of 4-week old A1 genotype tobacco were harvested and then immersed in a 1%
165 v:v Triton X100 detergent solution with 1 mM CaCl_2 to dissolve the cell content (Calba et al.,
166 1999). The detergent solution was renewed periodically for 28 days. The detergent was then
167 removed by washing the material for 15 days with a 1 mM CaCl_2 solution. The entire
168 treatment was carried out at 4°C .

169 Zinc-cell wall complexes were conditioned prior to sequential extractions as follows.

170 Three g of cell walls were placed in 1 L of nutrient solution (the same as the one used for the
171 hydroponic culture without Fe-EDTA) containing $100 \text{ }\mu\text{M ZnSO}_4$, then shaken end over end
172 for 24 hours. Other Zn-cell wall complexes were prepared for EXAFS analysis (see the
173 EXAFS section).

174

175 *Sequential extractions of whole roots and isolated cell walls of roots of tobacco*

176 The CECR was determined by sequential extractions by adapting the procedure of Dufey and
177 Braun (1986). Roots of A1 and C5 genotype tobacco plants from culture n°1 were harvested.
178 Each root sample was made of the roots of 5 plants grown in the same container. An aliquot
179 was oven dried at 105°C, digested and analyzed for total Zn. The remainder (25 mg ± 8 mg
180 dry weight) was shaken end over end in 5 mL of 10 mM CuSO₄ during 30 min. The initial pH
181 of the solution was 4.8. The suspension was then filtered, and Ca and Zn concentration in the
182 filtrate were measured. Copper is supposed to displace all cations associated to the cell walls
183 and to saturate the CECR. The roots were then briefly rinsed with a solution containing
184 0.1mM CuSO₄ to reduce the excess Cu in the interstitial volume of roots before to be shaken
185 end over end in 50 mL of 100 mM HCl during 20 min to extract Cu, the suspension was
186 filtered, and Cu concentration in the filtrate was measured. The acidic extraction is supposed
187 to desorb Cu from the cell walls. The CECR was thus estimated from the amount of desorbed
188 Cu, by considering Cu as a divalent cation.

189 Sequential extractions were performed on whole roots of A1 tobacco from culture n°2
190 (Table 1) and on isolated cell walls. Samples (22 mg ± 7 mg dry weight for roots and 32 mg ±
191 5 mg dry weight for cell walls) were treated with 10 mM CuSO₄, then 100 mM HCl as
192 described above. This procedure was realized at 25°C and at 4°C. Other extraction procedures
193 were tested at both temperatures. The first one involved three successive extractions in 10
194 mM CuSO₄ during 30 min, and then an extraction in 50 mL of 100 mM HCl during 20 min.
195 The second one involved an extraction in 5 mL ultra pure water during 2 hours, followed by
196 an extraction in 5 mL of 10 mM CaCl₂ during 2 hours. The third one involved an extraction in
197 50 mL of 10 mM EDTA pH 7 during 2 hours. For all procedures, after each extraction, the
198 root suspension was filtered over an ashless filter paper (Whatman 40), and elemental
199 concentrations were determined in the filtrate and in the extracted roots.

200 Roots of C5 genotype tobacco from culture n°3 (Table 1) were treated at 25°C
201 following the CuSO₄/HCl procedure, and aliquots of non-extracted, CuSO₄-extracted and
202 CuSO₄/HCl-extracted roots were kept for EXAFS analyses.

203

204 *Chemical analyses of plants and solutions*

205 Samples (shoots, whole roots, isolated cell walls of roots and root residues after extractions
206 depending on the experiment) were weighed, oven-dried at 105°C and digested in a 1:1
207 mixture of hot concentrated HNO₃ and HClO₄ (A.O.A.C., 1975). Ca, Zn and Cu
208 concentrations were determined in the digests and in the filtrates of the chemical extractions
209 by flame atomic absorption spectrometry (Varian SpectrAA-600, Australia). Malate, citrate
210 and oxalate concentrations were measured in the roots of A1 genotype tobacco at the end of
211 the preculture (4-week old plants). One g of fresh roots was put in 10 mL of HCl 100 mM at
212 60°C. After 20 min, solution was filtered over an ashless filter paper (Whatman 40) and the
213 supernatant was analyzed by High Pressure Ionic Chromatography (Dionex 4,000) using an
214 AS11 column. The elution was performed with a NaOH gradient, and the signal was detected
215 by conductimetry, and analyzed with a integrator Chromjet (Spectra-Physics) integrator.

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

219

220 *Zn K-edge EXAFS spectroscopy*

221 *Zn model compounds*

222 A variety of Zn-model compounds were used for the EXAFS data analysis. Zn-oxalate
223 dihydrate and Zn-citrate dihydrate were purchased from Alfa (Berkshire, UK). The
224 preparation of Zn-malate and Zn-sorbed hydroxylapatite were described previously (Sarret et

225 al., 2002; Panfili et al., 2005). The Zn-cysteine spectrum was provided by S. Beauchemin
226 (Beauchemin et al. 2004). The Zn-cell wall complexes containing 0.75, 1.4, 12.7 and 69.6
227 mmol kg⁻¹ d.w. Zn were prepared by placing 100 mg (dry weight) of isolated cell walls of
228 roots in 50 mL of 1.5, 6.1, 30.3 and 303 μM Zn(NO₃)₂ at pH 5.0, respectively, and shaking
229 end over end for 24 hours. Final pH values were 5.0, 5.4, 5.4, and 5.4, respectively. The
230 suspensions were then centrifuged, and the Zn loading was determined by difference between
231 initial and supernatant Zn concentrations.

232 For the Zn-cellulose complexes, 200 mg of cellulose (Sigma-Aldrich) were suspended in 60
233 mL of water and the pH was adjusted to 5.0. Two samples were prepared : after addition of 1
234 and 2 mL of 1.53 mM Zn(NO₃)₂ at pH 5.0, respectively, the suspensions were stirred during 3
235 hours at fixed pH 5.0 by adding 0.5M NaOH or HNO₃, then centrifuged. The Zn content in
236 the Zn-cellulose complexes was calculated as the difference between the amount of Zn
237 introduced and the amount of Zn measured in the supernatant: they were 1.27 and 3.82 mmol
238 kg⁻¹ d.w. Zn. For each Zn concentration, half of the Zn-cellulose samples was freeze-dried,
239 and half was kept in wet state for EXAFS analysis. For the Zn-pectin complexes, 166 mg of
240 pectin extracted from apples esterified at 70 to 75% (Fluka) were dissolved in 30 mL of
241 water, and the pH was adjusted to 5.0. Two samples were prepared : after addition of 0.4 and
242 0.9 mL of 4.31 mM Zn(NO₃)₂ at pH 5.0, respectively, the suspensions were stirred during 3
243 hours at fixed pH 5.0 by adding 0.5M NaOH or HNO₃. The Zn-pectin complexes were
244 directly freeze-dried because they could not be concentrated by centrifugation. Zn
245 concentrations were 15.29 and 7.65 mmol kg⁻¹ d.w. of pectin, respectively.

246

247 *EXAFS data acquisition and treatment*

248 Zinc K-edge EXAFS analyses were performed on untreated whole roots of A1 and C5
249 genotype tobacco grown for 4 days on artificial substrates as described above (culture n°4),

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

265

266 **Results**

267

268 *Accumulation of Zn in tobacco*

269 In culture n°1, A1 and C5 genotypes of tobacco were cultivated in hydroponics without Fe
270 and with 100 μM Zn. After 14 days of culture, total Zn uptake and Zn shoot content were
271 comparable for both genotypes: total uptakes were 1.1 ± 0.2 and 1.3 ± 0.2 μmol Zn per A1
272 and C5 plants, and shoot contents were 15.1 ± 1.7 and 15.9 ± 1.6 μmol Zn per g, respectively.
273 At the opposite, the Zn concentration of roots was larger for the ferritin overexpressor ($98.8 \pm$
274 5.2 $\mu\text{mol g}^{-1}$) than for the wild type (73.5 ± 5.8 $\mu\text{mol g}^{-1}$). For both genotypes, the pH of the

275 nutrient solution increased from 5.5 at the beginning, to 6.0 ± 0.1 after two days and to $7.0 \pm$
276 0.1 after 14 days of culture. The calculation of Zn speciation with SOILCHEM (Sposito and
277 Coves, 1988) suggests a minor precipitation at pH 7.0: At pH 5.5, calculated Zn species are
278 93% free Zn^{2+} and 6% $ZnSO_4$. At pH 7.0, they are 86% free Zn^{2+} , 6% $ZnSO_4$, 1% $ZnB(OH)_4$
279 and 6% precipitated Zn-phosphate.

280

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

282 The cation exchange capacity of the roots (CECR) was determined at different times of the
283 culture. It did not vary significantly between 0 and 14 days, and was comparable for the two
284 genotypes (Figure 1a). The mean value for the two genotypes and all exposure durations was
285 $32 \pm 3 \text{ cmol}_c \text{ kg}^{-1}$. The ratio of Ca extractable by $CuSO_4$ to the CECR ("Ca:CECR"), which
286 corresponds to the fraction of the CECR occupied by Ca, did not vary significantly (Fig. 1b).
287 The concentration of $Ca(NO_3)_2$ being kept the same ($1.02 \pm 0.05 \text{ mM}$) in the preculture and
288 culture solutions during the 14 days of culture, this steady-state was expected. Again, there
289 was no significant difference between the two genotypes. Calcium accounted for $52 \pm 10\%$ of
290 the CECR (mean value for the two genotypes and all exposure durations). On the contrary, an
291 increase in the fraction of the CECR occupied by Zn ("Zn:CECR") was expected because Zn
292 concentration increased from $0.5 \mu\text{M}$ in the preculture to $100 \mu\text{M}$ in the culture solution.
293 Cations exchange between the solution and roots is supposed to reach equilibrium within 48h
294 (Meychik et al., 2001). In our experiment, we observed a continuous increase in Zn:CECR
295 during the 14 days of culture (Fig. 1b). At the end of the culture, the sum of Ca:CECR and
296 Zn:CECR accounted for $90 \pm 5\%$ of the CECR for A1, and $110 \pm 5\%$ of the CECR for C5
297 tobacco.

298

299 *Comparison of different sequential extractions of Zn accumulated in roots of tobacco*

300 The accumulation compartments of Zn in A1 tobacco roots exposed to 100 μ M Zn for 4 days
301 (culture n°2) were studied by chemical extractions (Fig. 2). Various procedures were
302 compared : (i) 10 mM CuSO₄, then 100 mM HCl, (ii) three successive extractions with 10
303 mM CuSO₄, then 100 mM HCl, (iii) pure water, then 10 mM CaCl₂, and (iv) 10 mM EDTA.
304 Each procedure was done at 4°C and 25°C to evaluate the role of active Zn transport during
305 the treatments. Chemical extractions on whole roots and isolated cell walls of roots were
306 compared to distinguish the intra- and extra-cellular contributions.
307 On whole roots, water extracted about 20% of total Zn, and the CaCl₂ solution removed
308 another 20%. The one-step extraction with CuSO₄ yielded similar results as the water + CaCl₂
309 extraction (40 \pm 8 % and 39 \pm 5 respectively). The three-step CuSO₄ and the EDTA extraction
310 were slightly more efficient (52 \pm 4 % and 55 \pm 9% of total Zn, respectively). These data are
311 consistent with the occurrence of a distribution of affinity sites. The results obtained at 4°C
312 and 25°C were roughly similar except for the HCl treatment (extraction doubled at 25°C
313 compared to 4°C). This suggests that Zn transport through the cell membranes during the
314 water, CaCl₂, CuSO₄, and EDTA extractions was insignificant. This result also suggests that
315 cation diffusion within the roots is not significantly different at 4°C and at 25°C. In contrast,
316 HCl extractant is likely resulting in some damage of the integrity of cell membranes and
317 thereby in the release of intracellular Zn, as suggested for Cu by Iwasaki et al. (1990).

318

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

322

323 *Determination of Zn speciation in tobacco roots*

324 First, in order to give an idea of the sensitivity of EXAFS spectroscopy, Figure 3 shows the
325 spectra for various Zn reference compounds of interest for this study. The spectrum for Zn-
326 oxalate dihydrate presents a characteristic shoulder between 6.1 and 6.6 Å⁻¹. It is due to the
327 well ordered structure of this organic compound, Zn being bound to four carboxyl groups in a
328 planar configuration (Fig. 3). Citrate, malate and pectin contain hydroxyl and carboxyl
329 functional groups. The spectrum for Zn-citrate exhibits a weakly pronounced shoulder around
330 6.5 Å⁻¹, and the spectrum for Zn-malate is even smoother. This reflects an increase in disorder
331 from Zn-oxalate to Zn-citrate, and from Zn-citrate to Zn-malate. The spectra for Zn-pectin
332 and for Zn-cell walls at various Zn concentrations present strong similarities with Zn-malate,
333 which suggests a similar Zn local structure. Thus, in the cell walls and in Zn-pectin, the metal
334 is probably bound to hydroxyl and carboxyl groups. The spectra for Zn-cellulose (recorded in
335 freeze-dried and hydrated state) have a markedly higher frequency relative to Zn-pectin, and
336 present some similarities with aqueous Zn²⁺. This suggests an outer-sphere configuration, i.e.,
337 Zn being fully hydrated and bound to cellulose through weak interactions. This is consistent
338 with the fact that cellulose contains hydroxyl groups only, and that these groups are fully
339 protonated at pH 5.0, and deprotonate in alkaline conditions (pH > 10) (Smith and Martell,
340 1982). The structural parameters for Zn first coordination shell in these compounds were
341 determined. For Zn-cell walls and Zn-pectin, Zn-O distances were 1.99 and 2.00 Å,
342 respectively (Table 2). Considering typical Zn-O distances for tetrahedral and octahedral
343 coordination (1.95 to 2.0 and 2.0 to 2.2 Å, respectively, Sarret et al., 1998), this suggests that
344 the metal occupies both types of coordination sites in these samples. For Zn-cellulose, a Zn-O
345 distance of 2.07 Å was found, indicating an octahedral coordination. Figure 3 also shows the
346 spectra for an inorganic and organic Zn-phosphate, Zn-sorbed hydroxylapatite and Zn-
347 phytate, respectively. Zn is in tetrahedral coordination in both compounds (Table 2). The
348 similarity between the two spectra suggests that it may be difficult to distinguish between

349 mineral and organic Zn-phosphate, especially in case of a mixture of Zn species. Finally,
350 figure 3 shows the spectrum for Zn-sorbed ferrihydrite, which is used as a proxy for Zn in
351 ferritin (Briat and Lebrun, 1999).

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

370 The second (and most represented) Zn species identified was Zn-pectin and/or Zn-
371 malate. The similarity between the two EXAFS spectra prevents the positive identification of
372 one or the other compound (see above). Whole roots do contain malate (Table 3). However,
373 they are richer in oxalate and citrate (Table 3), and these two organic acids have a higher

374 affinity for Zn than malate ($\log K = 4.0$ for Zn oxalate, and 4.9 for Zn citrate, compared to 2.9
375 for Zn malate, Smith and Martell, 1982). Therefore, the presence of Zn-malate as major Zn
376 species is unlikely in these root samples. Zn-pectin which can be used as a proxy for Zn-cell
377 walls (see above) is more likely. Because of the similarity between Zn-pectin, Zn-malate and
378 Zn-citrate, this pool may contain a minor proportion of complexes of Zn with simple organic
379 acids complexes: it is referred to as "Zn-COOH/OH" in Table 2.

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

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

391

392 In order to get some insights on the localization of these three Zn species, whole roots
393 before and after extraction with CuSO_4 and $\text{CuSO}_4 / \text{HCl}$ were studied by EXAFS
394 spectroscopy (Fig. 4 and Table 2). For this purpose, whole roots of C5 genotype tobacco were
395 grown in hydroponics containing 200 μM Zn were used (culture n° 3, chemical extraction
396 experiment in Table 2). The speciation of Zn in this nutrient solution was similar to the one
397 calculated for the 100 μM Zn solution, with 92% free Zn^{2+} and 6% ZnSO_4 at pH 5.5, and 88%
398 free Zn^{2+} , 7% ZnSO_4 , 1% ZnB(OH)_4 and 3.5% of precipitated Zn-phosphate at pH 7.0. For

399 the untreated roots, the distribution of Zn species was comparable to what was found for
400 plants grown in solid substrates. Roots contained 81 ± 4 % Zn-COOH/OH, 6 ± 4 % Zn-
401 oxalate, and 6 ± 4 % Zn-phosphate. By multiplying these percentages with Zn total root
402 concentration, one obtains the molar concentration of Zn for each species, i.e., 39.9 ± 1.9 , 3.0
403 ± 2.0 and 3.0 ± 2.0 mmol Zn kg⁻¹, respectively. In the CuSO₄-extracted roots, the proportion
404 of Zn-COOH/OH species was decreased to 72 ± 10 % (23.3 ± 3.2 mmol Zn kg⁻¹). The Zn-
405 phosphate species identified in the untreated roots was absent, and the change in Zn-oxalate
406 concentration was within experimental error. These results indicate that the CuSO₄ extraction
407 removed all of the Zn-phosphate pool, about half of the Zn-COOH/OH pool, but did not alter
408 the Zn-oxalate pool.

409 The spectrum for the residues after the HCl extractions strongly differed from the other
410 root spectra, and could not be simulated by the three reference spectra used before. Its
411 frequency was intermediate between the untreated root spectrum and the Zn-cysteine, in
412 which Zn is bound to sulfur atoms. The first shell simulation showed that Zn was bound to 4.5
413 sulfur atoms at 2.28 Å, and 1.3 oxygen atoms at 2.13 Å (Figure 5 and Table 2). The presence
414 of sulfur atomic neighbours might suggest the binding of Zn by glutathione, phytochelatins or
415 metallothioneins. However, considering Zn concentration in the HCl-extracted roots (14.00
416 mmol kg⁻¹) and the CuSO₄-extracted roots (32.31 mmol kg⁻¹), S neighbours should have been
417 detected in the CuSO₄-extracted roots. As this was not the case, it is concluded that some
418 redistribution of Zn occurred during the HCl extraction. Thus, this acidic treatment, which
419 was supposed to desorb Cu from the cell walls, probably damaged a breakage of cellular
420 membranes and induced the subsequent release of intracellular proteins, leading to the
421 formation of Zn-S bonds. Note that this artefact was not observed for CuSO₄-extracted roots.
422

423 Discussion

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

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

441 Results of CECR showed a **continuous** increase in Zn:CECR during the culture, the sum
442 of Ca:CECR and Zn:CECR being close to 100% of the CECR after 14 days of culture. Three
443 interpretations can be proposed. Firstly, all cations originally occupying the CECR except Ca
444 might be progressively replaced by Zn. This seems unlikely. Secondly, part of intracellular Zn
445 might be released, either by active transport or by damage of the membrane during the CuSO₄
446 extraction. The temperature had no effect on Zn extraction by CuSO₄, thus invalidating the
447 possible role of an active transport. The efficiency of the CuSO₄ extraction was comparable to
448 the extraction with water and then CaCl₂, which are supposed to preserve the membrane

449 integrity. Moreover, EXAFS did evidence a damage of the membranes for the HCl-treated
450 roots, not for the CuSO₄-extracted ones. Therefore, the second hypothesis can be ruled out.
451 Thirdly, CuSO₄ may solubilize some Zn precipitated in the apoplasm. This hypothesis is
452 supported by EXAFS results. In order to avoid (or at least to limit) this precipitation of Zn,
453 next experiments were performed on roots after only 4 days of exposure to Zn (i.e., when Zn
454 occupies about 10 % of the CECR).

455 Results of the chemical extractions on whole roots and on isolated cell walls evidenced
456 a distribution of Zn affinity sites, from the least to the most strongly bound Zn: (i) soluble in
457 water, (ii) extractable by CaCl₂ or by CuSO₄ in one step, (iii) extractable by EDTA or by
458 CuSO₄ in three steps, and finally (iv) non-exchangeable Zn. In whole roots, this latter pool
459 represented 45 to 48% of total root Zn and may include intracellular Zn, and extracellular Zn
460 bound to high affinity sites or precipitated. In isolated cell walls, strongly bound Zn accounted
461 for a small fraction (4 to 5%) of Zn, as observed for *Thlaspi caerulescens* (Lasat et al., 1998)
462 and wheat (Hart et al., 1998).

463

464 The EXAFS analyses of tobacco roots showed that zinc was predominantly bound to
465 COOH/OH groups (40 to 80% of total root Zn). Only half of this pool was CuSO₄-
466 exchangeable. The second half might correspond to strong Zn-cell wall complexes and/or to
467 intracellular complexes with organic acids such as citrate and malate. Zn oxalate was found as
468 a minor component (0 to 30% of total Zn). It was not affected by the CuSO₄ treatment, which
469 suggests an intracellular localization. Oxalate was observed as free anion and as Ca-oxalate
470 crystals in the vacuoles of tobacco leaves (Wang et al., 1992, Bouropoulos et al., 2001). Ca-
471 oxalate crystals are also excreted through the trichomes of tobacco (Sarret et al., 2006). The
472 role of oxalate in the detoxification of Zn in fungi and lichens is well known (Dutton and
473 Evans, 1996, Adamo and Violante, 2000). In higher plants, oxalate has been shown to

474 detoxify Al, Sr, Pb and Cu (Franceschi and Nakata, 2005). The present study suggests a role
475 in Zn detoxification. Zn-phosphate was present as a minor component in whole roots (0 to
476 16% of total Zn). This species has been observed on the surface roots in hydroponics (Küpper
477 et al., 2000, Sarret et al., 2002), and inside the root cells of several crop species (Van
478 Steveninck et al., 1994). In the present case, Zn-phosphate was removed by CuSO_4 thus
479 suggesting an extracellular location. No relationship could be drawn between the distribution
480 of Zn species and Zn total concentration, plant genotype or to the composition of the growing
481 medium. Moreover, a relatively large dispersion in the percentages of Zn species was
482 observed between the samples. Further investigations are necessary to better interpret these
483 observations.

484 Because of the low Zn concentration of the roots EXAFS spectra could not be recorded
485 on hydrated samples, but on freeze-dried materials. The structure of the Zn-cell wall
486 complexes and Zn-phosphate precipitates should not be affected by this dehydrating treatment
487 (Guiné et al., 2006). At the opposite, Zn-organic acid complexes present in solution are likely
488 to be precipitated by the freeze-drying treatment. Considering the one identified in this study
489 (Zn-oxalate), results should not be affected though: indeed the spectra for solid Zn-oxalate
490 dihydrate and Zn-oxalate in solution are similar (Sarret et al., 1998). The occurrence of free
491 Zn^{2+} may be overlooked due to the freeze-drying treatment. However, Zn^{2+} is unlikely to be
492 present in significant amount in the cell walls and inside the cells because of the high
493 concentration of ligands in these compartments. Free Zn^{2+} was observed in the xylem sap of a
494 hyperaccumulating plant (Salt et al., 1999). However, xylem sap certainly accounts for a
495 minor fraction of total root Zn. Therefore, the freeze-drying treatment should not modify
496 significantly the distribution of Zn species in the roots.

497 In conclusion, the combination of chemical extractions and EXAFS spectroscopy,
498 generally used for the study of soils and sediments, proved as an interesting approach for

499 plants. It overcame the relatively low sensitivity of EXAFS for organic complexes and for
500 mixed species, and enabled the identification of intracellular and extracellular Zn species.
501 Among our findings, Zn-phosphate was found in the apoplasm of the tobacco roots although
502 the P concentration in culture solution was 5 μM only, and intracellular Zn oxalate was
503 identified in these roots.

504

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512

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641 Table 1: Culture conditions and investigations

Culture number	Growing medium	Zn concentration in the medium	Duration of Zn exposure (days)	Genotype	Investigations
1	Hydroponic	100 μM ZnSO ₄	2, 4, 7, 14	A1, C5	CECR, CuSO ₄ -extractable Ca, CuSO ₄ -extractable Zn
2		100 μM ZnSO ₄	4	A1	Chemical extractions
3		200 μM ZnSO ₄	4	C5	Zn K-edge EXAFS on non-extracted roots and residues after extraction
Solid substrates					
4	Agarose	49 g L ⁻¹ of ferrihydrite containing 0.2% Zn	4	A1, C5	Zn K-edge EXAFS on non-extracted roots
4	Agarose + Zn- hectorite	49 g L ⁻¹ of hectorite containing 0.2% Zn	4	A1, C5	Zn K-edge EXAFS on non-extracted roots
4	Agarose	1500 μM ZnSO ₄	4	A1, C5	Zn K-edge EXAFS on non-extracted roots

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

Samples	Zn conc. (mmol kg ⁻¹ , d.w.)	Linear Combination Fits				First shell simulation				
		Distribution of Zn species (molar % of total Zn) ^a				Structural parameters				
		Zn oxalate	Zn-COOH/OH	Zn phosphate	NSS ^b	Atom	R (Å) ^c	CN ^d	σ^2 (Å ²) ^e	NSS ^a
Zn oxalate dihydrate						O	2.07	6.0	0.007	1.7
Zn citrate dihydrate						O	2.03	5.5	0.010	0.5
Zn malate						O	2.01	4.2	0.001	1.6
Zn-pectin	7.65 and 15.29					O	2.00	4.6	0.009	1.3
Zn-isolated cell walls	0.76 to 69.58					O	1.99	4.3	0.009	0.4
Aqueous Zn ²⁺						O	2.07	6.0	0.009	1.4
Zn-cellulose	1.27 to 3.82					O	2.07	6.0	0.006	1.9
Zn-sorbed hydroxylapatite	152.9					O	1.97	4.0	0.008	0.2
Zn phytate						O	1.96	3.9	0.008	0.3
Zn cysteine						S	2.35	4.5	0.007	1.2

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
<i>Chemical extraction experiment</i>										
C5, hydroponics	49.23	6 ± 4	81 ± 4	6 ± 4	2.4	O	2.01	4.2	0.010	0.7
Same root, CuSO ₄ -extracted	32.31	20 ± 6	72 ± 10	0	3.7	O	2.02	4.7	0.010	1.3
Same root, CuSO ₄ /HCl- extracted ^e	14.00					{ S	2.11 2.29	1.4 4.7	0.006 0.006	0.8

^a 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 $1.1 \times NSS_{best}$. ^b $NSS = \frac{\sum [k^3 \chi(k)_{exp} - k^3 \chi(k)_{fit}]^2}{\sum [k^3 \chi(k)_{exp}]^2} * 100$. ^c Interatomic distance (Å). ^d Coordination number. ^e Debye-Waller disorder factor (Å²). ^f No satisfactory linear combination fit was obtained with the three components for this spectrum.

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

Organic anion	Concentration in roots (mmol kg ⁻¹ DW)
Malate	154 ± 46
Oxalate	497 ± 56
Citrate	187 ± 37

643
644 Table 4: Concentration of Zn in shoot and root of other plant species grown in hydroponics

Plant species	Exposure duration, days	Zn Concentration in solution, μM	Zn Concentration in shoots, mmol kg ⁻¹ DW	Zn Concentration in roots, mmol kg ⁻¹ DW	References
White Mustard	8	105	0.8	5	from Fargosova, 2001
Wheat	8	100	4	59	from van Steveninck et al., 1993
Soybean	14	40	14	79	from Fontes and Cox, 1998
Rye grass	15	1000	22	132	from Monnet et al., 2001

Barley	10	10	1	10	from Brune et al., 1994
Barley	10	400	19	145	from Brune et al., 1994
<i>Silene vulgaris</i> Zn sensitive / tolerant	7	100	19 / 13	150 / 120	after Harmens et al., 1993*
<i>Silene vulgaris</i> Zn sensitive / tolerant	14	150	23 / 10	-	after Chardonens et al., 1999*
<i>Thlaspi caerulescens</i>	16	10	46	<15	after Shen et al., 1997*
<i>Thlaspi caerulescens</i>	16	500	431	185	after Shen et al., 1997*
<i>Thlaspi ochroleucum</i>	16	10	31	46	after Shen et al., 1997*
<i>Thlaspi ochroleucum</i>	16	500	215	431	after Shen et al., 1997*
<i>Arabidopsis halleri</i>	28	100	31	77	after Zhao et al., 2000*
Tobacco A1 / C5	14	100	15 / 16	74 / 99	this study

646 * Values of Zn concentration were read on graphs

647 **Figure 1a.** Variation of the cation exchange capacity of roots (CECR) for A1 and C5
648 genotype tobaccos. The horizontal line corresponds to the average CECR for A1 and C5. **b.**
649 Variation of the CuSO₄-extracted Ca : CECR ratio (white bars) and of the CuSO₄-extracted
650 Zn : CECR ratio (hatched bars). For both graphs, tobacco were cultivated for 14 days in
651 hydroponics with 100 μM ZnSO₄, and errors bars represent standard deviations.

652

653 **Figure 2.** Comparison of four types of sequential extractions at 4 and 25°C on roots of
654 tobacco cultivated for 4 days in hydroponics with 100 μM ZnSO₄ (R), and at 25°C on isolated
655 root cell walls of tobacco incubated for 24 hours in hydroponics with 100 μM ZnSO₄ (CW).
656 Res: residual Zn. Values are normalized to Zn total content, which ranges between 45 and 69
657 mmol kg⁻¹ for the roots, and between 39 and 96 mmol kg⁻¹ for the cell walls. Errors bars
658 represent standard deviations.

659

660 **Figure 3.** Zn K-edge EXAFS spectra for Zn reference compounds. Values in parentheses
661 indicate the Zn content, in mmol kg⁻¹, dry weight.

662

663 **Figure 4.** Zn K-edge EXAFS spectra for some Zn reference compounds (Zn content, in mmol
664 kg⁻¹, dry weight in parentheses), and for roots of A1 and C5 genotype tobacco grown on
665 artificial substrates and on hydroponics, and for the residues after the CuSO₄ and HCl
666 treatment. Dashed lines are linear combination fits using reference spectra (proportions of the
667 species given in Table 2).

668

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

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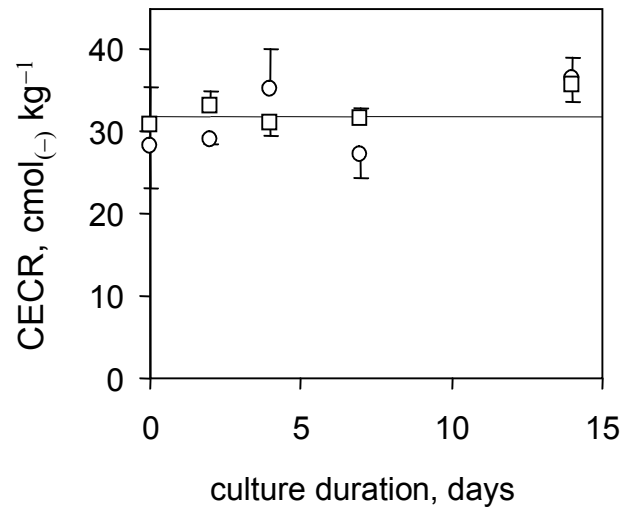
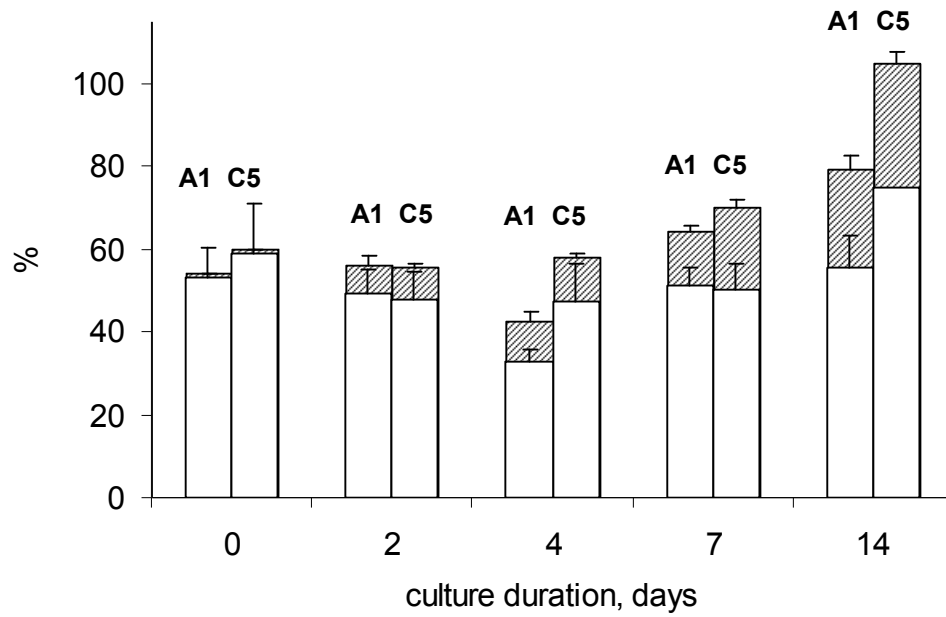
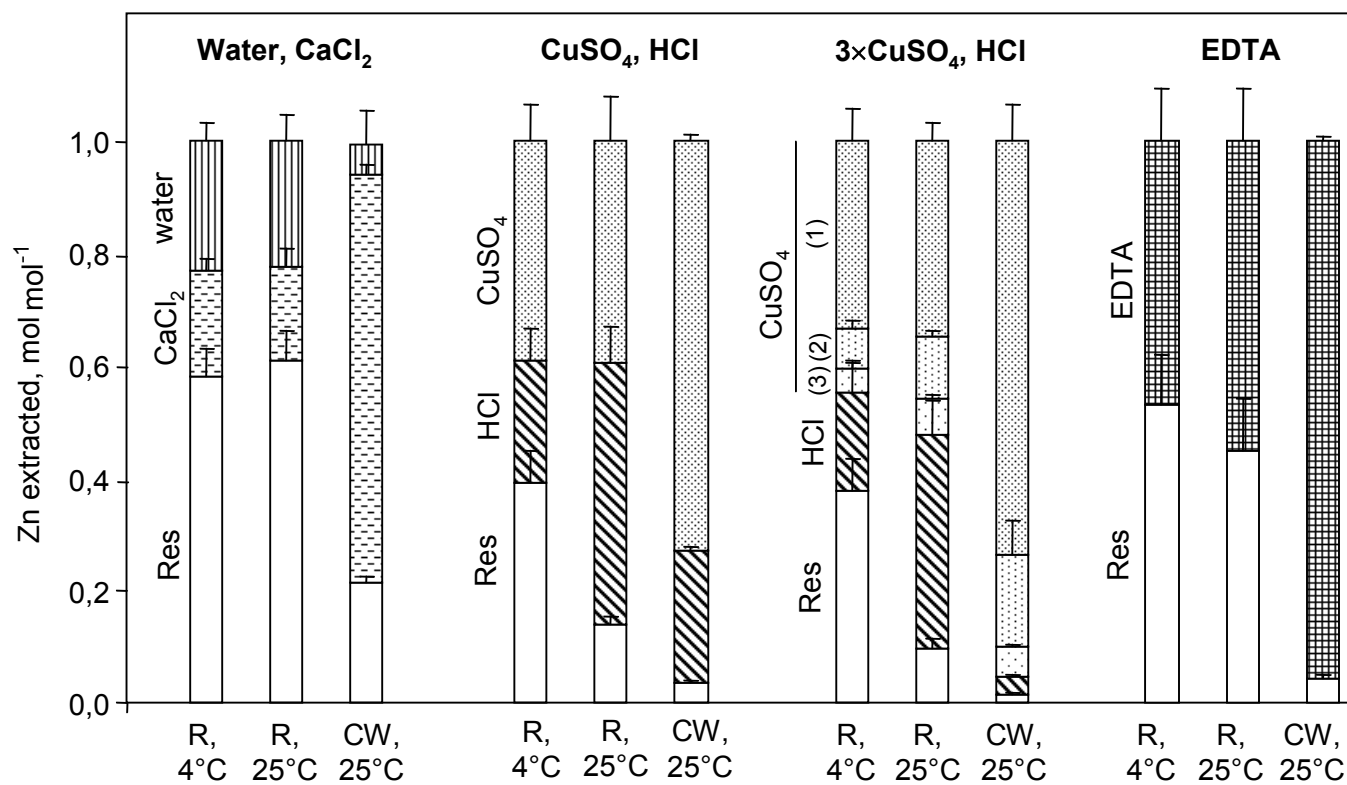
**Figure 1a****Figure 1b**

Figure 2



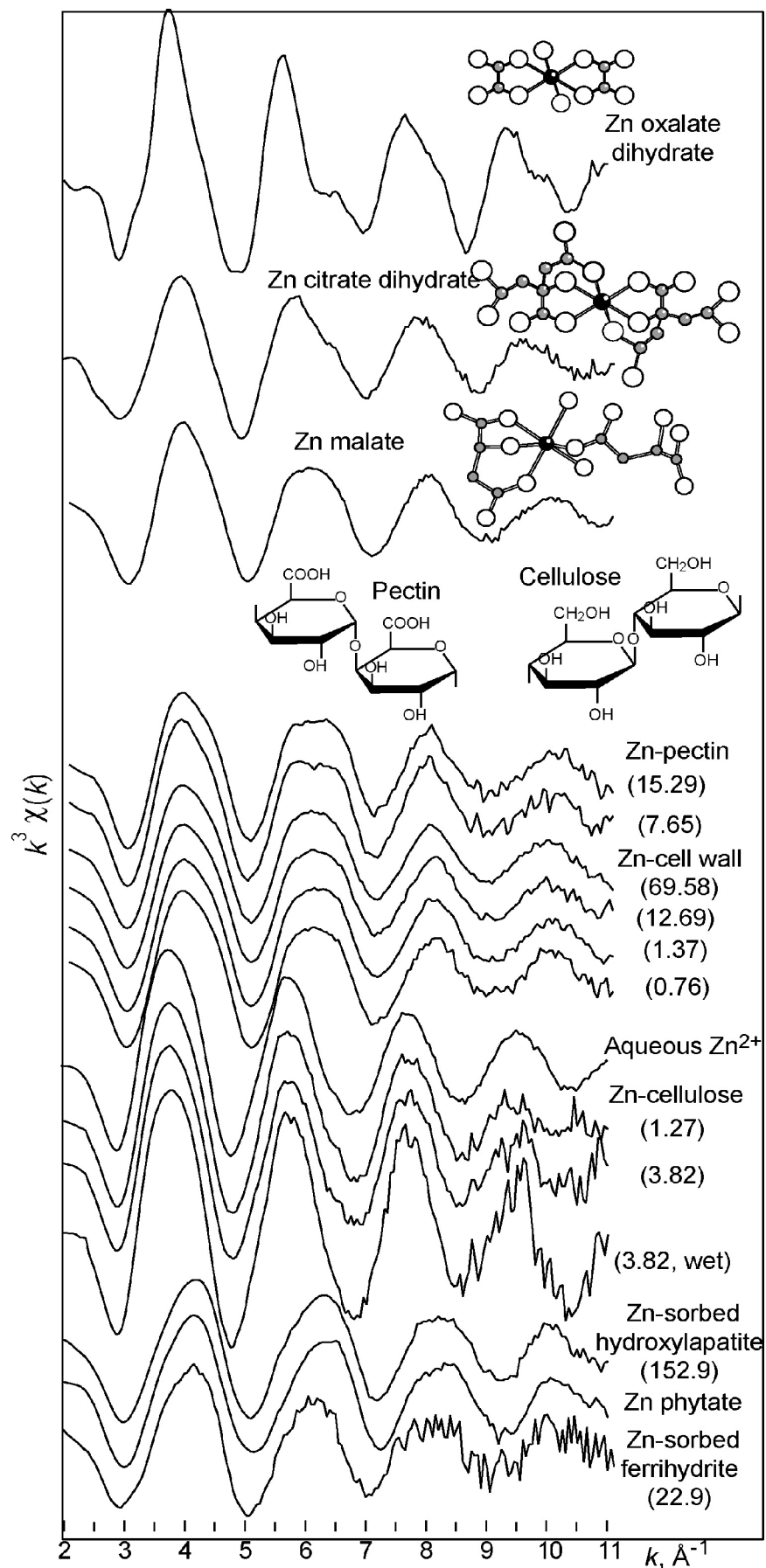


Figure 3

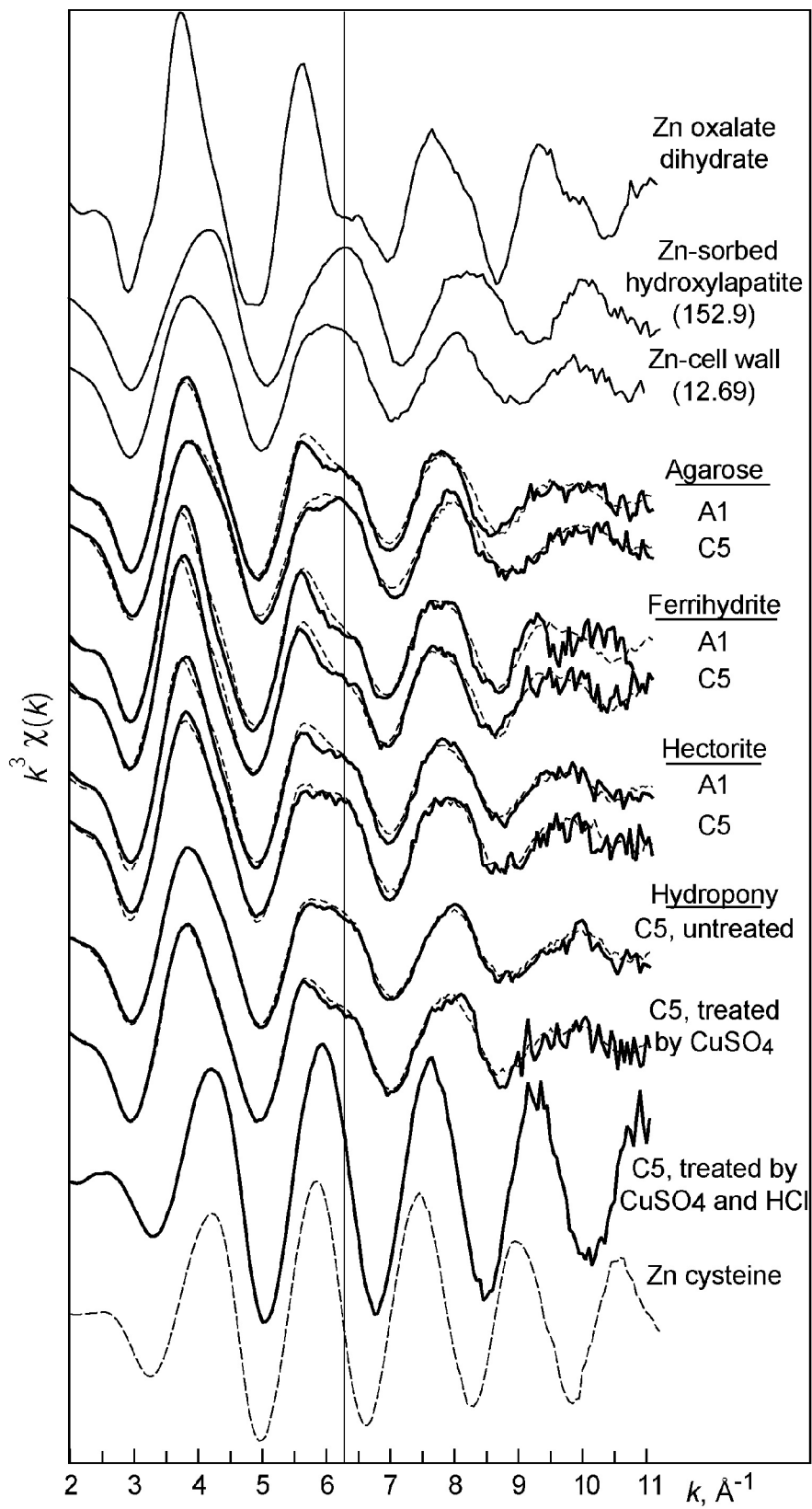
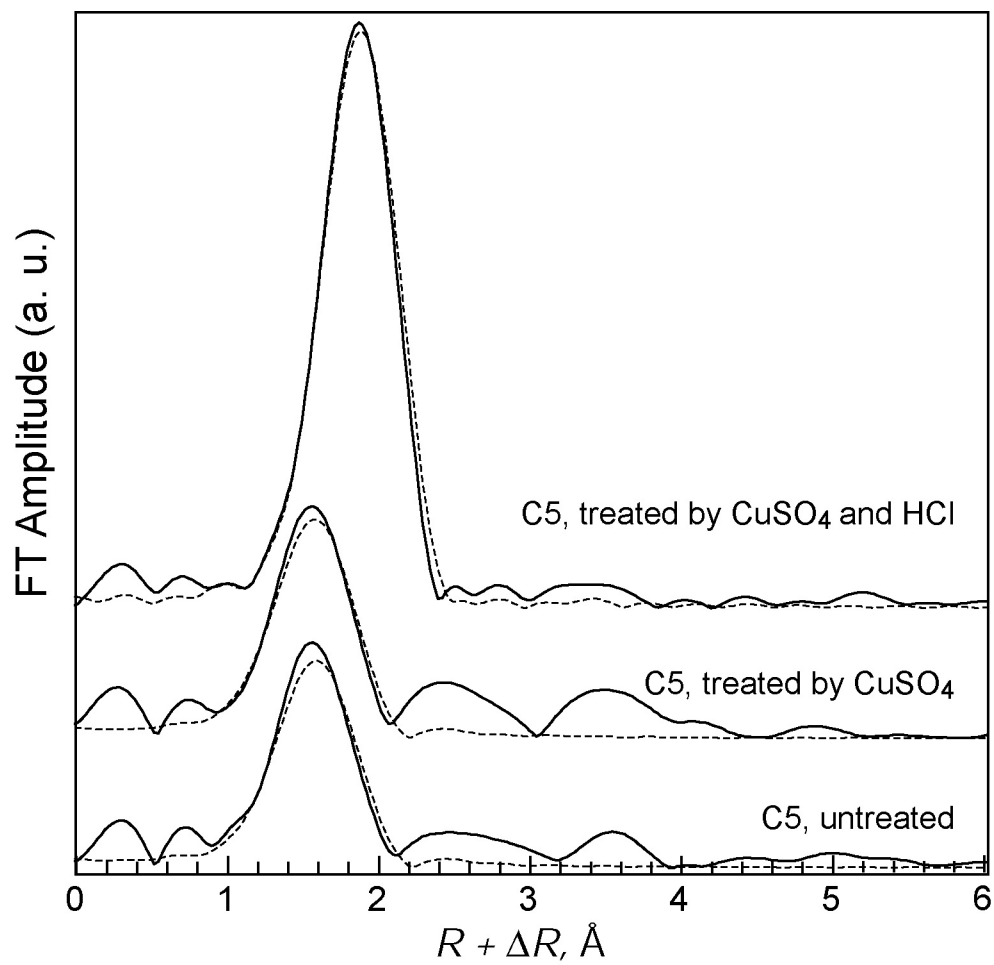


Figure 4

**Figure 5**