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1 **Innovative combination of spectroscopic techniques to reveal nanoparticle fate in a crop plant**

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25 **Abstract**

26 Nanotechnology is the new industrial revolution of our century. Its development leads to an increasing
27 use of nanoparticles and thus to their dissemination. Their fate in the environment is of great concern
28 and especially their possible transfer in trophic chains might be an issue for food safety. However, so
29 far our knowledge on this topic has been restricted by the lack of appropriate techniques to
30 characterize their behavior in complex matrixes. Here, we present in detail the use of cutting-edge
31 beam-based techniques for nanoparticle *in situ* localization, quantification and speciation in a crop
32 plant species (*Lactuca sativa*). Lettuce seedlings have been exposed to TiO₂ and Ag nanoparticles and
33 analyzed by inductively coupled plasma spectrometry, micro particle induced X-ray emission coupled
34 to Rutherford backscattering spectroscopy on nuclear microprobe, micro X-ray fluorescence
35 spectroscopy and X-ray absorption near edge structure spectroscopy. The benefits and drawbacks of
36 each technique are discussed, and the type of information that can be drawn, for example on the
37 translocation to edible parts, change of speciation within the plant, detoxification mechanisms, or
38 impact on the plant ionome, are highlighted. Such type of coupled approach would be an asset for
39 nanoparticle risk assessment.

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45 **Keywords:** nanoparticle; micro X-ray fluorescence spectroscopy; X-ray absorption near edge
46 spectroscopy; micro-particle induce X-ray emission/Rutherford backscattering spectroscopy;
47 multivariate statistical analysis

48

49 **1. Introduction**

50 Nanotechnologies are developing fast with increasing number of applications in fields like medicine,
51 sustainable development or plant protection products. They represent promising solutions to current
52 issues such as more efficient disease treatment on targeted organs or decreased inputs of pesticides
53 in the environment [1]. These new applications also imply an increased dissemination of nanoparticles
54 (NPs, particles with at least one dimension below 100 nm) in ecosystems. Still, their fate in the
55 environment is largely unknown mainly because of the lack of adapted techniques to assess their
56 behavior in complex matrices (soil, plant or animal).

57 Two types of complementary techniques can be used to investigate the fate of NPs: bulk and imaging
58 techniques. The most often used technique for the quantification of metallic NP on bulk samples is
59 inductively coupled plasma – mass or atomic emission spectrometers (ICP-MS, ICP-AES) [7–9].

60 Although ICP techniques have very low detection limits (down to $\text{ng}\cdot\text{kg}^{-1}$ [10]), and provide an essential
61 information, they cannot provide spatial information. Thus, in the case of plant roots, they average
62 adsorbed and absorbed elements from different tissue layers which can result in distorted data.
63 Sample preparation can be an issue if proper acidic digestion of NPs has not been established. Often,
64 standard digestion protocols do not take into account the particulate form of NPs, which may render
65 them less soluble, and thus can lead to underestimation of NP content. Localizing NPs inside biological
66 tissues requires 2D techniques such as scanning and transmission electron microscopy (SEM, TEM)
67 coupled with an energy dispersive X-ray spectrometer (EDS) [2–4]. SEM is often used to investigate the
68 fate of NPs on organism surface (for instance to visualize carbon nanotubes sticking out of root
69 epidermis [5]) or on cross-sections but has a low sensitivity (detection limit around $1000\text{ mg}\cdot\text{kg}^{-1}$ [6]).

70 TEM has a very good lateral resolution allowing investigations at the cellular to sub-cellular scale, but
71 it is not suited for localization of metal at the organ scale. More important, sample preparation (water
72 substitution, resin embedding, staining, coating) can generate artifacts and elemental relocation.
73 Existing cryotechniques (high pressure freezing, cryosubstitution) are highly valuable, but not
74 commonly used in this field. These technical limitations represent a major bottleneck to advance our

75 understanding of NP fate in the environment.

76 To overcome these issues, the combination of both bulk analyses and 2D techniques is a powerful
77 approach. In particular, micro beam-based techniques are useful but underused tools in biology [11–
78 13]. These techniques are based on - but not restricted to - synchrotron radiation (photon) or ion
79 (proton) beams. On synchrotron, elemental distribution can be studied by micro-X-ray fluorescence
80 (μ XRF) and chemical state by X-ray absorption near edge structure spectroscopy using a micro-focused
81 beam (μ XANES) or in full-field mode. On nuclear microprobes, metal distribution and *in situ*
82 quantification can be obtained by combining micro-particle induced X-ray emission (μ PIXE) and
83 Rutherford backscattering spectroscopy (μ RBS). These techniques are already well-known and widely
84 used in chemistry, physics or material science but their development in biology and environmental
85 sciences is more recent [12,13]. Latest developments in sample environment, detector sensitivity and
86 spatial resolution had allowed their use for biological samples.

87 In this study, we report the complementarity of chosen beam-based techniques to determine the fate
88 of two different types of NPs in a crop plant: lettuce (*Lactuca sativa*). Plants are a major component of
89 ecosystems: they are at the interface between air, water and soil and at the base of the human food
90 chain. We chose to study the behavior of two metal-containing NPs widely produced[1]: titanium
91 dioxide (TiO_2) and silver (Ag) NPs. Moreover, these NPs have also been selected for their contrasted
92 solubilities [14].

93 Lettuce seedlings were exposed in hydroponics to different concentrations of TiO_2 and Ag-NPs. After 7
94 days, plantlets were analyzed by a combination of techniques: ICP-MS and ICP-AES, μ PIXE/RBS, μ XRF
95 and XANES. Emphasis has also been settled on sample preparation for the different techniques
96 (optimization of the digestion procedure, preparation and observation of samples in cryogenic
97 conditions). This innovative approach permitted to deepen our knowledge about NP fate in plants and
98 to obtain original data about their impact on plant ionome.

99

100 **2. Material and Methods**

101 **2.1. Plant culture & NP characterization**

102 Lettuce seeds (*Lactuca sativa*, var. Laitue Romaine) were exposed for one week to 0, 10, 100, 1000
103 mg.L⁻¹ Ag or TiO₂-NPs in a modified Hoagland solution. Those concentrations are representative of an
104 acute contamination of the environment [15] and permitted to test the detection limits of the
105 employed techniques. Uncoated Ag (≈ 40 nm) and TiO₂ (anatase, 4 nm) NPs were used. An Ag⁺ exposure
106 condition was also set-up to account for Ag-NP dissolution. More details on plant culture and NP
107 characterization are available in the supplementary data and Figure S1 (Appendix).

108

109 **2.2. Metal quantification by ICP-AES and ICP-MS**

110 Usual digestion protocols (using hot HNO₃) might not be stringent enough to mineralize NPs as well as
111 plant tissues, leading to the underestimation of metal concentrations. To obtain reliable data, effort
112 was put to find out the best digestion protocol for both types of NPs testing the influence of several
113 parameters (temperatures, type of acids, ratio NP powder/acid volume). Ag-NPs were easily
114 mineralized regardless of the chemicals and the temperature conditions used. The following protocol,
115 which was also efficient to mineralize plant tissue (white cabbage), was chosen: 1 mL HNO₃, 1.5 mL
116 HClO₄ at 70°C during 24 h. For TiO₂-NPs, we chose a microwave assisted digestion using 5 ml H₂SO₄, 3
117 ml of HNO₃ and 1 ml of H₂O₂. Digestion yields of the different protocols are gathered in Table S1
118 (Appendix). Roots of three individuals per conditions were harvested, weighted and analysed for Ti
119 and Ag concentrations on an ICP-AES for Ag-NPs and using an ICP-MS for TiO₂-NPs. More details are
120 available in the supplementary data (Appendix).

121

122 **2.3. Sample preparation for beam-based techniques**

123 Beam-based techniques permit to have access to localized information. Sample preparation is a crucial
124 step and if not properly done can lead to redistributions and changes in the speciation of elements
125 [16]. To prevent this, samples were prepared under cryogenic conditions. First, roots and stems were
126 thoroughly washed with deionized water to take off NPs lightly bound to the surface. Then samples

127 were cut in small pieces (2 mm) and immersed in a droplet of resin (Tissue Teck Sakura) and
128 immediately cryo-fixed by plunging the sample in isopentane cooled with liquid nitrogen. The small
129 size of the sample and droplet of resin allows rapid freezing of the whole tissue. Finally, samples were
130 cut in thin cross-sections (20 μm) using a cryo-microtome (Leica). Cross-sections were mounted on
131 appropriate sample holder for synchrotron experiments or freeze-dried (48h, -52°C , 0.01 mbars) for
132 nuclear microprobe and XANES full-field analyses.

133

134 **2.4. Proton beam-based techniques – *In situ* quantification by $\mu\text{PIXE/RBS}$**

135 Samples (prepared as specified in 2.3) were analyzed in freeze-dried state under vacuum on the AIFIRA
136 nuclear microprobe [17]. For this experiment, beamline was operated with a proton source at 3 MeV
137 with a beam focused to 2.5 μm and a current intensity of 500 pA. Mapping of low-Z elements (H, C, N
138 and O) was performed using RBS, in parallel with PIXE for the quantification of elements heavier than
139 Mg. Detection limit is around 100 $\mu\text{g.g}^{-1}$. Data processing was performed using several softwares:
140 Supavisio [18], SIMNRA [19] and GUPIX [20]. More details are available in the supplementary data
141 (Appendix).

142

143 **2.5. Synchrotron based techniques – Distribution by μXRF and chemical speciation by XANES**

144 Synchrotron experiments were carried out on three beamlines: ID21 [21] and ID22 [22] at the
145 European Synchrotron Radiation Facility (ESRF, Grenoble, France) and LUCIA at SOLEIL (Saint Aubain,
146 France).

147 To avoid beam damage, acquisitions were performed under cryogenic conditions on ID21 and LUCIA.
148 μXRF maps were recorded with various step sizes (from 0.1 to 3 μm) with a dwell time from 100 to
149 2000 ms. μXRF data were fitted using PyMCA software [23].

150 Ag L_{III} -edge (3.33 to 3.45 keV energy range, 0.5 eV step) and Ti-K edge (4.96 to 5.05 keV energy range,
151 0.5 eV step) μXANES spectra were recorded in regions of interest of the fluorescence maps. The beam
152 position was slightly moved from one spectrum to another to avoid radiation damage. At least 10

153 spectra of 30s were averaged for each point of interest, after checking the absence of radiation
154 damage. XANES spectra on reference compounds were recorded during previous experiments [24,25]
155 (Figure S2, Appendix).

156 Full-field measurements were performed at the ID21 XANES full-field end-station [26]. The achievable
157 pixel size is 0.32 μm . Measurements were recorded on freeze-dried samples using the Si(111)
158 monochromator with an energy step of 0.3 eV from 3.33 to 3.42 keV for Ag L-edge; and Si(220) with
159 an energy step of 0.3 eV from 4.95 to 4.98 keV and 1 eV from 4.98 to 5.06 keV for Ti K-edge. Data
160 processing was made using the SIFT_PyOCL procedure [27] and TXM-Wizard [28].

161 XANES data treatment was performed using Athena software [29] according to Larue *et al.* [24,25].

162 More details are available in the supplementary data (Appendix).

163

164 **2.6. Statistical analysis**

165 To achieve normality and homoscedasticity, data were \log_{10} transformed. They were compared using
166 one-way ANOVA (for Ti and Ag concentrations in roots as measured by ICP) or two-way ANOVA (for
167 plant fresh biomass), with organ (roots or leaves) and treatment (control, TiO_2 -NPs, Ag ionic and Ag-
168 NPs) as main effects, and the interaction (organ x treatment). Post hoc tests (pairwise T-tests with
169 Bonferroni correction, and Tukey tests) were applied to detect differences between groups of samples.
170 Differences were considered significant when $p \leq 0.05$.

171 To analyze $\mu\text{PIXE/RBS}$ quantitative results and the effect of the different NP concentrations on the root
172 (divided in epidermis, parenchyma and vascular cylinder) ionome (comprising Ca, Cl, Cu, Fe, K, Mn, Mg,
173 Na, P, S, Si and Zn concentrations) we used linear discriminant analysis (LDA) and principal component
174 analysis (PCA). Those techniques are known as part of a group of exploratory data analyses which
175 permit to accommodate the small number of biological replicates due to beamtime restrictions. Data
176 were first $\log_{10(+1)}$ transformed, then standardized. For LDA, the homogeneity between within-group
177 covariance matrices were tested *prior* the analysis.

178 All statistical analyses were performed using the R statistical software [30], with the vegan [31] and
179 MASS [32] packages.

180

181

182 **3. RESULTS and DISCUSSION**

183 **3.1. Toxicity symptoms and ICP metal concentrations**

184 Exposure to the highest NP concentration (1000 mg.L⁻¹) affected the plant fresh weight ($F=3.48$,
185 $P=0.041$), but no interaction between NP treatment and organs could be detected ($F=0.90$, $P=0.46$),
186 indicating that both roots and leaves were affected similarly by the exposure to NPs (Figure S3A,
187 Appendix). Plants showed reduced fresh weight in roots and leaves when exposed to Ag-NPs (roots:
188 176.60 ± 78.78 mg; leaves: 1165.87 ± 350.73 mg) and Ag⁺ (roots: 150.97 ± 54.30 mg; leaves: $1172.70 \pm$
189 625.73 mg) in comparison to control (roots: 349.83 ± 121.82 mg; leaves: 1312.70 ± 328.77 mg) and
190 TiO₂-NP treated plants (roots: 322.67 ± 95.54 mg; leaves: 1586.63 ± 433.67 mg). Plants exposed to Ag
191 (ions and NPs) were displaying early senescence signs and brownish roots (Figure S3B, Appendix).

192 These results are consistent with previous studies on various plants reporting no acute phytotoxicity
193 symptoms following TiO₂-NP exposure [33–37] but decreased root length and biomass after Ag-NP
194 treatment for rice [38] or ryegrass [39]. A study published on the comparative effect of Ag-NPs vs. TiO₂-
195 NPs on tomato also highlighted different phytotoxicity profiles [40]. It is also interesting to notice that
196 the condition accounting for Ag-NP dissolution led to the same growth inhibition that the Ag-NP
197 condition itself. It brings to light the usual question dealing with Ag-NP toxicity: does it come from the
198 NPs or from the dissolution of Ag⁺ ions? According to several studies Ag as ions would be more toxic
199 than Ag as NPs [41–43]. However, NP toxicity would come for a large part from the release of Ag ions
200 during exposure but the nanoparticulate form would also play a specific role in the phytotoxicity
201 process: negatively charged NPs might diffuse more easily than cations within organic matrices [43],
202 and internalized Ag-NPs might then release Ag⁺ inside the biological compartments [44–46]. Within
203 the framework of our experiment (high Ag concentrations, short-term exposure), our results rather

204 support the hypothesis of toxicity coming primarily from Ag ions. This conclusion is in good agreement
205 with a study showing that, if the soluble Ag fraction is high, NPs will not add measurable additional
206 toxicity but at low Ag ionic fraction the nanoparticulate form will increase the phytotoxicity [44].
207 After exposure, metal concentrations in plants were assessed by ICP-AES and ICP-MS. Roots of control
208 seedlings contained low level of both metals (Ti and Ag < 6 mg.kg⁻¹). After a 7-day exposure to 1000
209 mg.L⁻¹ NPs, it was significantly higher: 238.0 ± 54.9 (F=128.2, P=1.2.10⁻⁵) and 1600.5 ± 271.3 (F=49.42,
210 P=0.002) mg.kg⁻¹ fresh weight (FW) of Ag and Ti respectively (Figure S4, Appendix).
211 Ti root concentration was more than 6 times higher than Ag root concentration when exposed to the
212 same initial NP concentration. This trend was not seen in the other comparative study between Ag and
213 TiO₂-NPs on tomato in which Ag concentration in roots was higher [40]. This difference could be linked
214 to the difference in exposure media. Tomato seedlings were exposed in ultrapure water and lettuces
215 in this study in Hoagland solution; this will impact NP behaviour in suspension (agglomeration and
216 dissolution) and thus their interaction with plants. Moreover, different species might also have
217 different uptake as suggested by a study comparing Ti uptake in wheat, common bean and curly dock
218 [34] showing root concentration ranging from 2460 to 5280 mg.kg⁻¹. Additionally, no information is
219 available on the digestion protocol used for ICP measurements so direct comparison might not be
220 pertinent (Table S1, Appendix). Another interesting fact is that the same growth inhibition of the two
221 Ag treatments noticed before (Figure S3, Appendix) is not linked to similar Ag concentrations in lettuce
222 roots. Ag concentration in roots of seedlings exposed to Ag⁺ was significantly lower (82.3 ± 5.2 mg.kg⁻¹
223 FW, F=128.2, p=1.2.10⁻⁵) than in plants exposed to Ag-NPs (Figure S3A, Appendix). This result suggests
224 that not only Ag⁺ ions first dissolved from NPs in the medium are taken up by lettuce roots. Several
225 hypotheses can be proposed: (i) either NPs can penetrate plant roots creating “channels” (as it has
226 been suggested for carbon nanotubes and tomato seed coat [47]) that will facilitate other ion/NP
227 internalisation and internalized NPs would release ions directly into cells [46], or (ii) NP interaction
228 with the root surface enhances their dissolution at the vicinity of the root and subsequently the uptake

229 of Ag⁺ [39,42] with possible later Ag reduction inside organisms [48], or (iii) a large part of NPs and
230 their secondary products are sorbed on the root surface.

231 Finally, these ICP measurements suggest that there is no apparent link between toxicity symptoms
232 (more toxicity for Ag) and metal accumulation in roots (more Ti accumulated).

233 However, ICP-AES and ICP-MS provide bulk analyses and do not distinguish between metal adsorbed
234 on the root surface vs. internalized inside the roots. To shed light on this aspect, a spatially resolved
235 and quantitative approach is needed.

236

237 **3.2. *In situ* quantification by μ PIXE/RBS**

238 Lettuce roots were exposed to Ag⁺ (10 mg.L⁻¹), Ag-NPs and TiO₂-NPs (10 to 1000 mg.L⁻¹); root and stem
239 cross-sections were imaged by μ PIXE (Figure S5 - Appendix, Table 1). The Ag⁺ treatment lead to
240 undetectable level of Ag using this technique and was discarded from the analysis. μ RBS analysis in
241 parallel made it possible to obtain reliable local quantification by area of interest in the samples (Figure
242 S5, S6 - Appendix). Differently to ICP-AES/ICP-MS analyses, results are expressed in mg.kg⁻¹ dry weight
243 (DW). For rough estimation, one could consider 10% of dry matter for lettuce roots.

244 Considering results averaged on the whole cross-section including the root surface, μ PIXE/RBS and ICP-
245 AES/ICP-MS results are in good agreement, evidencing a different behavior between the TiO₂ and Ag
246 NPs (Figure S6, Appendix). In the analyzed cross-sections, Ti was 12 times more concentrated than Ag:
247 25390 and 2096 mg.kg⁻¹ DW respectively after the 1000 mg.L⁻¹ treatment. The difference between Ti
248 and Ag concentration at the cross-section scale is due to a high Ti adsorption on root epidermis in
249 comparison with the parenchymal concentration (for Ti p<0.05, for Ag p=0.26): 71455 \pm 10083 mg
250 Ti.kg⁻¹ in the epidermis area vs. 5888 \pm 5670 mg Ag.kg⁻¹. So using a 2D mapping technique and focusing
251 on the contribution of each tissue type (epidermis vs. parenchyma vs. vascular cylinder), the conclusion
252 is different. If the epidermis area is discarded, one can conclude that lettuce root had taken up more
253 Ag than Ti with approximately 878 \pm 399 mg Ag.kg⁻¹ DW contained in the parenchyma and vascular
254 cylinder compared to 99 \pm 89 mg.kg⁻¹ DW for Ti (Figure S6, Appendix). This phenomenon was also

255 suggested to explain the high Ti concentration in *Vicia faba* [35], bean [34], wheat [34,36] and rapeseed
256 [37]. In terms of risk assessment, these μ PIXE/RBS results suggest that Ag is more worrying since it is
257 taken up by lettuce roots in higher amount and thus is more susceptible to translocate towards the
258 edible parts. Inversely, Ti is found aggregated at the root surface but a small amount is internalized. If
259 one had only considered ICP-AES/ICP-MS data, the conclusion would have been the reverse.

260 The leaves were not analyzed by ICP-AES, but stem cross-sections for the plants exposed to 1000 mg.L⁻¹
261 were analyzed by μ PIXE to identify a possible translocation of Ag and Ti towards the leaves (Figure
262 S6, Appendix). Ti was detected in all stem tissues: epidermis: 283 ± 292 mg.kg⁻¹, parenchyma: 27 ± 15
263 mg.kg⁻¹ and vascular cylinder: 48 ± 39 mg.kg⁻¹. Its distribution was uneven with for instance a hot spot
264 of Ti in the stem vascular cylinder at 2254 mg.kg⁻¹ DW and high variations within replicates. Ag was not
265 detected in the stem even if its concentration in roots was higher than Ti concentration. An explanation
266 to this phenomenon could be the chemical form of the detected element. If Ag is dissolved inside
267 plants then Ag ions would be more mobile and distributed homogeneously in plant tissue leading to a
268 low local Ag concentration, below detection limit. Inversely, Ti due to its limited dissolution would
269 remain as NPs and form hot spots of aggregated NPs thus reaching locally the detection limit of μ PIXE
270 technique.

271 To evaluate the most appropriate exposure condition for this type of experiment, plants were also
272 exposed to 10 and 100 mg.L⁻¹ TiO₂ and Ag-NPs. At 100 mg.L⁻¹ Ag was still detectable in the parenchyma
273 of lettuce roots with approximately the same concentrations as when exposed to 1000 mg.L⁻¹ but was
274 under detection limits in the vascular cylinder. At the lowest exposure concentration (10 mg.L⁻¹), Ag
275 was always below detection limits. Inversely, Ti was detectable in all used concentrations with the
276 existence of Ti overaccumulation on root epidermis. This comparison with lower concentrations
277 confirms results obtained at the highest concentration and also validates our hypothesis of the lower
278 detectability of Ag in comparison to Ti. Even if Ag is more taken up by lettuce than Ti, it becomes under
279 detection limit for lower doses whereas Ti is still detectable.

280 Interestingly, there was no significant difference in Ti concentrations between the 100 and the 1000
281 mg.L⁻¹ treatments (p=0.96). This can be explained by the higher accumulation of Ti at the root surface
282 in the 1000 mg.L⁻¹ exposure which might act as a physical barrier limiting the diffusion and uptake of
283 Ti inside the root tissues (lower Ti concentration in both the parenchyma and vascular cylinder). This
284 effect was not visible for Ag-NP exposure in which concentrations in the tissues increased with Ag
285 exposure (p=5.73.10⁻⁹).

286 A question arising from the high accumulation of Ti on the root surface is its potential influence on
287 plant ionome [49]. Very little has been done so far to investigate the impact of NP exposure on plant
288 nutrition (*Elodea Canadensis* [34], *Arabidopsis thaliana* [50]) and presently there is no study on crop
289 plants. A major advantage of μ PIXE is the possibility to access the distribution of all elements
290 permitting the identification of co-localizations or changes in elemental concentrations.

291 To analyze the effect of different NP concentrations on the elemental accumulation in roots, we used
292 multivariate statistics analysis techniques, namely LDA and PCA. Both techniques are widely used in
293 multivariate statistics [51], LDA being applied to discriminate classes of data, and PCA to identify
294 relationships between variables, without taking into account classes or structures in the data. Across
295 all conditions (NP concentrations and plant tissues), a clear discrimination (LD1: 0.8086) between the
296 control and NP-exposed roots could be detected by LDA (Figure 1A), with Cu (eigenvalue=2.53, Ctrl: 202
297 mg.kg⁻¹, Ag-NP: 39 mg.kg⁻¹, TiO₂-NP: 42 mg.kg⁻¹), Fe (eigenvalue=-1.96, Ctrl: 22 mg.kg⁻¹, Ag-NP: 38 mg.kg⁻¹,
298 TiO₂-NP: 40 mg.kg⁻¹), Mn (eigenvalue=1.50, Ctrl: 195 mg.kg⁻¹, Ag-NP: 25 mg.kg⁻¹, TiO₂-NP: 59 mg.kg⁻¹)
299 and Ca (eigenvalue=-1.30, Ctrl: 9923 mg.kg⁻¹, Ag-NP: 22374 mg.kg⁻¹, TiO₂-NP: 213482 mg.kg⁻¹) being
300 among the most important elements for the discrimination of the groups on the first discriminant axis.

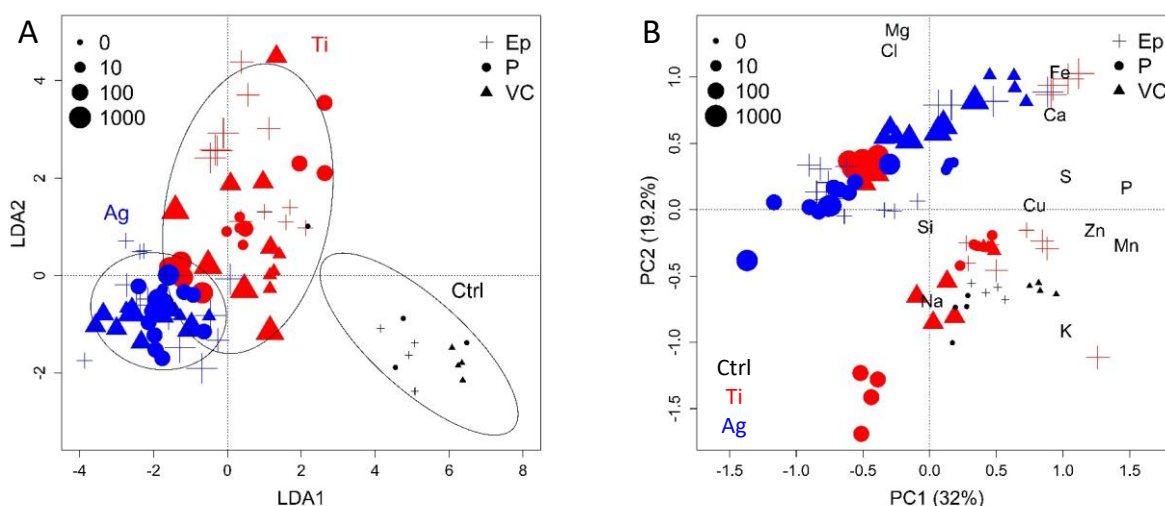
301 A less clear discrimination between the Ag and TiO₂ treatments could be detected (as seen by the
302 partial overlap of the 90% tolerance intervals in Figure 1A), indicating a possible common effect of both
303 NPs on the root elemental concentrations. ICP measurements on *Arabidopsis* exposed to Ag-NPs
304 showed a decreased concentration for K, Fe and Zn [50]. Though the direct comparison is difficult
305 because of the very different exposure conditions (2 mg.L⁻¹ in agar medium vs. 10 to 1000 mg.L⁻¹ in

306 hydroponics), LDA on lettuce roots also highlighted a disrupted Fe homeostasis with an eigen value of
307 -1.96 whereas the two other elements (K and Zn) were not modified which suggest a species-
308 dependent effect.

309 To gain deeper insights on the effects of the different NPs and the relationships between elements, a
310 PCA was performed (Figure 1B). Overall, exposure to Ag-NPs led to a decrease in the concentration of
311 several elements (such as Cu, Zn, Mn, P, S and K) indicating a large disruption of the root ionome,
312 whereas common effect of Ag-NPs and TiO₂-NPs could be seen but only when plants were exposed to
313 1000 mg.L⁻¹. The highest concentration of TiO₂-NPs also led to a higher accumulation of Fe, P, S and Ca
314 in the root epidermis, not seen at the lower concentrations. Those higher concentrations in the
315 epidermis are correlated with decreased concentrations in both the parenchyma and vascular cylinder
316 of plants treated with 1000 mg.L⁻¹ TiO₂-NPs, leading to a similar profile as to plants exposed to Ag-NPs.
317 The same analyses were also performed after excluding the epidermis from the data set, to consider
318 only the internal tissues (Figure S7A, B - Appendix). They provided the same clustering of data, and led
319 to the same conclusions on the changes in root ionome.

320

321 *Figure 1. Root ionome analyzed by μ PIXE/RBS. A. LDA statistical analysis of elemental concentrations in roots. B.*
322 *PCA statistical analysis of elemental concentrations in roots. Elements included in the analysis are Na, Mg, Si, P,*
323 *Cl, K, Ca, Mn, Fe, Cu and Zn (Ti and Ag excluded). For the figure in color, the reader is referred to the web version*
324 *of this article.*



325

326

327 To conclude, μ PIXE/RBS experiment evidenced a similar impact of Ag-NPs on plant ionome at all
 328 exposure concentrations, whereas TiO_2 -NPs displayed a different impact at very high concentration
 329 (1000 mg.L^{-1}) as compared to lower concentrations ($<100 \text{ mg.L}^{-1}$). Even without visible acute toxicity
 330 symptoms at one week exposure, high concentrations of TiO_2 -NPs may lead to a long-term toxicity by
 331 acting as a physical barrier disrupting plant homeostasis.

332

333 3.3. Metal speciation by XANES

334 To investigate the influence of metal chemical form in plants on their fate, metal speciation *in situ* was
 335 determined by XANES. To perform μ XANES *in situ*, elemental distribution was first mapped by
 336 synchrotron radiation based- μ XRF (Table 1). The detection limit of μ XRF is lower than for μ PIXE, around
 337 $1 \mu\text{g.g}^{-1}$ in the tissues [52]. The limit to assure a good XANES data quality is one or two orders of
 338 magnitude higher. Thus, the highest exposure condition was chosen for synchrotron experiments
 339 (1000 mg.L^{-1} in the exposure medium). Distribution maps of metals in root and stem cross-sections
 340 obtained after exposure to $1000 \text{ mg TiO}_2\text{-NPs.L}^{-1}$ and $1000 \text{ mg Ag-NPs.L}^{-1}$ are presented in Figure 2.

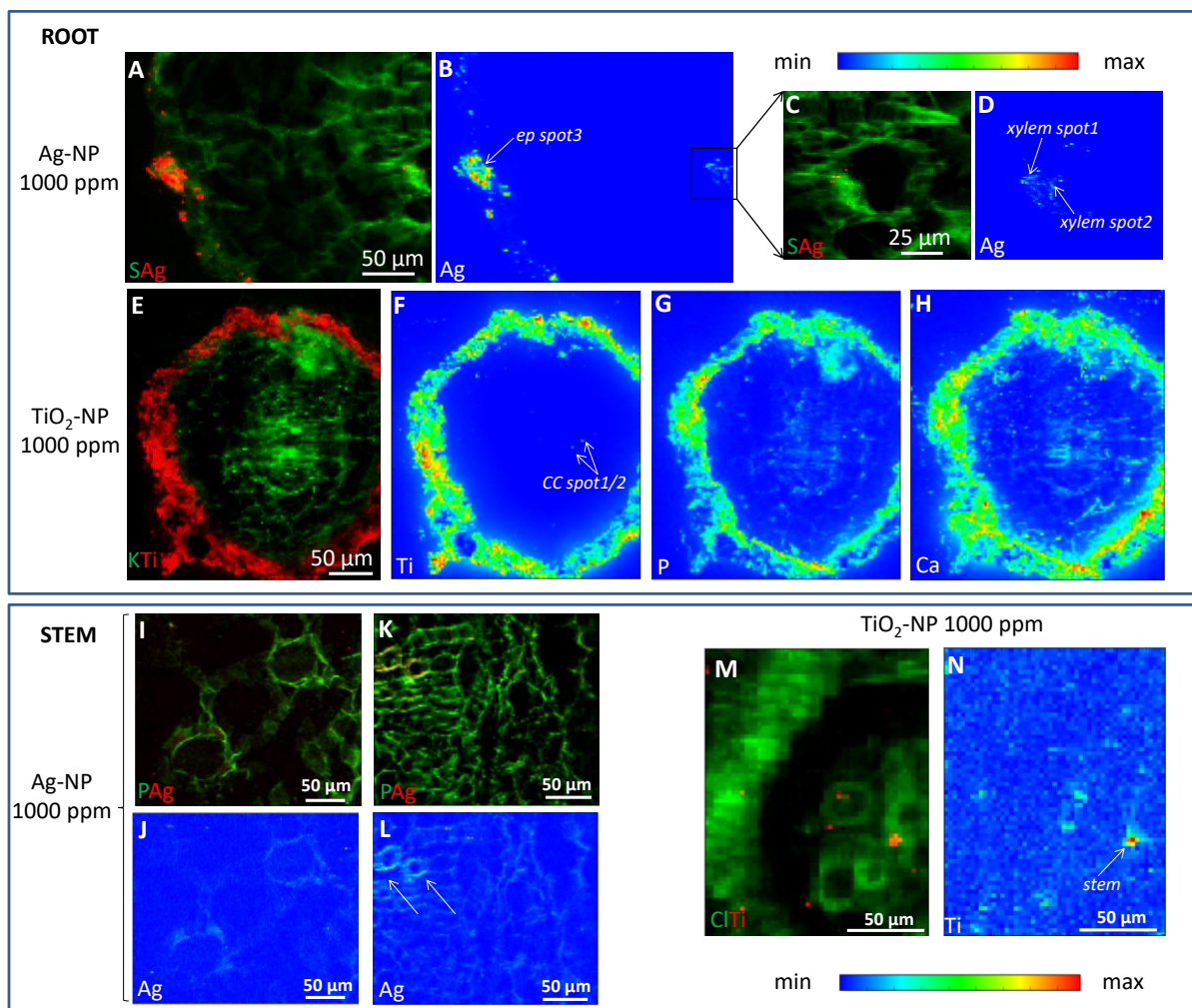
341 μ XRF results agreed with μ PIXE results, showing a continuous layer of Ti at the root surface associated
 342 with Ca and P (Figure 2E, F, G, H) which explains the high contribution of Ca and P on the PC1 of the
 343 previous PCA analysis (Figure 1B). Likewise, hot spots of Ti were detected inside roots, in the

344 parenchyma and in the stem (Figure 2E, F). Ag appeared as agglomerates on root epidermis (Figure 2A,
345 B). Moreover, the high sensitivity of μ XRF also permitted to detect Ag in the root vascular bundle
346 (Figure 2C, D) as well as in vascular bundles in the stem (Figure 2I, J, K, L). The presence of Ag in the
347 root vascular cylinder was further confirmed by μ XRF analysis of a freeze-dried root cross-section at
348 the hard X-ray nanoprobe beamline ID22 (excitation at 29.5 KeV, above Ag K-edge) (Table 1, Figure
349 S8A, B – Appendix). Exciting above the Ag K-edge enhances the sensitivity for Ag due to the higher
350 fluorescence yield of the K emission lines together with the higher photon flux provided at this
351 beamline. However at this high energy other relevant elements such S, P, Cl, K and Ca are poorly
352 detected. These data support the hypothesis that Ag-NPs were transferred through the vascular
353 system to the edible tissue of lettuces. For TiO_2 -NPs the conclusion needs to be mitigated since this Ti
354 also exists as a geogenic element in earth crust. Translocation of both TiO_2 and Ag-NPs in other plant
355 species and transfer to edible parts of crops including cucumber [53,54], tomato [40] and rice [38] have
356 already been demonstrated.

357

358 *Figure 2. μ XRF analysis of lettuce roots and stems exposed for 7 days in hydroponics to 1000 mg.L^{-1} Ag-NPs or*
359 *1000 mg.L^{-1} TiO_2 -NPs. Two color maps show Ag (A, C, I, K) or Ti (E, M) (red) superimposed to endogenous elements*
360 *(S, P, K or Cl in green). Elemental distribution maps in temperature color for Ag (B, D, J, L), Ti (F, N), P (G) and Ca*
361 *(H). Color bars represent the fluorescence counts. Maps were acquired on ID21 and LUCIA. Arrows indicate spots*
362 *where μ XANES spectra were recorded. For the figure in color, the reader is referred to the web version of this*
363 *article.*

364



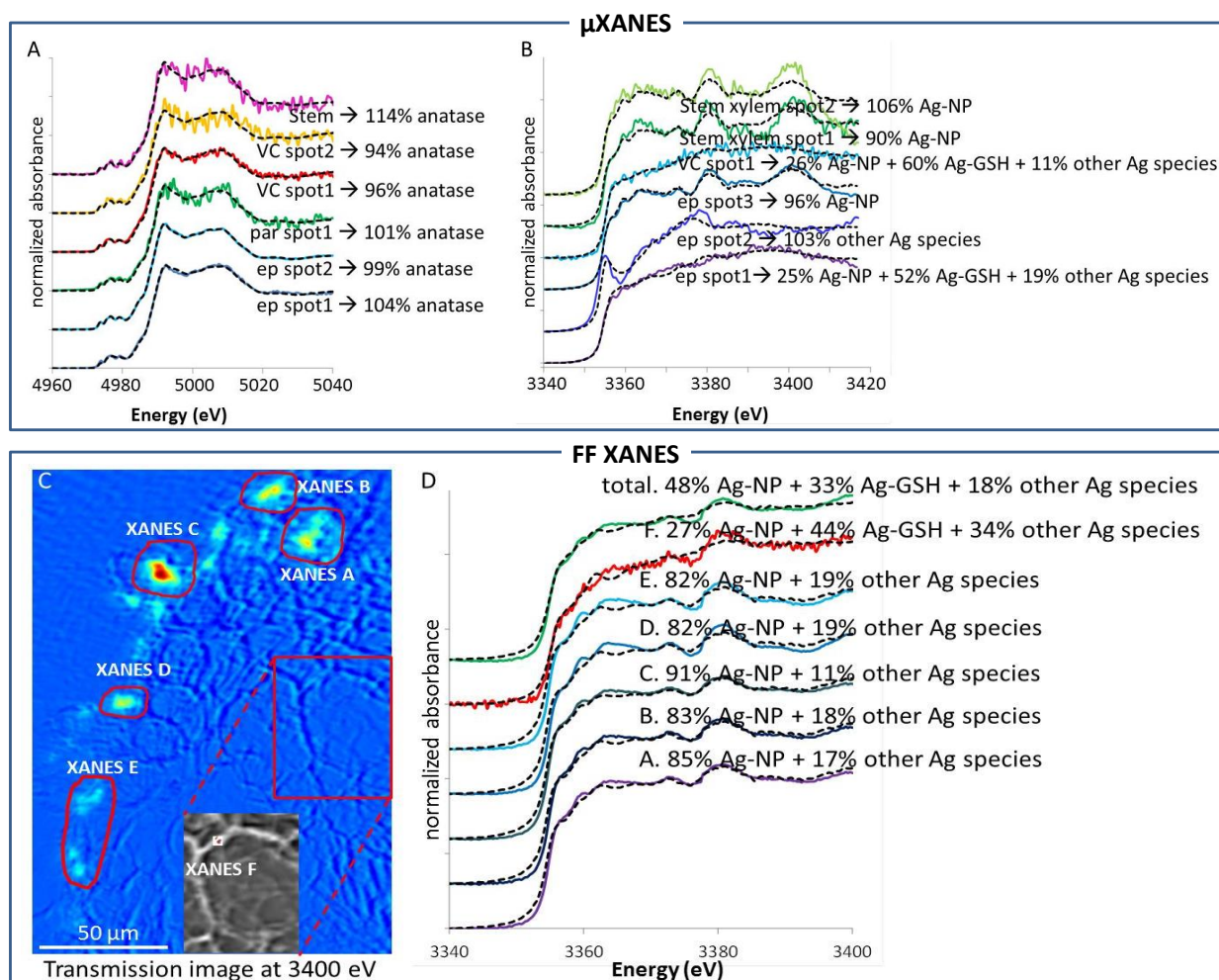
365

366

367 Metal speciation was determined from these maps (arrows in Figure 2 and data not shown) by μ XANES
 368 and using linear combination fitting of reference compounds (Table 1). Ti in the epidermis as well as in
 369 the root parenchyma and vascular cylinder was present as anatase. It was also transferred to stems in
 370 this chemical state (Figure 3A, Figure S9A, B - Appendix). The stability of anatase NPs in the plants is
 371 consistent with previous publications [36,37,53,54]. Inversely, Ag was present in roots under two
 372 oxidation states from 100% Ag^0 to 100% monovalent Ag and in association with different ligands
 373 regardless of the tissue (Figure 3B). The predominant ligand identified by linear combination fitting is
 374 a thiol-containing molecule (glutathione). This molecule is known to be involved in plant detoxification
 375 mechanisms and has been shown to increase in concentration in wheat exposed to Ag-NPs [55]. Ag-
 376 thiol complexes have already been detected in lettuce leaves after foliar exposure to Ag-NPs [24]. Ag

377 in leaves was too diluted to obtain exploitable XANES spectra. Likewise, no good quality XANES spectra
 378 could be obtained for roots exposed to lower NP concentrations (10 and 100 mg.L⁻¹).

379
 380 *Figure 3. XANES analysis in situ of Ti and Ag in root (VC: root vascular cylinder, par: root parenchyma, ep: root*
 381 *epidermis) and stem cross-sections of lettuce in different acquisition modes: μ XANES (Ti in A and Ag in B) and in*
 382 *XANES full-field mode (transmission map of a root cross-section in C with selected area for XANES extraction,*
 383 *corresponding spectra in D. “total” corresponds to all extractible spectra of the analyzed area). In plain lines*
 384 *experimental data, in dotted lines the fit and reference compound contributions to the linear combination fits.*
 385 *For the figure in color, the reader is referred to the web version of this article.*



386
 387
 388 To have a better understanding of Ag internalization mechanisms, a root cross-section was analyzed
 389 by full-field XANES (Figure 3C, D, Table 1). This technique permits to obtain 2D speciation of an element

390 of interest over a large area but is restricted to samples with high concentrations of this element and
391 to thin cross-sections since the signal is recorded in transmission mode. At the moment this acquisition
392 mode runs at room temperature only, so root cross-sections were freeze-dried before analysis. The
393 overall signal of the root cross-section showed a contribution of three forms of Ag, half of the signal
394 came from Ag-NPs, and the other half from other Ag species with 33% being complexed by a thiol-
395 containing molecule. A spatial segregation seemed to exist between the epidermis and the
396 parenchyma. Indeed, Ag at the root surface showed only a moderate dissolution (between 10 and 20%)
397 whereas Ag in the parenchyma displayed a higher dissolution (78%, XANES F). These speciation results
398 corroborate the hypothesis stated after μ PIXE/RBS analysis: Ag inside root tissue was mainly present
399 as monovalent Ag, with a small contribution from elemental Ag (as Ag-NPs). These results do not
400 permit to distinguish the two internalization scenarios stated before: only ions are internalized vs. Ag-
401 NPs are internalized and dissolved inside the cells. Indeed, plant exposure to ionic Ag also led to the
402 formation of Ag⁰ in plant tissues [24,56,57]. Since TiO₂-NPs can be internalized, it is conceivable that
403 Ag-NPs are internalized as well. Thus, the two pathways probably co-exist: both Ag ions and Ag-NPs
404 are taken up by roots, with later dissolution of Ag-NPs inside the tissue and resulting Ag⁺ ions are partly
405 scavenged by the detoxification system. XANES analysis confirmed different behaviors for the two NPs:
406 Ti was detected in plant tissues as agglomerated but unchanged NPs, unlike Ag which was present as
407 Ag-NPs and secondary species including Ag⁺ bound to thiol ligands.

408

409 Table 1: Summary of spectroscopic techniques employed in this study

Technique	Instrument	Information	Characteristics
μ XRF	Synchrotron (ID21 and LUCIA)	Distribution at the Ti K and Ag L edge	Very sensitive ($1 \mu\text{g}\cdot\text{g}^{-1}$) In association with μ XANES Cryogenic conditions
μ XRF	Synchrotron (ID22*)	Distribution at Ag K edge	K-edge more sensitive Better lateral resolution (sub-cellular scale) No information on light elements Freeze-dried samples
μ XANES	Synchrotron (ID21)	Speciation	<i>in situ</i> Cryogenic

Full-field XANES	Synchrotron (ID21)	Speciation	<i>in situ</i> Fast acquisition on large area Concentrated and thin samples (in transmission) Freeze-dried samples
μ PIXE/RBS	nuclear microprobe (AIFIRA)	Distribution + quantification	Sensitivity (100 $\mu\text{g.g}^{-1}$) Local quantification For all elements (H \rightarrow U) Freeze-dried samples

410 * Since our experiment, ID22 nanoimaging beamline has been upgraded and relocated at ID16 (NINA).

411

412 Conclusion

413 This study highlights the interest of using spectroscopic beam-based techniques to analyze the fate of
414 NPs in the environment and especially in biological matrixes. It permitted to bring new and original
415 information that are mandatory for understanding the fate of NPs in plants, which are key information
416 for risk assessment and food safety. For instance, μ PIXE/RBS and μ XRF showed that Ag-NPs were more
417 efficiently taken up by roots than TiO₂-NPs, whereas considering only ICP-MS/AES data, one could have
418 had a reverse conclusion because of the presence of TiO₂-NPs adsorbed on the root surface. Ag and Ti
419 were observed in the stem, suggesting a transfer of the NPs and/or their secondary products to the
420 edible parts of lettuce. The study of the speciation by XANES demonstrated different behaviors for
421 those two NPs: Ti was detected in root tissues as agglomerated but unchanged NPs, unlike Ag which
422 was present as Ag-NPs and secondary species including Ag⁺ bound to thiol ligands. Another interesting
423 piece of information is related to NP toxicity. In our exposure conditions, TiO₂-NPs were not acutely
424 toxic however, at high concentrations they had a clear impact on root homeostasis by disrupting Fe,
425 Ca and P uptake which may lead to long-term toxicity. On the other hand, Ag-NPs were phytotoxic at
426 all concentrations tested and altered to a higher extent root ionome. Obviously, those results have to
427 be mitigated since exposure conditions are not representative of chronic contaminations. This
428 represents the main limit of those techniques nowadays: their limit of detection which restricted, until
429 recently, studies to exposure concentrations mimicking an acute contamination of the environment.
430 Many other spectroscopic techniques exist that are equally useful and bring complementary data for
431 instance at the sub-cellular level (nanoSIMS) or in depth profiling (ToF-SIMS) or on the bio-

432 macromolecule distribution (μ FTIR). Finally coupling those spectroscopic methods with biochemical
433 and molecular information would be a very powerful and promising approach in the future.

434

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444

445 **APPENDIX A. SUPPLEMENTARY DATA**

446 Supplementary data to this article can be found online at doi:

447

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