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Abstract: The broad use of silver nanoparticles (AgNPs) in daily life products enhances their possibilities to reach the environment. Therefore, it is important to understand the uptake, translocation and biotransformation in plants and the toxicological impacts derived from these biological processes. In this work, Lactuca sativa (lettuce) was exposed during 9 days to different coated (citrate, polyvinylpyrrolidone, polyethylene glycol) and sized (60, 75, 100 nm) AgNPs at different concentrations (1, 3, 5, 7, 10, 15 mg L-1). Total silver measurements in lettuce roots indicated that accumulation of AgNPs is influenced by size and concentration, but not by nanoparticle coating. On the other hand, nanosilver translocation to shoots was more pronounced for neutral charged and large sized NPs at higher NP concentrations. Single particle inductively coupled plasma mass spectrometry analysis, after an enzymatic digestion of lettuce tissues indicated the dissolution of some NPs. Ag K-edge X-ray absorption spectroscopy analysis corroborated the AgNPs dissolution due to the presence of less Ag-Ag bonds and appearance of Ag-O and/or Ag-S bonds in lettuce roots. Toxicological effects on lettuces were observed after exposure to nanosilver, especially for transpiration and stomatal conductance. These findings indicated that AgNPs can enter to edible plants, exerting toxicological effects on them.

HIGHLIGHTS

- *Lactuca sativa* were exposed to different AgNPs at different concentrations
- Accumulation of AgNPs depends on their size and concentration
- NP characteristics and concentration has an influence on their transport to shoots
- Appearance of Ag-O/Ag-S bonds indicated the dissolution of some NPs in roots
- Transpiration and stomatal conductance were affected after being exposed to AgNPs

Uptake, translocation and ligand of silver in *Lactuca sativa* exposed to silver nanoparticles of different size, coatings and concentration

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Abstract

The broad use of silver nanoparticles (AgNPs) in daily life products enhances their possibilities to reach the environment. Therefore, it is important to understand the uptake, translocation and biotransformation in plants and the toxicological impacts derived from these biological processes. In this work, *Lactuca sativa* (lettuce) was exposed during 9 days to different coated (citrate, polyvinylpyrrolidone, polyethylene glycol) and sized (60, 75, 100 nm) AgNPs at different concentrations (1, 3, 5, 7, 10, 15 mg L^{-1}). Total silver measurements in lettuce roots indicated that accumulation of AgNPs is influenced by size and concentration, but not by nanoparticle coating. On the other hand, nanosilver translocation to shoots was more pronounced for neutral charged and large sized NPs at higher NP concentrations. Single particle inductively coupled plasma mass spectrometry analysis, after an enzymatic digestion of lettuce tissues indicated the dissolution of some NPs. Ag K-edge X-ray absorption spectroscopy analysis corroborated the AgNPs dissolution due to the presence of less Ag-Ag bonds and appearance of Ag-O and/or Ag-S bonds in lettuce roots. Toxicological effects on lettuces were observed after exposure to nanosilver, especially for transpiration and stomatal conductance. These findings indicated that AgNPs can enter to edible plants, exerting toxicological effects on them.

Keywords: silver nanoparticles, Lactuca sativa, inductively coupled plasma optic emission spectrometry, single particle inductively coupled plasma mass spectrometry, X-ray absorption spectroscopy.

1. Introduction

Silver nanoparticles (AgNPs) are one of the most commercialized nanoparticles due to their antibacterial and physicochemical properties given by their nanosize (1-100 nm). This fact, increases their chance to reach the environment [1-4]. Consequently, living organisms are exposed to these particles generating a growing concern in the risks that can pose to ecosystem and human health. AgNPs can enter into vegetal species through roots or aerial parts of the plant [4,5]. Inside the plant, AgNPs are susceptible to suffer transformation processes that can induce toxicity effects, especially the dissolution of metallic silver from nanoparticles that is considered to partly explain nanoparticles toxicity [6-9]. To understand their behavior and the toxicological effects in plants, several studies have been performed. Most of them have been conducted with *Arabidopsis thaliana* as model plant because it has a short generation time, small size, high seed production and its fully sequenced genome is published [3]. From these works, it has been confirmed that AgNPs can be accumulated in *A. thaliana* plants cultivated in different media such as agar [10,11], Hoagland's [12,13] or soil [14]. It has been demonstrated that their accumulation can decrease plant chlorophyll content affecting its photosynthesis [10,15] and can provoke root, shoot and leaf growth reduction [10,13-15]. In addition, several studies have found an increase of ROS species, oxidative stress, cellular structural damages or gene alterations when *A. thaliana* has been exposed to AgNPs [10-12,15-18]. Diverse authors have corroborated that toxicity of nanosilver to *A. thaliana* depends on particle concentration [11,16], particle size [13] and silver species (AgNPs or Ag(I)) [13,16]. Because of the negative effects that nanosilver can exert to *A. thaliana*, it was also considered essential to evaluate the toxicity of these particles in aquatic [19,20], wetland [21,22] and edible plants [7,26-38]. Among the studied crop plants, some works have been performed with

Lactuca sativa (lettuce), which is common in farmlands and as foodstuff. Barrena et al. [29] observed that uncoated AgNPs (2 nm) can modify lettuce seed germination. Larue et al. [39] determined that foliar exposure of *L. sativa* to uncoated AgNPs (38.6 nm) at different concentration levels (1, 10, 100 μg g^{-1}) did not lead to changes in biomass, protein content and photosynthetic parameters. Nevertheless, Ag agglomerates were detected by micro X-ray fluorescence over the surface of lettuce leaves including stomata. Moreover, it was determined by X-ray absorption near edge structure spectroscopy, that lettuce leaves contained a mixture of AgNPs and secondary species such as Ag-glutathione. Doolette et al. [40] demonstrated that AgNPs (40 nm) and silver sulfide nanoparticles (152 nm) uptake by lettuce from dosed soils depends on nanoparticles dissolution. The bioavailability of Ag from these particles increases with the application of ammonium thiosulfate fertilizer. Furthermore, a low translocation of Ag from roots to shoots in lettuce was also reported. These previous studies with lettuce informed about the uptake, transport and toxicity of AgNPs, but none of them assessed the impact of nanosilver characteristics on these biological processes. Cvjetko et al. [9] stated that AgNPs toxicity in *Allium cepa* is directly correlated to particle size, charge and/or coating. Thus, in order to better understand the behavior of nanosilver in lettuces, the effect of these particles in relation to their characteristics has also to be studied. The aims of this work were to: (1) assess the concentration effect on uptake and translocation of AgNPs and Ag(I) in lettuce tissues; (2) evaluate the uptake and translocation of different types of AgNPs (size/coating) and Ag(I) in *L. sativa* roots and shoots; (3) evaluate the toxicity effects of AgNPs and Ag(I) by some *L. sativa* plant physiological parameters; (4) determine silver form in roots and shoots from the studied lettuce plants; and (5) investigate Ag speciation and ligand environment in the model crop plant by Ag K-edge X-ray absorption spectroscopy (XAS).

2. Material and methods

2.1 Chemicals, materials and apparatus

Well characterized (AgNPs characteristics supplied by the manufacturer NanoComposix) commercial solutions of sodium citrate (2 mM) stabilized silver nanoparticles (citrate-AgNPs) of 60 and 100 nm; polyvinylpyrrolidone coated AgNPs (PVP-AgNPs) of 75 and 100 nm stabilized with sodium citrate (2 mM); and polyethylene glycol coated AgNPs (PEG-AgNPs) of 100 nm dissolved in water were purchased from NanoComposix (San Diego, USA). These AgNPs commercial stock solutions were used to dose plant growth medium for assessing nanosilver absorption by lettuces. An ionic silver stock solution of 1000 ± 2 mg L^{-1} (Merck KGaA, Darmstadt, Germany) was also employed for spiking plant growth medium, and for preparing inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS) standard solutions. The Hoagland solution (nutrient solution) was prepared with the following reagents: zinc sulfate monohydrate ($ZnSO_4 \cdot H_2O$, Panreac, Barcelona, Spain), molybdenum trioxide (MoO_3, Panreac, Barcelona, Spain), calcium nitrate tetrahydrate ($(CaNO_3)_2 \cdot 4H_2O$, Panreac, Barcelona, Spain), ammonium iron (III) sulfate dodecahydrate ($NH_4Fe(SO_4)_2 \cdot 12H_2O$, Panreac, Barcelona, Spain), manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, Panreac, Barcelona, Spain), potassium nitrate (KNO_3, Merck KGaA, Darmstadt, Germany), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, Panreac, Barcelona, Spain), boric acid (H_3BO_3, Merck KGaA, Darmstadt, Germany), ammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Sigma-Aldrich, St. Louis, USA) and magnesium sulfate ($MgSO_4$, Sigma-Aldrich, St. Louis, USA). In addition, for preparing the Hoagland solution, sodium hydroxide (NaOH, Panreac, Barcelona, Spain) was used to adjust the pH. High purity water obtained from a Milli-Q purification system (Millipore Corp., Bedford, MA) was employed to prepare

115 the Hoagland solution and to dilute stock solutions and sample digests. Acetone and
116 calcium carbonate ($CaCO_3$, Panreac, Barcelona, Spain) were employed to determine
117 chlorophyll and carotene contents. For the microwave acid digestion of lettuce tissues
118 analytical grade hiperpur quality nitric acid (HNO_3 69%, Ag 0.1 ng g^{-1}, Panreac,
119 Barcelona, Spain) and hydrogen peroxide (H_2O_2 30%, Sigma-Aldrich, St. Louis, USA)
120 was used. Macerozyme R-10 enzyme from *Rhizopus* sp. (Serva Electrophoresis GmbH,
121 Heidelberg, Germany) and citric acid (Panreac, Barcelona, Spain) were utilized to
122 digest plant tissues for AgNPs extraction.
123 Ovan magnetic stirrers (Barcelona, Spain) were employed to agitate the lettuce growth
124 media (Hoagland solution). An ultrasonic bath J.P. Selecta (Barcelona, Spain) was
125 employed to break down the possible agglomerated AgNPs.
126 Leaf gas exchange parameters were measured using a portable open-circuit infrared gas
127 analyzer system (CIRAS-2, PP-Systems Inc. Amesbury, USA) at about 400 mg L^{-1} of
128 CO_2 equipped with a leaf chamber cuvette (PLC6–18mm of diameter, PP-systems Inc.
129 Amesbury, USA). Chlorophyll fluorescence of the adaxial surface of attached leaves
130 was measured using a portable modulated fluorimeter PAM-2100 (Heinz Walz GmbH,
131 Effeltrich, Germany). Lettuce leaf chlorophylls and carotenoids concentration were
132 determined employing a spectrophotometer Genesys 6 (Thermo electron corporation,
133 Massachusetts, USA).
134 The acid digestion of vegetal tissue samples was carried out with a Berghof Speedwave
135 Xpert microwave digestion system (Eningen, Germany) equipped with an innovative
136 sensor technology (temperature and pressure). The enzymatic digestion of lettuce tissue
137 samples was performed with an incubator Ecotron (Infors HT, Bottmingen,
138 Switzerland) equipped with a temperature sensor. The separation of plant tissues from
139 the supernatant during enzymatic digestion procedure was done with a Rotofix 32A

centrifuge (Hettich-Zentrifugen, Lauenau, Germany).

2.2 Plant culture

Lactuca sativa plantlets obtained from a garden center (Can Morera, Girona, Spain) were cleaned with tap water, especially their roots since they contained organic matter. After the cleaning procedure, the plantlets were completely rinsed with Milli-Q water. Lettuces were cultivated in polypropylene containers containing 100 mL of Hoagland solution [41] (pH ~5.7) contaminated with AgNPs or Ag(I) depending on the test to be performed as shown in **Table 1**. The growing period was 9 days with 16h/8h light/dark regime at controlled temperature (21 ± 2 ºC). In addition, hydroponic media were under constant magnetic stirring during the growing period.

2.3 Measurement of physiological parameters

After lettuces cultivation and exposure to AgNPs or Ag(I), the physiological parameters (leaf gas exchange, chlorophyll fluorescence and photosynthetic pigment content) of fully-developed lettuce leaves were measured to determine if these emerging contaminants have a toxicological effect on the studied plant. Data were analyzed separately for both tests, and one-way ANOVAs were applied to test whether different Ag(I) or AgNPs concentration or coated AgNPs were statistically different from controls.

2.3.1 Gas-exchange measurements

Foliar gas exchange parameters, including transpiration rates (E), stomatal conductance (g_s) and net photosynthetic rates (A) were measured in an attached fully-developed leaf from three (or four) different *L. sativa* plants per treatment in the concentration and coating experiments performed (see **Table 1**). These parameters were analyzed using a portable open-circuit infrared gas analyzer system. Intrinsic (A/g_s; WUE) and instantaneous (A/E; WUE_i) water use efficiencies were also calculated.

2.3.2 Chlorophyll *a* fluorescence measurements

Measurement of chlorophyll *a* fluorescence provides an insight about the health of the photosynthetic system. Components of chlorophyll *a* fluorescence were quantified with a portable modulated fluorometer PAM-2100 (Heinz Walz GmbH, Effeltrich, Germany) using 4 plants per treatment in the concentration effect test and 2-4 plants per treatment in the coating effect test. Measurements were performed on one exposed and fully-developed leaf per plant. After a dark-adaptation period of at least 30 min, we obtained the potential photochemical efficiency of PSII or *Fv*/*Fm*, where *Fv* was the variable fluorescence calculated as *Fv = Fm-Fo*, being *Fo* the minimum and *Fm* the maximum dark-adapted fluorescence. The actual photochemical efficiency of PSII in the light-adapted state was estimated as: $\Delta F/Fm' = (Fm'-F)/Fm'$, where *F* is the steady-state fluorescence yield under the given environmental conditions, and *Fm'* is the maximum level of fluorescence obtained during a saturating flash of light. From this index, we calculated the apparent electron transport rate (*ETR*) as: $ETR = \Delta F/Fm' \times PAR \times 0.84 \times 0.5$, where *PAR* was the incident photosynthetically active radiation (expressed in µmol m^2 s^{-1}), 0.84 was the assumed coefficient of absorption of the leaves, and 0.5 was the assumed distribution of absorbed energy between the two photosystems [42]. Finally, the non-photochemical quenching coefficient (*NPQ*), which is a measure of the thermal dissipation of excess energy, was determined as $NPQ = (Fm-Fm')/Fm'$.

2.3.3 Chlorophyll and carotenoid concentration

To determine leaf concentration of chlorophylls (*Chl*) and carotenoids (*Car*), 0.1 g of fresh lettuce leaves per plant were ground with a mortar in presence of a little quantity of liquid nitrogen, 10 mg $CaCO_3$ to buffer the solution and 1 mL of acetone (100%). Then, 7 mL of 80% acetone were added in aliquots. When the sample was colorless and totally ground, it was transferred into 15 mL centrifuge tube, being centrifuged at 6000

rpm during 5 min. The resultant supernatant was transferred to another centrifuge tube and it was diluted to 10 mL with 80% acetone. Finally, the absorbance was measured at 470, 646.6 and 663.6 nm wavelengths using 80% acetone as blank sample. Leaf concentrations of chlorophyll *a* and *b*, total chlorophylls, and carotenoids were then estimated using Porra et al. [43] equations for the chlorophylls and Lichtenthaler and Wellburn [44] equation for the carotenoids, as in Verdaguer et al. [45]. The content of pigments was finally expressed as mg g^{-1} dry weight (DW) and the ratio *Car*/*Chl a+b* was calculated.

2.4 Plant treatments

After measuring plant physiological parameters, lettuces were submitted to different sample treatments depending on the analysis to be performed. The sample treatments applied are described in the following sections:

2.4.1 Total sample digestion

In order to determine the total silver content accumulated in plant tissues (shoots and roots) a microwave acid digestion was performed. For that, lettuces were washed 3 times with Milli-Q water to remove the residual dosing solution and then the shoots were separated from the roots. Afterwards, shoots and roots were dried in the oven during 24 h at 60-70 ºC. After 24 h, the dried samples were weighed, placed in a polytetrafluoroethylene (PTFE) reactor and were ground with a glass rod. Next, 9 mL of HNO_3 (69%) and 1 mL of H_2O_2 (30%) were added in each PTFE reactor. After capping the vessels, the vegetal samples were digested into the microwave following a digestion program consisting on a first step of 5 min to reach 180 ºC, a second step of 10 min at 180 ºC and a third step of 15 min of cooling to room temperature [46]. Once at room temperature, plant digests were transferred to polystyrene tubes and were diluted to 20 mL with Milli-Q water. Finally, the samples were stored at 4 ºC until their analysis by

ICP-OES or ICP-MS. Data were analyzed separately for concentration, coating and size tests. One-way ANOVAs were applied to all these tests separately to determine whether different Ag(I) or AgNPs concentration, different coated AgNPs and different sized AgNPs were statistically different between each other.

2.4.2 Enzymatic sample digestion

An enzymatic digestion was performed to verify the presence of AgNPs after being accumulated in lettuce tissues. As in microwave acid digestion procedure, lettuces were washed 3 times with Milli-Q water before carrying out the enzymatic digestion. After removing the residual dosing solution, the shoots were separated from the roots and then both vegetal tissues (roots and shoots) were cut in small pieces using scissors. Then, the tissues were homogenized in 8 mL of citrate buffer (2 mM) adjusted to optimum pH range (3.5-7.0) for Macerozyme R-10 in accordance with manufacturer's information. Afterwards, 2 mL of Macerozyme R-10 (1 g of enzyme powder in 20 mL of Milli-Q water) were added. Macerozyme R-10 from *Rhizopus* sp. is a multi-component enzyme containing pectinase 0.5 unit/mg, hemicellulose 0.25 unit/mg and cellulose 0.1 unit/mg, which enables the digestion of vegetal tissues and the extraction of the nanoparticles. After adding the Macerozyme R-10, the samples were shaken in an incubator at 37 ºC during 24 h. Afterwards, the plant digests were settled during 1 h and then were filtered by gravity to remove the small pieces of plant tissues [47]. Finally, the samples were filtered through 0.45 μm cellulose acetate filter (Whatman, Filterlab, Barcelona, Spain) and diluted to 20 mL with Milli-Q water.

2.4.3 XAS sample preparation

The fresh roots were rinsed with Milli-Q water, frozen in liquid nitrogen, homogenized and fixed onto Cu holders for measurements under cryo conditions.

2.5 Analysis of plant digests

The plant tissue digests obtained from the two digestion methods were analyzed with two different instruments as described below:

2.5.1 Measurement of total silver content in lettuce tissues

Lettuce root digests obtained from microwave acid digestion were analyzed with an ICP-OES system (Agilent 5100 Vertical Dual View, Agilent Technologies, Tokyo, Japan) in order to determine the total silver content accumulated in the roots. This instrument is equipped with a concentric glass nebulizer, a double pass glass cyclonic spray chamber, an echelle polychromator wavelength selector and a charge-coupled device (CCD) detector. The measurement conditions were: 1200 W RF power, axial plasma configuration, 12 L min^{-1} plasma gas flow rate, 0.7 L min^{-1} nebulizer flow rate, 25 s of stabilization time, 1 s of reading time and 3 replicates for each reading. The silver wavelength used was 328.068 nm.

Silver concentrations in lettuce root and shoot tissues below the detection limit of ICP-OES (lettuce tissue digests obtained from enzymatic and microwave acid digestions) were determined by an ICP-MS 7500 c (Agilent Technologies, Tokyo, Japan) equipped with a Babington nebulizer, a double pass scott nebulizer chamber, an octopole reaction collision cell system (ORS), a quadrupole analyzer and an electron multiplier detector. Measurement parameters were: 1500 W RF power, 15 L min^{-1} plasma gas flow rate, 1.1 L min^{-1} nebulizer flow rate, 20 s of stabilization time, integration time of 0.1 s and 3 readings per replicate. The isotope monitored was ^{107}Ag.

2.5.2 Detection of AgNPs in lettuce tissues

Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) was employed for analyzing the plant tissue digests obtained by enzymatic digestion in order to determine the chemical form of silver (AgNPs or Ag(I)) after being accumulated in these plants. Prior to SP-ICP-MS analysis, the total silver content was determined using

ICP-OES or ICP-MS system to adjust the silver concentration in sample digests at <1 μg L^{-1}. SP-ICP-MS analysis was carried out with an ICP-MS 7500 c (Agilent Technologies, Tokyo, Japan) using a 10 ms dwell time and a total measurement time of 60 s. The raw data obtained by the analysis performed was treated using Excel software.

2.6 XAS analysis

The samples were measured at CLÆSS beamline of Synchrotron ALBA in Barcelona, Spain. The samples were fixed on the Cu holders and mounted on a finger cooled by a He cryostat. Ag K-edge EXAFS spectra were measured in fluorescence mode using CdTe detector (Amptek, USA) with a total collection time of ~2 h in consecutive runs, to ensure the useful interval up to k = 10 $Å^{-1}$. The spectra were analyzed with IFFEFIT software [48]. Statistical analysis of Ag neighborhood in plants was performed by XLSTAT software.

3. Results and discussion

3.1 Evaluation of plant growing medium conditions

A test to evaluate the best conditions for accumulating the maximum quantity of silver inside lettuce tissues (roots and shoots) during the growing process was performed before carrying out the experiments of this study. For this test, lettuce plants were cultivated during 9 days (16h/8h light/dark, 21 ± 2 ºC) in 100 mL hydroponic medium containing 75 nm PVP-AgNPs (1 mg L^{-1}) under two different conditions: without agitation and magnetic stirring (~200 rpm). After 9 days of cultivation, lettuce roots and shoots were submitted to an acid digestion following the procedure described previously (section 2.4.1). Then, the resulting sample digests were analyzed by ICP-OES or ICP-MS to determine the total silver content in lettuce tissues. As can be seen in **Fig. A1** from **Appendix**, the highest total silver concentration was obtained in lettuce roots and shoots cultivated in nutrient medium submitted to magnetic stirring agitation. This

290 could be due to the fact that AgNPs tend to be deposited at the bottom of the growing
291 tank when no stirring is used during lettuce cultivation. For this reason, it was decided
292 to agitate the Hoagland medium with magnetic stirrers during lettuce cultivation.

293 **3.2 Physiological parameters**

294 **3.2.1 Gas-exchange**

295 The analyses of gas exchange parameters in *L. sativa* revealed that plants were sensitive
296 to the different 75 nm PVP-AgNPs concentrations (0, 3, 5 and 10 mg L^{-1}) since the
297 transpiration rate and stomatal conductance were affected. Specifically, plants subjected
298 to the different AgNPs concentrations showed lower transpiration rate values than
299 control ones (see **Table 2**), but the effect was not in dose dependent manner, since there
300 were not statistically significant differences between the tested concentrations
301 (statistical data not shown). The stomatal conductance was also reduced in lettuce
302 plants, but only when they grew under 3 and 5 mg L^{-1} AgNPs concentration, although
303 the same tendency was observed by plants under 10 mg L^{-1} ($F_{1,6}$= 5.511, p = 0.057).
304 Hence, the presence of 75 nm PVP-AgNPs in the culture medium affected negatively
305 lettuce plant water relations being reduced the transpiration rates and stomatal
306 conductance by nearly half of that of control plants (49.4% ± 6.5 and 53% ± 4.2,
307 respectively). Lower transpiration rates agree with lower stomatal conductance.
308 Stomatal closure could be the consequence of a direct PVP-AgNPs effect on guard
309 cells, since nanosilver particles, through the xylem stream water, can reach leaves and
310 thus stomata [49]. In fact, other authors stated that in cut roses nanosilver particles also
311 reduced the stomatal conductance and transpiration rate [50]. Nevertheless, when those
312 particles are coated with PVP, results could vary. For instance, in willow and hybrid
313 poplar cuttings the transpiration was unaffected by PVP-AgNPs [51], although values
314 obtained were also lower than those of controls. The decrease in stomatal conductance

could also be a consequence of an indirect effect due to an interference of PVP-AgNPs with root water uptake. In the treated lettuce plants of this study, much more nanosilver particles were found in roots than in shoots, which would support a stomatal closure due to a disruption of water uptake. Accordingly, in *A. thaliana*, AgNPs caused imbalance in the levels of water affecting the transcript levels of aquaporins [10]. A decrease in root water uptake could provoke plant water deficit, reduction of mineral nutrients, rising ABA in leaves and promoting stomatal closure [52,53]. Nevertheless, more studies are needed to assess in lettuce the relationship between PVP-AgNPs aquaporin root cell, water uptake, ABA and mineral nutrients levels, and stomata movement. Photosynthetic rates did not vary in lettuce plants treated with 75 nm PVP-AgNPs in comparison to control ones. Likely, because of the low light intensity conditions that limits plant CO_2 fixation. Neither the water use efficiency (*WUE* and WUE_i) of treated plants differed from control ones.

Regarding results from the coating effect test, only data for the transpiration rate and stomatal conductance was provided. In this case, the instrument was not sensitive enough to make the photosynthesis measurements under low light exposure. Transpiration rate and stomatal conductance only diminished in *L. sativa* plants growing under Ag(I) (about three-fold reduction of E and g_s) and 100 nm PVP-AgNPs (about 1.5-fold reduction of E and g_s), in comparison to control ones (see **Table 3**), being the most intense the effects for Ag(I). These results highlight the negative effect of Ag(I) on plant water relations, which could agree with a potent inhibitor effect of silver on plasma membrane aquaporins of root cells [54]. Moreover, the results suggest that coated nanosilver particles reduce the negative effect of Ag(I). PVP, PEG and citrate are used as coatings in order to promote stability, avoid aggregation of AgNPs and decrease

interaction between proteins [55] so, root water uptake disruption could, likely, be alleviated by these stabilizing compounds.

3.2.2 Chlorophyll fluorescence parameters and leaf chlorophyll and carotenoid concentrations

In the concentration effect test, there were no significant differences among lettuce plants grown under 0, 3, 5 and 10 mg L^{-1} of 75 nm PVP-AgNPs in the parameters estimated from the chlorophyll fluorescence measurements (*Fv*/*Fm*, *ETR* and *NPQ*). The high values of *Fv*/*Fm* obtained in all the treatments (between 0.81 and 0.82) indicate that plants did not experience photoinhibition under any of the different 75 nm PVP-AgNPs concentrations assayed, which was expected taking into account the low light intensity (below 50 mol m^2 s^{-1}) applied to the plants. Accordingly, treatments did neither affect the leaf amount of chlorophylls and carotenoids nor the ratio carotenoids/chlorophylls (data not shown).

Interestingly, in the coating effect test, we found significantly (p = 0.032) higher *Fv*/*Fm* values in plants treated with 100 nm PVP-AgNPs compared to controls (see **Fig. A2** from **Appendix**) which was in agreement with the significantly lower carotenoid/chlorophyll ratio observed in these plants (see **Fig. A3** from **Appendix**). Enhanced photochemical efficiency (*Fv*/*Fm*) indicates that a higher number of reaction centers are able to accept electrons and carry out photosynthesis, which would be in accordance with a lower need for photoprotection *via* an increased carotenoid/chlorophylls ratio. In plants treated with 100 nm PEG-AgNPs, the leaf content of *Chl a* (p = 0.011), *Chl b* (p = 0.004), total *Chl* (p = 0.009) and carotenoids (p = 0.012) was more than twice that of controls (see **Fig. A4** from **Appendix**) and, though not significant, *Fv*/*Fm* values also tended to be higher compared to controls. These results are opposite to those reported on *Vigna subterranean* [56] or *Brassica* sp. [57],

364 but they are in agreement with Sharma et al. [58], who found enhanced quantum
365 efficiency and more chlorophyll content in *Brassica juncea* leaves treated with silver
366 nanoparticles. Salama [59] demonstrated a concentration-dependent effect of silver
367 nanoparticles on the leaf amount of chlorophylls and carotenoids of *Phaseolus vulgaris*
368 and *Zea mays*. Indeed, increasing concentration of silver nanoparticles from 20 to 60 mg
369 L^{-1} led to enhanced growth, photosynthetic pigment, carbohydrate and protein contents,
370 while concentrations over 60 mg L^{-1} induced an inhibitory effect on these parameters.
371 Yang et al. [53] observed that the contents of Fe and Cu, which play a role in the
372 photosynthesis process, were lowered when *Triticum aestivum* L. plants were exposed
373 to 200 and 2000 mg kg^{-1} AgNPs. Additionally, Rui et al. [60] determined that the
374 expression of antioxidant isoenzymes localized in the chloroplast and/or cytosol (Fe-
375 superoxide dismutase and Cu/Zn-superoxide dismutase) increase in AgNPs treated roots
376 and pods of peanuts because of stress conditions for the plant. Finally, citrate-AgNPs
377 and Ag(l) treatments did not modify significantly the studied parameters in relation to
378 controls.

379 **3.3 Total silver content in lettuce tissues**

380 As it is stated in the Introduction, nanoparticle properties and their concentration in the
381 environment greatly influence plant uptake and translocation, as well as toxicity for the
382 plant. In order to better understand the behavior of AgNPs in lettuce tissues (roots and
383 shoots), total silver concentration was determined in vegetal samples after being
384 exposed to different types of AgNPs (size and coating) and at different nanosilver
385 concentrations treatments. The results obtained for the different tests performed are
386 shown in the following points:

387 **3.3.1 AgNPs and Ag(I) concentration effect**

In order to determine the amount of AgNPs that lettuces (root and shoot tissues) can uptake, accumulate and translocate, Hoagland solution was spiked at different nanosilver concentrations (concentration range: 3-15 mg L^{-1}). In addition, Ag(I) was also tested at the same concentration levels to determine if there were differences in respect to nanosilver.

As it is shown in **Fig. 1a**, AgNPs and Ag(I) were trapped by lettuce roots because there was an increase in the silver content in these tissues in relation to controls. Total silver accumulated in lettuce root tissues increased in dose-dependent manner for both silver forms. No significant differences (p (3 mg L^{-1}) = 0.2754; p (5 mg L^{-1}) = 0.0520) were observed between the two silver species uptake at low concentrations but at concentrations above 7 mg L^{-1} significant differences were observed (p (10 mg L^{-1}) = 0.0403; p (15 mg L^{-1}) = 0.0031) showing a higher Ag(I) uptake by root tissues in respect to 75 nm PVP-AgNPs. Cvjetko et al. [7] and Vinković et al. [27] observed also differences between Ag(I) and AgNPs uptake by root tissues at higher concentrations with *Allium cepa* and *Capsicum annuum* L. plants. It is worth mentioning that the error bars were bigger at 10-15 mg L^{-1} AgNPs or Ag(I) concentration, probably because roots could not accumulate more silver and they started to suffer changes in their metabolism due to toxicological effects (e.g. a minor growth of the lettuce plant was observed).

Previous studies demonstrated that AgNPs can be accumulated in roots and be transported to aerial parts of the plant [53,57,60]. For this reason, the total silver content in lettuce shoots was also determined after an acid microwave digestion. The results showed that both silver chemical species (75 nm PVP-AgNPs and Ag(I)) accumulated in lettuce roots were transported to shoot tissues that did not contain silver as could be seen in control sample (see **Fig. 1b**). Nevertheless, the content of silver accumulated in lettuce roots was mainly 5000-fold higher than in lettuce shoots. The same trend was

determined in *Triticum aestivum* L. plants exposed to 20, 200 and 2000 mg kg^{-1} AgNPs [53]. Statistical differences at some concentration levels (e.g. p (3 mg L^{-1}) = 0.0215; p (15 mg L^{-1}) = 0.0028) were observed between 75 nm PVP-AgNPs and Ag(I), the latter being more effectively transported to lettuce aerial parts. Vinković et al. [27] observed also this difference with *Capsicum annuum* L. plants and the reason of this dissimilarity was explained by the aggregation behavior of AgNPs, which at the studied concentrations easily aggregated and consequently they were less efficiently transported. Additionally, it was observed that the silver concentration accumulated in lettuce shoots increased in dose-dependent manner for both silver forms up to 7 mg L^{-1}, especially 75 nm PVP-AgNPs. At concentrations higher than 7 mg L^{-1} the shoot tissues probably contained less silver because they started to suffer toxicological effects and their metabolism was affected (e.g. deadness of lettuce shoots as a consequence of necrosis was observed) (see **Fig. 1a**). Indeed, the hydric status of lettuce leaves (E, g_s) was already affected by 75 nm PVP-AgNPs at a concentration of 3 mg L^{-1}.

3.3.2 AgNPs coating effect

AgNPs are synthesized with different surface coatings to improve their stability. The coat around nanoparticles modifies their surface characteristics (e.g. surface charge) and consequently their intrinsic behavior. Furthermore, under environmental conditions surface coatings are susceptible to suffer changes. For instance in their superficial charge, which is an important parameter affecting the interactions with other components [7,61]. In this work three different 100 nm coated AgNPs were tested at 1 mg L^{-1} concentration: PVP-AgNPs (negatively charged at pH 7), citrate-AgNPs (highly negatively charged at pH 7) and PEG-AgNPs (neutral at pH 7) [62]. In **Fig. 2a** it can be seen that the different coated AgNPs tested were similarly accumulated in lettuce roots (p = 0.9514). So, surface charge seems to have no effect on AgNPs uptake by lettuce

roots although, as commented above (section 3.2.1) can affect plant physiology in a different manner. Cvjetko et al. [7] studied the uptake of PVP-AgNPs and citrate-AgNPs by *Allium cepa* roots and observed a similar silver uptake, especially at higher concentrations (50 and 75 μM). In addition, Ag(I) uptake by lettuce roots was also tested to check if there were differences in respect to nanosilver. The results obtained demonstrated that Ag(I) concentration in root samples was higher although no statistical differences were observed (p = 0.1237). Published studies with other edible plants also determined that Ag accumulation was lower in treatments with AgNPs than with $AgNO_3$ [7,27,57] .

Total silver content in lettuce shoots was also determined. Although no statistical differences (p = 0.7700) were observed between total silver concentrations in lettuce shoots for all treatments performed (see **Fig. 2b**), it seems that neutral surface charged AgNPs (PEG-AgNPs) and Ag(I) were more easily transported from roots to shoots compared to negatively charged AgNPs (PVP- and citrate-AgNPs). These results are in agreement with other works in which is confirmed that translocation of Ag(I) from roots to shoots is higher than AgNPs [27,63]. Additionally, a stronger reduction in transpiration and stomatal conductance due to the presence of Ag(I) inside plants was already observed in comparison with other treatments (lowest E and g_s values) (see **Table 3**).

3.3.3 AgNPs size effect

AgNPs can be synthesized at different sizes. This particle characteristic could also influence their behavior in the environment. For this reason, the size effect on AgNPs uptake by lettuce roots and their translocation to shoots was studied with two different sized PVP-AgNPs (75 and 100 nm) and citrate-AgNPs (60 and 100 nm). The results obtained showed that small sized AgNPs (75 nm PVP-AgNPs and 60 nm citrate-AgNPs) were more absorbed by lettuce roots than large sized AgNPs (100 nm PVP- and

citrate-AgNPs) (see **Fig. 3a** and **3c**), observing statistical differences between the tested particle sizes (p_{PVP} = 0.0001; $p_{citrate}$ = 0.0037). The same tendency was observed in a bioaccumulation study of different sized AgNPs (20, 30-60, 70-120 and 150 nm) in *Oryza sativa* L. cv *KDML 105* root tissues [23]. This supports the hypothesis that roots have a higher tendency of trapping smaller AgNPs rather than bigger ones. However, it is worth mentioning that in this work the same AgNPs mass concentration is used, so the number of particles present in the solutions of 75 nm PVP-AgNPs and 60 nm citrate-AgNPs is higher than the solutions of 100 nm PVP- and citrate-AgNPs. Therefore, the probability of smaller AgNPs being absorbed by lettuce roots is higher than for the bigger ones.

The total silver content in lettuce shoots was also determined. The concentration of large sized AgNPs (100 nm PVP- and citrate-AgNPs) was higher than small sized AgNPs (75 nm PVP-AgNPs and 60 nm citrate-AgNPs) (see **Fig. 3b** and **3d**), exhibiting statistical differences between different sized PVP-AgNPs (p = 0.050) but none for citrate-AgNPs (p = 0.1501). These results are in agreement with Thuesombat et al. [23] that also determined the total silver content in *Oryza sativa* L. cv *KDML 105* leaf tissues of different sized AgNPs (20 nm, 30-60, 70-120 and 150 nm) and they concluded that 20 nm AgNPs were less accumulated in rice aerial parts than 150 nm AgNPs. This tendency was also confirmed by calculating the translocation factor (*TF*) with the following equation [64]:

$$\boldsymbol{TF = \ C_{shoot}/C_{root}} \qquad (\mathbf{1})$$

where C_{shoot} is the total silver concentration in lettuce shoots and C_{root} is the total silver concentration in lettuce roots. The *TF*s obtained were higher for large sized AgNPs (100 nm PVP-AgNPs: 0.0099; 100 nm citrate-AgNPs: 0.0068) than small sized AgNPs (75 nm PVP-AgNPs: 0.0004; 60 nm citrate-AgNPs: 0.0019). These results indicate that

large sized AgNPs could be transported more efficiently from root tissues to shoot tissues than small sized AgNPs.

3.4 AgNPs detection in plant tissues by SP-ICP-MS

SP-ICP-MS is a viable methodology for detecting nanoparticles at levels down the ng L^{-1} in liquid samples, differentiating them from other forms of the same element. To obtain reliable results, just one nanoparticle must be measured during each reading period. So sufficiently diluted metal-rich particle suspensions and adequate data acquisition frequency are required [65,66]. In this study, an enzymatic digestion was carried out in order to maintain intact the AgNPs accumulated in lettuce tissues for their determination by SP-ICP-MS. The nanoparticles transformation inside lettuce tissues was assessed at different concentrations and using AgNPs with different coatings and sizes. The results obtained from these three different tests performed are shown below:

3.4.1 AgNPs concentration effect

Before carrying out the SP-ICP-MS analysis of vegetal tissues, the total amount of silver present in the enzymatic plant digests was determined to know the silver extracted by enzymatic digestion from lettuce roots and shoots.

After knowing the silver concentrations in lettuce enzymatic digests (shoots and roots); sample dilution was performed, when it was necessary (concentration $\leq$1 μg L^{-1}), to obtain an adequate sample to be analyzed by SP-ICP-MS. The time resolved plots obtained for the different concentration levels studied (3, 5 and 10 mg L^{-1}) are shown in **Fig. 4**. A large number of peaks above the baseline were observed in **Fig. 4c** and **4e**, indicating the presence of AgNPs in lettuce roots except for **Fig. 4a** where less peaks were observed (3 mg L^{-1}). This could probably be because the presence of AgNPs is smaller at 3 mg L^{-1} and the probability to get all of them partially or totally dissolved is higher than at 5 and 10 mg L^{-1}. Therefore, the amount of Ag(I) is higher in 3 mg L^{-1} in

513 comparison with 5 and 10 mg L^{-1}, where the presence of Ag(I) was also determined
514 (baseline counts around 100-120). This was also reflected by the calculations carried out
515 to estimate the concentration of both metal forms (AgNPs and Ag(I)) in which it was
516 observed that at 5 and 10 mg L^{-1} the percentage of AgNPs was higher than at 3 mg L^{-1}
517 (data not shown). Furthermore, the histogram representations (see **Fig. 4d** and **4f**) of all
518 the concentration levels tested showed a first distribution at low pulse intensity values
519 corresponding to dissolved silver and a second distribution at high pulse intensity values
520 corresponding to nanosilver except for **Fig. 4b** (3 mg L^{-1} concentration level) where
521 both distributions (AgNPs and Ag(I)) were overlapped. Ag K-edge EXAFS also
522 confirmed the dissolution of AgNPs, where significantly higher proportion of -S and -O
523 ligands was observed at 3 mg L^{-1} (**Table 4**, **Fig. 8**).
524 In lettuce shoots the presence of AgNPs was also confirmed at higher concentration
525 levels (see **Fig. 5**). At 3 mg L^{-1} the baseline counts were shifted to higher values in
526 respect to baseline (around 40-50 counts) and the signal was practically constant (see
527 **Fig. 5a**). Additionally, the histogram representation showed one distribution at low
528 pulse intensity corresponding to Ag(I) (see **Fig. 5b**). At 5 and 10 mg L^{-1} the presence of
529 Ag(I) was also confirmed but some peaks above the baseline were observed indicating
530 the presence of AgNPs as well (see **Fig. 5c**, **5e**). Although there were some AgNPs in
531 lettuce shoots at 5 and 10 mg L^{-1}, the percentage of AgNPs quantified by SP-ICP-MS
532 was insignificant in comparison with Ag(I) percentage (data not shown). Therefore,
533 their presence was lower than in root tissues. The presence of Ag(I) in all concentration
534 levels, because of AgNPs dissolution, could explain the lower results obtained in the
535 different physiological parameters evaluated (E, g_S and Fm/Fv) in respect to controls
536 (see **Table 2** and **Fig. A2** from **Appendix**), indicating that the toxicity of AgNPs could
537 be partially explained by the presence of Ag(I) as some authors stated [8,9]. However,

AgNPs toxicity to lettuces cannot be only a consequence of their dissolution, since in 5 mg L^{-1} treatment more Ag-Ag bonds were observed by EXAFS analysis (**Table 4**).

3.4.2 AgNPs coating effect

As in the concentration test, the total amount of silver in lettuce tissues was determined by ICP-MS before SP-ICP-MS analysis. Silver was detected in plant roots but not in plant shoots; thus, in this latter case the SP-ICP-MS analysis was not carried out.

After diluting the lettuce root enzymatic digests to a proper concentration (≤1 μg L^{-1}), time resolved plots obtained for all the coated AgNPs tested shown several peaks above the baseline indicating the presence of AgNPs in root tissues (see **Fig. 6a**, **6c**, **6e**). However, the presence of dissolved silver was higher in roots treated with citrate-AgNPs (baseline around 50 counts) and PEG-AgNPs (baseline around 25 counts) (see **Fig. 6a** and **6e**) in comparison with roots treated with PVP-AgNPs (baseline around 10 counts) (see **Fig. 6c**). This was also observed in histogram representation, where the first distribution corresponding to Ag(I) was at higher pulse intensity, especially for citrate-AgNPs, compared to PVP-AgNPs. Additionally, the percentage of estimated PVP-AgNPs was higher than PEG-AgNPs and citrate-AgNPs (data not shown). The same behavior was observed with Ag K-edge EXAFS (see **Table 5**). Thus, in biological environment, PVP-AgNPs would be more stable and less susceptible to suffer transformations than citrate-AgNPs and PEG-AgNPs. On the other hand, citrate-AgNPs chlorophyll (*Chl a* and *Chl b*) and carotenoid values obtained were similar to Ag(I) (see **Fig. A4** from **Appendix**) but the E and g_s were more similar to those obtained for PEG-AgNPs (see **Table 3**). Therefore, more experiments are needed to understand citrate- and PEG-AgNPs dissolution and to confirm that they can behave similarly to Ag(I).

3.4.3 AgNPs size effect

In this test, as in coating effect test, silver was only detected in lettuce root enzymatic digests during ICP-OES analysis. So, lettuce shoots were not analyzed by SP-ICP-MS. Previous to SP-ICP-MS analysis, the samples were diluted to an adequate concentration ($\leq$1 μg L^{-1}). From 75 nm PVP-AgNPs time resolved plot, it was observed that silver signal was constant and shifted to higher values in respect to baseline (around 100 counts) (see **Fig. 7a**), indicating that AgNPs were dissolved to Ag(I). On the other hand, 100 nm PVP-AgNPs time resolved plot showed several peaks above the baseline, evidencing the presence of silver nanoparticles in lettuce roots (see **Fig. 7c**).

Histogram representations showed only one distribution corresponding to Ag(I) in the case of 75 nm PVP-AgNPs (see **Fig. 7b**) and two distributions corresponding to Ag(I) (first distribution) and AgNPs (second distribution) in the case of 100 nm PVP-AgNPs (see **Fig. 7d**). Furthermore, the percentage of Ag(I) estimated for the smaller particles was higher than for the bigger ones (99% and 26%, respectively). These results are in agreement with EXAFS analysis, because a higher number of Ag-Ag bonds were found for 100 nm NPs than for 75 nm NPs (see **Table 6**). Therefore, larger particles are more stable than smaller ones in lettuce roots. This fact supports the hypothesis that smaller NPs are more prone to get dissolved than bigger NPs as it is stated by some authors [67].

3.5 Speciation and ligand environment of Ag in lettuce plant

The prevailing feature in EXAFS spectra of all samples is the signal of the neighbor Ag atoms in the NPs: it follows the spectral features of bulk Ag metal up to 5 Å. The contribution of low-Z organic ligands is hardly noticeable beneath the signal of ~12 strongly scattering Ag neighbors. Two candidate ligands are identified: sulfur at 2.4 Å, and oxygen at 2.3 Å (see **Fig. 8**). In the Feff model constructed from the first 5 scattering paths of Ag metal lattice and single paths of O and S ligands, the number of

587 required parameters is at par with the number of freedom degrees of the usable part of
588 the spectrum. Statistically stable values of the parameters can thus only be gained in
589 collective fitting, preferably of related groups of spectra (studying e.g. concentration
590 effect, coating effect, size effect), so that common values can be assumed for some
591 parameters. When parameter values for a given sample from fitting in different groups
592 are compared, their reliability can be assessed. Those pertaining to the Ag neighbors
593 within the NP are highly reliable, with the spread in the percentage range. The
594 parameters of the S-shell vary typically for 25%, and those of O-shell more than 50%.
595 When compared to the initial AgNPs added to the nutrient solution, the Ag
596 neighborhood in plant roots is characterized by lower number of Ag neighbors,
597 indicating leaching of Ag(I), which makes the AgNPs smaller (see **Tables 4**, **5**, **6**). Ag
598 ions are then bound to -S or -O ligands, but *de novo* formation of Ag-Ag bonds is also
599 possible.
600 In the concentration effect study, agglomerative hierarchical clustering analysis of Ag
601 neighborhood in plants *vs* intact 75 nm PVP-AgNPs shows that the Ag neighborhood at
602 5 and 10 mg L^{-1} resembles that of intact AgNPs (see **Fig. 9a**), while at 3 mg L^{-1} the Ag
603 neighborhood is significantly different, with higher number of -O and -S ligands due to
604 higher level of AgNPs dissolution. A higher number of Ag-Ag bonds are seen at 5 mg
605 L^{-1} than at 3 and 10 mg L^{-1} (see **Table 4**), which correlates with increased plant
606 physiological parameters as WUE_i and WUE and lowered E and g_s (see **Table 2**),
607 possibly as a direct effect of AgNPs on the membrane systems of plant cells.
608 In the coating effects study, PVP coated 100 nm NPs show the highest stability (53%
609 Ag-Ag bonds) (see **Table 5**), again correlating with lower E and g_s (see **Table 3**).
610 Agglomerative hierarchical clustering confirms higher similarity of Ag neighborhood in
611 the lettuce roots exposed to PEG and citrate coated AgNPs (see **Fig. 9b**), characterized

612 by a lower number of Ag-Ag bonds, while higher number of O-ligands is seen in roots
613 exposed to 100 nm PVP-AgNPs.
614 In the size effects study, 100 nm PVP-AgNPs show better stability than 75 nm NPs,
615 with higher percentage of Ag-Ag bonds (53% vs 38%) (see **Table 6**) correlating with
616 lower root and higher shoot Ag concentration. This indicates that more stable AgNPs
617 are more easily transported from the roots to the shoots, since silver ions probably react
618 already with the cell components in the root system.

619 **4. Conclusions**

620 The AgNPs accumulation and biotransformation processes in plants and their
621 subsequent toxicological effects are governed by several factors, among them
622 nanoparticle physicochemical properties. The results showed that nanosilver absorption
623 by lettuce roots and their translocation to shoots were concentration dependent
624 processes. Furthermore, the accumulation of these emerging pollutants in lettuce tissues
625 at low exposure concentrations was similar to Ag(I), but at high concentration levels
626 Ag(I) was accumulated at a greater extent. Nanosilver concentration tests also indicated
627 through SP-ICP-MS analysis that at 3 mg L^{-1} these emerging pollutants were more
628 prone to get dissolved compared to 5 mg L^{-1} and 10 mg L^{-1}. The same results were
629 obtained by Ag K-edge EXAFS analysis of plant roots due to higher number of Ag-S
630 and Ag-O bonds at low concentrations were found, pointing to a higher level of AgNPs
631 dissolution at lower nanoparticle concentration. Although transpiration rate values and
632 stomatal conductance of lettuces were reduced after being exposed to AgNPs,
633 toxicological effects were not concentration dependent in the different exposure levels
634 tested in this work.
635 Particle coatings were found to have no effect on nanosilver uptake by lettuce roots.
636 Nevertheless, 100 nm PEG-AgNPs and Ag(I) seemed to be more transported to aerial

parts of the edible plant than negatively charged nanoparticles. SP-ICP-MS and Ag K-edge EXAFS analysis demonstrated that PVP coated nanosilver is more stable in lettuce roots than PEG- and citrate-AgNPs. Toxicological studies showed that Ag(I) has a higher negative impact on transpiration and stomatal conductance in respect to other studied AgNPs, although the differences on toxicological effects that differently coated AgNPs can exert in lettuce are not clear. On the other hand, particle size has an effect on their absorption by lettuce roots and their translocation to shoots. Small sized AgNPs were more accumulated by roots, meanwhile large sized nanosilver were more efficiently transported to lettuce aerial parts. Ag K-edge EXAFS results indicated that 100 nm PVP-AgNPs were more stable because the presence of Ag-Ag bonds was higher in comparison with 75 nm PVP-AgNPs. SP-ICP-MS results also showed the dissolution of smaller tested AgNPs and the maintenance of nanoform for bigger NPs in lettuce roots. Although this work gives an overview of the parameters that can influence the AgNPs accumulation, transformation and the toxicological effects derived from these processes to lettuce plants, more research is needed to understand the behavior and risks that can pose these emerging contaminants to edible plants.

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7.6 References

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List of tables

Table 1 Lettuce growing conditions in the different tests performed (control = 0 mg L^{-1}).

Test	Silver form in lettuce growing media	Concentration of silver in lettuce growing media
AgNPs and Ag(I) concentration effect	75 nm PVP-AgNPs Ag(I)	0, 3, 5, 7, 10 and 15 mg L^{-1}
AgNPs coating effect	100 nm PVP-AgNPs 100 nm citrate-AgNPs 100 nm PEG-AgNPs	0, 1, 3 mg L^{-1}
AgNPs size effect	75 nm PVP-AgNPs 100 nm PVP-AgNPs 60 nm citrate-AgNPs 100 nm citrate-AgNPs	0, 1, 3 mg L^{-1}

Table 2 Overall means ± standard errors for the studied leaf physiological parameters in *Lactuca sativa* plants grown under four different 75 nm PVP-AgNPs concentration (0, 3, 5 and 10 mg L^{-1}). The sample size used was n = 4 for each concentration. p-values for each physiological parameter were the result of comparing each AgNPs concentration with the control one (0 mg L^{-1}) by one-way ANOVA tests. The significance level considered was $p \leq 0.05$ (* indicate a p-value marginally significant; E: transpiration rate; g_s: stomatal conductance; A: photosynthetic rate; WUE_i: instantaneous water use efficiency; WUE: intrinsic water use efficiency; *ns*: not significant; p: p-values).

	0 mg L^{-1}	3 mg L^{-1}	p	5 mg L^{-1}	p	10 mg L^{-1}	P
E (mmols H_2O m^{-2} s^{-1})	0.86 ± 0.03	0.45 ± 0.13	0.022	0.33 ± 0.15	0.013	0.53 ± 0.13	0.045
g_s (mmols H_2O m^{-2} s^{-1})	54 ± 4.26	24.75 ± 7.55	0.015	21.75 ± 10.83	0.032	29.50 ± 9.53	0.057*
A (µmols CO_2 m^{-2} s^{-1})	0.60 ± 0.07	0.50 ± 0.09	*ns*	0.48 ± 0.08	*ns*	0.50 ± 0.14	*ns*
WUE_i (A/E)	0.69 ± 0.07	1.92 ± 1.04	*ns*	3.38 ± 2.21	*ns*	1.09 ± 0.35	*ns*
WUE (A/g_s)	0.01 ± 0.00	0.04 ± 0.02	*ns*	0.06 ± 0.05	*ns*	0.02 ± 0.01	*ns*

Table 3 Overall means ± standard errors for the *Lactuca sativa* leaf transpiration rate (*E*) and stomatal conductance (g_s) from plants grown under 3 mg L^{-1} Ag(I) or AgNPs coated with different compounds (PEG, citrate or PVP). The sample size used was $n = 4$ for controls and citrate-AgNPs and $n = 3$ for PEG-AgNPs and PVP-AgNPs. For Ag(I) $n = 2$ because two of the four initial plantlets died before ending the experiment. *p*-values given for each physiological parameter are the results of comparing each treatment with the control one (0 mg L^{-1}) by one-way ANOVA tests. The significance level considered was $p \leq 0.05$ (*ns*: not significant; *p*: *p*-values).

	0 mg L^{-1}	**Ag(I)**	***p***	**Citrate-AgNPs**	***p***	**PVP-AgNPs**	***p***	**PEG-AgNPs**	***p***
***E* (mmols H_2O m^{-2} s^{-1})**	1.22 ± 0.09	0.46 ± 0.04	0.004	1.39 ± 0.15	*ns*	0.78 ± 0.09	0.018	1.01 ± 0.19	*ns*
***g_s* (mmols H_2O m^{-2} s^{-1})**	47.00 ± 4.02	16.00 ± 1.00	0.007	54.25 ± 7.39	*ns*	27.00 ± 4.04	0.019	42.67 ± 9.24	*ns*

Table 4 Best fit model parameters of Ag K-edge EXAFS spectra measured on frozen-hydrated plant roots exposed to 75 nm PVP-AgNPs at different concentrations. *Rf* – relative frequency, *R* the distance between Ag and the neighbor atom (Ag, S or O), σ^2 – the width of the radial distribution of the neighbors. *E0* – effective zero energy of the photoelectron. Estimated errors in units of the last decimal place are given in parentheses.

AgNPs concentration	**75 nm PVP-AgNPs added to the nutrient solution**	**Roots 3 mg L^{-1}**	**Roots 5 mg L^{-1}**	**Roots 10 mg L^{-1}**
***Rf* (Ag)**	**0.76(2)**	**0.38(3)**	**0.57(3)**	**0.35(3)**
***R* (Ag) [Å]**	2.875(2)	2.866(2)	2.872(2)	2.863(2)
σ^2Ag [Å^2]	32(6)	32	32	32
***Rf* (S)**	**0****	**0.35(10)**	**0.0(10)**	**0.11(10)**
R* (S) [Å]	-	2.430(11)	2.430	2.430
σ^2S [Å^2]*	-	72(30)	72	72
***Rf* (O)**	**0.18(20)**	**0.60(20)**	**0.29(20)**	**0.22(20)**
R* (O) [Å]*	2.300	2.300	2.300	2.300
σ^2(O) [Å^2]**	72	72	72	72
***E0* [eV]**	1.2(3)	0.6(3)	1.4(3)	1.0(3)

*- Constrained to common value, **- fixed value

Table 5 Best fit model parameters of Ag K-edge EXAFS spectra measured on frozen-hydrated plant roots exposed to 100 nm PVP, citrate and PEG coated AgNPs. *Rf* – relative frequency, *R* the distance between Ag and the neighbor atom (Ag, S or O), σ^2 – the width of the radial distribution of the neighbors. *E0* – effective zero energy of the photoelectron. Estimated errors in units of the last decimal place are given in parentheses.

AgNPs concentration, coating and size	**Roots 3 mg L^{-1} PVP, 100 nm**	**Roots 3 mg L^{-1} citrate, 100 nm**	**Roots 3 mg L^{-1} PEG, 100 nm**
***Rf* (Ag)**	**0.54(3)**	**0.38(2)**	**0.34(2)**
***R* (Ag) [Å]**	2.868(3)	2.867(2)	2.869(2)
σ^2Ag [Å^2]	32(5)	32	32
***Rf* (S)**	**0.18(8)**	**0.20(8)**	**0.21(8)**
R* (S) [Å]	2.430(12)	2.430	2.430
σ^2S [Å^2]*	72(30)	72	72
***Rf* (O)**	**0.34(15)**	**0.19(15)**	**0.16(15)**
R* (O) [Å]*	2.300	2.300	2.300
σ^2(O) [Å^2]**	72	72	72
***E0* [eV]**	5.8(9)	0.0(8)	1.0(7)

*- Constrained to common value, **- fixed value

Table 6 Best fit model parameters of Ag K-edge EXAFS spectra measured on frozen-hydrated plant roots exposed to 75 and 100 nm PVP-AgNPs at different concentrations. *Rf* – relative frequency, *R* the distance between Ag and the neighbor atom (Ag, S or O), σ^2 – the width of the radial distribution of the neighbors. *E0* – effective zero energy of the photoelectron. Estimated errors in units of the last decimal place are given in parentheses.

AgNPs concentration, coating and size	**Roots 3 mg L^{-1} PVP, 75 nm**	**Roots 3 mg L^{-1} PVP, 100 nm**
***Rf* (Ag)**	**0.38(3)**	**0.53(3)**
***R* (Ag) [Å]**	2.866(2)	2.868(2)
σ^2Ag [Å^2]	37(5)	32(5)
***Rf* (S)**	**0.35(10)**	**0.18(10)**
R* (S) [Å]	2.430(12)	2.430
σ^2S [Å^2]*	72(30)	72
***Rf* (O)**	**0.60(22)**	**0.34(20)**
R* (O) [Å]*	2.300	2.300
σ^2(O) [Å^2]**	72	72
***E0* [eV]**	0.6(8)	5.8(9)

*- Constrained to common value, **- fixed value

List of figures

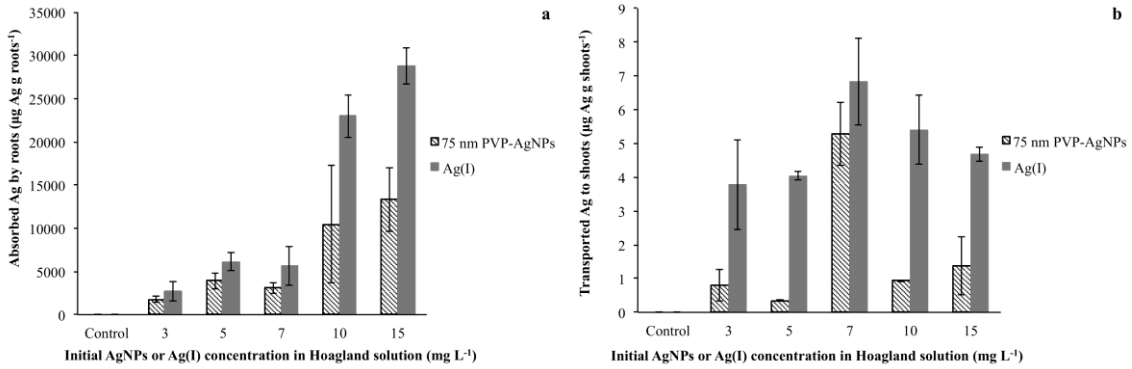


Fig. 1 Total silver concentrations in lettuce roots (**a**) and lettuce shoots (**b**) obtained from concentration effect test (control (0), 3, 5, 7, 10 and 15 mg L^{-1}) with 75 nm PVP-AgNPs and Ag(I) (error bars: standard deviation (n = 3)).

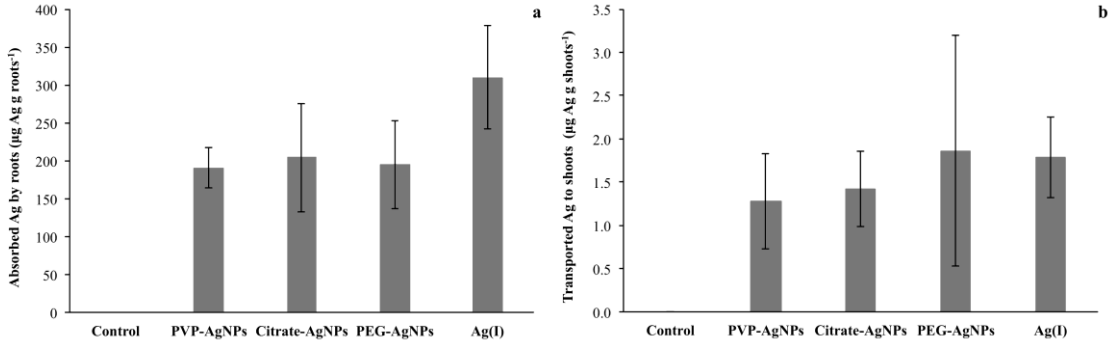
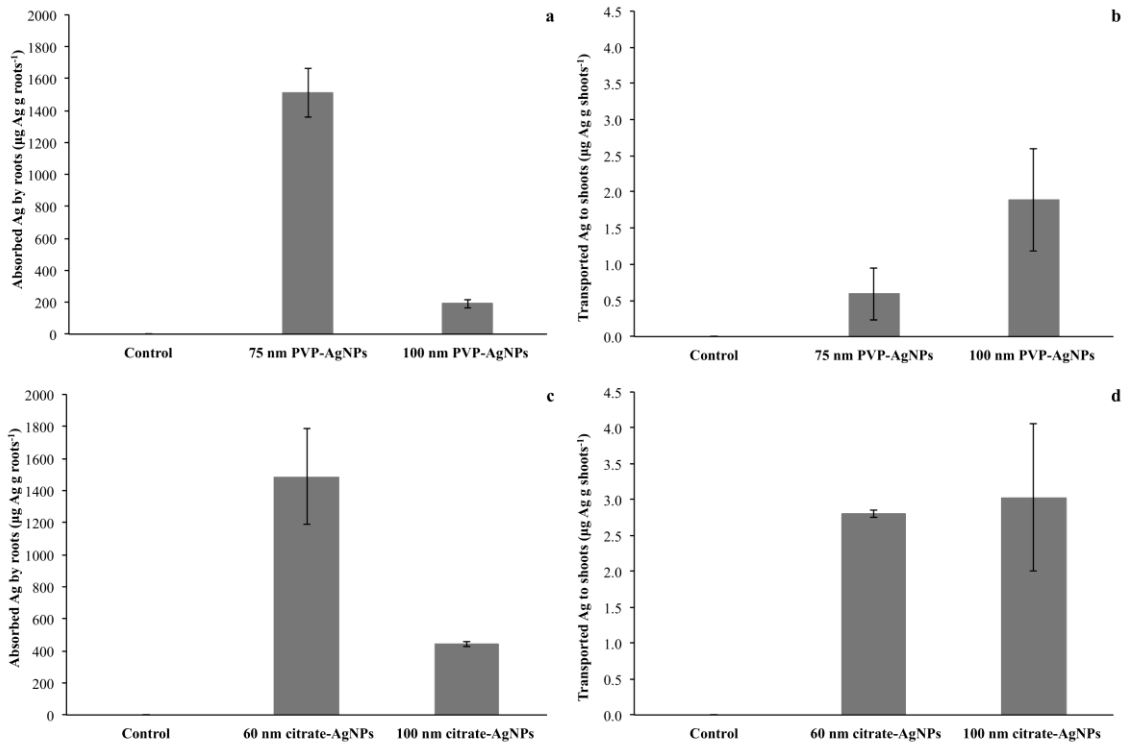
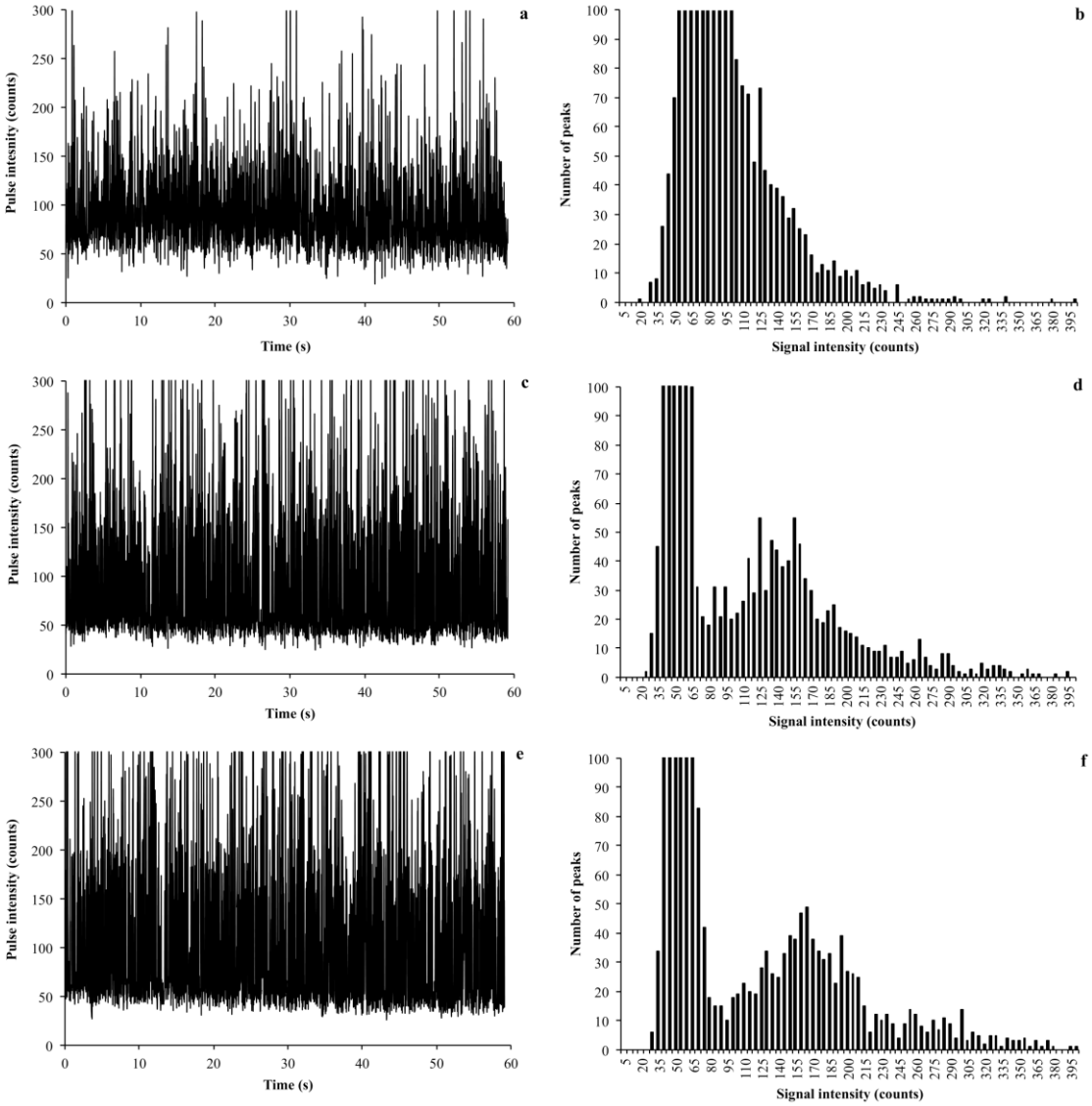


Fig. 2 Total silver content in lettuce tissues (**a** roots; **b** shoots) resulting from coating effect test using different coated 100 nm AgNPs (PVP-, citrate- and PEG-AgNPs) at 1 mg L^{-1} concentration in Hoagland solution (error bars: standard deviation (n =3)).



994

995 **Fig. 3** Content of silver in vegetal roots (**a** PVP-AgNPs; **c** citrate-AgNPs) and shoots (**b** PVP-
996 AgNPs; **d** citrate-AgNPs) after performing particle size effect test at 1 mg L^{-1} concentration
997 (error bars: standard deviation (n = 3)).



998

999 **Fig. 4** SP-ICP-MS time resolved plots and histograms of lettuce roots contaminated with 75
1000 nm PVP-AgNPs at different concentration levels (**a-b** 3 mg L^{-1}; **c-d** 5 mg L^{-1}; **e-f** 10 mg L^{-1}).
1001

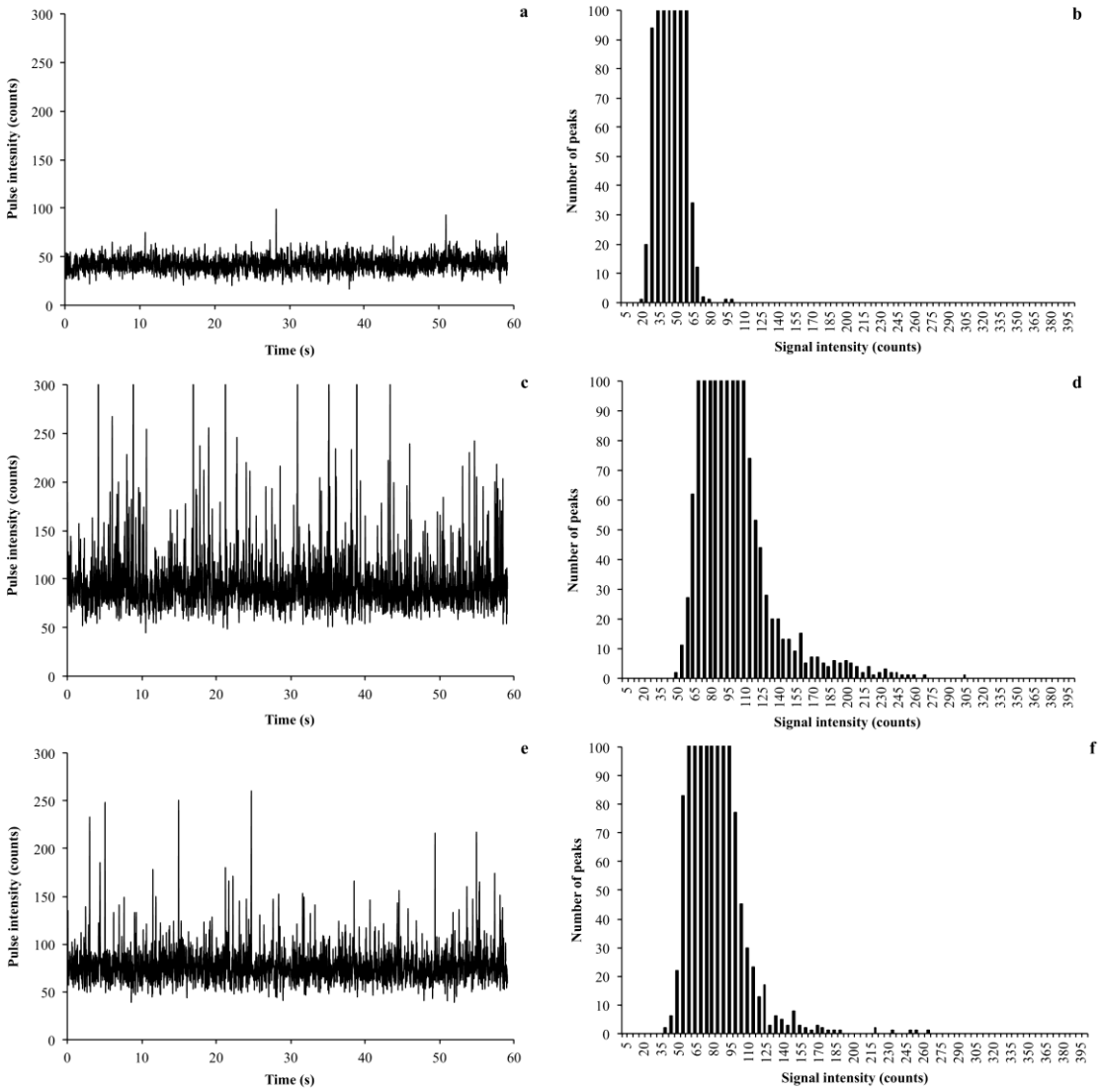
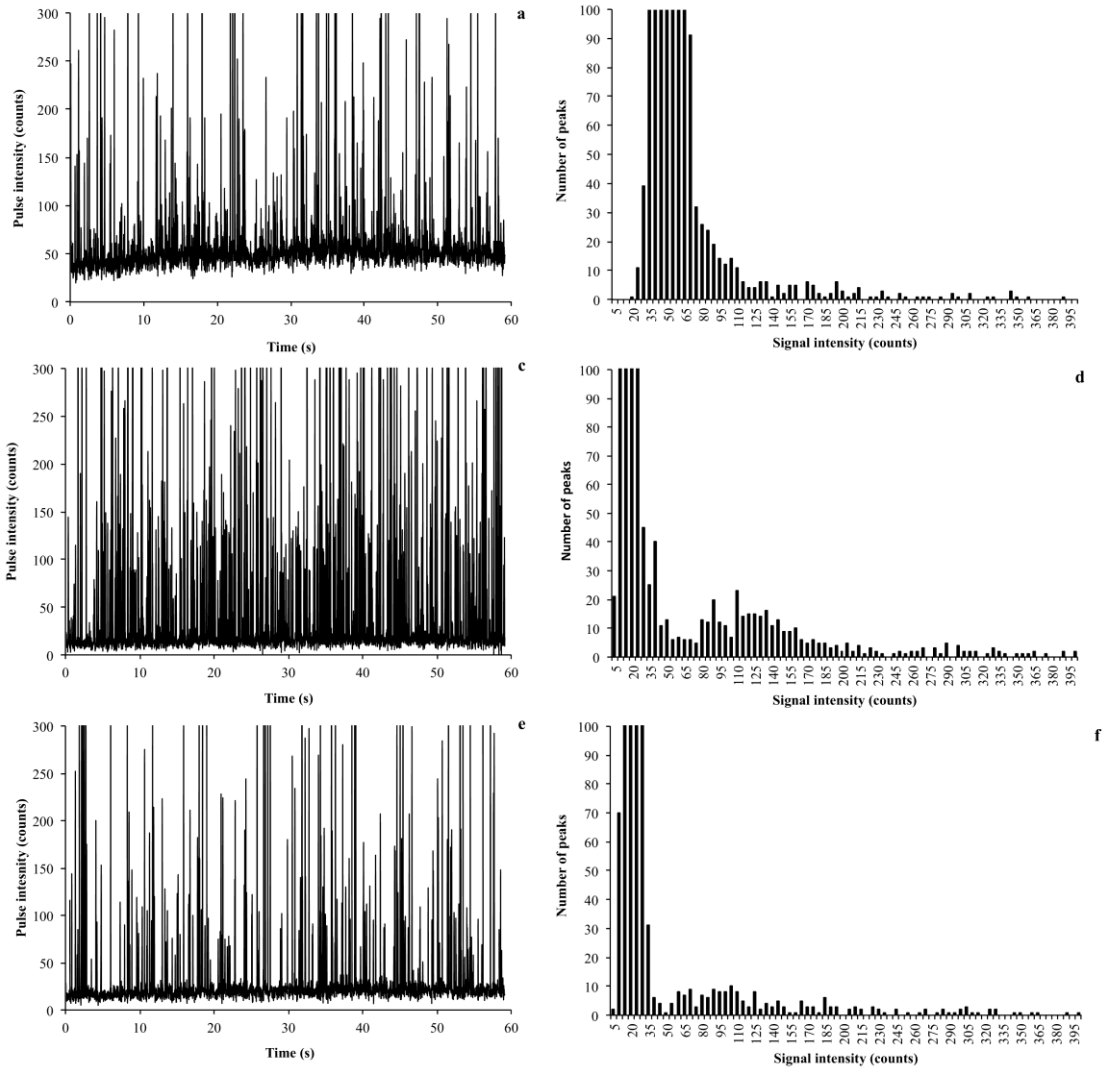


Fig. 5 SP-ICP-MS time resolved plots and histograms of lettuce shoots contaminated with 75 nm PVP-AgNPs at different concentration levels (**a-b** 3 mg L^{-1}; **c-d** 5 mg L^{-1}; **e-f** 10 mg L^{-1}).



1006

1007 **Fig. 6** SP-ICP-MS time resolved plots and histograms of lettuce roots contaminated
1008 with different coated AgNPs at 1 mg L^{-1} (**a-b** citrate-AgNPs **c-d** PVP-AgNPs; **e-f** PEG-AgNPs).

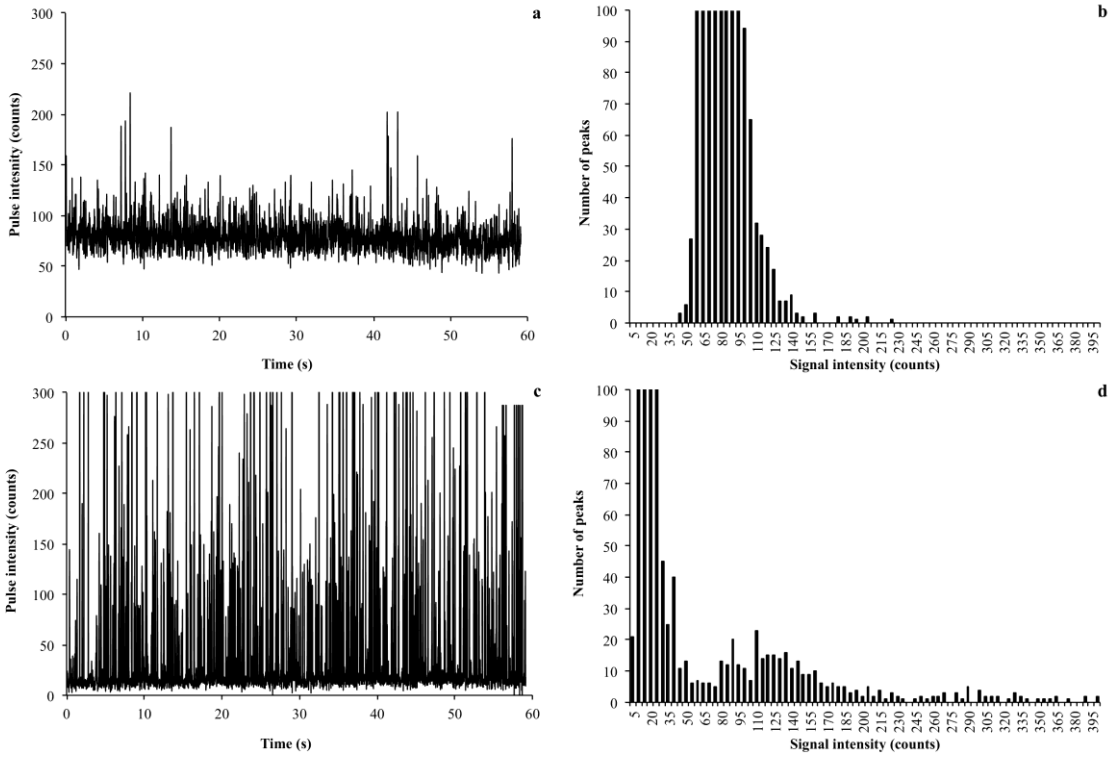


Fig. 7 SP-ICP-MS time resolved plots and histograms of lettuce roots contaminated with different sized AgNPs at 1 mg L^{-1} (**a-b** 75 nm PVP-AgNPs **c-d** 100 nm PVP-AgNPs).

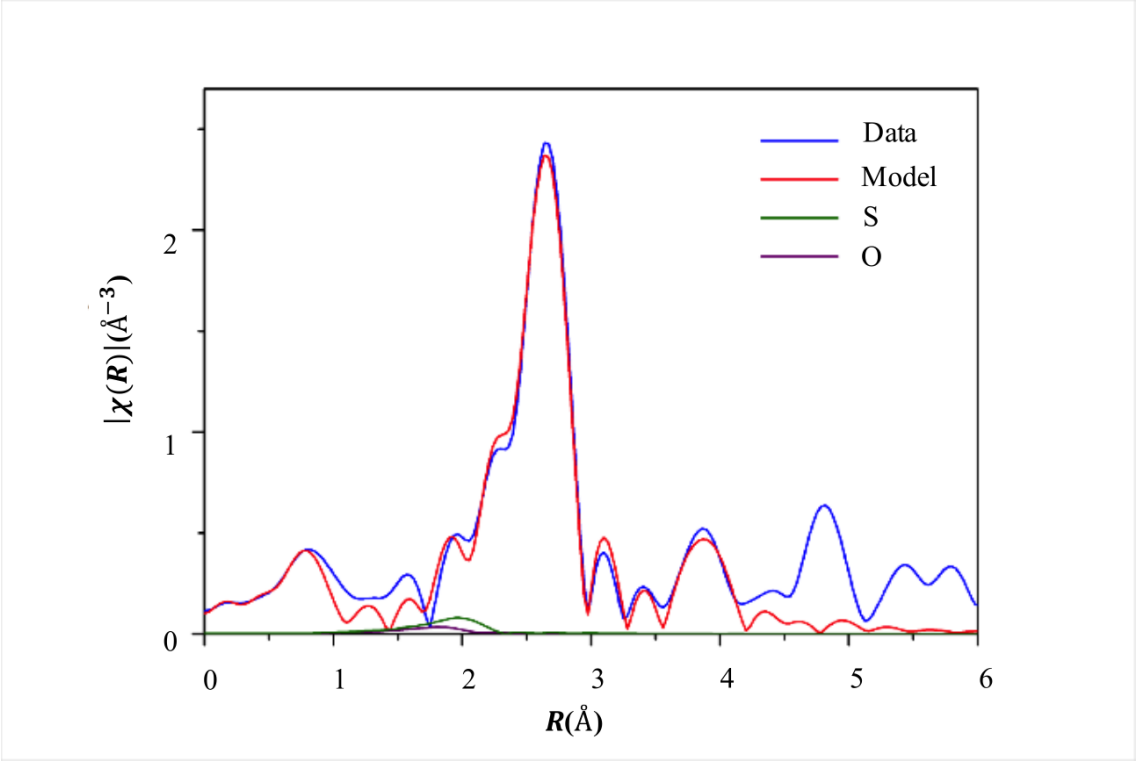


Fig. 8 Fourier transform magnitudes of the k3 weighted Ag K-edge EXAFS of plant roots exposed to 3 mg L^{-1} of PVP-AgNPs.

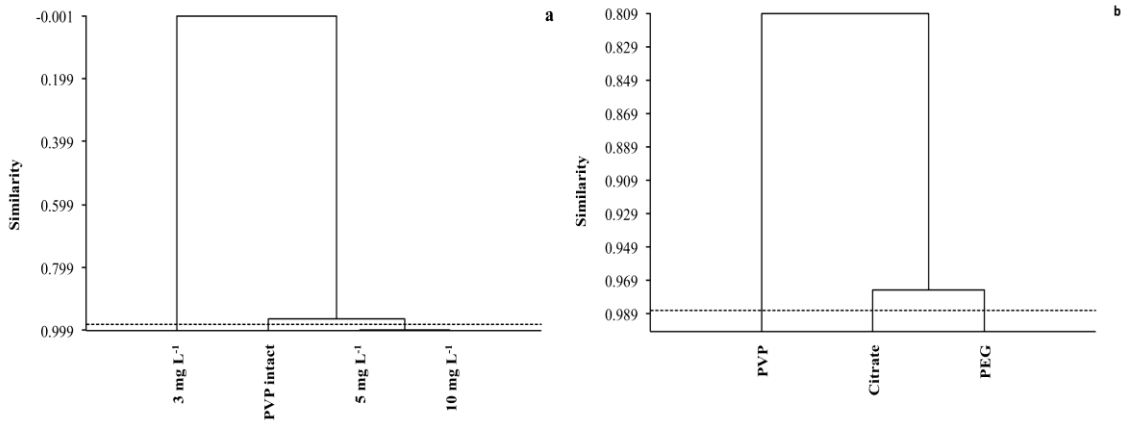


Fig. 9 Agglomerative hierarchical clustering analysis based on Pearson's correlation coefficient, of Ag neighborhood as obtained by Ag K-edge EXAFS analysis in concentration effect study, where the lettuces were exposed to 3, 5, 10 mg L^{-1} of 75 nm PVP-AgNPs and PVP intact (Ag neighborhood in raw 75 nm PVP-AgNPs) (**a**), and coating effect study where the lettuce plants were exposed to 3 mg L^{-1} of 100 nm PVP, citrate and PEG coated AgNPs (**b**).

Supplementary Material

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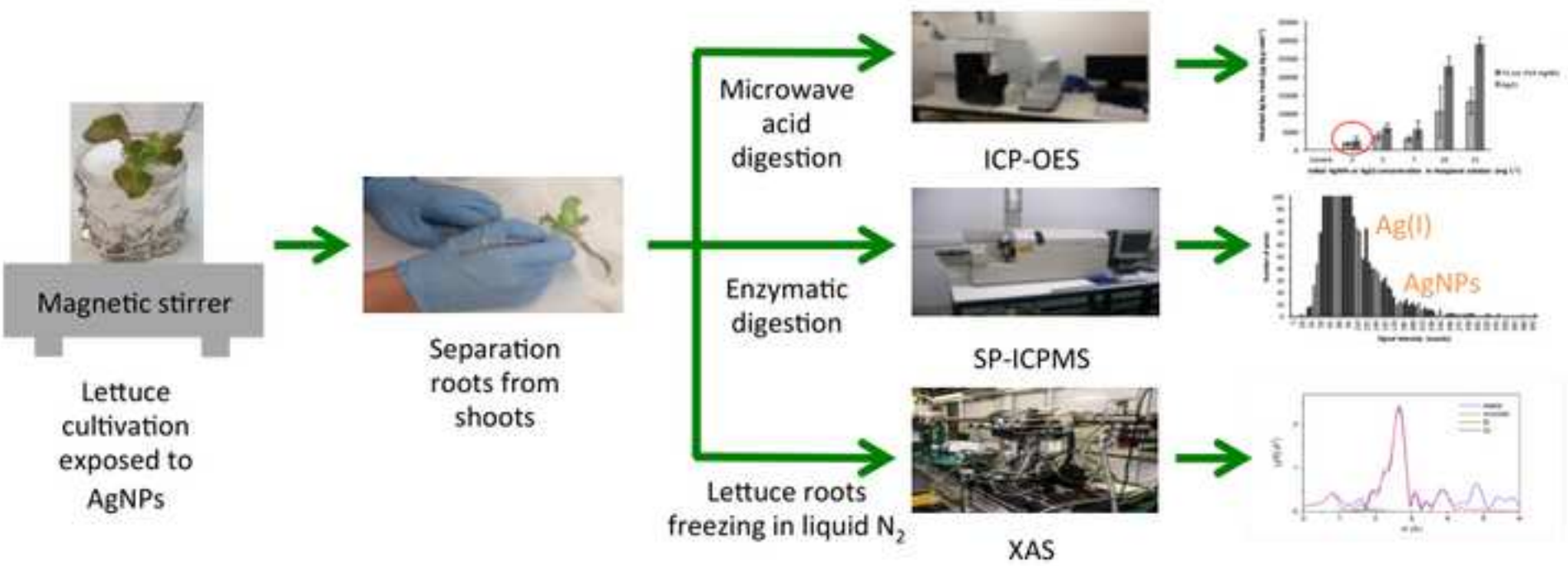
Graphical Abstract



Abstract

The broad use of silver nanoparticles (AgNPs) in daily life products enhances their possibilities to reach the environment. Therefore, it is important to understand the uptake, translocation and biotransformation in plants and the toxicological impacts derived from these biological processes. In this work, *Lactuca sativa* (lettuce) was exposed during 9 days to different coated (citrate, polyvinylpyrrolidone, polyethylene glycol) and sized (60, 75, 100 nm) AgNPs at different concentrations (1, 3, 5, 7, 10, 15 mg L^{-1}). Total silver measurements in lettuce roots indicated that accumulation of AgNPs is influenced by size and concentration, but not by nanoparticle coating. On the other hand, nanosilver translocation to shoots was more pronounced for neutral charged and large sized NPs at higher NP concentrations. Single particle inductively coupled plasma mass spectrometry analysis, after an enzymatic digestion of lettuce tissues indicated the dissolution of some NPs. Ag K-edge X-ray absorption spectroscopy analysis corroborated the AgNPs dissolution due to the presence of less Ag-Ag bonds and appearance of Ag-O and/or Ag-S bonds in lettuce roots. Toxicological effects on lettuces were observed after exposure to nanosilver, especially for transpiration and stomatal conductance. These findings indicated that AgNPs can enter to edible plants, exerting toxicological effects on them.