Ag nanoparticles and wheat roots: a complex interplay

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Graphical Abstract (TOC)

ABSTRACT

Agricultural soils are major sinks of silver nanoparticles in the environment, and crops are directly exposed to these emerging contaminants. A clear picture of their chemical transformations, uptake and transport mechanisms, and phytotoxic impacts is still lacking. In this work, wheat plants were exposed to pristine metallic (Ag-NPs) and sulfidized (Ag₂S-NPs) silver nanoparticles and ionic Ag. Data on Ag distribution and speciation, phytotoxicity markers and gene expression were studied. A multi-technique and multi-scale approach was applied combining innovating tools at both laboratory and synchrotron. Various chemical transformations were observed on the epidermis and inside roots,

26 even for Ag₂S-NPs, leading to an exposure to multiple Ag forms, which likely evolve over time. Genes
27 involved in various functions including oxidative stress, defense against pathogens and metal
28 homeostasis were impacted in different ways depending on the Ag source. This study illustrates the
29 complexity of the toxicity pattern for plants exposed to Ag-NPs, the necessity of monitoring several
30 markers to accurately evaluate the toxicity, and the interest of interpreting the toxicity pattern in
31 light of the distribution and speciation of Ag.

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1. INTRODUCTION

Agricultural soils are pointed as a major sink for engineered nanoparticles (NPs) due to the application of sewage sludge¹, which contains various types of engineered nanomaterials (ENMS) and their secondary products². Current regulations concerning sewage sludge application focus on their content of certain metals, none of them consider ENMS. The use of nanopesticides creates another, not precisely quantified, input of NPs in soils³. Silver nanoparticles (Ag-NPs) are one of the most prevalent metallic nanoparticles in consumer products due to their biocidal action⁴. Sulfidation is a major transformation of Ag-NPs that starts during the use of the products (e.g., laundry washing for textiles)² and is completed during wastewater treatment³, making silver sulfide (Ag₂S) the main Ag-species in sewage sludge⁵. Due to the extremely low solubility of microcrystalline Ag₂S, sulfidation has been considered as a natural antidote against Ag-NPs toxicity⁶. This opinion requires refinement since in sludges and soils Ag₂S precipitate in presence of other compounds (mostly organics) potentially forming nanometric and multiphasic Ag-S forms whose solubility is not known². Moreover, a recent study evidenced the dissolution of Ag₂S NPs in aquatic media⁷.

Contrarily to other metals, natural Ag enrichments in soils are rare⁸, so there is no known plant species, which has evolved to cope with Ag toxicity. The toxicity of Ag-NPs is supposed to be primarily due to the release of Ag⁺ ions. Besides this, it could be a toxicity related with the characteristics of the nanoparticles (size, specific surface area, surface charge) that are not exerted by the bulk Ag.⁹⁻¹¹ As for other ENMs, the transfer of Ag-NPs inside plant tissues has been observed^{9,12}, but it is difficult to conclude on their direct transfer since it could result from Ag⁺ reduction inside plants. Various toxicity symptoms and up-regulation of genes linked with oxidative stress have been observed¹³. So far, most studies on the impact and transfer of Ag-NPs in plants have been done using pristine Ag-NPs. Recent opinions in nanoecotoxicology point the need of using environmentally realistic experimental conditions¹¹, including the use of low doses (micromolar range or lower)¹¹ and the study of aged NPs, i.e., Ag₂S-NPs. With only three published studies, data on transfer of Ag₂S-NPs in

60 plants and possible phytotoxicity is extremely scarce¹⁴⁻¹⁶. In a recent work, Wang et al. (2017) have
61 found that Ag₂S-NPs reduce the growth of cucumber and wheat plants, a process that seems to be
62 related with an interference with the ethylene signaling pathway, the plant defense system and
63 upward water transport¹⁶.

64 This study aimed at clarifying the changes in speciation of Ag-NPs and Ag₂S-NPs in wheat and the
65 uptake and transfer pathways, and at evaluating the phytotoxic impacts in light of Ag distribution and
66 speciation. Wheat, a cereal used worldwide for food supply, was exposed to low doses, and a multi-
67 technique and multi-scale approach was applied combining X-ray micro and nano-computed
68 tomography (μ and nano-CT), micro X-ray fluorescence (μ -XRF) and micro X-ray absorption
69 spectroscopy (μ -XANES), toxicity markers and gene expression.

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71 2. MATERIALS AND METHODS

72 2.1 Nanoparticles and chemicals

73 Metallic silver nanoparticles (Ag-NPs) were provided by NanoAmor, Nanostructured and Amorphous
74 Materials Inc. (USA). Ag₂S nanoparticles (Ag₂S-NPs) were produced by the sulfidation of the Ag-NPs as
75 stated in Levard et al. (2011)¹⁷ (detailed information is given in SI).

76 Pristine and sulfidized nanoparticles (Ag-NPs and Ag₂S NPs) were thoroughly characterized in powder
77 and in suspension (additional information is provided in SI). AgNO₃ powder was provided by Sigma-
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83 2.2 Plant culture

84 Wheat (*Triticum aestivum* L.) plants were grown in hydroponics as described in SI. They were
85 randomly selected to be treated as follow follow: i) control, no Ag addition; ii) 30 μM Ag-NP; iii) 30
86 μM Ag₂S-NPs and 4) 30 μM AgNO₃.

87 Plants were harvested after 3 weeks of treatment. Roots and shoots were separated and thoroughly
88 rinsed with ultrapure water and Ethylenediaminetetraacetic acid (EDTA) 20mM and fresh weights
89 were recorded. Samples for phytotoxicity studies were immediately frozen in liquid nitrogen and
90 stored at -80°C until analysis. Samples for synchrotron analysis and for X-ray computed-tomography
91 were keep fresh until specific sample preparation.

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93 2.3 Silver distribution and speciation in roots cross sections (synchrotron $\mu\text{-XRF}$ and $\mu\text{-XANES}$)

94 Fresh roots were prepared for synchrotron analyses in cryogenic conditions as stated in Larue et al.
95 (2016)¹⁸. $\mu\text{-XRF}$ and Ag L_{III}-edge $\mu\text{-XANES}$ measurements were performed on root thin cross sections
96 (20-30 μm) on the scanning X-ray microscope at ID21 beamline of the ESRF under cryo-conditions
97 using a vibration-free cryo-stage, passively cooled by a liquid nitrogen dewar. Detectors included a
98 Si₃N₇ diode for I₀ and a Silicon Drift Detector (X-flash Bruker 80mm² active area) for the emitted X-ray
99 fluorescence. Focusing was realized using Kirkpatrick-Baez mirrors system. The photon flux was
100 $3.07 \times 10^{10} \text{ ph} \cdot \text{s}^{-1}$ at 3.45 keV with a beam size of 0.4 x 0.6 μm . $\mu\text{-XRF}$ maps were recorded with various
101 step sizes (from 0.3 x 0.3 μm^2 to 4 x 4 μm^2) with an incident energy of 3.45 keV, and a dwell time of
102 100 ms. Elemental maps presented in this paper were obtained after fitting each pixel XRF spectrum
103 using PyMCA software¹⁹ as described in Castillo-Michel et al. (2016)²⁰.

104 In the areas of interest identified in the $\mu\text{-XRF}$ maps, Ag L_{III}-edge $\mu\text{-XANES}$ spectra were recorded by
105 single point acquisition method (3.33 to 3.45 keV energy range, 0.5 eV step). When Ag aggregates
106 were too small, the drift of the beam during acquisition did not allow to collect clean spectra. In

these areas, μ -XANES mapping was applied. In this case, regions of interest of 3-3.5 μm x 4.5-5 μm were scanned with a step size of 0.5 μm at different energies across the Ag L_{III} -edge. Maps were recorded in fluorescence mode in energy steps of 3eV from 3.32 to 3.34 keV; 0.5 eV from 3.34 to 3.38 and 1eV from 3.34 to 3.40 keV. A stack of the obtained 107 XANES maps was performed with MATLAB routines (R2016, version 9.0) in order to get a μ -XANES spectra for each 0.5x0.5 μm^2 pixel of the examined area. μ -XANES spectra were extracted by PyMCA software¹⁹. The possibility of averaging all the μ -XANES spectra collected in the scanned area enables to get a good quality XANES spectra of small NP aggregates.

Bulk XANES spectra of reference compounds were recorded on unfocused mode during previous experiments²¹ in the same conditions as samples. Ag solid state reference compounds include Ag⁰ foil, Ag⁰ nanoparticles (Ag-NPs), Ag chloride (AgCl), Ag nitrate (AgNO₃), Ag phosphate (Ag₃PO₄) and Ag carbonate (Ag₂CO₃). Solution samples include Ag-malate (10 mM AgNO₃ and 100 mM malate, pH 5.5) and Ag-GSH (10 mM AgNO₃ and 100 mM GSH, pH 5.5, prepared in anoxia). 30% glycerol was added to the solutions to avoid formation of ice crystals. Additionally, the spectra of the solid state Ag₂S-NPs and of Ag diethyldithiocarbamate (C₅H₁₀AgNS₂, Ag-DEDTC) were recorded for this experiment.

μ -XANES and XANES spectra were treated by using ATHENA software²². Principal components Analysis (PCA) and Target Transformations (TT) were used to identify the main Ag species preset in the samples. Then, their proportion in the samples was determined by Least-Squares Combination Fitting (LCF) of the experimental spectra. Details about these analysis are provided in SI.

2.4 Silver concentrations

Immediately after harvest, shoots and roots were separated, fresh weights were recorded and they were frozen in liquid N₂ and lyophilized. After acid digestion Ag concentrations were determined by Inductively Coupled Plasma Mass Spectrometry ICP-MS (Elan DRC II Perkin Elmer). Further details are provided in SI.

2.5. 3D distribution of Ag-NPs in the roots using multi-scale X-ray computed-tomography (coupled μ and nano-CT)

Before 3D imaging, fresh roots were soaked in successive ethanol solutions (from 30% to 100% vol) and dehydrated by critical point drying consisting in the replacement of ethanol by CO₂ in the supercritical state²⁰.

3D imaging at the micro-scale (μ -CT) was performed with a Micro XCT-400 X-ray microscope (Zeiss XRadia) using various magnification achieving an isotropic voxel size from 2.9 to 0.62 μm in a field of view of 1024x1024x1024 voxels (3D pixels). Scans were acquired at 40 kV and 250 μA . Between 1601 and 2501 projections were collected with an exposure time ranging from 4s to 10s per projection (depending on the magnification) through a 360° rotation.

The roots were also scanned in 3D at the nano-scale using an UltraXRM-L200 3D X-ray microscope (Zeiss Xradia) equipped with a copper X-ray source (rotating anode) producing a polychromatic beam with a maximum intensity at energy of 8.048 keV (Cu K α -X ray emission) and Fresnel zone plate providing a spatial resolution of 150 nm. Scans were recorded phase contrast mode with 901 projections from -90 to 90° with an angle step of 0.2° and an exposure time of 60 s per projection giving a total scanning time of 17 h. The field of view (FOV) was 65x65x65 μm^3 with an isotropic voxel of 63.5 nm. The FOV position of the nano-CT scan was centered at region of interest previously identified by μ -CT. Reconstruction of the 3D images was performed using a Zeiss XRadia software (XM Reconstructed-Parallel beam-9.0.6445 software) based on a filtered back projection algorithm.

Avizo 8.0 software was used for reconstructed dataset visualization.

As X-ray computed-tomography did not provide direct chemical and speciation information, 2D chemical mapping was performed on the same sample. The goal was to validate the identification of the Ag distribution in the samples by μ -CT and nano-CT. μ -XRF maps of the same roots and the same

regions were recorded on ID21 as described above. In this case, measurements were not performed in cryogenic conditions and the photon flux was $3.1 \times 10^{10} \text{ ph} \cdot \text{s}^{-1}$ at 3.45 keV.

2.6 Phytotoxicity

2.6.1 Estimation of lipid peroxidation: malondialdehyde (MDA).

Lipid peroxidation was evaluated on fresh tissues by the quantification of thiobarbituric acid reactive species (TBARS) by a modified method of Reilly and Aust as stated in Pradas del Real. (2014)²³. Additional information is provided in SI.

2.6.2. Real-time polymerase chain reaction (RT-qPCR)

Silver and other metals have been reported to impact the expression of genes involved in oxidative stress response, defense against pathogens, metal homeostasis, salt stress and plant growth²⁴⁻²⁶.

Quantitative analysis of the gene expression of selected genes was performed by reverse-transcription real time PCR (RT-qPCR). Studied genes were selected to screen the potential interference of NPs with main plant vital processes: oxidative balance (catalase, iron superoxidase dismutase, glutathione peroxidase), homeostasis (Phosphoethanolamine N-methyltransferase), pathogen defense (Pathogen-inducible ethylene-responsive element-binding protein) and growth (ETTIN-like auxin response factor). The expression of the Metallothionein-like protein was also studied to shed light on NPs transport processes. Target-specific PCR primers for these genes were designed using Primer-BLAST²⁷. Encoded genes names, accession number in NCBI database (National Center for Biotechnology: <https://www.ncbi.nlm.nih.gov/>) and the list of primers used are given in table S1.

RNA was isolated from plant roots by using RNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. RNA concentration and purity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) by measuring absorbance at 230, 260 and 280 nm. RNA was reverse transcribed into cDNA using the SuperScript II Reverse first strand kit (Invitrogen). Quantitative PCR was performed in a MX3005P multiplex quantitative PCR system (Stratagene) using MESA Blue qPCR Mastermix for SYBR Assay Low ROX (Eurogentec). We used the following thermal cycling steps: 95°C for 5 min, then 95°C for 15 s, 55°C for 20 s and 72°C for 40 s 40 times and finally 95°C for 1 min, 55°C for 30 s and 95°C for 30 s for the dissociation curve. PCR efficiencies were experimentally checked for compliance using a mix of all samples, with a quality criterion of 2 ± 0.3 , and a theoretical value of 2 was used for calculations. Actin, 18sR and GAPDHR were chosen as housekeeping genes for normalization. Cq threshold was determined using the Mx-Pro 3.20 software (Stratagene) with default settings. mRNA expression analysis, normalization and statistical analysis were performed using the $\Delta\Delta Cq$ method as described previously⁹. The RNA level modulation of selected genes was analyzed based on Ct comparison using REST 2009 (Qiagen). Data are expressed as percentages of the corresponding control $\pm$ standard deviation.

3. RESULTS AND DISCUSSION

3.1 Effect of initial Ag form on distribution and speciation within roots

Distribution and speciation of Ag in roots were obtained by combining several highly complementary imaging tools (i.e. μ -CT, nano-CT, μ -XRF and μ -XANES). μ -CT provides the in-situ 3D distribution of dense voxels in intact samples and in relatively large field-of-views (FOV). Virtual cross-sections (various orientation or thickness) can be extracted from the obtained reconstructed volume. However, μ -CT does not provide elemental information, to validate that dense voxels can be attributed to Ag accumulation regions, μ -XRF mapping of the same FOV was performed. The good similarity in Ag-rich pixels (red pixels in Figure 1b or purple pixels in Figure 5b when Ag is co-localized

with S) and dense voxels (brilliant voxels in Figure 1a and 5a) distribution contributes to validate the identification of Ag accumulation regions. μ -CT is sensitive to accumulation regions with high local Ag concentration (i.e. such as Ag-NPs aggregates). However, it does not allow to discriminate Ag when it is homogeneously distributed in the plant matrix (i.e. single Ag-NPs or released Ag^+). Recent technical developments allow performing 3D imaging with nanometer spatial resolution using X-ray laboratory sources (nano-CT). Coupling μ -CT (spatial resolution at micrometer scale and relatively large FOV) with nano-CT (spatial resolution of 150 nm and small FOV) offers the opportunity to further study the Ag internalization processes. Synchrotron-based μ -XRF provides 2D elemental information with high-sensitivity (low femtogram/ μm^2)²⁰ and can be coupled (as in this study) to μ -XANES spectroscopy to obtain speciation information.

The surface of plant roots exposed to AgNO_3 (Ag^+) contained localized accumulation zones as shown by μ -CT (Figure 1a and 1b, animations 1 and 2 in supporting information). These accumulation zones were not present in the control root (Figure S4.a and S5.a in SI). The 3D reconstructed image (Figure 1a) and the virtual cross sections (Figure 1b and animation 2 in SI) showed that these accumulation zones had a variety of sizes (a few tens to 100 μm in diameter). In contrast, μ -XRF revealed an homogenous presence of Ag in root epidermal cells (Figure 1.b d). μ -XRF analyses also revealed the presence of Ag in the cytoplasm of cortex cells, in the endodermis and in the central cylinder (Figure S2 in SI). Ag was also detected in intercellular spaces of the cortex, in accumulation regions of several microns in diameter (Figure 1.e).

The speciation of Ag at the cortex region was determined by μ -XANES spectroscopy. In spot 1, Ag was bound to thiol (84%) and O ligands (modelled by AgNO_3) (16%) (AgNO_3 -cortex 1 in Figure 2). Chelation by thiol ligands is a well-known detoxification mechanism inside cells, but thiol ligands are also present in the apoplast²⁸⁻²⁹. Given the high affinity of Ag(I) for thiols, the formation of Ag-thiols in the extracellular compartment is not surprising. In spot 2, a slight oscillation on the spectra at 3670-3380 eV was indicative of the presence of metallic Ag as minor species. Thus, part of Ag(I) was

reduced into Ag(0). The reduction of ionic Ag into Ag-NPs in plants exposed to AgNO₃ has been reported previously³⁰⁻³¹, and proposed as a green technology for the production of Ag-NPs¹⁷. In parallel, a significant Ag translocation from roots to shoots was measured (Figure S3.a). The presence of Ag both in the apo-and symplast and in the central cylinder suggests that both types of transport pathways, apo- and symplastic, are used by Ag ions.

For roots exposed to Ag-NPs, μ -CT and μ -XRF show the presence of localized Ag accumulation regions with a size of 1-4 μ m adhering on the epidermis (Figure 4.a, d, e). Metallic Ag was the major Ag species of these accumulation regions (Figure. 2, root 3; Figure S4.e-f in SI, 80% and 97% respectively). At higher spatial resolution, nano-CT revealed that these Ag-NPs accumulated preferentially in discontinuities between root epidermal cells (Figure S5.b, c). In addition, root architecture and morphology were impacted since an absence of secondary roots and a proliferation of root hairs were observed exclusively for this treatment (Figure S5.a). Many Ag-NPs were fixed on root hairs (Figure 4. a, Animations 3 and 4 in supporting information, Figures S6.b and S7.b in SI).

A higher Ag root and shoot content was observed for this treatment compared to the AgNO₃ treatment (Figure. S3), suggesting a nano-specific accumulation mechanism different to what was observed for the AgNO₃ exposure. Our observations support two points of entry previously suggested in the literature. On one hand, high local concentration of metals in the epidermis was shown to cause rupture of this tissue³². Such ruptures in which Ag-NPs are preferentially accumulated (Figure S4.b, c) might facilitate the transfer of Ag inside roots. On the other hand, root hairs which have a thin cell wall, and take up nutrients by transport, diffusion, and endocytosis³³ are considered as potential points of entry of NPs¹².

In the epidermis Ag was mostly present as metallic Ag (root 3, Figure S4 in SI). However, a biotransformation of Ag was evidenced inside the roots where Ag was homogeneously distributed in the cell walls of the cortex (purple areas in Figure 4.d), as a mixture of Ag-thiol species and other

ionic Ag species (86 and 14% respectively, Figure S2, f). Contrary to what was observed in the epidermis, no Ag(0) was observed inside roots. Similar speciation results were observed in the walls of the endodermis (77 and 26% respectively, Figure 4.c). Thus, Ag-NPs were completely dissolved and complexed by organic ligands. Ag was detected inside cells of the cortex, in the endodermis and in the central cylinder. These observations suggest both apoplastic and symplastic transfer of Ag in monovalent form.

In the case of roots treated with Ag₂S-NPs, μ -XRF shows that Ag is mainly colocalized with S (purple pixels in Figure 5b, resulting in mixing blue (S) and red (Ag)). μ -CT and nano-CT reveals that these Ag accumulation regions, with a size from 3 to 8 μ m are mostly on the root surface (Figure 5.a, c, b and 3D animations 5 and 6 in SI). μ -XANES showed that Ag at root surface was mainly present as Ag₂S, with proportions ranging from 62% to 89% depending on the sample (Figure 2). Secondary Ag chemical forms were also identified, including metallic Ag (up to 40%, arrows in Figure 5e). In the secondary root (figure S4 in SI), no metallic Ag was found, but 13% (point 2-2) to 26% (point 2-1) of Ag was bound to thiols. These results provide evidence that Ag₂S-NPs were dissolved, and then Ag⁺ ions were either complexed to thiol containing molecules or reduced to elemental Ag. These results contradict the general idea that Ag₂S is highly insoluble, which is based on the solubility constant of macrocrystalline Ag₂S ($K_{sp} = 5.92 \cdot 10^{-51}$ ³⁴). The partial dissolution of Ag₂S could be favored by the root exudation³⁵. Wheat plants exudate organic acids and phytosiderophores, this process is enhanced in case of nutrient deficiency or metal stress³⁶⁻³⁸. In a recent study with Alfalfa plants exposed to Ag₂S-NPs, the authors suggested that Ag₂S could be partially dissolved in the acidic environment of root border cells¹⁵. However, to our knowledge, this is the first work that clearly shows this process. Plants and plant extracts have been used to reduce Ag(I) to Ag(0) for the green synthesis of Ag-NPs³⁹, however this is the first time that the reduction of Ag₂S-NPs is observed in the rhizosphere

(endorhizosphere and rhizoplane in this case) of living plants. The identification of the reducing agent would require further investigations.

μ -XRF together with μ -CT showed that Ag_2S -NPs were especially concentrated at the base of the lateral roots (Figure 5.a,d). During the development of lateral roots, the newly formed root breaks through the cortex and endodermis. Some authors suggest that this zone may provide a direct access for NPs to the xylem⁴⁰. Nano-CT analysis were also performed in the base of lateral roots (data not shown), however the high disorder of cell layers made impossible the clear discrimination of internal and surface areas. A few Ag-rich spots were detected inside the root only by μ -XRF (Figure 5.e, red arrows). Except these few internal Ag_2S -rich spots, very low Ag signal was detected in the cell walls and cytoplasm of the cells by μ -XRF (Figure S2 in SI) and no signal was detected in the endodermis and the central cylinder. This result is consistent with the low Ag root and shoot content measured for this treatment (Figure S3.a). Thus, despite a partial dissolution of Ag_2S -NPs near roots, Ag transfer remained limited at the present exposition time.

3.2. Phytotoxicity and gene expression response

Both Ag uptake in roots and translocation in shoots increased in the order $\text{Ag}_2\text{S-NP} < \text{AgNO}_3 < \text{Ag-NPs}$ exposure (Figure S3.a). Plant growth reduction was correlated with Ag accumulation in tissues with the highest effect observed in the Ag-NPs treatment (Figure S3. b). However, finer phytotoxicity markers and impacts on gene expression were specific for each Ag source. Previous studies showed that the phytotoxicity of Ag-NPs was associated with reactive oxygen species production and lipid peroxidation¹³. However, no information is available about plants exposed to Ag_2S -NPs. Lipid peroxidation was evaluated by the quantification of thiobarbituric acid reactive substances (TBARS) (figure S3, c). It was the highest for the AgNO_3 treatment, likely due to the high concentration in Ag^+ . Surprisingly, lipid peroxidation for the Ag-NPs and Ag_2S -NPs treatments was lower than the control it was thus not correlated with growth reduction.

As mentioned above, root architecture was impacted in the Ag-NPs treatment. Secondary roots did not develop and a proliferation of roots hairs was observed. The presence of Ag-NPs aggregates on root hairs might have an impact on their functions. Previous studies showed a loss or absence of root hairs for plants exposed to Ag-NPs, but this effect may be due to the high dose used¹². Root hair proliferation is generally related with nutrient or water deficiency. The presence of Ag in the cell wall, as observed in our study (Figure 4.c, d), has been suggested to affect the function of nutrient transporter proteins¹³. In parallel, exposure to Ag₂S-NPs and AgNO₃ has been shown to down regulate the expression of aquaporins¹⁶. Exposure to Ag-NPs was not tested in this latter study so it could be related to root hair proliferation. Finally, changes in root architecture and morphology, are very likely to influence the entry of Ag in nanoparticulate and ionic form.

In order to further investigate the phytotoxicity induced by the different sources of Ag, the expression of selected genes in the roots of wheat plants was studied by real-time polymerase chain reaction (RT-qPCR) (Figure 3). The genes encoding the enzymes catalase (CAT), superoxide dismutase (FeSOD) and glutathione peroxidase (GPX) were studied because of their key role in the oxidative stress. Each NP treatment induced changes in the expression of different genes. Ag₂S-NPs lead to the overexpression of CAT and FeSOD whereas Ag-NPs induced a drop in the expression of FeSOD but an increase in that of GPX. Previous studies found up-regulated expression of CAT and SOD in *Vigna radiata*²⁴ and *Arabidopsis thaliana*²⁵⁻²⁶ exposed to pristine Ag-NPs. Taken together with the TBARS results and the localization and speciation of Ag, our results suggest that plants exposed to Ag-NPs and Ag₂S-NPs are able to control the oxidative stress by upregulating the enzymes related with radical scavenging, possibly due to the slow release of Ag⁺ from the nanoparticles. Exposure to AgNO₃, which induced a strong lipid peroxidation, did not induce any modulation of the expression of these genes. A possible explanation could be that the oxidative stress exceeds to capability of the enzymatic machinery, which is then not induced.

We investigated the expression of the gene encoding metallothionein (Wali). Metallothioneins (MTs) regulate metal homeostasis, and are also involved in the scavenging of reactive oxygen species (ROS)⁴¹. Literature regarding the production of MT's in plants exposed to Ag is scarce, with only one publication reporting the overexpression of MT in wheat plants exposed to Ag⁺ and Ag-NPs⁹. In the present study, however, we found overexpression of Wali for AgNO₃ and Ag₂S-NPs treatments while it was moderately but significantly down-regulated for the Ag-NPs treatment. It is suggested that the synthesis of MTs in plants could be related with the detoxification of sulfide (HS⁻), which is phytotoxic, although it has not been clearly demonstrated yet⁴². Since the speciation study evidenced some dissolution of Ag₂S-NPs, the overexpression of MT found for the Ag₂S-NPs treatment may be a response to a release of Ag⁺, but also of sulfide.

The three treatments also induced a down-regulation of the Phosphoethanolamine N-methyltransferase (PEAMT), encoding a protein related with the salt stress, and an up-regulation of the Pathogen-inducible ethylene-responsive element-binding protein (PIEP), encoding a protein involved in the response to pathogens. Both results are opposite to that found for genes with similar functions in a previous study with *A. thaliana*²⁵.

We also investigated the effect of the different treatments in the auxin-like response factor (ETT1). Auxins are hormones that control cell proliferation and growth in plants, and are involved in root branching. A crosstalk between ROS and auxin production in plant response to metal stress has been evidenced⁴³. In the present work, we did find an overexpression of the EET1 in the roots of AgNO₃ treated plants but no impacts were found for the two NP treatments. Thus, the absence of root branching in the Ag-NPs treatment is not related to the expression of this gene.

4. ENVIRONMENTAL IMPLICATIONS

It has been generally hypothesized that Ag-NPs may act as "Trojan Horses" entering living organisms and then releasing Ag⁺ over time causing toxicity⁴⁴. This has been recently proposed as the

mechanism by which Ag₂S-NPs could be toxic to wheat and cowpea. In the present work, although the Trojan horse scenario is very likely to take place, a more complex scheme was evidenced. As illustrated in the synthetic Figure 6, interconversions of Ag forms outside and inside the plant, leading to a mixture of Ag species, were observed. The distribution of Ag species probably evolves over time, leading to a complex exposition pattern for plants, also exposed to HS⁻ in the case of Ag₂S-NPs. The conflicting results usually seen in the literature concerning the toxicity of silver nanoparticles are generally ascribed to differences in exposure conditions, structural properties of nanoparticles and plant species. This evolving Ag speciation may represent an additional source of variability and conflicting results.

Data from this study showed drastic different responses of the plant depending on the starting nanoparticles, highlighting the importance to include transformed nanoparticles in ecotoxicological studies.

Although the uptake and translocation of Ag was lower for the Ag₂S-NPs exposure compared to the Ag-NPs exposure, phytotoxicity symptoms were observed. These results show that the sulfidation of Ag-NPs is not a perfect antidote to toxicity and that Ag₂S-NPs are not as stable as expected when exposed to plant roots. In agricultural soils, the rhizospheric activity of plants might partly dissolve Ag₂S causing some impacts on crop quality and yield, and more generally on ecological services.

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FIGURES

Figure 1. Roots of wheat plants exposed to AgNO_3 : a) 3D reconstructed image by μ -CT (voxel size $1\ \mu\text{m}$) b) virtual 2D slice ($1\ \mu\text{m}$ thickness) extracted from a). μ -XRF tricolor maps of entire root ($1\ \text{px} = 1\ \mu\text{m}$) (c), of cross section ($1\ \text{px} = 1\ \mu\text{m}$) (Ep=epidermis, Cx=cortex, Ed= endodermis, Pe=pericycle, Xy=xylem) (d) and of a zoom in the cortex ($1\ \text{px} = 0.5\ \mu\text{m}$) (e). Extremity of white arrows in e) indicate points where μ -XANES spectra were collected.

Figure 2: Linear combination Fitting (LCF) of the μ -XANES spectra collected in the different points of the samples (dotted lines), experimental spectra (solid lines). Results for the Linear Combination Fitting (LCF) of all samples are given in table S2 of SI. And Ag references used (solid lines) and reconstruction of these references by Target Transformation (dotted lines) for the three components determined by Principal Components Analysis (PCA). Reference of AgNO_3 is a proxy for Ag^+ species ligated to O ligands. Ag-GSH (Ag-glutathione) and Ag-DEDTC (Ag- diethyldithiocarbamate) were indistinguishable and were used as proxy of Ag-Thiol. The spectra of Ag0 foil and Ag-NPs were also indistinguishable and were used as references for metallic Ag.

Figure 3. Gene expression quantified by qPCR in roots of plants exposed to AgNO₃ (blue), Ag₂S-NPs (green) and Ag-NPs (pink). Results are expressed as fold change regards control. They represent the average of 3 analytical replicates $\pm$ standard deviation, (*p < 0.05). CAT (catalase), FeSOD (iron superoxidase dismutase), GPX (glutathione peroxidase), Wali (Metallothionein-like protein) PEAMP (Phosphoethanolamine N-methyltransferase), PIEP (Pathogen-inducible ethylene-responsive element-binding protein), ETT1 (ETTIN-like auxin response factor).

Figure 4. Roots of wheat plants exposed to Ag-NPs, μ -XRF tricolor maps of a) entire root (1 px = 1 μ m) (1 px = 1 μ m), (b) root cross section (Sf=surface, Ep=epidermis, Cx=cortex, Ed= endodermis, Pe=pericycle, Xy=xylem) (b), and of a zoom in the endodermis (c) and cortex (d) (1px = 0.5 μ m). Red arrows show preferential accumulation sites for Ag-NPs. Extremity of white arrows in c) and d) indicate points where μ -XANES spectra were collected.

Figure 5. Roots of wheat plants exposed to Ag_2S -NPs: a) 3D reconstructed image by μ -CT (voxel size 0.9 μm), b) virtual μ -CT slice (1 μm thickness) extracted from a), c) nano-CT reconstructed image of the primary root surface (white square in a)) (voxel size of 63.5 nm). μ -XRF maps of entire root (1 px = 1 μm) d), of a cross section (e) (Sf=surface, Ep=epidermis, Cx=cortex, Ed= endodermis, Pe=pericycle, Xy=xylem). Red arrows indicate equivalent Ag accumulation regions in images. White arrows in e) indicate points where μ -XANES spectra were collected.

Figure 6. Synthesis of the observed changes in Ag speciation that take place inside and outside wheat roots depending on the starting materials, proposed transfer pathways and induced effects on plants.