Ag nanoparticles and wheat roots: a complex interplay

Supporting Information (SI)

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1. MATERIALS AND METHODS

1.1 Nanoparticle characterization

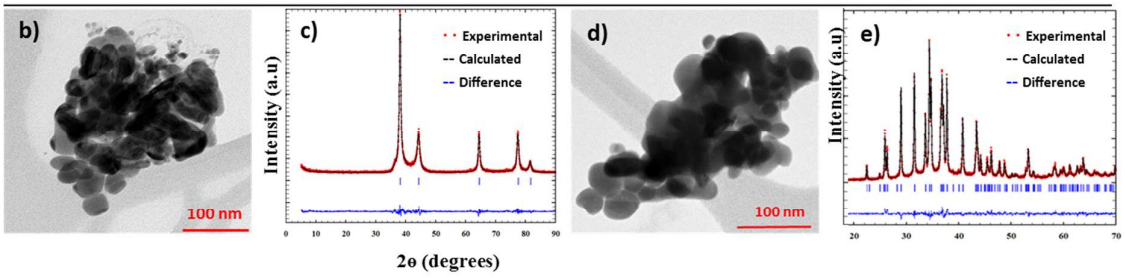
Metallic silver nanoparticles (Ag-NPs) were provided by NanoAmor, Nanostructured and Amorphous Materials Inc. (USA). These Ag-NPs have a coating of polyvinylpyrrolidone (PVP). Ag_2S nanoparticles (Ag_2S-NPs) were produced by the sulfidation of the PVP-Ag-NPs as stated in Levard et al. (2011)[1]. Briefly, a suspension of 1mM PVP-Ag-NPs (pH=7) was mixed with a 1mM Na2S in a 0.01M NaNO3 electrolyte. After 3 days of reaction, solutions were centrifuged, washed three times with MQ water and dried. The total sulfidation of PVP-Ag-NPs to Ag_2S-NPs was tested by X-ray Difraction (XRD)

Pristine metalllic (Ag-NPs) and sulfided silver nanoparticles (Ag_2S-NPs) were thoroughly characterized in powder and in suspension. Nanoparticles stock suspensions were prepared in double distilled water at 10 mM of Ag concentration and sonicated for 45 min in a ultrasonication bath (Bioblock Scientific 88169). Shape and nominal diameter were determined by Transmission Electron Microscopy (TEM-TECNAI OSIRIS). Specific Surface Area (BET) was determined by the

Volumetric Gas (N_2) Adsorption Method at 77K (BELSORP-max, BelJapan.Inc). Coherent domain size was determined by X-Ray diffraction (XRD). XRD patterns were recorded with a Bruker D5000 powder diffractometer equipped with a SolX Si(Li) solid state detector from Baltic Scientific Instruments using CuKα1+2 radiation. Intensities were recorded at 0.04°, 2-theta step intervals from 15 to 70° (5 s counting time per step). Rietveld refinement of the powder diffraction patterns were carried out using the FullProf package[2]. In suspensions of 100 mgPVP-Ag-NPs·L^{-1} in ultrapure water, the hydrodynamic diameter was measured by Dynamic Light Scattering (DLS), Z potential was also measured over a range of pH from 2 to 10 (Zetasizer Nano ZS Malvern Instruments). In the same suspensions, ionic Ag^+ concentration was measured with an ion specific silver/sulfide Ion selective electrode (Thermo Scientific) calibrated with a series of $AgNO_3$ standard solutions.

Results of the characterization are presented in figure S1.

a)	nominal diameter (nm)	Especific surface area ($m^2 \cdot g^{-1}$)	Coherent domain size (nm)	Hydrodynamic diameter (nm)	Zeta potential (mV)	ionic Ag^+ ($mg \cdot L^{-1}$)
PVP-Ag-NPs	52±1	6.3	23±3	61±10	-0.7	<0.01
nano Ag_2S	42±5	4.2	57±5	*	-17	<0.01



* Hydrodynamic diameter and ionic Ag+ were measured in MQ water suspensions and Zeta potentials were determined in the Hoagland Nutrient solution

Figure S1 : Ag-NPS and Ag_2S-NPs characterization: a) Summary of the main characteristics (shape and nominal diameter were investigated in 120 particles), b) TEM image of the PVP-Ag-NPs, c) Experimental and Rietveld refined X-ray diffraction patterns of PVP-AgNPs, d) TEM image of the Ag_2S-NPs, e) Experimental and Rietveld refined X-ray diffraction patterns of Ag_2S-NPs

1.2. Plant culture

Seeds of wheat (*Triticum aestivum* L.) were germinated in moisture vermiculite after sterilization on NaClO 15% for 10 min. Once the cotyledon was developed, plants were transferred to a hydroponic system. The hydroponic system consisted on glass pots containing 2L of the corresponding solution. 12 plants were placed in each pot by floating in a polystyrene cover. Plants were acclimated 3 days in ¼ strength Hoagland Nutrient solution containing the following constituents: 0.75 mM KNO_3, 0.5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.25 mM $NH_4H_2PO_4$, 0.13 mM $MgSO_4{\cdot}7H_2O$, 12.5 µM NaCl, 6.25 µM H_3BO_3, 0.5 µM $ZnSO_4{\cdot}7H_2O$, 0.5 µM $MnSO_4{\cdot}H_2O$, 0.03 µM $CuSO_4{\cdot}5H_2O$, 0.13 µM $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 5 µM Fe(III)-EDTA. Then, plants were randomly selected to be treated as follow: i) control, no Ag addition; ii) 30 µM Ag-NP; iii) 30 µM Ag_2S-NPs and 4) 30 µM $AgNO_3$. The system was continuously aerated and agitated in order to keep the nanoparticles in suspension. Solutions were replenish with MQ water daily and totally renewed twice a week.

Plants were harvested after 3 weeks of treatment. Roots and shoots were separated and thoroughly rinsed with MQ water and EDTA 20mM and fresh weights were recorded. Samples for phtytotoxicity studies were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Samples for synchrotron analysis and for X-ray computed-tomography were keep fresh until specific sample preparation.

1.3 Silver distribution and speciation in roots cross sections (synchrotron µ-XRF and µ-XANES)

µ-XANES and XANES spectra were treated by using ATHENA software[3], backgrounds were subtracted and spectra were normalized. Principal components Analysis (PCA) of all the spectra was performed as stated in Ressler et al. (2000)[4]. The number of components needed to describe the system was set in 4 based in the statistical IND indicator. Then, the Ag model compounds that constitute the most probable components of the original XANES spectra were identified by Target Transformations (TT). Ag^0, Ag-NPs, Ag_2S, Ag-GSH, Ag- DEDTC and $AgNO_3$ were the reference compounds better reconstructed by the TT.

Once main Ag species were identified, their proportion in the samples was determined by Least-Squares Combination Fitting (LCF) of the experimental spectra. The XANES spectra for Ag^0 and Ag-NPs were indistinguishable so Ag^0 was selected to perform the LCF. Similarly, the XANES spectra of Ag-GSH and Ag-DEDTC were also very similar giving the same results in the LCF so they were considered as proxy for Ag-Thiol.

The quality of the fits was quantified by the normalized sum-squares residuals NSS = $\Sigma(\mu \text{ experimental} - \mu \text{ fit})^2 / \Sigma (\mu \text{ experimental})^2 \times 100$, where μ is the normalized absorbance. An additional reference compound from the list of the most probable determined by TT, was added to the LCF when decreased the NSS by more than a 10%.

1.4 Silver concentrations

Immediately after harvest, shoots and roots were separated, fresh weights were recorded and they were frozen in liquid N2 and lyophilized. Then, samples were mineralized in a HNO3 and HF mixture at 120°C for 4h. After acid evaporation, samples were diluted in HNO3 5% until analysis. Ag concentrations were determined by Inductively Coupled Plasma Mass Spectrometry ICP-MS (Elan DRC II Perkin Elmer). NIST 2782 reference material (Industrial Sludge) was used to test the efficiency of the process. The recovery percentages were: 105 ± 2% for Ag.

1.5. Phytotoxicity

1.5.1 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)[5]. Briefly, fresh samples (0.1 g) of roots were homogenized on ice with 1 mL of deionised water and centrifuged at 16,000 g for 10 min. The supernatants were removed, and the pellets were re-suspended in 500 µL of solution containing 0.01% butylated hydroxy-toluene (BHT) in 80% ethanol. Then, 900µL of TBA (2.57×10^{-2}M), TCA (9.18×10^{-1}M) and HCl (3.20 M) were added to each sample. Samples were

vortexed, incubated in a water bath at 70◦C for 30 min, cooled on ice and then centrifuged at 16,000 g for 10 min. The absorbance of supernatants was measured at 532 nm. Absorbance at 600 nm was subtracted from this measure to eliminate the interference of soluble sugars in the samples. Absorbances were determined by UV–vis light spectrophotometer (Perkin Elmer, Lambda 35).

2. RESULTS

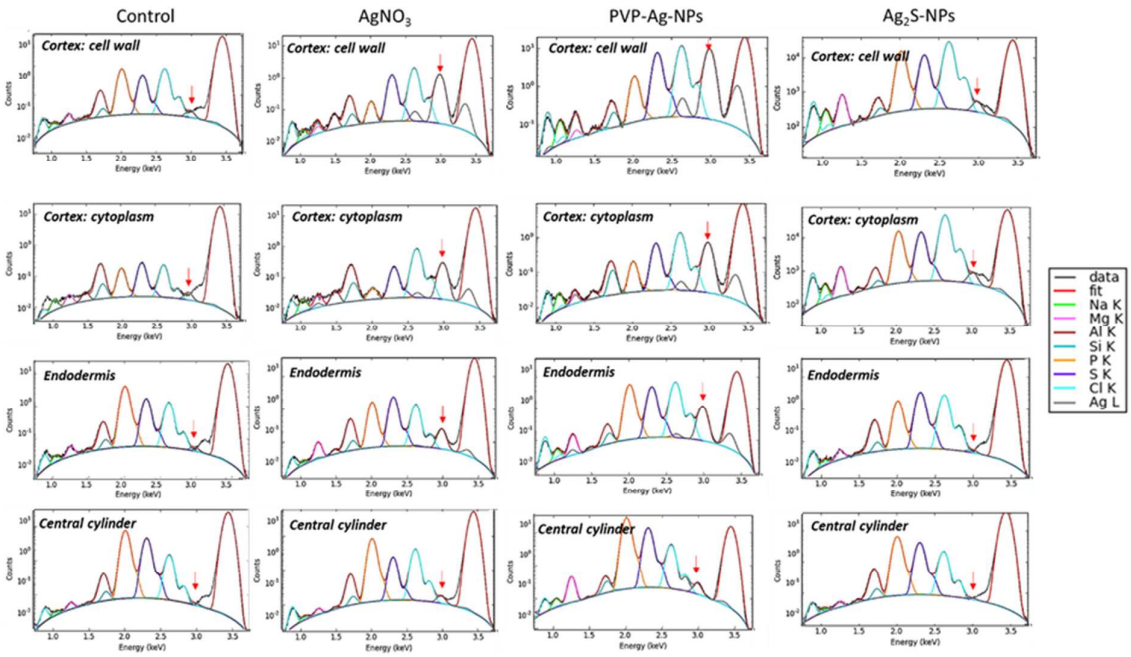


Figure S2: Fitted µ-XRF spectrum extracted from the cell wall and the cytoplasm of the cortex of root exposed to $AgNO_3$ (root $AgNO_3$), Ag-NPs (root Ag-NP-1.f), and Ag_2S-NPs (Ag_2S-2)

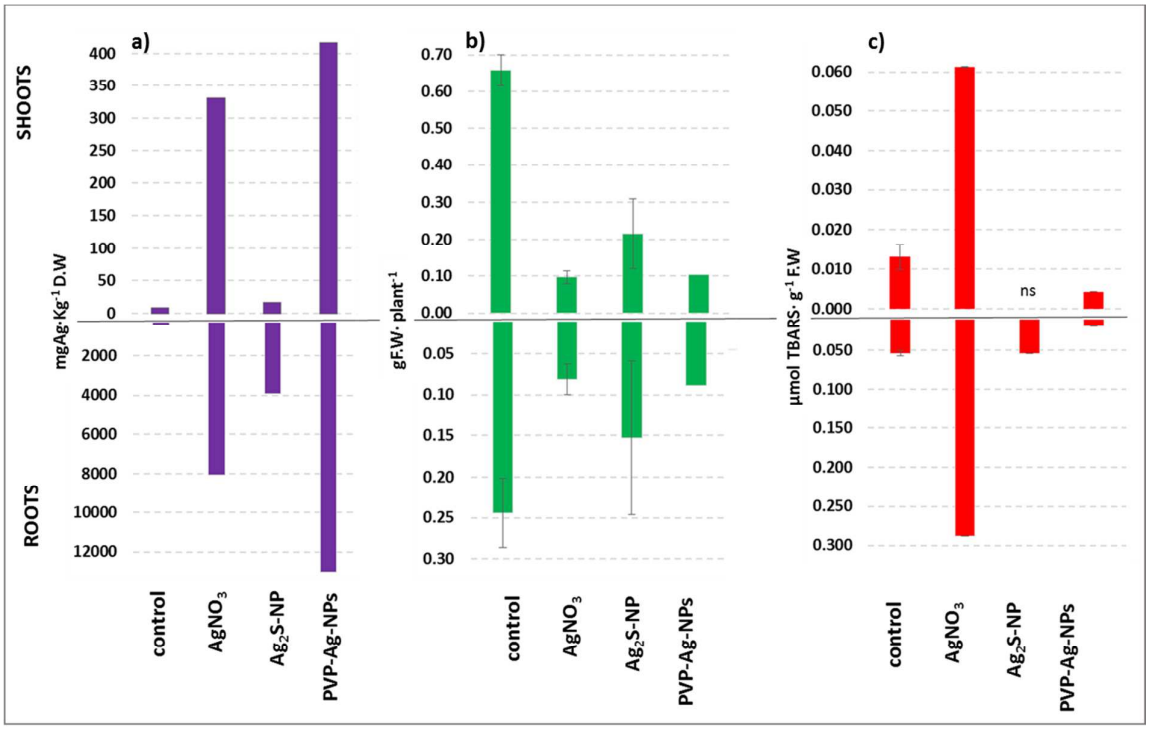


Figure S3: Phytotoxicity: a) silver concentrations in shoots and roots of wheat plants exposed to the different Ag sources (no replicates); b) biomass(g FW·plant^{-1}) (n=3, biological replicates) and c) oxidative stress as Thiobarbituric acid Reactive species (µmol TBARS·g^{-1} F.W, n=3, biological replicates)

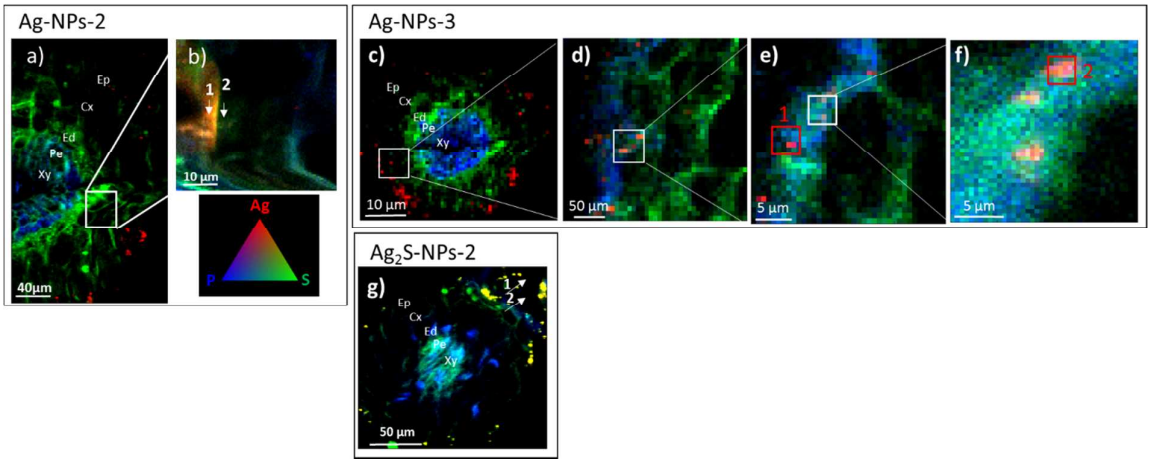


Figure S4. Roots of wheat plants exposed to different sources of Ag. Ag-NPs-2: a) µ-XRF tricolor map of a frozen hydrated root cross section, b) µ-XRF map of the area indicated in map a, white narrows indicate points where µXANES were collected. Ag-NPs-3: a) µ-XRF tricolor map of a frozen hydrated

111 root cross section, b) µ-XRF map of the area indicated in map a, c) µ-XRF map of the area indicated in
112 map b, red squares indicate areas where µ-XANES were collected by the µ-mapping mode. g) Ag_2S-
113 NP: µ-XRF tricolor map of a cross section a fresh lateral root, narrows indicate points where XANES
114 were collected. Silver in red, Sulfur in green and phosphorous in blue.

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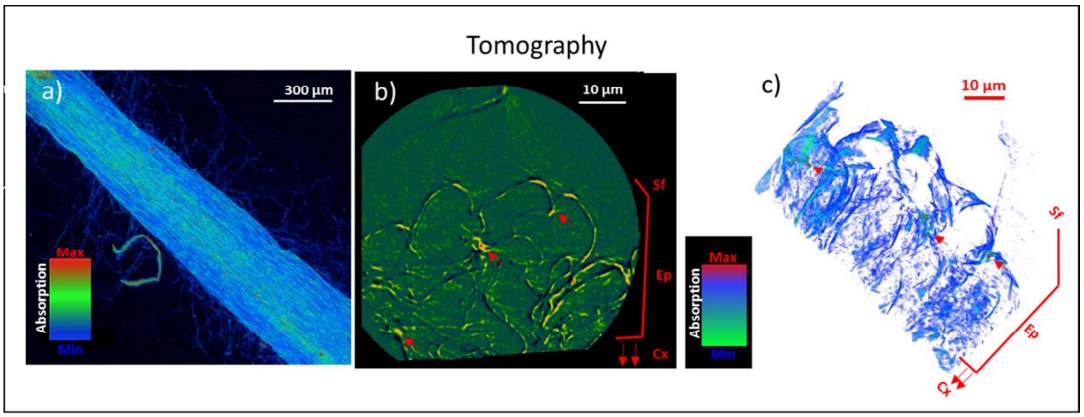

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117 **Figure S5.** Roots of wheat plants exposed to Ag-NPs a) 3D reconstructed image by µ-CT (voxel size 0.9
118 µm), b) virtual nano-CT slice extracted from a), c) nano-CT reconstructed image of the root surface
119 (voxel size of 63.5 nm). Red arrows indicate zones of Ag accumulation.

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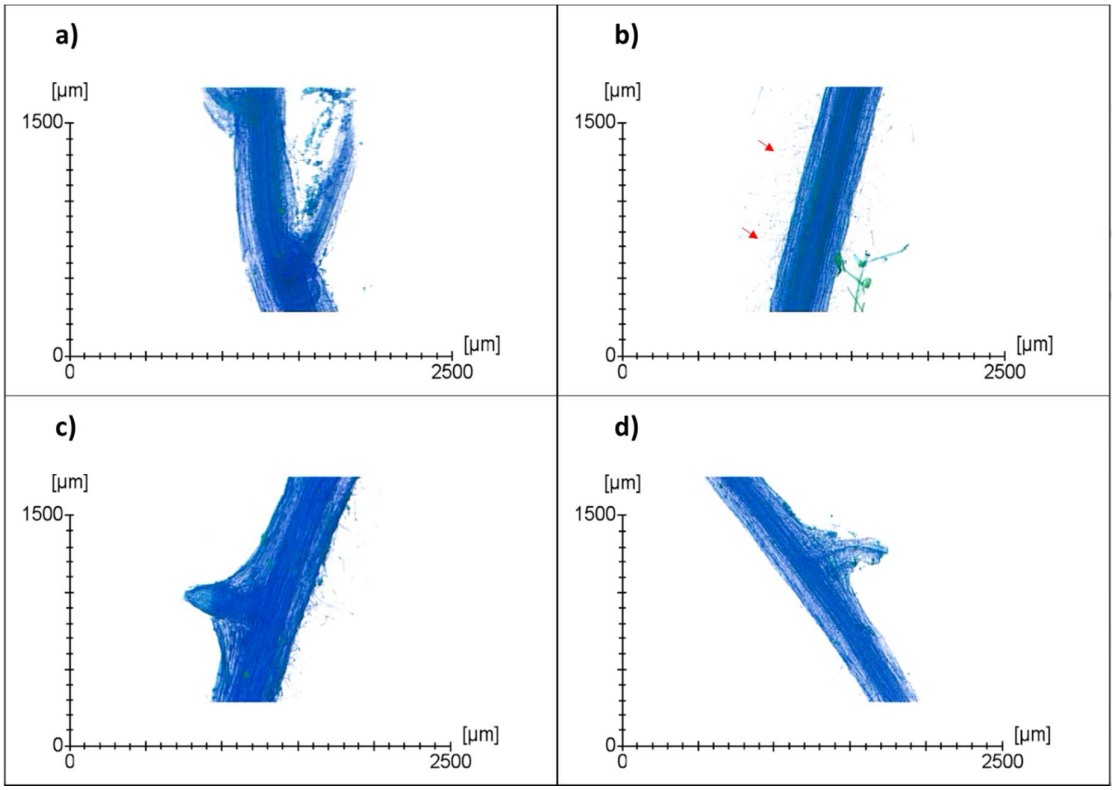

Figure S6: 3D reconstructed image of dehydrated roots by µ-CT. a) control, b) Ag-NPs, c) $AgNO_3$, d) Ag_2S-NPs.

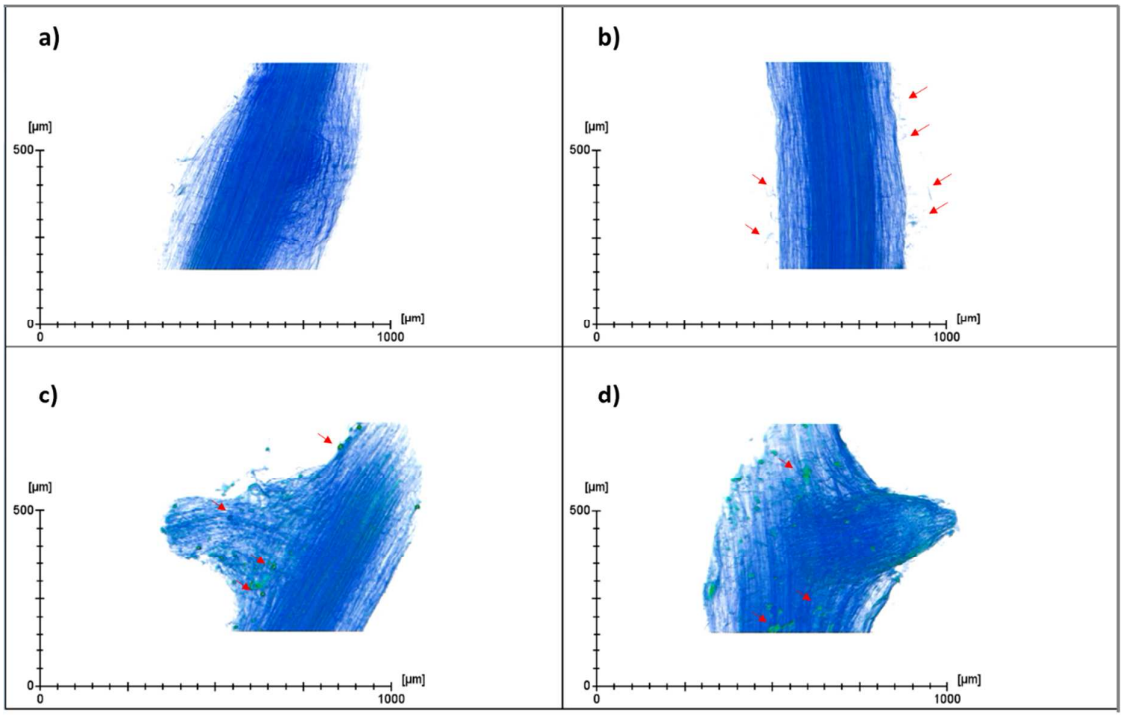

Figure S7: 3D reconstructed image of dehydrated roots by µ-CT (voxel size 0.9 µm). a) control, b) Ag-NPs, c) Ag_2S-NPs, c) $AgNO_3$. Red arrows show root hairs and/or Ag accumulation zones

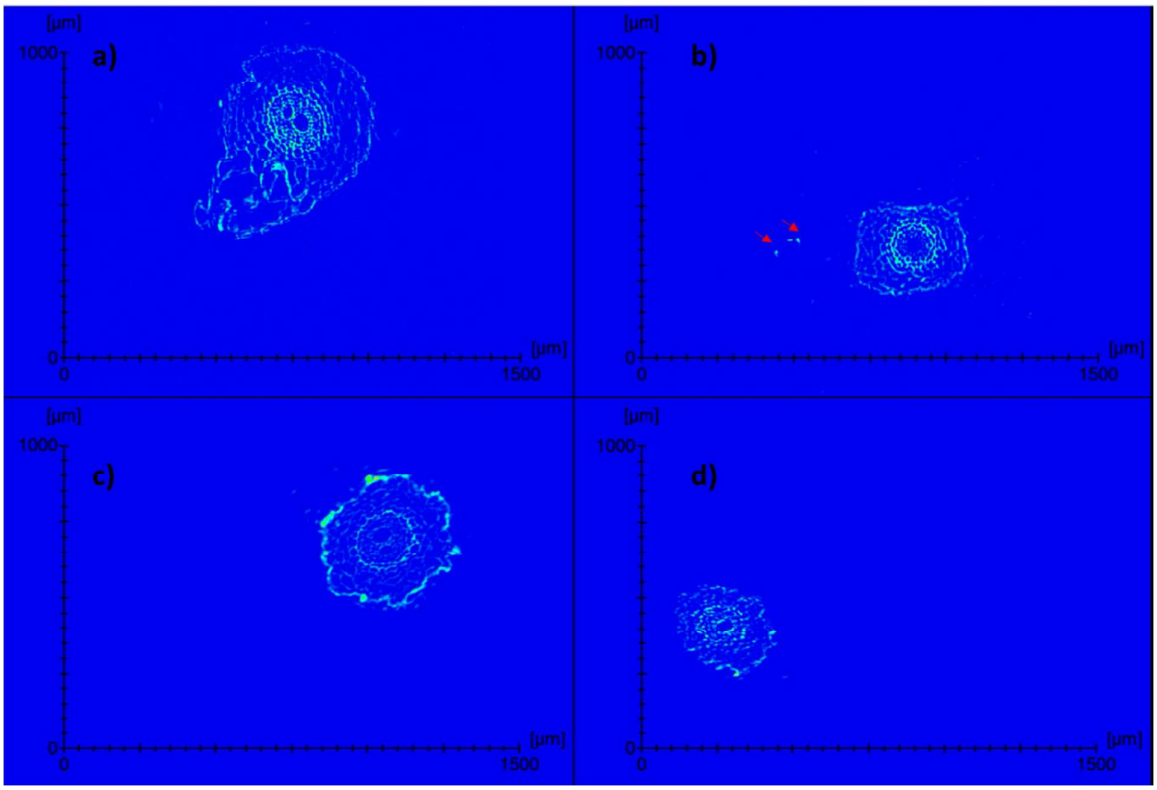

Figure S8: virtual µCT slice (1 µm thickness) from dehydrated roots by µ-CT. a) control, b) Ag-NPs (red narrows indicate Ag accumulation in root hairs), c) Ag_2S-NPs (red narrows indicate Nps aggregates), c) $AgNO_3$.

Table S1: List of genes which expression have been studied by qPCR. Encoded genes names, accession number in NCBI database (National Center for Biotechnology: https://www.ncbi.nlm.nih.gov/) and the list of primers used.

Gene name	Encoded gene name	Gene ID	Forward primer	Reverse primer
Catalase	CAT	D86327.1	GGACTATGAGGAGCGGTTCG	TTGTTGTGGTGGGAGCACTT
Iron superoxide dismutase	FeSOD	JX398977.1	TGGTTGGGTTTGGCTTGTCT	TCCCCAAAAGCAAGTGGGTT
Glutathione peroxidase 1	GPX	FJ797431.1	CACGACTTCACCGTCAAGGA	TTGGAGTTGGTCAAGCCACA
Metallothionein-like protein (wali1)	Wali	L11879.1	TGCAACCCCTGCAACTGTTA	ACACACAAGGACACCAAGGG
Phosphoethanolamine N-methyltransferase (PEAMT2)	PEAMT	FJ803924.1	TTGCTGAAGACCGCACTGAT	ATAGTCCTCCTGGCCGAAGT
Pathogen-inducible ethylene-responsive element-binding protein (PIEP1) mRNA	PIEP	EF583940.1	CGAATCTACCGGGATGGTGG	ATGATCACCCGTCATCGTCG
ETTIN-like auxin response factor (ETT1-alpha)	ETT1	AY376128.1	GCGATCGACGTCCAACAATG	AACCAGCTAAATGGCCTCCC
Actin (ACT1)*	Actin	AF326781	AAATCTGGCATCACACTTTCTAC	GTCTCAAACATAATCTGGGTCATC
18S rRNA*	18SR	GI223036846	CAAGCCATCGCTCTGGATACATT	CCTGTTATTGCCTCAAACTTCC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *	GAPDH	EF592180	TTTTCACCGACAAGGACA	AAGAGGAGCAAGGCAGTT

Table S2: Ag species distribution (%) plant roots obtained by Linear Least squares Combination Fitting of Ag L_{III} edge XANES spectra

	% of Ag species					
SAMPLE	**Ag-Np**	**Ag_2S-NP**	**Ag-Thiol**	**$AgNO_3$**	**SUM**	**NSS(%)**
AgNO3-Cortex 1			84	16	100	0.12
AgNO3-Cortex 2	14		76	12	102	0.21
Ag2S-NP-Epiderm-main root 1	40	62			102	0.02
Ag2S-NP-Epiderm-main root 2	17	84			101	0.13
Ag2S-NP-Epiderm-leteral root 1		75	26		101	0.04
Ag2S-NP-Epiderm-leteral root 2		89	13		102	0.21
Ag-NP-Epidermis (3-2)	80		24		104	0.52
Ag-NP-Epidermis (3-1)	97				97	0.18
Ag-NP-Endodermis (2-1)			92	1	93	0.29
Ag-NP-Endodermis (2-2)			85	16	101	0.45
Ag-NP-Cortex (1-2)			86	14	100	0.18
Ag-NP-Endodermis (1-1)			77	26	103	0.38

Proportions are expressed as mean percentage (%) calculated for these fits. Residual between fit and experimental data: $NSS = \Sigma [k^3 \chi_{exp} - k^3 \chi_{fit}]^2 / \Sigma [k^3 \chi_{exp}]^2 \times 100$).

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