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RESEARCH ARTICLE

Phytotoxic effects of silver nanoparticles in tobacco plants

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Abstract

The small size of nanoparticles (NPs), with dimensions between 1 and 100 nm, results in unique chemical and physical characteristics, which is why they are implemented in various consumer products. Therefore, an important concern is the potential detrimental impact of NPs on the environment. As plants are a vital part of ecosystem, investigation of the phytotoxic effects of NPs is particularly interesting. This study investigated the potential phytotoxicity of silver nanoparticles (AgNPs) on tobacco (*Nicotiana tabacum*) plants and compared it with the effects of the same $AgNO_3$ concentrations. Accumulation of silver in roots and leaves was equally efficient after both AgNP and $AgNO_3$ treatment, with predominant Ag levels found in the roots. Exposure to AgNPs did not result in elevated values of oxidative stress parameters either in roots or in leaves, while $AgNO_3$ induced oxidative stress in both plant tissues. In the presence of both AgNPs and $AgNO_3$, root meristem cells became highly vacuolated, which indicates that vacuoles might be the primary storage target for accumulated Ag. Direct AgNP uptake by root cells was confirmed. Leaf ultrastructural studies revealed changes mainly in the size of chloroplasts of AgNP-treated and $AgNO_3$-treated plants. All of these findings indicate that nano form of silver is less toxic to tobacco plants than silver ions.

Keywords Silver nanoparticles · *Nicotiana tabacum* · Oxidative stress · Comet assay · Antioxidant enzymes · Ultrastructure

Introduction

Nanoparticles (NPs) are defined as structures that have at least one dimension between 1 and 100 nm, which results in their unique chemical and physical characteristics. These unique features make them highly attractive for implementation in products for wide application (Benn et al. 2010). The increasing production and exploitation of NPs raises important concerns regarding their release to water, soil, and air (Peralta-Videa et al. 2011) and therefore possibly a harmful effect on the environment and, consequently, human health (Maynard et al. 2011; Beer et al. 2012; Colman et al. 2013).

Petra Cvjetko and Mira Zovko equally contributed to this work.

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Among the different available NPs, silver nanoparticles (AgNPs) are the most widely applied due to their well-known antibacterial and antifungal effects, as well as their plasmonic and opto-electrical properties (Pokhrel et al. 2012). Studies performed in a variety of organisms indicate AgNP toxicity, which may be ascribed to different mechanisms, including the disruption of the integrity of the cell membrane (Suresh et al. 2010), damage and binding of the proteins and/or DNA (Arora et al. 2009; Domazet Jurašin et al. 2016), and formation of reactive oxygen species (ROS) (Hsin et al. 2008; Vinković Vrček et al. 2016).

The potential ways for terrestrial plants to be exposed to AgNPs are possible leaching from nano-enabled products (Pirela et al. 2015), intentional sub-surface release for environmental remediation (Tran et al. 2013), irrigation using contaminated surface water (Pokhrel and Dubey 2013), land applications of contaminated biosolids (Colman et al. 2013), or waste water effluent discharge (Farkas et al. 2011). As plants are primary producers and a vital part of ecosystem, the phytotoxic effects of NPs should be given particular attention in future studies. Toxicological studies that investigated the impact of AgNPs on plants suggest their uptake, accumulation, and translocation in different plant organs and thus consequences on growth and development processes (Yin et al. 2012; Dimkpa et al. 2013; Vinković et al. 2017; Cvjetko et al. 2017). Moreover, evaluations of oxidative damage to lipids, proteins, and DNA molecule as well as changes in the activity of plant hormones or antioxidant enzymes in plants treated with AgNPs (Dimkpa et al. 2013; Mirzajani et al. 2013; Yasur and Rani 2013; Vinković et al. 2017; Cvjetko et al. 2017) suggest that oxidative stress could have an important role in the phytotoxicity of AgNPs. Therefore, it is important to increase our knowledge about mechanisms of AgNP toxicity to ensure a controlled and safe implementation of AgNPs in a variety of agricultural products.

In this study, we investigated the potential phytotoxicity of citrate-coated AgNPs on an important crop plant, tobacco (*Nicotiana tabacum* L.), which is also a commonly used model organism in the research of abiotic stress (Gichner et al. 2004; Garnier et al. 2006; Peharec Štefanić et al. 2012; Tkalec et al. 2014). Adult tobacco plants were simultaneously exposed to the same concentrations of citrate-coated AgNPs and $AgNO_3$. Comparison of phytotoxic effects was performed for AgNP-exposed and $AgNO_3$-exposed plants due to the dissolution behavior of AgNPs (Lowry et al. 2012), and therefore, their toxicity is often related to the released Ag^+ (Kawata et al. 2009; Kim et al. 2009; Vinković Vrček et al. 2016; Vinković et al. 2017). Roots and leaves were analyzed separately to obtain information on silver uptake, induction of oxidative stress, as well as ultrastructural changes in plant tissue.

Materials and methods

Synthesis, characterization, and stability evaluation of AgNPs

AgNPs coated with citrate were prepared by reduction of $AgNO_3$ with sodium citrate as reported previously (Milić et al. 2015; Cvjetko et al. 2017). Purification of freshly prepared AgNP suspensions was performed immediately after synthesis by two washing steps with Milli-Q® (Millipore, 18.2 MΩ cm resistivity) water using 20-min centrifugation at 15,790×*g*, after which AgNPs were resuspended in Milli-Q® water and stored at 4 °C protected from light until use.

The physicochemical characteristics and stability of AgNPs were evaluated using a UV-Vis spectrophotometer (CARY 300, Varian Inc., Australia), Zetasizer Nano ZS (Malvern, UK), and a monochromated TF20 (FEI Tecnai G2) transmission electron microscope (TEM) as described by Cvjetko et al. (2017). For determination of the possible silver dissolution in Milli-Q® water, AgNP suspension was ultrafiltrated through Millipore Amicon Ultra-4 3K membranes. Total silver concentration in the AgNP suspension and the filtrates was determined in acidified solutions (10% *v*/*v* HNO_3) using an Agilent Technologies 7500cx inductively coupled plasma mass spectrometer (ICP-MS) (Agilent, Waldbronn, Germany). Furthermore, the 2 μL of AgNP stock solution was pipetted on a Formvar®/Carbon copper grid, air-dried, and examined by TEM.

Plant material and culture treatments

For this experiment, the *Nicotiana tabacum* L. cv Burley was used as a model plant. Plant material was cultivated as previously described (Peharec Štefanić et al. 2012; Tkalec et al. 2014). Briefly, seeds were surface sterilized with 50% (*v*/*v*) sodium hypochlorite and thoroughly washed with deH_2O. For seed germination and plant growth, 300-mL Erlenmeyer flasks were filled with 50-mL of Murashige and Skoog (1962) nutrient medium supplemented with 500 mg L^{-1} MES [2-(*N*-morpholino)ethanesulfonic acid], 1.5 g L^{-1} sucrose, and 2.2 g L^{-1} Phytagel (pH 5.6) (Gichner et al. 1999). Two tobacco seeds were placed on the surface of the culture medium in each of the 100 flasks and left to germinate (approximately 5 days). Germinated seeds were grown for 2 months in the same Erlenmeyer flask in the conditions of the growth chamber (16/8 light/dark cycle, light intensity of 90 mE m^{-2} s^{-1}, and temperature of 24 °C) in order to obtain adult plants with a fully developed root system and shoots with differentiated leaves. For exposure to silver applied in either nanoparticle (AgNP) or ionic ($AgNO_3$) form, plants of similar size (six plants per each treatment and control) were transferred to the Milli-Q® water supplemented with either AgNPs or $AgNO_3$ applied in the following concentrations:

25, 50, 75, 100, or 500 μM. Control plants were cultured in Milli-Q® water devoid of silver. Plants were exposed to treatment for 7 days. Leaves and roots were analyzed separately. Experiments were performed in three biological replicates, each with six technical replicates.

Determination of Ag content

In order to remove the AgNPs that adhered to root tissue, roots of adult plants were washed with 0.01 M HNO_3 and rinsed with ultrapure Milli-Q® water. Roots and leaves were dried in a microwave oven for 24 h at 80 °C until constant weight was obtained. The tissue was powdered using mortar and pestle and acid digested with a mixture of 65% HNO_3 and 37% H_2O_2 (ratio 3:1, respectively) in a microwave oven (ETHOS SEL Milestone, Shelton, CT, USA) according to the EPA 3051a method. The samples were cooled and subsequently diluted with 1% (*v*/*v*) HNO_3 until a final volume of 50 mL was obtained. For determination of the total Ag concentration, an ELAN DRC-e ICP-MS (PerkinElmer, USA) instrument was applied. The calibration curve obtained with a set of standards of known concentrations was used to calculate the silver concentration. The detection limit and limit of quantification (LOQ) were 0.05 and 0.1 mg kg^{-1}, respectively. Spike recovery tests were 95.2 and 95.6% for leaves of AgNP-treated and $AgNO_3$-treated plants, respectively, and 96.6 and 96.8% for roots of AgNP-treated and $AgNO_3$-treated plants, respectively.

ROS determination

ROS level was determined by fluorescence microscopy by application of fluorescent dye dihydroethidium (DHE) as described previously (Cvjetko et al. 2017). After the treatment with AgNPs, roots were thoroughly washed with water and subsequently incubated at room temperature in the dark for 30 min in 10 μM DHE solution. Leaf fragments were taken from the exposed plants and incubated in 10 μM DHE solution under the same conditions as described for the roots. Afterwards, the tissue was thoroughly washed, transferred to the microscopic slides, and analyzed with Olympus BX-51 (Olympus, Tokyo, Japan) fluorescence microscope coupled to the high-resolution camera Olympus DP70 (Olympus, Tokyo, Japan). Excitation wavelengths were 450–490 nm, and emission wavelengths were 520 nm or more. The obtained images were analyzed for fluorescence intensity with computer software Lucida 6.0 (Wirral, UK) by manually marking regions of interest (ROI) in 100 randomly chosen cells per treatment. The presented results are expressed according to the relative intensity compared to control tobacco roots or leaves.

Protein extraction

Proteins were extracted by grinding 200 mg of leaves and 400 mg of roots (all fresh tissue) in 1 mL of 50 mM potassium phosphate buffer, pH 7.0. The 50 mg of insoluble polyvinylpyrrolidone (PVP) was added to the plant material prior to grinding. The homogenates were centrifuged for 15 min at 20,000×*g* at 4 °C, after which supernatants were collected and re-centrifuged for 60 min at 20,000×*g* at 4 °C. Protein concentration was measured according to the Bradford (1976) method. Bovine serum albumin was used as a standard. These supernatants were subsequently used for carbonyl quantification and assays of enzymatic activity.

Malondialdehyde and protein carbonyl content

The lipid peroxidation level was determined by measuring the content of malondialdehyde (MDA), according to the modified method of Heath and Packer (1968). A total of 200 mg of fresh leaves and 400 mg of fresh roots were homogenized in 1.3 mL of 0.3% (*w*/*v*) 2-thiobarbituric acid (TBA) prepared in 10% (*w*/*v*) trichloroacetic acid (TCA), and incubated at 95 °C for 30 min. The cooled mixtures were then centrifuged for 1 h at 20,000×*g* and + 4 °C. The absorbance of the supernatant was measured at 532 nm. For correction of nonspecific turbidity, subtraction of the absorbance recorded 600 nm was done. For calculation of lipid peroxide content, a molar absorption coefficient for MDA (155 mM^{-1} cm^{-1}) was used and the content was expressed as micromole per gram of fresh weight.

The reaction with 2,4-dinitrophenylhydrazine (DNPH) was used for protein carbonyl quantification according to Levine et al. (1990). A total of 200 μL of protein supernatants was combined with 300 μL of 10 mM DNPH in 2 M HCl and incubated for 1 h at room temperature protected from light. Afterwards, the proteins were precipitated with 500 μL of cold 10% (*w*/*v*) TCA. Samples were cooled at − 20 °C and then centrifuged for 10 min at 20,000×*g* and + 4 °C. In order to remove excess reagent, the pellets were washed with 500 μL of ethanol/ethylacetate (1/1 *v*/*v*) three times. The precipitated proteins were dissolved in 6 M urea in 20 mM potassium phosphate buffer (pH 2.4) in an ultrasonic bath. Absorbance was measured at 370 nm. For protein recovery estimation, the absorbance of each sample was measured at 280 nm. Protein carbonyl content was calculated using a molar absorption coefficient for aliphatic hydrazones of 22 mM^{-1} cm^{-1} and expressed as micromole per milligram of proteins.

Comet assay

The Comet assay was performed according to Gichner et al. (2004) with previously published modifications (Balen et al. 2011; Cvjetko et al. 2017). Briefly explained, after the

mechanical isolation of nuclei in 400 mM Tris-HCl (pH 7.5) at 4 °C, they were mixed in equal volumes (50 μL) with low melting point agarose (LMP, 1% (*w*/*v*)). After 10 min of denaturation (for DNA unwinding) and 20 min of electrophoresis at 0.8 V cm^{-1} and 300 mA in the freshly prepared buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13), slides were neutralized, air-dried, and subsequently stained with 70 mL ethidium bromide (20 mg mL^{-1}) for 5 min. A computerized image analysis system (Komet version 5, Kinetic Imaging Ltd., Liverpool, UK) was used to measure the tail DNA percentage (% tDNA) as the primary measure of DNA damage.

Assays of enzymatic activities

All enzymatic assays were done at 25 °C, and all spectrophotometric analyses were conducted in a UV/visible spectrometer (ATI UNICAM UV4, Cambridge, UK).

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was determined by following the method of Beauchamp and Fridovich (1971). The reaction mixture was composed out of 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 0.1 M EDTA, and 2 mM riboflavin supplemented with different volumes of enzyme extracts in 50 mM phosphate buffer (pH 7.8). All mixtures were kept for 8 min in a 15 W light box, after which the formazan formation produced by NBT photo reduction was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to generate 50% inhibition of the NBT reduction rate. Activity was expressed as units of SOD activity per milligram of protein.

Pyrogallol peroxidase (PPX, E.C. 1.11.1.7) activity was evaluated by measuring the absorbance increase at 430 nm as a result of the pyrogallol oxidation ($\varepsilon = 2.6$ mM^{-1} cm^{-1}), by following the method of Nakano and Asada (1981). The reaction mixture was composed out of 50 mM potassium phosphate buffer (pH 7.0), 20 mM pyrogallol, and 1 mM H_2O_2, to which 20 μL of enzyme extract was added. The activity of PPX was calculated as micromole of purpurogallin (product of pyrogallol oxidation) per minute per milligram of protein.

For ascorbate peroxidase (APX, E.C. 1.11.1.11) activity, the decrease in absorbance at 290 nm ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) was estimated (Nakano and Asada 1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM ascorbate, and 0.12 mM H_2O_2, supplemented with 180 mL of enzyme extract. The activity of APX was expressed as micromole of ascorbate oxidized per minute per milligram of protein.

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease in absorbance at 240 nm ($\varepsilon =$ 36 mM^{-1} cm^{-1}) every 10 s during 2 min, as described by Aebi (1984). The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2, to which 50 μL of enzyme extract was added. CAT activity was calculated as micromole of decomposed H_2O_2 per minute per milligram of protein.

Microscopic analyses

For localization of AgNPs in plant cells and ultrastructural analyses of 100 μM AgNPs and 100 μM $AgNO_3$-treated tobacco plants, small pieces of tobacco tissue (root and leaf) were fixed with 1% (*w*/*v*) glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) for 1 h at + 4 °C, washed twice with cold 50 mM cacodylate buffer (pH 7.2) and post-fixed with 1% (*w*/*v*) osmium tetroxide in the same buffer for 1 h at + 4 °C, followed by a 10-min wash in ice-cold water. After dehydration in a graded series of ethanol, the tissue was embedded in Spurr's resin. Semi-thin sections of fixed material were stained with mixture of 2% (*w*/*v*) toluidine blue and 2% (*w*/*v*) borax and examined using a light microscope. Ultrathin sections were stained with 2% (*w*/*v*) uranyl acetate and 2% (*w*/*v*) lead citrate and examined using a FEI Morgagni 268D electron microscope for ultrastructural study and monochromated TF20 (FEI Tecnai G2) TEM for confirmation of AgNP localization in the tobacco cells.

Statistical analysis

All results represent the mean values ± standard errors (SE) of three biological replicates, each with six technical replicates. The results of each assay were compared by analysis of variance (ANOVA), followed by Duncan test using the STATISTICA 12.0 (Stat Soft Inc., USA) software package. Differences among means were considered statistically significant at $p \leq 0.05$.

Results

Characterization and stability evaluation of AgNPs

Characterization of purified citrate-coated AgNPs was performed in Milli-Q® water using the UV-Vis spectroscopy, TEM, dynamic light scattering (DLS), and electrophoretic light scattering (ELS). The position of the SPR peak at 415 nm in UV-Vis spectra confirmed the nanosized dispersion in Milli-Q® water for citrate-coated AgNPs. DLS measurements showed bimodal volume size distribution where the larger AgNP population (~ 80%) had a size of 61.2 ± 33.9 nm and the smaller (~ 20%) was characterized by 13.8 ± 4.9 nm in size. The recorded TEM micrographs were in accordance with DLS data revealing the presence of spherical and rod-like AgNPs (Fig. 1a). Electron dispersive X-ray (EDX) analysis confirmed that all of the detected particles contained silver (Fig. 1b, c). The ζ potential measurements revealed a negative surface charge of citrate-coated AgNPs

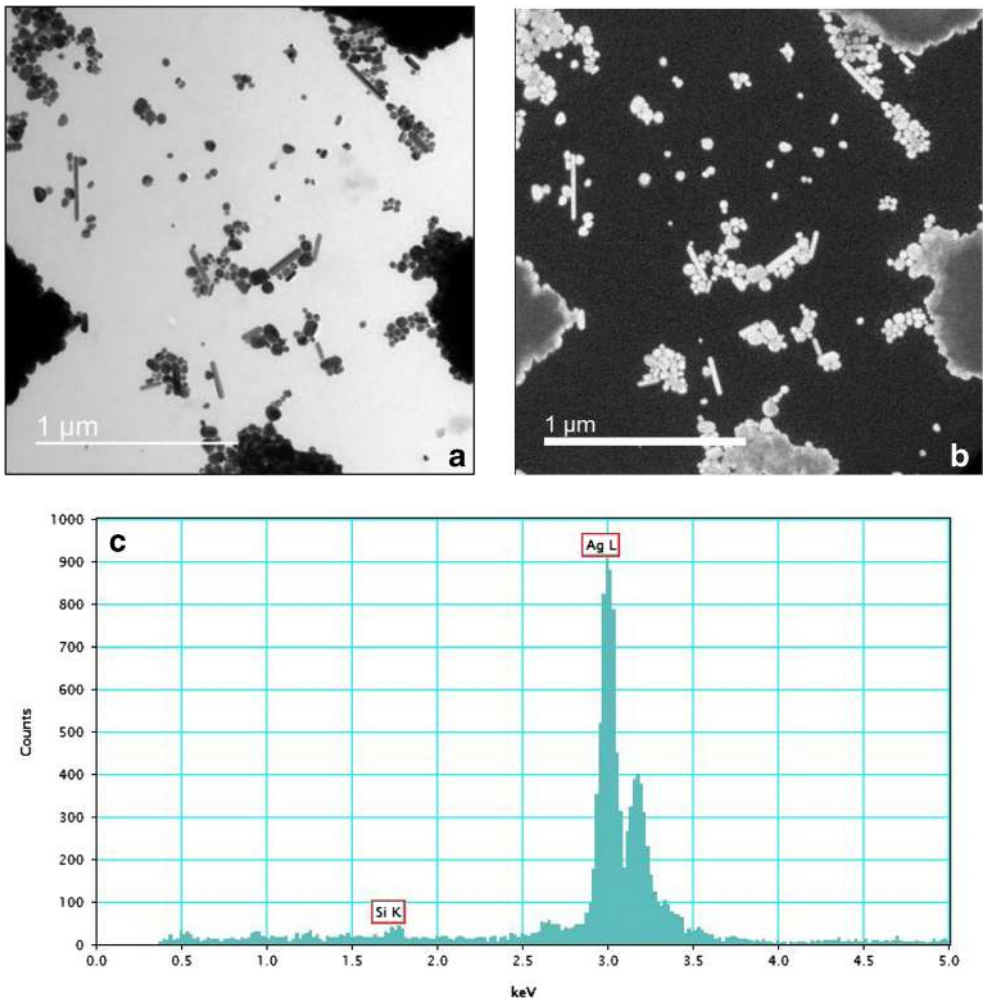


Fig. 1 Citrate-coated AgNPs investigated by transmission electron microscopy. **a** TEM image. **b** Bright field image. **c** Energy-dispersive X-ray spectrum

characterized by a ζ potential value of − 39.8 ± 3.4 mV. This value indicates strong electrostatic stabilization of the AgNPs through the ionization of the polar citrate carboxyl groups on the surface. Determination of silver dissolution in Milli-Q® water showed a level of free Ag^+ in AgNP suspension lower than 0.5%.

Silver uptake

Roots of tobacco plants exposed to either AgNPs or $AgNO_3$ exhibited a significant increase in Ag uptake, which was of similar values at corresponding AgNP and $AgNO_3$ concentrations. Ag accumulation elevated linearly with the increase of the applied concentration in both types of treatments, although there was no significant difference between the treatments. Exposure to the 500 μM AgNPs resulted with the highest value, which however did not significantly differ compared to the corresponding $AgNO_3$ treatment (Table 1).

Ag uptake in leaf tissue was significantly lower (100 to 30 times for AgNP treatments and 70 to 30 times for $AgNO_3$ treatments) compared to roots, although a similar pattern was obtained. Ag accumulation linearly increased after exposure to either AgNPs or $AgNO_3$, and it was not significantly different between corresponding AgNPs and $AgNO_3$ treatments. Significantly, the highest values were recorded in leaves of plants treated with the highest concentration (500 μM) of both AgNPs and $AgNO_3$ (Table 1).

In situ ROS formation

Among the applied AgNP and $AgNO_3$ treatments, only exposure to 100 and 500 μM $AgNO_3$ resulted with significantly higher ROS formation compared to the control in root tissue (Table 2), while similar values to control were obtained in leaves in all of the treatments (Table 3).

Effect on lipid peroxidation

In root tissue, no significant difference in MDA content was observed in AgNP treatments compared to control (Table 2). A significant increase in lipid peroxidation level was detected in roots of plant exposed to the majority of $AgNO_3$ treatments,

Table 1 Silver content (mg g^{-1} DW) in roots and leaves of tobacco plants after treatment with AgNP and $AgNO_3$

Treatment	Roots		Leaves	
	AgNP	$AgNO_3$	AgNP	$AgNO_3$
Control	< 0.0001a	< 0.0001a	< 0.0001a	< 0.0001a
25 μM	1.247 ± 122.01b	1.121 ± 136.24b	0.012 ± 0.002b	0.018 ± 0.004b
50 μM	1.395 ± 351.54b	1.450 ± 436.16b	0.014 ± 0.002b	0.021 ± 0.007b
75 μM	1.712 ± 80.86bc	1.741 ± 134.45bc	0.019 ± 0.001b	0.023 ± 0.004b
100 μM	1.742 ± 192.87bc	1.747 ± 150.02bc	0.036 ± 0.003c	0.038 ± 0.005c
500 μM	2.480 ± 141.97d	2.399 ± 310.64cd	0.079 ± 0.005d	0.082 ± 0.004d

Values are the means ± SE of three biological replicates, each with six technical replicas. If values are marked with different letters, the means are significantly different ($p \leq 0.05$) according to Duncan test. In control samples, Ag was detected below the instrument's limit of quantification (LOQ < 0.0001 mg g^{-1})

in comparison to the control tissue. Higher MDA content was found in all $AgNO_3$ treatments compared to the corresponding AgNP treatments, although at 25 and 500 μM, the values were not significantly different (Table 2).

In leaves, significantly elevated MDA content compared to the control was found only in plants exposed to the highest (500 μM) AgNP concentration, while $AgNO_3$ induced it at 100 and 500 μM (Table 3). Higher MDA content in $AgNO_3$ treatment than in the corresponding AgNPs treatment was found at 100 μM (Table 3).

Effect on protein oxidation

Exposure to AgNPs did not induce a difference in protein carbonyl content in root tissue after exposure to any of the applied concentrations in comparison to control plants (Table 2). On the contrary, the carbonyl content was significantly increased after exposure to $AgNO_3$ compared to control as well as corresponding AgNP treatments, starting from the lowest applied concentration (25 μM) (Table 2).

In leaf tissue, AgNP treatments did not significantly increase protein carbonyl content, while exposure to $AgNO_3$ resulted with significantly elevated carbonyl content compared to the control in all of the treatments with the exception of the lowest applied concentration (25 μM) (Table 3). However, there were no differences between $AgNO_3$ and AgNP treatments.

Effect on DNA

None of the applied AgNP concentrations induced increased DNA tail in roots of the adult plants compared to the control (Table 2). On the contrary, $AgNO_3$ treatments induced significant DNA damage in root tissue at all of the applied concentrations (Table 2).

As for the leaves, among the investigated treatments, only 100 and 500 μM $AgNO_3$ induced a significant increase in DNA tail compared to the control value (Table 3).

Effect on antioxidant enzyme activity

In root tissue, AgNP treatments did not induce significant changes in SOD activity compared to the control, even though after exposure to 100 and 500 μM, lower values were obtained (Table 2). $AgNO_3$ applied at 25, 50, and 75 μM resulted with higher values, although after the treatment with 75 μM $AgNO_3$, it was not statistically different compared to the control. At 25 and 50 μM, SOD activities in $AgNO_3$-treated plants were higher than in the corresponding AgNP treatments (Table 2). In leaves of plants exposed to either AgNPs or $AgNO_3$, no significant changes in SOD activity were measured in any of the tested concentrations (Table 3).

None of the applied AgNP concentrations induced changes in PPX activity in root tissue compared to the control (Table 2). On the contrary, all of the applied $AgNO_3$ concentrations significantly induced PPX activity compared to the control as well as to the corresponding AgNP concentrations (Table 2). In leaf tissue, AgNP treatments reduced the PPX activity when applied at the highest concentrations (100 and 500 μM) compared to the control as well as corresponding $AgNO_3$ treatments, while exposure to $AgNO_3$ at 50 and 75 μM concentrations resulted in an increase of PPX activity (Table 3).

AgNP and $AgNO_3$ had a similar impact on root APX activity; treatments with lower concentrations (25, 50, and 75 μM) exhibited similar values as the control (Table 2), while higher concentrations (100 and 500 μM) significantly decreased the APX activity. However, a decrease was somewhat more pronounced in AgNP compared to $AgNO_3$ treatments. In leaves, no significant changes in APX activity were measured after exposure to either AgNPs or $AgNO_3$ applied at any of the tested concentrations (Table 3).

In root tissue, $AgNO_3$ and particularly AgNP treatments in general induced an increase in CAT activity especially after exposure to 25, 50, 75, and 100 μM AgNPs and 25, 50, and 75 μM $AgNO_3$ (Table 2). However, at 100 μM, higher CAT activity was measured after exposure of plants to AgNPs than

Table 2 Contents of reactive oxygen species (ROS), malondialdehyde (MDA), protein carbonyl, and % tail DNA as well as differences in specific activities of superoxide dismutase (SOD), pyrogallol peroxidase (PPX), ascorbate peroxidase (APX), and catalase (CAT) in roots of tobacco plants 7 days after exposure to 25, 50, 75, 100, and 500 μM AgNPs and $AgNO_3$

	Conc. (μM)	ROS (% of control)	MDA (μmol g^{-1} FW)	Protein carbonyl (μmol mg^{-1} prot.)	% Tail DNA	SOD activity (U mg^{-1} prot.)	PPX activity (U mg^{-1} prot.)	APX activity (U mg^{-1} prot.)	CAT activity (U mg^{-1} prot.)
Control	0	100 ± 1.15a	2.85 ± 0.27a	0.11 ± 0.01a	5.37 ± 0.45a	2.69 ± 0.64ab	16.23 ± 0.69a	0.38 ± 0.02a	0.04 ± 0.01a
AgNP	25	116.54 ± 3.64a	3.25 ± 0.23ab	0.12 ± 0.01a	5.62 ± 0.37a	1.96 ± 0.40ab	13.87 ± 0.75a	0.38 ± 0.03a	0.09 ± 0.02bc
	50	117.64 ± 6.11ab	2.97 ± 0.34a	0.12 ± 0.01a	6.37 ± 0.50a	3.00 ± 0.62ab	13.11 ± 1.08a	0.31 ± 0.05ab	0.10 ± 0.01bc
	75	114.07 ± 6.83ab	3.09 ± 0.27a	0.13 ± 0.01a	6.46 ± 0.48a	1.69 ± 0.71ab	13.09 ± 1.40a	0.34 ± 0.05a	0.11 ± 0.02bc
	100	106.11 ± 2.16a	2.79 ± 0.34a	0.13 ± 0.01a	6.84 ± 0.48a	0.90 ± 0.15a	13.64 ± 0.91a	0.19 ± 0.02c	0.08 ± 0.01b
	500	109.95 ± 4.57a	2.83 ± 0.38a	0.13 ± 0.01a	6.36 ± 0.46a	0.73 ± 0.12a	12.83 ± 0.96a	0.18 ± 0.03c	0.05 ± 0.01ab
$AgNO_3$	25	114.51 ± 5.64ab	3.32 ± 0.28bc	0.21 ± 0.01b	9.40 ± 0.64b	5.38 ± 0.66cd	20.02 ± 1.14b	0.38 ± 0.03a	0.07 ± 0.01b
	50	116.59 ± 4.58ab	3.58 ± 0.13bc	0.25 ± 0.01bc	12.56 ± 0.75d	5.72 ± 0.92d	26.81 ± 2.17c	0.30 ± 0.03ab	0.06 ± 0.01b
	75	116.34 ± 6.39ab	3.75 ± 0.19c	0.24 ± 0.1bc	10.73 ± 0.69bc	3.63 ± 0.71bcd	21.84 ± 1.12bc	0.27 ± 0.03ab	0.06 ± 0.01b
	100	131.35 ± 4.12b	3.42 ± 0.19bc	0.24 ± 0.01bc	11.47 ± 0.72cd	2.66 ± 0.72ab	20.78 ± 0.82b	0.26 ± 0.03bc	0.04 ± 0.01a
	500	128.12 ± 8.54b	3.2 ± 0.20abc	0.27 ± 0.01bc	11.02 ± 0.55bcd	2.32 ± 0.93ab	21.95 ± 2.19b	0.26 ± 0.03bc	0.04 ± 0.01a

Values are the means ± SE of three biological replicates, each with six technical replicas. If values are marked with different letters, the means are significantly different ($p \leq 0.05$) according to Duncan test

Table 3 Contents of reactive oxygen species (ROS), malondialdehyde (MDA), protein carbonyl, and % tail DNA as well as differences in specific activities of superoxide dismutase (SOD), pyrogallol peroxidase (PPX), ascorbate peroxidase (APX), and catalase (CAT) in leaves of tobacco plants 7 days after exposure to 25, 50, 75, 100, and 500 μM AgNPs and $AgNO_3$

	Conc. (μM)	ROS (% of control)	MDA (μmol g^{-1} FW)	Protein carbonyl (μmol mg^{-1} prot.)	% Tail DNA	SOD activity (U mg^{-1} prot.)	PPX activity (U mg^{-1} prot.)	APX activity (U mg^{-1} prot.)	CAT activity (U mg^{-1} prot.)
Control	0	100 ± 1.03a	7.29 ± 0.53a	0.07 ± 0.01a	3.08 ± 0.23a	1.19 ± 0.14a	3.46 ± 0.38bc	0.12 ± 0.01a	0.17 ± 0.01de
AgNP	25	95.33 ± 0.80a	8.37 ± 0.59ab	0.09 ± 0.01ab	3.39 ± 0.24abc	1.17 ± 0.09a	3.48 ± 0.31bc	0.13 ± 0.02a	0.15 ± 0.01cd
	50	93.60 ± 2.33a	8.84 ± 0.77abc	0.09 ± 0.01ab	3.76 ± 0.25abc	1.54 ± 0.03a	3.87 ± 0.36bcd	0.14 ± 0.02a	0.12 ± 0.01bc
	75	95.97 ± 2.50a	8.91 ± 0.39abc	0.09 ± 0.01ab	3.69 ± 0.27abc	1.79 ± 0.07a	3.16 ± 0.15b	0.13 ± 0.02a	0.10 ± 0.01ab
	100	93.61 ± 1.68a	9.23 ± 0.93abc	0.10 ± 0.01ab	3.25 ± 0.26ab	1.38 ± 0.18a	1.61 ± 0.07a	0.13 ± 0.02a	0.10 ± 0.01a
	500	91.88 ± 2.28a	10.22 ± 0.47bcd	0.09 ± 0.01ab	3.19 ± 0.27ab	1.43 ± 0.16a	1.61 ± 0.06a	0.13 ± 0.02a	0.10 ± 0.01ab
$AgNO_3$	25	93.67 ± 3.70a	8.13 ± 0.43a	0.09 ± 0.01ab	3.16 ± 0.26a	1.54 ± 0.13a	4.34 ± 0.22cd	0.13 ± 0.15a	0.28 ± 0.01fg
	50	94.81 ± 2.47a	8.22 ± 0.18ab	0.11 ± 0.01b	3.82 ± 0.30abc	1.61 ± 0.0 a	4.60 ± 0.51d	0.12 ± 0.15a	0.28 ± 0.01fg
	75	95.54 ± 3.38a	9.27 ± 0.41abc	0.12 ± 0.01b	3.59 ± 0.31abc	1.73 ± 0.29a	5.16 ± 0.50d	0.12 ± 0.13a	0.32 ± 0.01g
	100	93.77 ± 2.41a	11.23 ± 0.45d	0.10 ± 0.01b	4.10 ± 0.32bc	1.58 ± 0.12a	4.49 ± 0.41cd	0.11 ± 0.09a	0.26 ± 0.01ef
	500	91.52 ± 2.03a	10.56 ± 0.36cd	0.10 ± 0.01b	4.24 ± 0.32c	1.64 ± 0.14a	3.93 ± 0.34bcd	0.11 ± 0.06a	0.23 ± 0.01ef

Values are the means ± SE of three biological replicates, each with six technical replicas. If values are marked with different letters, the means are significantly different ($p \leq 0.05$) according to Duncan test

to $AgNO_3$. In leaves, all of the applied AgNP concentrations significantly decreased the CAT activity. On the contrary, the effect of $AgNO_3$ treatments on CAT activity was concentration dependent; lower concentrations of $AgNO_3$ (25 and 50 μM) had no significant impact on CAT activity, 75 μM $AgNO_3$ significantly induced the CAT activity, while at 100 and 500 μM, reduced values were obtained compared to the control (Table 3). At all of the applied concentrations, CAT activity was higher in plants exposed to $AgNO_3$ than in those exposed to AgNPs.

Microscopic study and in situ AgNP localization

Microscopy observations revealed that the 100 μM concentration of both AgNP and $AgNO_3$ treatments induced the high vacuolization of root cells compared to the control (Fig. S1; Fig. 2a–c), and due to large vacuoles, only nuclei could be observed within the cells (Fig. 2b, c). Moreover, root cells of $AgNO_3$-treated plants were partly destroyed, while nuclei were highly damaged (Fig. 2c). After exposure to AgNP treatment, the AgNPs were visible as black dots mainly near the cell wall and inside the root cells (Fig. 3a). Therefore, root cells were further examined by TEM-EDX. Figure 3 shows that AgNPs were localized in the root cells and in the intermembrane space (Fig. 3b–e). The EDX scan confirmed that the particles found in the TEM images contained silver (Fig. 3f), which proves the direct uptake of AgNPs and their accumulation in the root cells.

Leaf semithin sections showed no significant changes in the cell organization, except the difference in the leaf thickness (Fig. S2). As for the leaf TEM study, changes were mainly revealed in the size of chloroplasts of both AgNP-treated

Fig. 3 Localization of AgNPs, at the ultrastructural level, in the tobacco root cells from the 100 μM AgNP-treated plants. TEM images of silver nanoparticles (**a**, **b**, **d**), bright field images (**c**, **e**), and energy-dispersive X-ray spectrum (**f**). AgNPs silver nanoparticles indicated by arrows

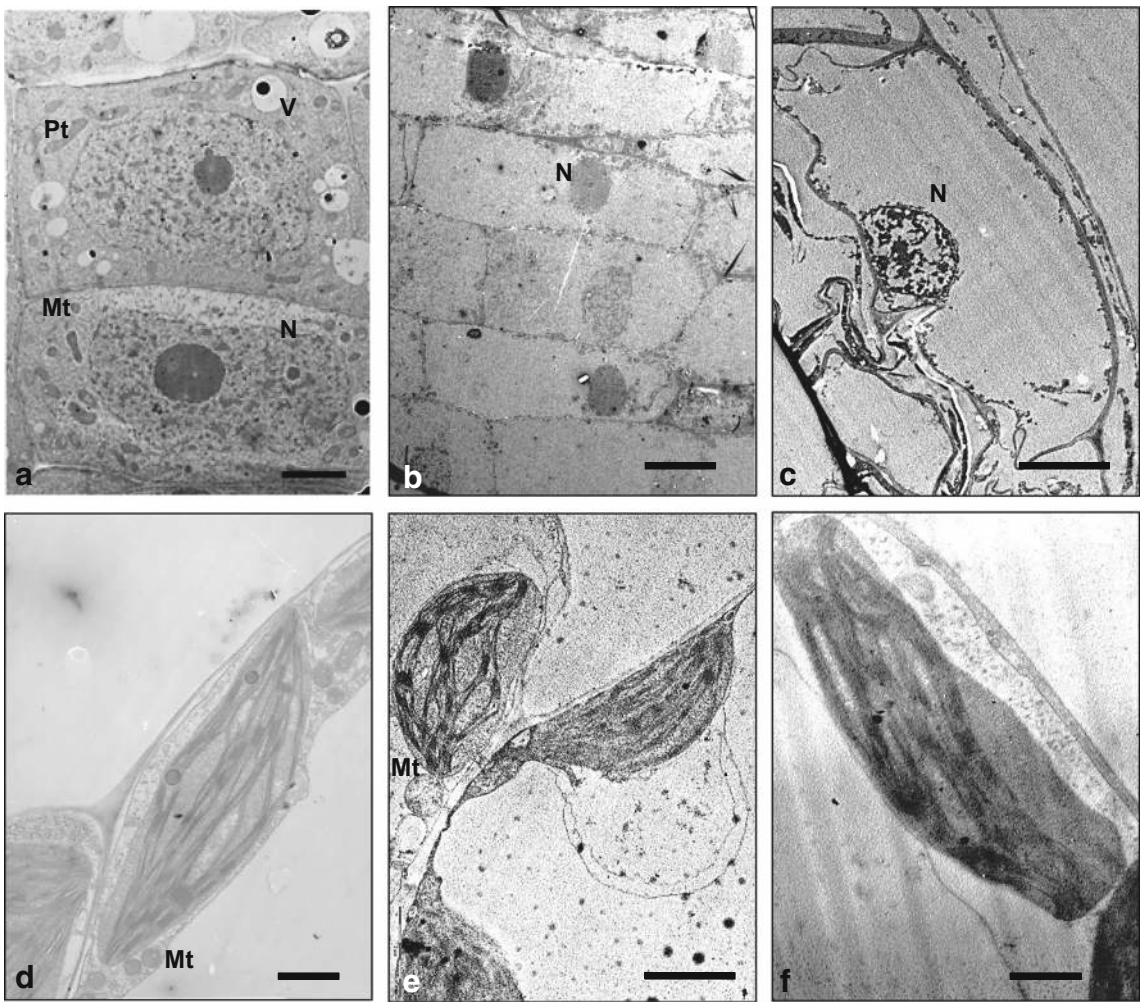
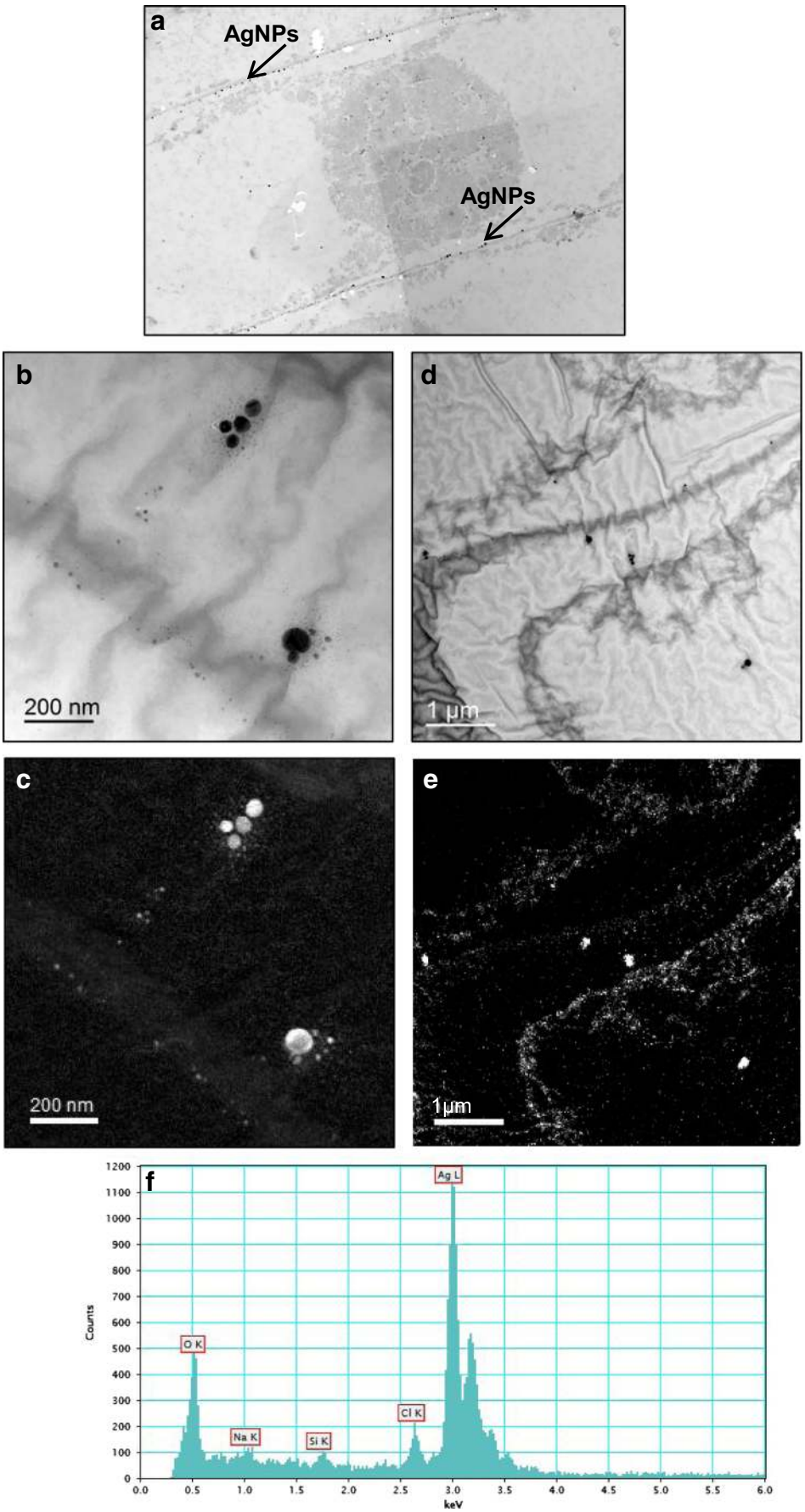


Fig. 2 Ultrastructure of root and leaf cells. Root cells from **a** control plant (bar = 2 μm), **b** plant treated with 100 μM AgNPs (bar = 5 μm), and **c** plant treated with 100 μM $AgNO_3$ (bar = 5 μm). Leaf cells from **d** control plant (bar = 1 μm), **e** plant treated with 100 μM AgNPs (bar = 2 μm), and **f** plant treated with 100 μM $AgNO_3$ (bar = 1 μm). N nucleus, V vacuole, Mt mitochondrion, Pt plastid



and $AgNO_3$-treated plants; after exposure to AgNPs, chloroplasts were smaller (Fig. 2e) compared to chloroplasts in the control cells (Fig. 2d), while those found in leaf cells of $AgNO_3$-treated plants were bigger (Fig. 2f) than the control ones. After exposure to AgNPs, chloroplasts were somewhat swollen and ruptured, although the thylakoid system in all of the examined chloroplasts was well developed with no significant changes between treatments and control. In the leaf tissue, AgNPs could not be detected.

Discussion

In our experiment, the Ag concentration in roots was 30 to 100 times higher than in leaf tissue after both AgNP and $AgNO_3$ treatments, which indicates the predominant accumulation of Ag in the roots of tobacco plants. Shtangeeva et al. (2011) reported that after exposure of wheat plants to Ag_2SO_4 and $AgNO_3$, Ag was mostly accumulated in the roots, while concentrations of Ag in leaves remained at the control level. Moreover, it was shown that *Arabidopsis* roots treated with AgNPs may bio-accumulate up to 10 times more Ag than shoots (Geisler-Lee et al. 2014).

Studies performed on several plant species suggest that Ag accumulation was often found to be much lower in treatments with AgNPs compared to $AgNO_3$ (Yasur and Rani 2013; Pokhrel and Dubey 2013; Vinković et al. 2017; Cvjetko et al. 2017). However, in the current study, Ag accumulation in roots and leaves of tobacco plants was equally efficient after both AgNP and $AgNO_3$ treatments. Geisler-Lee et al. (2012) reported that at lower corresponding concentrations of AgNPs and $AgNO_3$, Ag accumulation in roots of *Arabidopsis* plants was higher in AgNP treatments compared to those by $AgNO_3$, while at higher concentrations, $AgNO_3$ induced a similar Ag uptake as AgNPs. Moreover, Qian et al. (2013) found higher Ag uptake in leaves of *Arabidopsis* plants exposed to the AgNPs compared to the values obtained after the treatment with the same $AgNO_3$ concentration. Nair and Chung (2014a) suggested that the possible reason for high Ag accumulation in AgNP-exposed plants might be the direct uptake of AgNPs by plants or that the AgNPs could get oxidized on the root surface as Ag^+, which could enter into the root tissue directly without dissolving in the solution. In our study, the direct AgNP uptake by root cells was confirmed by TEM and EDX analysis, although oxidation on the root surface cannot be excluded.

The high Ag concentration in the roots of AgNP-treated and $AgNO_3$-treated tobacco plants found in our study is in good correlation with the results of microscopy. To be more precise, the root tip cells were highly vacuolated after exposure to either 100 μM AgNPs or 100 μM $AgNO_3$ compared to the control, which indicates that vacuoles might be the primary storage for accumulated silver. High vacuolization of root tip cells after AgNP and $AgNO_3$ treatments was also reported for *Eruca sativa* (Vannini et al. 2014) and *Lolium multiflorum* (Yin et al. 2011), although in these studies, the effects were less severe in the root cells treated with $AgNO_3$. In our study, the ultrastructural analysis revealed black dots in the roots cells and EDX scan confirmed that these particles contained silver. In the studies of other authors, AgNPs were localized inside the vacuoles of rice (Mazumdar and Ahmed 2011) and *Brassica campestris* root cells (Mazumdar 2014) as well as in the plasmodesmata, cell wall, and middle lamella of *Arabidopsis* root cells (Geisler-Lee et al. 2012), which is in good correlation with our results.

Metal-induced stress very often results with increased ROS generation in plants (Balen et al. 2011; Tkalec et al. 2014), and several studies have reported that AgNPs and $AgNO_3$ may induce oxidative stress in plant cells (Jiang et al. 2014; Nair and Chung 2014b; Barbasz et al. 2016). In the current study, however, the exposure of tobacco plants to AgNPs and $AgNO_3$ induced an increase of ROS formation in root tissue only after exposure to 100 and 500 μM $AgNO_3$, while no changes were recorded after treatments with AgNPs. Furthermore, none of the applied AgNP concentrations induced a significant increase, either in MDA and protein carbonyl content or in tail DNA in tobacco plant roots. This is in accordance with the low ROS levels obtained after AgNP treatments, but is opposite to the results reported for wheat cultivars (Barbasz et al. 2016), rice (Nair and Chung 2014b), and *Arabidopsis* (Nair and Chung 2014a) exposed to AgNPs, where increased lipid peroxidation and protein oxidation were found. In our previous study, when the citrate-coated AgNPs were applied for treatments of *Allium cepa* roots, significantly higher MDA and protein carbonyl content as well as increase in ROS was obtained after treatments with 50, 75, and 100 μM (Cvjetko et al. 2017). As for the DNA damage, Vannini et al. (2014) found no changes at DNA level in wheat seedlings exposed to AgNP, while Cvjetko et al. (2017) also reported that treatments of *A. cepa* with citrate-coated AgNPs did not increase DNA damage, which corroborates our findings. Results of ROS formation and oxidative stress parameters indicate that sensitivity to AgNPs is very much dependent on the plant species. As for exposure to $AgNO_3$, the majority of the treatments induced significant increase in lipid peroxidation, protein oxidation, and DNA damage in root tissue compared to the control, which is in accordance with previously published results on toxicity of ionic Ag (Nair and Chung 2014a; Cvjetko et al. 2017). Taking into consideration that the root tissue exhibited similar Ag uptake after both AgNP and $AgNO_3$ treatments and that AgNP treatment did not induce elevated values of the oxidative stress parameters, it can be concluded that the Ag accumulated in root tissue after AgNP treatments remained mainly in the form of nanoparticles, which were of high stability and did not release ionic silver after entering the cells, thus showing much less toxicity compared to ionic Ag.

In leaves, none of the applied AgNP concentrations induced changes in ROS formation, MDA and protein carbonyl content, or DNA damage. As for exposure to $AgNO_3$, the most prominent effect was observed in protein carbonyl content, which significantly increased at almost all of the treatments, although the obtained values were much lower than in root tissue for corresponding concentrations. Moreover, lipid peroxidation and DNA damage occurred only at the two highest applied concentrations (100 and 500 μM). Since the Ag accumulation in leaves was many times lower than in roots after both AgNP and $AgNO_3$ treatments, the weaker effects found in leaf tissue were somewhat expected, thus confirming that the majority of the accumulated Ag remained in the root cells and that only a small portion of Ag was translocated to leaves. In several other studies, $AgNO_3$ was found to be more phytotoxic than AgNPs. For example, Nair and Chung (2014a) found increased ROS formation in $AgNO_3$-treated *Arabidopsis* seedlings compared to AgNP-treated ones, while Barbasz et al. (2016) reported that lipid peroxidation was higher in wheat callus exposed to $AgNO_3$ than AgNP treatments.

In roots, exposure to AgNPs in general did not induce significant changes in activity of SOD and PPX, while lower AgNP concentrations induced higher CAT activity. This is in good correlation with no measurable changes in oxidative stress parameters, indicating that AgNPs in higher concentrations induced mild oxidative stress, which could be efficiently alleviated by antioxidant enzymes. Contrary, $AgNO_3$ treatments resulted with elevated SOD, PPX, and CAT activity, which in line with increased values of oxidative stress parameters imply severe oxidative stress. Moreover, SOD and CAT activity after an initial increase at lower treatment concentrations showed a decline. These results are in good correlation with ROS content since the DHE test applied for ROS detection mostly measures formation of the superoxide radical (O^{2-}) (Cvjetko et al. 2017), which is neutralized by SOD. Hence, at higher $AgNO_3$ concentrations, insufficient SOD activity resulted with increased ROS formation. Under unstressed and mild stress conditions, the formation and removal of ROS are in balance due to proper activity of antioxidant enzymes. However, when ROS formation is too high, the defense system can be overwhelmed. When *Arabidopsis* plants were exposed to severe salt stress, a significant decrease in Cu-Zn-SOD activity was found, while exposure to mild salt stress resulted in increases of Cu-Zn-SOD activities (Alscher et al. 2002). Interestingly, for both types of treatments, APX activity decreased after exposure to 100 and 500 μM AgNPs and $AgNO_3$, which was more pronounced in AgNP treatments compared to $AgNO_3$. Cvjetko et al. (2017) also reported a decrease in APX as well as CAT activity in *A. cepa* roots treated with AgNPs and $AgNO_3$. On the other hand, increased CAT activity was recorded in *Spirodela polyrhiza* exposed to AgNPs (Jiang et al. 2014). Moreover, Hernandez-Viezcas et al. (2011) found that ZnO-NPs increased CAT activity in velvet mesquite roots, while APX activity remained at control values. Qian et al. (2013) reported that AgNPs and $AgNO_3$ induced the antioxidant capacity when *Arabidopsis* plants were exposed to relatively weak stress, but it was overwhelmed by continuous or high-intensity stress. Changes in activities of antioxidant enzymes in tobacco plants exposed to AgNP treatments might indicate stress imposed to roots although other parameters of oxidative stress do not imply so.

In leaf tissue, no changes were recorded in SOD and APX activities after either AgNP or $AgNO_3$ treatments, which is in line with the found unaltered ROS content. PPX activity increased significantly after 50 and 75 μM $AgNO_3$ treatments, but 100 and 500 μM AgNP treatments significantly decreased it. Although no AgNPs were detected in the leaves of exposed plants, treatment with 100 μM AgNPs induced alterations in leaf cell chloroplasts, mainly in size. The effects on chloroplast ultrastructure seemed to be a general stress response, because they have been described previously under different biotic and abiotic stress conditions (Hernández et al. 1995; Hernández et al. 2006; Popov et al. 2016). Moreover, Jiang et al. (2014) and Nhan et al. (2015) reported ultrastructural changes in chloroplasts of *Spirodela polyrhiza* exposed to AgNPs and cotton plants exposed to CeO_2-NPs, respectively. In our study, alterations in chloroplast ultrastructure correlated with the imbalance of the chloroplast antioxidant system, which was recorded as a strong decrease in PPX activity. In a study by Díaz-Vivancos et al. (2008), alterations in the chloroplast ultrastructure along with the decrease in the activities of APX, PPX, and CAT in pea plants subdued to biotic stress were recorded, although no changes in the oxidative stress parameters were observed, which is similar to the results obtained in our study. A decrease in PPX activity at higher AgNP concentrations, after an initial increase at lower concentrations, was recorded in leaves of *Pelargonium* plants (Hatami and Ghorbanpour 2013), while reduced CAT activity was noted in leaves of corn plants (Zhao et al. 2013) and green peas (Mukherjee et al. 2014) exposed to ZnO-NPs. Interestingly, although the antioxidant enzyme activities were not modified or were even reduced, AgNPs did not induce any significant oxidative stress in tobacco leaves, unlike $AgNO_3$. Since the silver concentration in leaves was similar in both treatments and much lower than in roots, it is possible that some of the changes observed in the leaves were just a consequence of the stressful events that took place in the roots.

Conclusion

Ag accumulated predominantly in the roots after exposure to both AgNPs and $AgNO_3$. The direct AgNP uptake by root cells was confirmed by TEM and EDX analyses. Root

meristem cells became highly vacuolated, which indicates that vacuoles might be the primary storage for accumulated silver. AgNPs were less phytotoxic for tobacco plants than $AgNO_3$ as treatments with $AgNO_3$, in general, induced oxidative stress in both tissues.

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