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RESEARCH ARTICLE

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Does plant growing condition affects biodistribution and biological effects of silver nanoparticles?

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Abstract

Among the many different types, silver nanoparticles (AgNPs) are the most commercialized and applied engineered nanoparticles in a wide range of areas, including agriculture. Despite numerous studies on their safety and toxicity of AgNPs, data on their effect and interactions with terrestrial plants are largely unknown. This study aimed to investigate the effect of growing conditions on the response of pepper plants (*Capsicum annuum* L.) to citrate-coated AgNPs. Growth parameters, biodistribution, and defence response were examined in peppers grown hydroponically or in soil substrate. In addition, the effects of nano and ionic form of silver were compared. The leaves and stems of peppers grown in substrate showed a higher bioaccumulation compared to hydroponically cultivated plants. The nano form of silver accumulated to a higher extent than ionic form in both leaves and stems. Both silver forms inhibited pepper growth to a very similar extent either through hydroponic or substrate growing settings. Unlike other studies, which investigated the effects of unrealistically high doses of AgNPs on different plant species, this study revealed that vascular plants are also susceptible to very low doses of AgNPs. Both silver forms affected all parameters used to evaluate oxidative stress response in pepper leaves; plant pigment and total phenolics contents were decreased, while lipid peroxidation and hydrogen peroxide lever were increased in treated plants. Similar biological effects of both nano and ionic Ag forms were observed for both substrate and hydroponic growing systems.

Additional keywords: phytotoxicity; pepper; plant uptake.

Abbreviations used: AgNP (silver nanoparticles); DAS (days after sowing); DLS (dynamic light scattering); ELS (electrophoretic light scattering); ENP (engineered nanoparticles); ICPMS (inductively coupled plasma mass spectrometer); NP (nanoparticles); ROS (reactive oxygen species); SRM (Standard Reference Materials); TBA (2-thiobarbituric acid); TBARS (2-thiobarbituric acid-conjugated substances); TEM (transmission electron microscopy); TW (tap water); UPW (ultrapure water).

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Introduction

The ever increasing progress of nanotechnology has brought about extensive debate about the risks and benefits of engineered nanoparticles (ENPs) for our lives and our environment (EC, 2014). Due to their growing production and widespread applications in many different products, a certain amount of ENPs ends up in aquatic, terrestrial and atmosphere environments. Despite numerous studies, data on the effect and behaviour of ENPs in these environments are still lacking (Bernhardt *et al.*, 2010). In particular, interactions of ENPs with plants are largely unknown. Besides the effect of the uptake and accumulation of ENPs in plant biomass on their fate and transport in the environment, information on the toxic effects of ENPs on plants are equally important from the perspective of environmental protection. Potential channels of exposure of terrestrial plants to ENPs include wastewater effluent discharge, leaching from

different nanoproducts, use of ENPs for environmental remediation, irrigation using contaminated surface water, land applications of contaminated biosolids and many others (Pokhrel & Dubey, 2013).

Nanotoxicological studies are nowadays more focused on microbial populations, algae, protozoa, mammalian cell lines, or animal models. Data on nanotoxicity in higher plants are still limited. Most of the studies evaluated the uptake, accumulation and biodistribution of ENPs (Gardea-Torresdey *et al.*, 2003; Lin & Xing, 2007, 2008; Judy *et al.*, 2011; Rico *et al.*, 2011; Yin *et al.*, 2011) or the effect of ENPs on different phenotypic changes in plants (Lin & Xing, 2007; Rico *et al.*, 2011). Only a few studies reported the response of plant tissues upon ENP accumulation by means of different plant biomarkers, such as antioxidative status and DNA damage (Cvjetko *et al.*, 2017, 2018). Several studies have been published on the hormonal responses in plants treated with NPs (Le *et al.*, 2014; Shukla *et al.*, 2014), among them our study on the cytokinin response of pepper plant to treatment with nanosilver (Vinković *et al.*, 2017). A plant's response to ENPs may be positive or negative (Monica & Crenomini, 2009). Depending on the type, different ENPs, like TiO_2, ZnO, Mg, Al, Pd, Cu, Si, C60 fullerenes, and carbon nanotubes, may cause either a reduction or increase of growth in higher plants (Monica & Crenomini, 2009; Bernhard *et al.*, 2010). Most metallic NPs have been shown to inhibit the development of plants at different stages (Lin & Xing, 2007). Silver nanoparticles (AgNPs) represent the most commercialized type of metallic NPs. It has now been well-established that AgNPs may release Ag ions that contribute to their biological toxicity (Bernhardt *et al.*, 2010). Thus, the ever increasing commercial use of silver, either in nano or ionic form, may contaminate wastewater systems with possible consequences on plant health, growth, and productivity if wastewater sludge is applied as a soil amendment (Lee *et al.*, 2012; Dimpka *et al.*, 2013). Despite these risks and the importance of plants in the food chain, investigations of the effects of AgNPs on plant growth and development are limited.

Detailed information on the impact of AgNPs in vascular plants is still missing (Vinković *et al.*, 2017). Several researchers have found that AgNPs inhibit the growth of *Lemna minor* (Gubbins *et al.*, 2011) and common ryegrass (Yin *et al.*, 2012), decrease the biomass and transpiration rates of zucchini (Stampoulis *et al.*, 2009), reduce plant biomass, plant tissue nitrogen content, and chlorophyll fluorescence in an aquatic macrophyte *Spirodela polyrhiza* (Jiang *et al.*, 2012). Also, they cause cytotoxicity and genotoxicity in *Allium cepa* root cells (Kumari *et al.*, 2009), and induce oxidative stress in *A. cepa* roots or in tobacco plants (Cvjetko *et al.*, 2017, 2018). By comparing the impact of nanoparticulate to the ionic form of Ag, some studies reported that AgNP toxicity is lower compared to free Ag^+ ions (Pokhrel & Dubey, 2013; Cvjetko *et al.*, 2017), while others demonstrated that the effect of AgNPs exceeded that of identical doses of dissolved Ag^+ ions (Yin *et al.*, 2011). Even though the mechanisms of AgNP toxicity have not been fully elucidated, they are very often explained due to the effects of dissolved Ag ions (Yin *et al.*, 2011; Dimpka *et al.*, 2013). Silver is known as a toxic trace metal. The effects of ionic Ag on plants *in vitro* have been documented by several hundreds of articles in the ISI Web of knowledge database from 1980 to date. In plants, heavy metals inhibit growth and development affecting important physiological processes such as transpiration, photosynthesis, electron transport, and cell division (Nagajyoti *et al.*, 2010). Another well-documented effect is the uncontrolled production of reactive oxygen species (ROS) causing oxidative stress, inactivation of enzymes, and DNA damage (Schützendübel & Polle, 2002; Sharma *et al.*, 2012; Cvjetko *et al.*, 2017). Indeed, the increased production of ROS is a common consequence of most abiotic and biotic stresses in plants at some stage of stress exposure (Schützendübel & Polle, 2002). Plants are generally protected against oxidative stress by a wide range of radical scavenging systems such as antioxidative enzymes peroxidase and catalase, as well as non-enzymatic phenolic compounds as reviewed by Michalak (2006).

The published studies on the phytotoxicity of AgNPs were conducted mainly in hydroponic systems, whereas only a few investigated plant exposure to ENPs in solid matrices (Lee *et al.*, 2012; Dimpka *et al.*, 2012, 2013). However, plant growth in hydroponics differs from growth in soil with regard to root structure, availability of solutes, modification of NP stability and transport by constituents of soil or water (Dimkpa *et al.*, 2013). For this reason, large differences in NP effects could be expected in the absence and presence of soil. The reported results showed that the impact of growing conditions on the effects of Ag in plants is directed either towards attenuated or simulated plant growth (Yin *et al.*, 2012).

With all of the above mentioned in mind, we aimed to expand knowledge on the environmental impacts of metallic NPs by investigating the response of pepper plants (*Capsicum annuum* L.) to citrate-coated AgNPs. Following our previous study on the effects of AgNPs on metal biodistribution, morphological parameters and hormonal responses in pepper plants grown hydroponically, this study aimed to examine whether the effects of AgNPs depend on growing conditions by evaluating the growth parameters, biodistribution

of Ag in leaves, stem and roots, and defence response in peppers initiated by Ag accumulation in leaves. To compare effects of the nano with the ionic form of silver, additional experiments were performed by treating pepper plants with silver nitrate. In both types of experiments, two different Ag concentrations were used, *i.e.* 0.1 and 1 mg/L, taking into account predicted concentrations of AgNPs in different environmental compartments ranging between 5 ng/kg and 1 mg/kg, and never exceeding 10 mg/kg (Fabrega *et al.*, 2011).

Material and methods

Synthesis and characterisation of AgNPs

Citrate-coated AgNPs were synthesized and purified as previously described (Milić *et al.*, 2015). Careful characterization and stability evaluation of AgNPs was performed by dynamic light scattering (DLS), electrophoretic light scattering (ELS), inductively coupled plasma mass spectrometer (ICPMS), UV-Vis spectroscopy and transmission electron microscopy (TEM). AgNPs were characterized at 1 mg/L under two different experimental conditions: in ultrapure water (UPW) and in the chlorine-free tap water (TW) used for plant watering/growing. The aim was to predict the colloidal stability and agglomeration behaviour of AgNPs during the experiments.

Total silver concentrations in the AgNPs colloidal suspensions were determined upon dilution in acidified solutions (10% HNO_3) using an Agilent Technologies 7500cx ICPMS (Agilent, Waldbronn, Germany). The formation of nanosized silver particles was verified by a Surface Plasmon Resonance peak measured using a UV-Vis spectrophotometer (CARY 300, Varian Inc., Australia). The size and charge of AgNPs were measured using a Zetasizer Nano ZS (Malvern, UK) equipped with a green laser (532 nm). The intensity of scattered light was detected at an angle of 173°. All of the measurements were conducted at 25 °C. Data processing was done by Zetasizer software 6.32 (Malvern instruments). Size is reported as hydrodynamic diameter (d_H), obtained as an average value of 10 measurements from the volume size distributions. The charge of AgNPs was evaluated by measuring electrophoretic ζ potential and reported as an average value of 5 measurements. Visualization of AgNPs was done using a Zeiss 902A TEM operated in bright field mode at an acceleration voltage of 80 kV. TEM samples were prepared by depositing a drop of the sample suspension on a Formvar® coated copper grid and air-dried at room temperature.

Dissolution of AgNPs in UPW and TW during 24 h was determined using an Orion 9616BNWP Sure-Flow™ Combination Silver/Sulfide Electrode (Thermo Scientific, USA) connected to a Seven Easy ISE meter (Mettler–Toledo, Switzerland) and centrifugal ultrafiltration (Millipore Amicon Ultra-4 3K) through a membrane with a nominal molecular weight limit of 3 kDa. Quantification of dissolved Ag ions after centrifugation in membrane filters for 30 min at 15000×*g* (Eppendorf Microcentrifuge 5417R, Eppendorf AG, Hamburg, Germany) was performed by ICPMS. For electrochemical detection of free Ag ions, the electrode was preconditioned before each experiment by immersion in a solution containing 0.01 mol/L Ag^+ for 3 h. Four calibration standards that bracket the expected sample concentration were prepared from 10 mg/L silver standard. Linear calibration was obtained over the whole range with a slope 59.3 mV/log [Ag^+]. Concentrations of Ag^+ were calculated from the obtained potential using the linear calibration line.

Plant exposure conditions

The block pepper plant (*Capsicum annuum* L.) was chosen due to its similarity to the tomato (*Solanum lycopersicum* L.), a USEPA recommended test plant (USEPA, 1996). Sweet pepper seeds Vedrana F1 were purchased from Enza Zaden Beheer B.V. (Enkhuizen, Netherland) and kept in the dark at 4 °C until use. The study was designed to explore the variation in pepper responses to various concentrations of the nano and ionic form of Ag under two different exposure scenarios: organic substrate *vs*. hydroponic growing. Both experiments took place at the same time during 2012 in a non-heated greenhouse in Osijek, Croatia. Seeding was performed on 15th March and plants were grown for 51 day.

In the organic substrate exposure scenario, pepper seeds were sown in polystyrene containers with 40 sowing places. Containers were filled with commercial substrate Brill Typ 3 (Gebr. Brill Substrate GmbH & Co). According to the manufacturer, the substrate is intended for the production of pepper and tomato transplants and comprises 65% white and 35% black peat. It is characterized by a pH of 5.5–6.0 and contains 500 g of NPK fertilizer/m^3. The total salt content of the substrate is 0.3–0.8 g/L. During the first 14 days after sowing (DAS), containers were watered with TW daily. Then, the watering of control plants continued using TW only, while the treated plants were watered with AgNPs or Ag^+ (in the form of $AgNO_3$) diluted in TW, each at two different concentrations (0.1 and 1 mg Ag/L). Thus, the experiment consisted out of 5 different variants where each variant had 4 repetitions with 10 plants per repetition. During the experiment, each plant was watered with at least 50 mL of TW or

treatment solution twice a day with an appearance of drainage up to 60% of a given quantity of water or solution. On the 30th, 35th and 40th DAS plants were fertigated with complex fertilizer Poly-Feed GG 20-20-20 + microelements (Haifa Group) in concentration of 0.20%. On DAS 34, watering was increased up to three times per day.

In the floating hydroponic exposure scenario, pepper seeds were also sown in polystyrene containers, filled with commercial substrate Brill Typ 3, and watered with TW daily. The treatments started when the pepper plants emerged and formed roots big enough to reach the bottom of the container (15th DAS). At this point, each container was placed into a separate vessel containing 2 L of TW (control plants) and/or 2 L of AgNPs or $AgNO_3$ diluted in the TW at two different concentrations (0.1 and 1 mg Ag/L). Each variant had 4 repetitions with 10 plants per repetition. All of the watering solutions were prepared and changed every two days. On the 25th, 30th, 35th and 40th DAS plants received nutrient enriched solution by dissolving complex fertilizer Poly-Feed GG 18-18-18 + ME (microelements) for soilless media (Haifa Group) in concentration of 0.20%. On DAS 33 and until the end of experiment, the vessels received fresh water or solution twice a day (17 days × 4 L = 68 L per 40 plants or 1.7 L per plant; 100 mL per plant daily). In both experiments, the treatment was finished on 49th DAS and plant material was sampled on 51st DAS. The pepper plants were grown until they developed 6-7 true leaves and formed the first flower buds.

At the end of each experiment, the leaves, stems and roots of control and treated pepper plants were identified, sorted, washed with distilled water, and surface-dried with filter paper. Plant heights and fresh weights of leaves, stems and roots were recorded. Then, fresh leaf biomass was subjected to analysis of pigment contents and total phenolics, while another part was oven dried at 80 °C for 48 h. The dried samples were analysed for total Ag content. Fine leaf powder obtained by maceration in liquid nitrogen was used for determination of total hydrogen peroxide content and lipid peroxidation rate.

Accumulation of silver in pepper plants

The total Ag concentration in the dried samples was measured by ICPMS after microwave digestion to assess the accumulation pattern of nano and ionic Ag forms. Verification of the accuracy and precision of the ICPMS method was performed using Standard Reference Materials (SRMs): NIST 1573a (tomato leaves) from the National Institute of Standards and Technology (NIST, USA) and Certified Reference Material No. 9 (Sargasso) from the National Institute for Environmental Studies (NIES, Japan). Digestion of pepper samples and SRMs was performed in closed-vessels with an UltraCLAVE IV Milestone digestion device (MLS GmbH Mikrowellen-Laborsysteme, Leutkirch, Germany) by addition of 5 mL of HNO_3 (65% suprapur, Merck, Darmstadt, Germany) to accurately weighed (0.25 g) dry samples in quartz digestion vessels. The method resulted in a total and simultaneous dissolution of samples and colourless digestives. A set of digestion blanks was also prepared and subjected to the same microwave procedure. After the vessels had cooled, deionised water was added to obtain an overall dilution of 200 (v/m). The ICPMS instrument was operated at conditions for general, high matrix analysis in an air-conditioned laboratory (20-22 °C). The instrument was tuned daily with an ICPMS tuning solution (Agilent Technologies, Japan) containing 10 µg/L of lithium, magnesium, yttrium, cerium, thallium and cobalt in 2% HNO_3 (w/v). Calibration standards were prepared daily from stock elemental standard solutions of 1000 mg Ag/L from Merck (Darmstadt, Germany). Both samples and standards were spiked with the 'internal standard stock solution' to the final concentration of 10 µg/L. For the purpose of contamination control, each series of measurements included a reagent blank. Each calibration curve was constructed linearly through zero after subtraction of the reagent blank.

The measured total Ag contents in the leaves, stems and roots of peppers were used to obtain the bioaccumulation factor (BF), which was calculated as % of applied Ag found in the DW of pepper parts.

Pigment content

Fresh pepper leaves were washed in distilled water and subjected to extraction in acetone before the determination of total carotenoid contents, chlorophyll *a* and *b*. Briefly, 1 g of average sample of pepper leaves were mixed with 40 mL of 100% acetone, and was homogenized for 2 min using the homogenizer PowerGen 125 (Fisher Scientific). The homogenate was filtered, and subsequently centrifuged at 2500 × *g* for 10 min. The supernatant was separated and used for further analysis. The absorbance of appropriate diluted extracts in acetone were read at 400-700 nm on the VARIAN Cary 50 UV-Visible Spectrophotometer. The amount of studied pigments was calculated according to Lichtenthaler & Wellburn (1983). All determinations were carried out in triplicate.

Total phenolic content

For the analysis of total phenolic content, pepper leaves were extracted in methanol by adding 1 g of

fresh leaves samples to 10 mL of 80% methanol (v/v). Extraction was carried out using an ultrasonic bath at 25°C for 30 min. Then the extracts were filtered through a nylon membrane filter of pore size 0.2 µm (Whatman Inc.). Total phenolic content in the leaf extracts was estimated spectrophotometrically according to the Folin-Ciocalteu method (Singleton & Rossi, 1965) using gallic acid (GA) as a standard for the calibration curve. The reaction was performed by mixing 20 µL of the methanol leaf extract, water to 1.6 mL, 0.1 mL Folin-Ciocalteu reagent and 0.3 mL sodium carbonate solution. After 1 h of incubation at 37 °C, absorbance was measured at 765 nm and compared to a GA calibration curve. Soluble phenolic content was expressed as mg GA equivalents per g of fresh weight (FW).

Lipid peroxidation

The lipid peroxidation rate was measured using the 2-thiobarbituric acid (TBA) reaction (Heath & Packer, 1968). The assay was performed by incubating the 0.5 mL of fresh leaves extract (0.2 g of macerated leaf powder extracted with 0.1% trichloroacetic acid) with 1 mL of the TBA reagent (0.5% thiobarbituric acid in 20% trichloroaceticacid) for 30 min in a water bath at 95 °C. The levels of TBA-conjugated substances (TBARS) were calculated using the extinction coefficient of 155 mM/cm from the data read at 532 nm after applying the correction read at 600 nm (for non-specific absorption) (Mukherjee & Choudhuri, 1983). The lipid peroxidation rate was expressed as nmol TBARS per g of FW.

Hydrogen peroxide content

The total hydrogen peroxide (H_2O_2) content in leaf tissue was evaluated as described by Mukherjee & Choudhuri (1983). Macerated leaf powder (0.2 g) was extracted with 1 mL of cold absolute acetone and centrifuged for 3 min at 1000 × *g* on 4 °C. Then, 400 µL of titanium oxysulphate and 500 µL of 25% ammonium hydroxide solution were added to the supernatant. The precipitated peroxide-titanium complex was solubilised with 1 mL of 2 M H_2SO_4. The absorbance of the supernatant was measured at 415 nm against blank. The total H_2O_2 content was determined using the standard curve plotted with a known concentration of hydrogen peroxide and expressed as nmol H_2O_2 per g of FW.

Statistical analysis

Factorial analysis of variance (ANOVA) was carried out and differences between treatments were evaluated by Fisher LSD test (p<0.05) using the SAS 9.0 statistical package. The data are reported in tables and figures as means with standard deviations in parentheses and error bars, respectively.

Results and discussion

The response of pepper plants to the nano and ionic form of Ag under two different exposure scenarios, floating hydroponic *vs.* substrate conditions, was evaluated by means of morphological parameters (plant height and masses of fresh leaves, stems and roots), biodistribution of Ag in plants, and levels of pigments, total phenolics, hydrogen peroxide and lipid peroxidation in pepper leaves.

Characterisation and stability evaluation of AgNPs

Before a critical interpretation of this study could commence, a careful characterisation and stability evaluation of AgNPs in both UPW and TW was needed. A physicochemical characterisation was performed using DLS, ELS, TEM and electrochemical techniques. Table 1 gives the hydrodynamic diameter (d_H), ζ potential values and polydispersity index (PdI) of citrate-coated AgNPs dispersed either in the UPW or TW.

DLS measurements showed that the volume size distribution of AgNPs in the UPW was bimodal, with particles characterised by a d_H value of 14.1 ±

Table 1. Hydrodynamic diameter (d_H) obtained from size distributions by volume (% mean volume), zeta potential (ζ) and polydispersity index (PdI) of citrate-coated silver nanoparticles in ultrapure water (UPW) and tap water (TW) used for watering of pepper plant after 1 h at 25 °C.

Parameter	UPW	TW
d_H (nm)	14.1 ± 8.7 (91%), 83 ± 38 (9%)	83 ± 23 (6%), 436 ± 124 (94%)
ζ (mV)	-28.7 ± 1.6	-3.4 ± 0.9
PdI	0.3	0.5
Released Ag^+ (%)	< 1.2	< 0.4

8.7 nm being dominant, while a minor population (< 10%) were particles larger than 50 nm. TEM analysis confirmed DLS results and revealed the presence of non-uniformly shaped NPs (Fig. 1a). The surface charge of AgNPs in the UPW was characterised by a negative ζ potential value (-28.7 ± 1.6 mV) due to the electrostatic stabilization of AgNPs with the polar citrate carboxyl groups. TEM images revealed the agglomeration behaviour of AgNPs in the TW showing the presence of differently sized agglomerates but also the presence of individual particles (Fig. 1b). In the TW, 94% of AgNPs had a d_H of 436 ± 124 nm and only a small AgNP population (6%) was smaller than 100 nm (Table 1).

The DLS technique can only approximately determine particle size, because light scattered on big particles or agglomerates hides any information about small particles. Thus, the d_H values obtained for AgNPs in the TW were the result of a collapse of the electrostatic diffuse layer at the AgNPs surface caused by a higher ionic strength of TW media. This was also obvious from the measured ζ potential of -3.4 ± 0.9 mV, the value close to the 0 mV (Table 1). A decrease in the absolute value of ζ potential by more than 25 mV in the TW as compared to the UPW decreased the interparticle repulsion of the AgNP dispersion, resulting in lower colloidal stability (Fabrega *et al.*, 2011).

To determine the dissolution behaviour of AgNPs in the UPW and TW, the concentration of free Ag^+ ions was measured in the AgNP suspensions during 24 h. Only ~ 1% of free Ag^+ was released in the UPW, while this amount was even lower in the TW (Table 1). The dissolution behaviour of citrate-coated AgNPs was comparable to our previously published data (Vinković *et al.*, 2017).

Biodistribution of AgNPs in pepper plants

Analysis of penetration and transport of NPs in plants is important from both the ecotoxicological and agricultural aspect of nanotechnological applications. The ICPMS analysis of pepper leaves and stems did not show significant differences in Ag accumulation in plants treated with the ionic or nanoparticulate form of silver either in hydroponic or in substrate conditions (Fig. 2a). Only roots of pepper plants showed higher BF for peppers treated with ionic Ag compared to the AgNPs (Fig. 2b).

The BF was high for roots with values ranging from 3 to a very high 9%. Completely different patterns in the accumulation of Ag were observed for different plant parts. Although one would expect that AgNPs or ionic Ag would be more bioavailable for transport to upper plant parts, this was not observed in this study. The leaves and stems of pepper plants grown in substrate showed a higher BF compared to hydroponically cultivated plants. The reason may be the complexation reactions of the nano or ionic Ag with substrate NP components, which increased their bioavailability such as humic substances, *e.g.* humic acid. It has been proved that humic acid can enhance uptake and translocation of certain nutrients in different plants species such as nitrogen, phosphorus, potassium, calcium, copper, manganese and zinc in maize (Eyheraguibel *et al.*, 2008) as well as nitrogen, phosphorus, iron and copper in tomato roots (Adani *et al.*, 1998). In addition, humic substances can improve uptake and translocation of heavy metals in plants as proved by Li *et al.* (2016). According to Chen *et al.* (2013), silver uptake by the algae is greater in the presence of humic acid without decreasing the growth that suggest that silver becomes less toxic in the presence of humic acid.

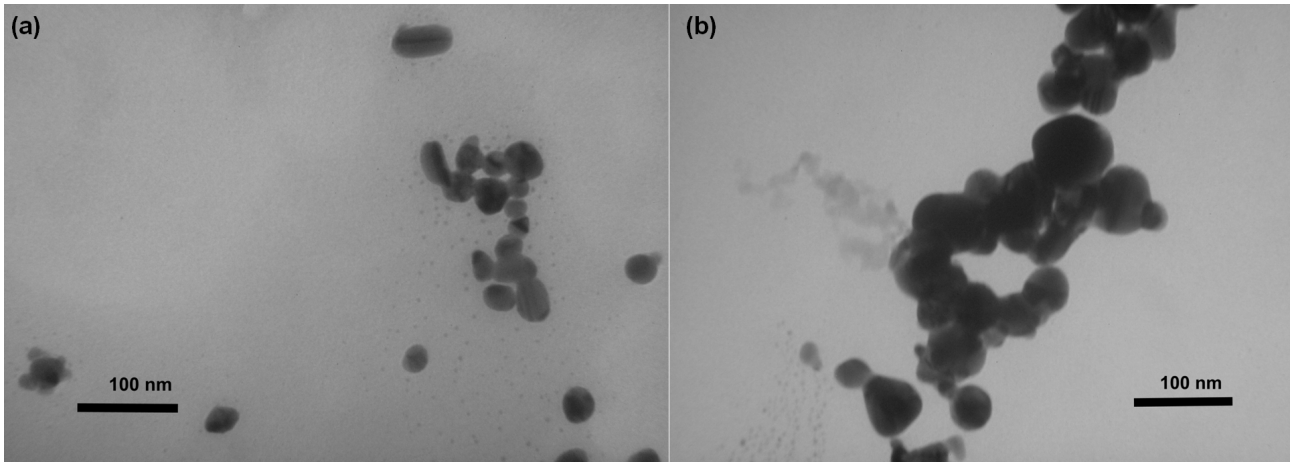

Figure 1. Transmission electron micrograph (TEM) of citrate-coated silver nanoparticles used for the treatment of pepper plants dispersed in (a) ultrapure water and in (b) tap water used for watering of pepper plant.

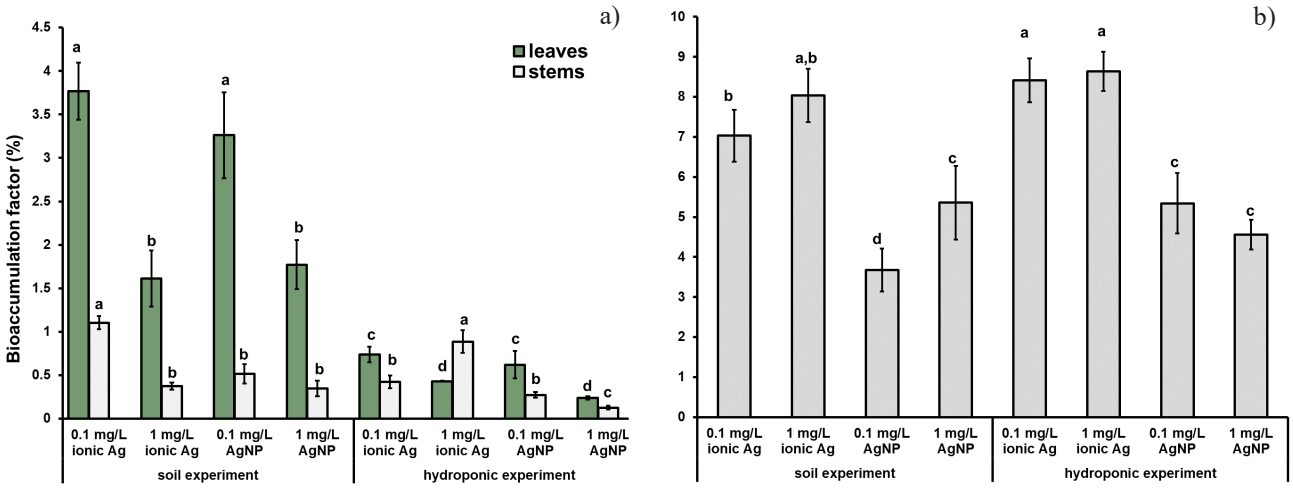

Figure 2. Uptake and distribution pattern of silver, given as bioaccumulation factors, in tissues of pepper plants grown in substrate or hydroponically and treated with different concentrations of AgNPs and Ag^+. Bioaccumulation factors for (a) leaves and stems, and (b) roots were calculated as ratio between found Ag levels and total Ag amount applied during a particular treatment. Values represent means of five replicates ± standard deviations. Different letters denote significant differences ($p < 0.05$) among treatments.

Only one exception from this pattern was found, *i.e.* stems of peppers grown in hydroponics and treated with ionic Ag (Fig. 2a). In addition, the BF in stems and leaves was lowered with an increasing concentration of AgNPs and ionic Ag, indicating that higher concentrations of Ag in watering solution does not necessary linearly increase Ag uptake and translocation of Ag. These results are contrary to recently published data regarding *Triticum aestivum* (Monica & Cremonini, 2009), *A. cepa* roots (Cvjetko *et al.*, 2017), or tobacco plants (Cvjetko *et al.*, 2018). However, an opposite dose-response uptake of AgNPs was found for *Brassica juncea* and *Medicago sativa* in a recently published study (Harris & Bali, 2008).

The calculated BFs were highest, as expected for roots of treated pepper plants, which showed a completely different BF-dose pattern compared to leaves and stems (Fig. 2b). In the substrate experiment, higher concentrations of AgNPs or ionic Ag led to a higher BF in roots, while there was no dose-response in roots of hydroponically cultivated peppers. Comparison of ionic *vs*. nanoparticulate Ag forms showed higher BF in roots of peppers treated with ionic Ag. This observation could be explained by the aggregation behaviour of AgNPs, which is expected to be more pronounced at higher concentration (>0.1 mg/L) lowering their uptake by plants. Although the mechanism of AgNP uptake in pepper plants cannot be drawn from these experiments, BF results for leaves and stems clearly indicate a similar accumulation for nanoparticulate and ionic Ag forms.

Effect of AgNPs on pepper growth

The effect of accumulated AgNPs or Ag^+ on plant growth was investigated by measuring the fresh weight of leaves, stems and roots, as well as the height of the treated compared to control pepper plants (Fig. 3).

Data obtained for plants grown hydroponically (Figs. 2 & 3) largely confirms the findings of our previously published study on the cytokinin response of peppers treated with nano and ionic Ag (Vinković *et al.*, 2017). Most studies conducted so far on ENP phytotoxicity and plant uptake were carried out employing hydroponic settings. Soil or substrate studies are needed as they more realistically represent the environmental fate of ENPs, although they cannot provide unambiguous answers due to the complicated nature of the soil or organic substrate matrix. In addition, the investigation of the phytotoxicity of metal-based ENPs is even more complex due to their potential dissolution and concurring effects of metallic ions. Interestingly, our results revealed that AgNPs and Ag^+ applied either through hydroponic or substrate settings inhibited pepper growth to a very similar extent. The same inhibition patterns were observed for all pepper growth parameters when comparing substrate and hydroponic conditions despite the higher height and FWs of leaves, stems and roots in peppers grown hydroponically. Significant differences between AgNPs and Ag^+ treatments were only observed for the FW of leaves and stems in hydroponically grown peppers treated with lower concentrations of AgNPs and Ag^+ (Fig. 3a-b), FW of roots in peppers grown in substrate and treated with higher concentrations of

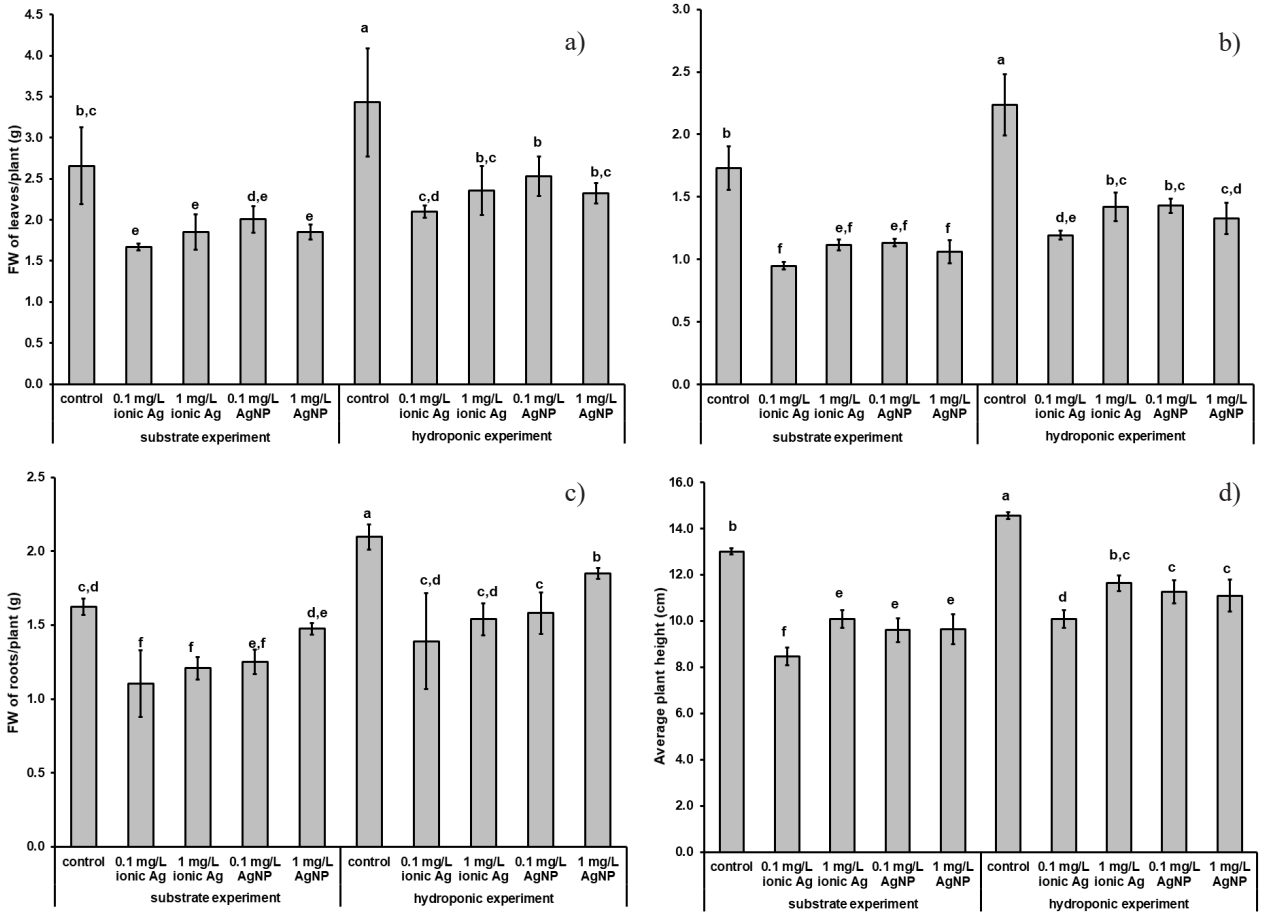

Figure 3. Effect of different concentrations of AgNPs and Ag^+ on fresh weights (FW) of (a) leaves, (b) stems and (c) roots, as well as on (d) heights of pepper plants cultivated either in substrate or hydroponically. Values represent the means of five replicates ± standard deviations. Different letters denote significant differences ($p < 0.05$) among treatments.

AgNPs and Ag^+ (Fig. 3c), and height of peppers treated with lower concentrations of AgNPs and Ag^+ (Fig. 3d). An almost identical pattern was observed in our study performed one year later in hydroponically grown peppers (Vinković *et al.*, 2017). However, very limited information on AgNP treatment in soil or substrate *vs*. hydroponic culture implied that the final outcome of AgNPs and Ag^+ exposures to plant growth depends on the plant species. For example, the annual ryegrass *Lolium multiflorum* responded differently to nano and ionic Ag depending on the growing conditions (Yin *et al.*, 2012). Thus, the negative growth response of ryegrass to treatment with AgNP or Ag^+ was observed in a pure culture, but it responded positively to both Ag forms in soil (Yin *et al.*, 2012). For *E. fistulosum* and *Carex* species, inhibition of root growth by both AgNPs and Ag^+ was observed in the pure culture experiment, while inhibition in soil was obtained only from AgNPs (Yin *et al.*, 2012). Our results indicated that even very low concentrations of AgNPs and Ag^+ (below 1 mg/L) inhibited the growth of pepper plants. Evaluation of dissolution behaviour even revealed a decreased release of free Ag^+ ions from AgNP surface in the TW compared to UPW (Table 1), thus implying that the toxicity of the nanoparticulate and ionic Ag forms is the same. However, it is extremely difficult to distinguish the mechanism of AgNP effect in plant tissues, especially in soil or substrate cultures. Many ligands present in soil or substrate like thiols, sulfide, chloride, or phosphate, may not only decrease the bioavailability of Ag^+, but also mitigate the biological effects of AgNPs by complexation and binding reactions (Reinsch *et al.*, 2012). In addition, it is well known that some plants are capable of reducing Ag^+ ions to AgNPs inside plant tissues (Harris & Bali, 2008). The interpretation of the final form of bioaccumulated Ag in our experiments was beyond the scope of this study. Even so, our findings provide important new information for understanding interactions between AgNPs and plant tissues. The potentially entangled nature of the equilibrium between AgNPs and Ag^+ in different environmental compartments including plant tissues requires complex, time consuming and methodologically demanding elucidations of the

detailed mechanism behind the biological response to AgNP treatments.

Oxidative stress response to AgNPs

It has been well-established that metals accumulated by plants and translocated to aboveground tissues may cause toxic effects at both biochemical and cellular level altering physiological and metabolic processes in plants (Michalak, 2006; Nagajyoti *et al.*, 2010). Inhibition of plant growth is the most obvious outcome of such toxic actions. Most of the studies published so far on the effects of metallic NPs on higher plants focus on biodistribution and plant growth response including NP effects on seed germination, root/shoot length, biomass etc. Only limited information on oxidative stress parameters, DNA damages, content of proteins and phenolics, hormonal response, photosynthesis parameters in plants treated with metallic NPs is available (Shukla *et al.*, 2014; Cvjetko *et al.*, 2017, 2018). Our previous study clearly showed that AgNPs induce abiotic stress in pepper plants, which was mediated by cytokinins (Vinković *et al.*, 2017). In this follow-up study, levels of plant pigments, total phenolics, hydrogen peroxide content and lipid peroxidation extent were determined in leaves of peppers treated with the nano or ionic form of Ag compared to control plants. In the substrate exposure scenario, plant pigments were affected by both nano and ionic silver (Table 2).

Interestingly, only the higher concentration of AgNPs decreased total carotenoid content and chlorophylls *a* and *b* in pepper leaves, while treatment with ionic Ag form was significant at both concentrations, *i.e.* 0.1 and 1 mg/L. Decrease in the level of photosynthetic pigments apparently blocked the photosynthetic process leading to pepper growth inhibition. Similar patterns in changes of plant pigments content were observed in hydroponic exposure scenario where lowest content of total carotenoid and chlorophylls *a* and *b* was recorded in plants treated with higher concentration of AgNPs (Table 2).

However, the mechanism of growth inhibition was obviously much more complex as plant biomasses decreased after treatment with the lower AgNP concentration (Fig. 3). Analysis of total phenol content showed the same pattern in plants grown hydroponically or in substrate (Fig. 4a). Unlike in the case of pigment content, treatment with lower concentrations of the nano or ionic form of Ag decreased total phenolics in leaves of peppers grown in substrate compared to control plants. In the case of hydroponically grown peppers, AgNP treatment decreased levels of total phenolics in leaves as compared to controls.

The effect of ionic Ag was dependent on concentration; lower concentrations had no significant effect, while the higher dose of ionic Ag increased total phenolics in leaves compared to controls. Thus, our results on the treatment of peppers with ionic Ag were similar to the elevated level of total phenol contents recorded in leaves of hydroponically grown *Bacopa monnieri* Linn. (Krishnaraj *et al.*, 2012). An affected level of total phenolics is typical in stressed plants (Sakihama *et al.*, 2002; Schützendübel & Polle, 2002). In stress conditions, plants alleviated the induced oxidative injury by different defence mechanisms. Plant phenolics are one of many antioxidant systems involved either in enzymatic or non-enzymatic antioxidant reactions (Sakihama *et al.*, 2002; Schützendübel & Polle, 2002). They can act as metal chelators, as antioxidants by donating electrons to other antioxidant defence

Table 2. Change in pigment levels of pepper leaves as a function of treatments with AgNPs or Ag^+, expressed in mg/g of fresh weight (FW). Pepper plants were cultivated either in substrate or hydroponically during 51 day. Values represent the mean of five replicates with standard deviations given in parentheses and different letters denote significant differences ($p < 0.05$) among treatments.

Cultivation	Treatment	Chlorophyll *a* (mg/g of FW)	Chlorophyll *b* (mg/g of FW)	Ratio of chlorophyll *a vs.* chlorophyll *b*	Total carotenoid content (mg/g of FW)
Substrate	Control	0.22 (0.05)^{a}	0.25 (0.04)^{a}	0.88 (0.12)^{a,b}	0.024 (0.012)^{a,b}
	0.1 mg/L Ag^+	0.14 (0.04)^{b}	0.18 (0.03)^{b,c}	0.75 (0.12)^{b}	0.011 (0.007)^{b,c}
	1 mg/L Ag^+	0.12 (0.03)^{b}	0.17 (0.04)^{c}	0.74 (0.04)^{b}	0.009 (0.002)^{c}
	0.1 mg/L AgNPs	0.22 (0.02)^{a}	0.21 (0.03)^{a,b}	1.02 (0.08)^{a}	0.032 (0.004)^{a}
	1 mg/L AgNPs	0.13 (0.05)^{b}	0.17 (0.03)^{c}	0.75 (0.17)^{b}	0.010 (0.004)^{b,c}
Hydroponics	Control	0.18 (0.02)^{a}	0.21 (0.02)^{a}	0.84 (0.07)^{b}	0.019 (0.002)^{b}
	0.1 mg/L Ag^+	0.11 (0.01)^{b}	0.16 (0.02)^{b}	0.72 (0.11)^{c}	0.011 (0.001)^{c}
	1 mg/L Ag^+	0.10 (0.03)^{b}	0.14 (0.04)^{b}	0.72 (0.06)^{c}	0.009 (0.001)^{d}
	0.1 mg/L AgNPs	0.19 (0.01)^{a}	0.19 (0.01)^{a}	0.99 (0.05)^{a}	0.029 (0.001)^{a}
	1 mg/L AgNPs	0.10 (0.01)^{b}	0.15 (0.01)^{b}	0.68 (0.04)^{c}	0.012 (0.001)^{c}

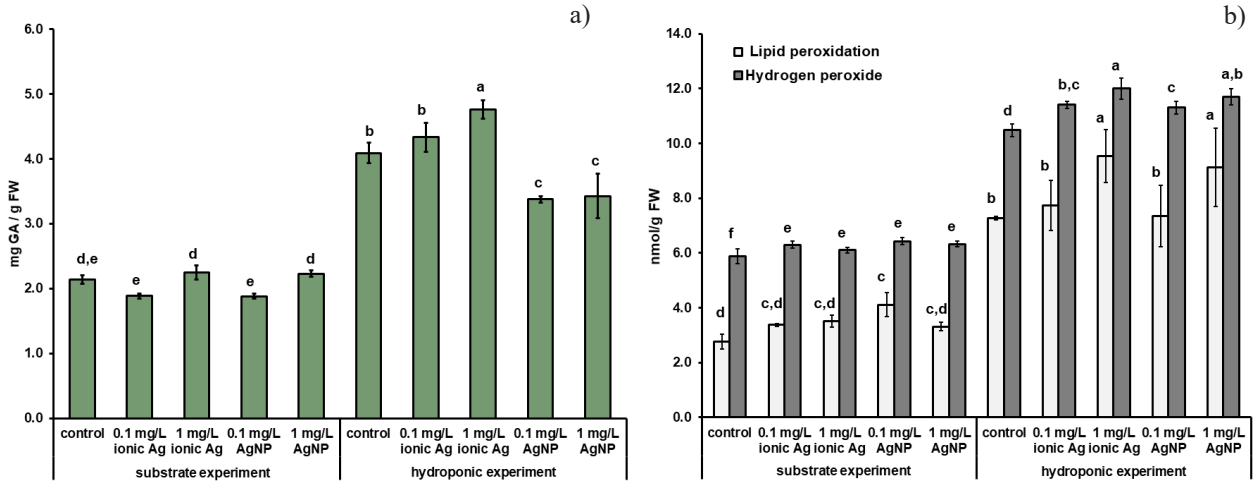

Figure 4. Effect of different concentrations of AgNPs and Ag^+ (a) total phenolic content (expressed as mg of gallic acid equivalent (GA) per gram of fresh weight), and (b) on lipid peroxidation extent (measured as the level of 2-thiobarbituric acid-conjugated substances (TBARS)) and hydrogen peroxide (H_2O_2) content in leaves of pepper plants cultivated either in substrate or hydroponically. The lipid peroxidation rate is expressed as nmol TBARS per g of fresh weight (FW), while the total H_2O_2 content is expressed as nmol H_2O_2 per g of fresh weight (FW). Values represent means of five replicates ± standard deviations. Different letters denote significant differences ($p < 0.05$) among treatments.

systems, or as prooxidants under certain conditions (Schützendübel & Polle, 2002). The balance between antioxidant and prooxidant characteristics of plant phenolics may be very complicated. Thus, the different phenolic response in peppers grown in hydroponic or substrate settings (Fig. 4a) may indicate that different mechanisms of phenolic actions are behind the plant response to treatment with nano or ionic Ag. As one of the phenolics actions in plant tissue may be induction of lipid peroxidation (Sakihama *et al.*, 2002), pepper leaves were analysed for lipid peroxidation rate (Fig. 4b). In addition, the level of H_2O_2 was determined in pepper leaves (Fig. 4b).

Both parameters showed the same pattern in both growing settings, although hydroponically grown peppers had higher levels of lipid peroxidation rates (Fig. 4b). Elevated levels of H_2O_2 were recorded in all of the treated groups either in hydroponically or in substrate growing conditions. There were no differences between ionic and nano Ag treatments (Fig. 4b). Similar accumulation of H_2O_2 was already observed in metal-exposed plants (Piqueras *et al.*, 1999; Schützendübel & Polle, 2002). Several studies have reported that both the nano and ionic form of Ag induce oxidative stress in plant tissues (Jiang *et al.*, 2014; Nair & Chung, 2014b; Barbasz *et al.*, 2016; Cvjetko *et al.*, 2018). Lipid peroxidation rate was evaluated as an additional biomarker for oxidative stress induction in plant leaves. A similar pattern observed for H_2O_2 levels was also detected for lipid peroxidation rates. Hydroponically grown peppers showed higher lipid peroxidation compared to substrate settings (Fig. 4b), while no significant differences was observed between nano and ionic Ag forms. There were no significant differences between control and treatment in peppers grown in substrate, except for the lower dose of AgNPs which elevated lipid peroxidation rate compared to control plants. Higher content of phenolics, H_2O_2 and lipid peroxidation rate found in hydroponic setting (Fig. 4.) can be due to mild hypoxic conditions in the root zone that was submerged in water or nutrient solution. Plants grown in a floating system may encounter problems of oxygen deficiency (hypoxia) at root level, as roots themselves gradually consume the oxygen dissolved in the nutrient solution (Lenzi *et al.*, 2011). Elevated content of ROS under oxidative stress is an integral part of many stress situations, including hypoxia and reaeration (Blokhina *et al.*, 2003) which in our study appeared every time when changing the solution in hydroponic setting. Accumulation of H_2O_2 under hypoxic conditions has been shown in the roots and leaves of *Hordeum vulgare*) and in wheat roots (Kalashnikov *et al.*, 1994; Biemelt *et al.*, 2000). Also, influence of hypoxia on higher lipid peroxidation rate has been detected in roots and shoots of wheat, oat, rice (Chirkova *et al.*, 1998) and corn leaves (Yan *et al.*, 1996). At the same time, plants can be tolerant to hypoxia without showing decrease in both growth and yield (Ferrante *et al.*, 2005; Lenzi *et al.*, 2008). Considering the silver toxicity, contrary to the recent study on tobacco plants (Cvjetko *et al.*, 2018), higher concentration of AgNPs or ionic Ag induced an increase in lipid peroxidation rates in pepper leaves (Fig. 4b). Similar results have been reported for other plant species

like wheat and rice (Nair & Chung, 2014; Barbasz *et al.,* 2016). Unlike other studies which reported a higher toxicity for AgNPs in some species (Stampoulis *et al.*, 2009) and lower in some other plant species (Pokhrel & Dubey, 2013; Vannini *et al.*, 2013; Yasur & Rani, 2013) compared to the effects of ionic Ag, our results do not reveal differences in the toxicity of the nano and ionic forms of Ag. The fact that AgNPs showed even lower free Ag^+ ions when dispersed in TW used for watering of pepper plants compared to ultrapure water (Table 1) indicates that AgNPs may be purely nano-related. Considering all possible transformation patterns of AgNPs and Ag^+ in different biological media, it is even more difficult to gain definitive answers on the mechanism of AgNP toxicity effects in vascular plants.

Similarly to other recent studies, this paper reports that AgNPs exhibit toxic effects in vascular plant species, much like those induced by the ionic metal form most probably caused by an analogous mechanism. Unlike other plant studies, which investigated effects of unrealistically high doses of AgNPs, our results revealed that vascular plants are also susceptible to very low doses of AgNPs. In addition, quite similar biological effects of both Ag forms were observed for both substrate and hydroponic growing systems. Currently, a definite mechanism of AgNPs toxicity in vascular plants cannot be determined as AgNP reactivity and the possible transformation patterns of both ionic and nano Ag forms tend to complicate the limited understanding of their phytotoxicity. Thus, to reach a conclusion on the mode of action of metal-based nanomaterials versus their free ions further investigations of various complimentary and measureable biomarkers need to be performed. Clearly, more work needs to be done to clarify the ecotoxicological effects of nanoparticle exposure in different growth mediums and under field conditions, as well as to characterize the potential risk associated with food chain contamination through agricultural species.

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