Silver nanoparticles affect germination and photosynthesis in tobacco seedlings

Renata Biba¹, Mirta Tkalec¹, Petra Cvjetko¹, Petra Peharec Štefanić¹, Sandra Šikić², Dubravko Pavoković¹, Biljana Balen¹*

¹ University of Zagreb, Department of Biology, Faculty of Science, Horvatovac 102a, 10000 Zagreb, Croatia
² Dr. Andrija Štampar Institute of Public Health, Department of Ecology, Mirogojska cesta 16, 10000 Zagreb, Croatia

Abstract – Extensive commercialization of silver nanoparticles (AgNPs) raises the risk of their accumulation in the soil-plant system. Once released into the environment, AgNPs are prone to chemical transformations, which make it hard to determine whether their phytotoxic effects are purely NP-related or a consequence of released Ag⁺ ions. In this study the effects of 25, 50, 75, 100 and 150 μM AgNPs and AgNO₃ on seed germination and early growth of tobacco (Nicotiana tabacum L.) seedlings were compared. Additionally, the effects on photosynthetic performance and pigment content were investigated. Germination rate and index values indicated delayed and slower germination in some AgNP treatments. Lower AgNP concentrations stimulated root growth, but induced a prominent reduction in fresh weight. By contrast, all AgNO₃ concentrations inhibited root growth but only the higher ones decreased fresh weight. Obtained results imply that the observed AgNP toxicity could be ascribed to NP form and can be correlated with high AgNP stability in the solid medium. On the other hand, the majority of AgNP and AgNO₃ treatments induced an increase in chlorophyll content that was accompanied by significantly lower values of relative electron transport rate and coefficient of photochemical quenching, implying an inhibition of the electron transport chain. A similar impact of AgNPs and AgNO₃ on photosynthesis can be correlated with lower stability of AgNPs in a liquid medium, resulting in AgNP aggregation and dissolution of Ag⁺ ions.

Keywords: chlorophyll fluorescence, germination, Nicotiana tabacum, photosynthetic pigments, silver ions, silver nanoparticles

Introduction

The rapid development and extensive commercialization of engineered nanomaterials (ENMs) have expanded their application in industry and daily life, raising serious concerns about their impact on the environment and human health (Scheringer 2008, Wiesner et al. 2009). Among the various types of ENMs, silver nanoparticles (AgNPs) are involved in nearly 25% of consumer products (Vance et al. 2015). Special physicochemical properties of Ag nanomaterials play a crucial role in their antibacterial activity, which is being utilized in the environmental, biomedical and industrial sectors (Deshmukh et al. 2019). Increased use of AgNPs raises the risk of their discharge into the environment and accumulation in the soil-plant system (Yang et al. 2017, Lv et al. 2019). Through plants AgNPs could be transported and accumulated in high trophic-level consumers, including humans (Rico et al. 2011, McKee and Filser 2016).

Previous studies have shown both positive and negative impacts of AgNPs on plant metabolisms, mainly depending on their concentration, size, shape and coating (Pallavi et al. 2016, Jasim et al. 2017, Cvjetko et al. 2017, 2018, Peharec Štefanić et al. 2019). Experimental methodology (growth medium, exposure method, exposure time) as well as plant system used (species and developmental stage) also significantly affect AgNP phytotoxicity (Yan and Chen 2019). AgNP suspensions are prone to chemical transformations (oxidation, dissolution, aggregation and agglomeration) (Levard et al. 2012, Gorham et al. 2014) making it...
harder to determine whether the effects of AgNPs are purely nanoparticle-related or a consequence of silver ion release from the nanoparticles. So far, research has provided evidence for both Ag+ ion- and AgNP-specific toxicity (Tkalec et al. 2019). Ag+ ions released from AgNPs can induce oxidative stress through excessive reactive oxygen species (ROS) production (Park et al. 2009), disturb cell function by binding to cell components and modifying their activities (Montes et al. 2017) and affect photosynthesis through competitive substitution of Cu+ ions in plastocyanin (Sujak 2005, Jansson and Hansson 2008). However, in some cases AgNPs proved to be more toxic than Ag+ ions at the same concentrations, due to the inhibition of apoplastic trafficking caused by the clogging of pores and barriers in the cell wall or plasmodesmata (Tripathi et al. 2017, Ruotolo et al. 2018).

High sensitivity to AgNPs caused by inhibition of photosynthetic processes was shown in several algae and plant species (Navarro et al. 2008, Oukarroum et al. 2012, Jiang et al. 2017, Dewez et al. 2018). Reduced photosynthetic activity and decreased ATP and NADPH synthesis can disturb the biochemical and physiological processes needed for cell growth, which in the end affects plant development (Gerst et al. 1994, Stirbet and Govindjee 2012). Measurement of chlorophyll a fluorescence together with the content of photosynthetic pigments can provide valuable insight into the mechanism of AgNP phytotoxicity, and coupled with germination percentage, root elongation and mass measurements, fast and reliable toxicity indicators, gives a more comprehensive view of the effects of AgNPs on plants (Wang et al. 2001, Dewez et al. 2018).

To elucidate the nature of AgNP-phytotoxicity, the effects of citrate-coated AgNPs and ionic Ag in the form of silver nitrate (AgNO3), both applied in concentrations of 25, 50, 75, 100 and 150 μM, on seed germination, early growth as well as on photosystem II (PSII) performance and the photosynthetic pigment content of tobacco (Nicotiana tabacum L. cv. Burley). Nutrient medium was sterilised and supplemented with AgNP or AgNO3 stock solutions to obtain 25, 50, 75, 100 and 150 μM concentrations and subsequently poured into Petri dishes (90 mm diameter) and left to solidify. Tobacco seeds were surface sterilised for 15 min using 50% (v/v) NaOCl (Kemika, Croatia), and then rinsed three times with sterile deionised H2O before being placed on the half strength MS medium. Control seeds were germinated on a medium devoid of AgNPs and AgNO3. Two days before the beginning of the experiment, Petri dishes with sown seeds were placed in cold stratification (+4 °C) to promote and synchronise seed germination (Kucera et al. 2005). Germination was monitored for 5 consecutive days, starting from the 3rd day after seed stratification. Seedlings were harvested after three weeks and used for measurements of root length as well as fresh and dry weight. All experiments were conducted two times. In each experiment, 100 seeds were sown for every treatment with either AgNPs or AgNO3 (200 seeds in total per treatment).

For analysis of effects of AgNPs and AgNO3 on photosynthesis and photosynthetic pigments and for measurement of Ag content, sterilized and stratified seeds were placed in sterile 100 mL Erlenmeyer flasks filled with 5 mL of liquid half strength MS medium and left to germinate on a shaker. Germinated seeds were grown for three weeks in the same Erlenmeyer flask, which was periodically supplemented with fresh sterile nutrient medium. After three weeks, the nutrient medium was replaced with fresh sterile liquid half strength MS medium supplemented with either AgNPs or AgNO3 in the above-mentioned concentrations. Seedlings were treated for 7 days.

During the experiments, all plant material was kept in the culture room at 24 ± 1 °C with 16/8 h light/dark cycles and 90 μmol m–2 s–1 light intensity.

Materials and methods

AgNPs and AgNO3 suspensions

All experiments were performed with commercial AgNPs with citrate coating (50 nm Citrate BioPure Silver Nanospheres, Nanocomposix, San Diego, CA, zeta potential of –47.8 mV). The concentration of AgNP stock solution was 9.27 mM. AgNO3 (Sigma Aldrich St. Lois, MO, USA) was dissolved in ultrapure water (ion-free Milli-Q water, 18.2 MΩ.cm resistivity, Merck Millipore, USA) and used as a 100 mM stock solution. Prior to treatments, the concentration of Ag in the AgNP and AgNO3 stock solution was determined by ELAN DRC-e inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer, USA). For preparation of treatment solutions of AgNPs, the concentration of silver was considered in calculations.

Plant material and culture treatments

In this study we used solidified half strength Murashige and Skoog (MS) nutrient medium (0.2% (w/v) Phytagel, 1.5% (w/v) sucrose; both Sigma Aldrich, USA) (Murashige and Skoog 1962) to study the effects of AgNPs and AgNO3 on the germination and early growth of tobacco (Nicotiana tabacum L. cv. Burley). Nutrient medium was sterilised and supplemented with AgNP or AgNO3 stock solutions to obtain 25, 50, 75, 100 and 150 μM concentrations and subsequently poured into Petri dishes (90 mm diameter) and left to solidify. Tobacco seeds were surface sterilised for 15 min using 50% (v/v) NaOCl (Kemika, Croatia), and then rinsed three times with sterile deionised H2O before being placed on the half strength MS medium. Control seeds were germinated on a medium devoid of AgNPs and AgNO3. Two days before the beginning of the experiment, Petri dishes with sown seeds were placed in cold stratification (+4 °C) to promote and synchronise seed germination (Kucera et al. 2005). Germination was monitored for 5 consecutive days, starting from the 3rd day after seed stratification. Seedlings were harvested after three weeks and used for measurements of root length as well as fresh and dry weight. All experiments were conducted two times. In each experiment, 100 seeds were sown for every treatment with either AgNPs or AgNO3 (200 seeds in total per treatment).

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During the experiments, all plant material was kept in the culture room at 24 ± 1 °C with 16/8 h light/dark cycles and 90 μmol m–2 s–1 light intensity.

AgNP stability in solid and liquid culture medium

AgNPs stability in the solid nutrient medium was analysed as previously reported (Peharec Štefanic et al. 2018). Briefly, one mL of half strength MS medium, solidified with Phytagel (agar substitute) and supplemented with AgNP stock solution to obtain a 150 μM concentration (the high-
AgNPs AFFECT TOBACCO GERMINATION AND PHOTOSYNTHESIS

Germination parameters

Seed germination was monitored for 5 days, each day at the same time. Seeds were considered germinated when the radicle emerged from the seed. Germination percentage was calculated using the formula:

\[ \text{Germination percentage} (\%) = \frac{\text{final number of germinated seeds \_total number of seeds}}{100} \]

To calculate germination index (GI) and germination rate (T50), daily counts of germinated seeds were used by employing the following formulas (Farooq et al. 2005):

\[ \text{GI} = \sum N_t \text{ (number of germinated seeds on the t day)/D_t} \text{ (germination days)} \]

\[ T_{50} = t_i + \left( \frac{\left[ \left( N/2-n_i \right) \right] \left( t_i - t_j \right)}{n_i - n_j} \right) \]

where N is the final number of germinated seeds, ni and nj are cumulative number of seeds germinated by consecutive counts at times ti and tj, considering that ni < N/2 < nj.

Growth parameters

Three weeks after exposure to AgNPs and AgNO3 on the solid half strength MS medium, seedlings were harvested and measured.

The length of the main root was measured using a ruler and root length reduction percentage (RLPR) was calculated using the formula by Zafar et al. (2015):

\[ \text{RLPR} \% = 100 \times \left( 1 - \frac{\text{root length stress}}{\text{root length control}} \right) \]

Seedlings fresh weight was measured and the fresh weight reduction percentage (FWPR) was calculated by employing the following formula (Zafar et al. 2015):

\[ \text{FWPR} \% = 100 \times \left( 1 - \frac{\text{fresh weight stress}}{\text{fresh weight control}} \right) \]

For dry weight measurements, seedlings were oven dried at 60 °C for 24 hours, and dry matter content (DMC) percentage was calculated using the formula:

\[ \text{DMC} \% = 100 \times \left( \frac{\text{dry weight}}{\text{fresh weight + dry weight}} \right) \]

Chlorophyll fluorescence

Chlorophyll a fluorescence measurement was carried out with a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). First the minimal fluorescence (F0) was determined in dark-adapted leaflets of three week old tobacco seedlings, followed by a short pulse of saturating light (3.000 µmol photons m⁻² s⁻¹) to induce maximum fluorescence (Fm). Then the leaflets were illuminated with actinic light (100 µmol photons m⁻² s⁻¹) to measure steady-state fluorescence (F) and maximum fluorescence (Fm) in light-adapted state. Maximum photochemical quantum efficiency of PSII (ΦPSII), effective quantum efficiency of PSII (ΦφPSII), nonphotochemical quenching (NPQ) and coefficient of photochemical quenching (qP) were calculated according to Maxwell and Johnson (2000). The relative electron transport rate (rETR) was calculated from the ΦPSII and photosynthetic photon flux density (Genty et al. 1989) and shown in the Results section.

Photosynthetic pigment analysis

Pigments were extracted from freeze dried leaf samples by homogenization in 96% ice-cold acetone with 0.3 mg mL⁻¹ CaCO₃. A high performance liquid chromatography (HPLC, Perkin Elmer, USA) with the diode array detector and non-endcapped Zorbax ODS column (4.6 × 250 mm, 5 µm particle size) preceded by an ODS guard column, both from Agilent, was used for separation. The pigments were eluted using a method described by Thayer and Bjorkman (1990)
with a slight modification: 100% solvent A (acetonitrile: methanol:water 84:12:4) for the first 2 min followed by a 14 min linear gradient to 100% solvent B (methanol:ethyl acetate 68:32) which continued isocratically for the next 9 min. The column was re-equilibrated in solvent A for 10 min prior to the next run. The flow rate was 1 mL min⁻¹.

The pigments neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, β-carotene, chlorophyll a and chlorophyll b were detected by their absorbance at 440 nm and quantified against known amounts of standards (DHI Water and Environment, Denmark).

Determination of Ag content

For determination of Ag content, whole three week old seedlings exposed to 25, 50, 75, 100 and 150 µM concentrations of AgNPs and AgNO₃ in a liquid half strength MS medium were taken. Firstly, they were washed with 0.01 M HNO₃ to remove AgNPs potentially adsorbed on the root surface, and subsequently rinsed with ultrapure water. Washed plant material was dried at 80 °C for 24 h and prepared for analysis as previously reported (Cvjetko et al. 2017, Peharec Štefanić et al. 2018). Determination of the total Ag concentration was done using an ELAN DRC-e ICP-MS (Perkin Elmer, USA). To calculate the Ag concentration, a calibration curve obtained with a set of standards of known concentrations was applied. Detection limit and limit of quantification (LOQ) were 0.05 µg g⁻¹ and 0.1 µg g⁻¹, respectively. Spike recovery tests were 96.6% and 96.8% for seedlings exposed to AgNPs and AgNO₃, respectively.

Statistical analysis

Statistical analysis was performed using the STATISTICA 13.0 (TIBCO) software package. The data were analysed by one-way ANOVA followed by the least significant difference (LSD) test. Differences between means were considered statistically significant at P ≤ 0.05.

Results

AgNPs stability in culture media

The results of the stability analyses obtained by spectrophotometric absorbance measurements of 150 µM AgNPs in solid and liquid half strength MS media are presented in Fig. 1. In the solid medium used for germination and early growth of tobacco seeds, a reduction of the absorption maximum (absorption of 0.8) and a small peak shift towards lower wavelengths (peak maximum at 422 nm) were recorded immediately after medium solidification (zero minute) compared to the absorption maximum obtained for the 150 µM AgNP solution in ultrapure water (absorption of 1.3 and peak maximum at 425 nm) (Fig. 1A). The intensity of the surface plasmon resonance (SPR) peak and its position remained relatively constant over 5 days suggesting little agglomeration of AgNPs had occurred. Obtained results indicate that in the solid medium AgNPs were encapsulated, which prevented their aggregation and/or the dissociation of Ag⁺ ions.

AgNP stability measurements in the liquid half strength MS medium, used for seedling growth for photosynthesis and photosynthetic pigment analyses, revealed stronger reduction of the absorption maximum compared to solid medium at zero minute (absorption of 0.5) and a peak shift towards lower wavelengths (peak maximum at 419 nm) compared to the absorption maximum obtained for the 150 µM AgNP solution in ultrapure water (absorption of 1.3 and peak maximum at 425 nm) (Fig. 1B). Moreover, an exponential decrease of the SPR intensity was observed over a period of 5 h, thus suggesting rapid agglomeration of AgNPs, although the position of the SPR peak remained relatively constant. After a 5 h period, no SPR peak could be detected, which indicates that AgNPs were completely aggregated. Additional DLS and TEM analysis of 150 µM AgNPs in the liquid half strength MS medium corroborated these findings (On-line Suppl. Figs. 1, 2). DLS analysis con-

Fig. 1. Stability analyses of AgNPs by UV-Vis absorption spectra in: A – solid half strength MS medium supplemented with 150 µM AgNPs recorded over a period of five days, B – liquid half strength MS medium supplemented with 150 µM AgNPs recorded over a period of seven days. Arrows indicate wavelengths of peak maximums.
confirmed the strong aggregation of AgNPs, which started almost immediately after the 150 µM AgNP solution in the liquid half strength MS medium was prepared; the appearance of nanoparticles with sizes larger than 100 nm was observed after 15 min and it continued progressively until 24 h when nanoparticles below 200 nm were no longer present (On-line Suppl. Fig. 1). The charge of AgNPs remained negative with a slight shift towards less negative values (On-line Suppl. Fig. 1). TEM analysis exhibited evident AgNP aggregation in exposure solutions of all concentrations, which was the most prominent in 150 µM AgNPs solution (On-line Suppl. Fig. 2).

**Effects on germination and early growth**

None of the applied concentrations of either AgNPs or AgNO₃ had a significant influence on germination percentage compared to control. Moreover, no significant difference was observed in the treatments with AgNPs and AgNO₃ of the corresponding concentrations (Fig. 2A). Germination rate was increased after exposure to all AgNP treatments in comparison to control, although significantly only at 50 µM concentration, while exposure to AgNO₃ had no significant effect. Comparison of treatments with corresponding concentrations of AgNPs and AgNO₃ revealed significant difference only for the 50 µM concentration (Fig. 2B). Germination index decreased after treatments with AgNPs compared to control, although significantly different values were obtained only at 50 and 100 µM concentrations (Fig. 2C). By contrast, no significant difference was obtained after exposure to AgNO₃. Corresponding concentrations of AgNPs and AgNO₃ were not significantly different (Fig. 2C).

Root growth was significantly enhanced after exposure to lower AgNP concentrations (25 and 50 µM) compared to control.
control, while treatments with 75, 100 and 150 µM AgNPs significantly reduced root elongation in a dose-dependent manner (Fig. 3A, Fig. 4). On the contrary, exposure to all AgNO₃ concentrations induced a significant reduction in root growth in comparison to control (Fig. 4). Comparison of treatments with AgNPs and AgNO₃ of the corresponding concentrations revealed a significant difference between them at 25 and 50 µM concentration (Fig. 3A).

All AgNP concentrations applied induced a significant reduction in fresh weight compared to control, unlike AgNO₃-treatments, among which only higher applied concentration (75, 100 and 150 µM) significantly decreased fresh weight content in comparison to control (Fig. 3B). A significant difference between the corresponding AgNP- and AgNO₃-treatments was obtained only for the 25 µM concentration, at which AgNPs induced a more prominent reduction in fresh weight than ionic Ag (Fig. 3B).

Compared to control, dry matter content increased after the majority of the treatments, being significantly different after exposure to 75 and 100 µM AgNPs and 50, 75, 100 and 150 µM AgNO₃. No significant difference was noticed between the corresponding treatments with AgNPs and AgNO₃ (Fig. 3C).

Effects on photosynthesis and photosynthetic pigments

Measurements of chlorophyll a fluorescence showed that none of the applied concentrations of either AgNPs or AgNO₃ had a significant influence on F₅/₇₃ compared to control (Tab. 1). On the other hand, relative electron transport rate and qP decreased markedly after exposure to all AgNP and AgNO₃ treatments, except for the lowest AgNP concentration (25 µM), where the observed decrease was not statistically different from control value (Tab. 1). Moreover, AgNO₃ applied in all concentrations, except the lowest one, had a more negative effect on both parameters than the corresponding AgNP treatments, leading to a reduction of over 50% (Tab. 1). Non-photochemical quenching was also significantly lower after exposure to all AgNP and AgNO₃ treatments, except for the 25 µM AgNPs, where the observed decrease was not statistically different from the control value (Tab. 1). There was no difference between AgNP and AgNO₃ treatments of corresponding concentrations (Tab. 1).

The majority of the applied concentrations of both AgNPs and AgNO₃ increased the content of total chlorophylls compared to control (Tab. 2). The only exceptions were the highest concentration of AgNPs and the lowest concentration of AgNO₃, where the values were similar to those of control. In both types of treatments, the highest concentration of total chlorophylls was measured after exposure to 50 µM concentration, although the value was higher in seedlings exposed to AgNPs compared to AgNO₃ (Tab. 2). On the other hand, higher concentrations of AgNPs (100 and 150 µM) resulted in lower pigment content than the corresponding concentrations of AgNO₃. Compared to control, chlorophyll a/b ratio was decreased after treatments with lower AgNP concentrations (25, 50 and 75 µM) as well as with the 50 µM AgNO₃ (Tab. 2).

Regarding total carotenoids, exposure to 25, 50 and 75 µM AgNPs significantly increased their content compared to control, while among treatments with AgNO₃, the 50, 75 and 100 µM concentrations resulted in significant augmentation (Tab. 2). Comparison of corresponding AgNP and AgNO₃ concentrations revealed that significantly lower values were measured in seedlings exposed to 100 and 150 µM AgNPs than in the corresponding concentrations of AgNO₃.

### Tab. 1. Contents of Ag in dry weight (DW) and chlorophyll fluorescence parameters (expressed as % of control) in tobacco seedlings after 7 days of exposure to 0, 25, 50, 75, 100 and 150 µM AgNPs and AgNO₃. Fv/Fm – maximum fluorescence in dark-adapted state, rETR – relative electron transport rate, qP – coefficient of photochemical quenching, NPQ – non-photochemical quenching. Values are the means ± standard error of three different experiments, each with three replicas. If values are marked with different letters, the treatments are significantly different at P ≤ 0.05 (LSD test); capital letters mark the differences among AgNP concentrations as well as control, small letters mark the differences among AgNO₃ concentrations as well as control, while asterisks mark the differences among different treatments of the same concentration. * - Ag content was below the limit of quantification (< 0.1 µg g⁻¹). ns – no significant difference.

| Conc (µM) | Ag (µg g⁻¹ DW) | Fv/Fm | rETR | qP | NPQ |
|-----------|----------------|-------|------|----|----|
| control   | 0              | 100.00 ± 0.82 | 100.00 ± 4.46 | 100.0 ± 5.17 | 100.0 ± 4.33 |
| AgNP      | 25             | 180.03 ± 28.67 | 98.57 ± 0.64 | 83.04 ± 7.44 | 89.30 ± 6.68 |
|           | 50             | 214.85 ± 30.88 | 98.33 ± 0.46 | 60.76 ± 5.85 | 64.78 ± 6.99 |
|           | 75             | 270.26 ± 18.93 | 98.13 ± 0.73 | 57.38 ± 3.37 | 58.91 ± 3.49 |
|           | 100            | 358.60 ± 28.02 | 98.94 ± 0.73 | 60.76 ± 5.06 | 61.46 ± 5.47 |
|           | 150            | 404.98 ± 19.20 | 100.15 ± 0.97 | 65.82 ± 9.69 | 63.04 ± 9.39 |
| AgNO₃     | 25             | 76.92 ± 16.52 | 99.26 ± 0.67 | 76.66 ± 4.46 | 75.81 ± 4.70 |
|           | 50             | 152.19 ± 23.33 | 98.43 ± 1.24 | 46.72 ± 7.44 | 44.84 ± 4.91 |
|           | 75             | 204.72 ± 15.11 | 99.17 ± 0.84 | 42.14 ± 5.85 | 41.56 ± 4.28 |
|           | 100            | 230.87 ± 27.13 | 99.64 ± 1.32 | 38.75 ± 3.37 | 39.69 ± 7.93 |
|           | 150            | 296.03 ± 15.24 | 99.17 ± 0.98 | 36.89 ± 5.06 | 35.17 ± 6.12 |

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Table 2. Contents of photosynthetic pigments and their ratios expressed as % of control in tobacco seedlings after 7 days of exposure to 0, 25, 50, 75, 100 and 150 µM AgNPs and AgNO₃ in a liquid half-strength MS medium. VZA - violaxanthin, zeaxanthin and antheraxanthin (xanthophyll cycle pool), chl/β - chlorophyll/β, car/chl - carotenoids/chlorophyll, VZA/chl - VZA/chlorophyll. Values are the means ± standard error of three different experiments, each with three replicas. If values are marked with different letters, the treatments are significantly different at P ≤ 0.05 (LSD test); capital letters mark the differences among AgNP concentrations as well as control, small letters mark the differences among AgNO₃ concentrations as well as control, while asterisks mark the differences among different treatments of the same concentration.

| Conc (µM) | Total carotenoids | Total chlorophyll | VZA | Neo-xanthin | Lutein | β-carotene | Chl/β | Car/Chl | VZA/Chl |
|----------|------------------|------------------|-----|-------------|--------|------------|--------|---------|---------|
| 0        | 100 ± 2.94       | 100 ± 1.63       | 100 | 100 ± 1.30  | 100    | 100 ± 1.76 | 100   | 100     | 100     |
| AgNP     | 25               | 120.31 ± 3.23   | 121.27 ± 2.28 | 115.71 ± 3.42 | 108.34 ± 3.34 | 101.97 ± 2.15 | 100.70 ± 2.53 | 100.00 ± 3.36 | 94.86 ± 2.14 |
|          | 50               | 130.51 ± 2.52   | 146.02 ± 2.97 | 121.28 ± 2.77 | 115.71 ± 3.42 | 101.97 ± 2.15 | 100.70 ± 2.53 | 100.00 ± 3.36 | 94.86 ± 2.14 |
|          | 75               | 120.88 ± 2.40   | 116.10 ± 1.24 | 95.50 ± 2.93 | 92.47 ± 1.78 | 97.12 ± 1.32 | 97.12 ± 1.32 | 97.12 ± 1.32 | 97.12 ± 1.32 |
|          | 100              | 121.78 ± 2.16   | 121.78 ± 2.16 | 115.49 ± 2.95 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 |
|          | 150              | 117.79 ± 2.30   | 117.79 ± 2.30 | 115.49 ± 2.95 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 |
| AgNO₃    | 25               | 99.96 ± 4.34    | 101.97 ± 2.15 | 103.54 ± 3.33 | 108.34 ± 3.34 | 101.97 ± 2.15 | 100.70 ± 2.53 | 100.00 ± 3.36 | 94.86 ± 2.14 |
|          | 50               | 122.92 ± 5.15   | 122.78 ± 2.11 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 |
|          | 75               | 120.31 ± 2.16   | 121.78 ± 2.16 | 115.49 ± 2.95 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 |
|          | 100              | 117.79 ± 2.30   | 117.79 ± 2.30 | 115.49 ± 2.95 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 |
|          | 150              | 117.79 ± 2.30   | 117.79 ± 2.30 | 115.49 ± 2.95 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 | 120.97 ± 2.39 | 105.95 ± 2.67 |

AgNPs AFFECT TOBACCO GERMINATION AND PHOTOSYNTHESIS

Similar results as those of total carotenoids were obtained for xanthophylls involved in the xanthophyll cycle (violaxanthin, zeaxanthin and antheraxanthin, VZA), although the differences among the AgNP and AgNO₃ treatments were significant only for 100 and 150 µM concentrations (Tab. 2). Other xanthophylls, neoxanthin and lutein, followed a similar trend; namely, exposure to lower AgNP concentrations (25, 50 and 75 µM) significantly increased their content compared to control, while treatments with AgNO₃ resulted in a significant increase with all tested concentrations, except the lowest one (25 µM) (Tab. 2). Comparison of corresponding AgNP and AgNO₃ treatments revealed that the two highest AgNP concentrations resulted in significantly lower pigment content than treatments with 100 and 150 µM AgNO₃. In contrast, β caroten content was increased only after the treatment with 75 µM AgNPs, while the two highest concentrations significantly lowered its value.

Among all tested treatments only exposure to 100 µM AgNPs resulted in a lower carotenoid/chlorophyll ratio in comparison to the control (Tab. 2). Moreover, seedlings exposed to higher AgNP concentrations (100 and 150 µM) had reduced carotenoid/chlorophyll ratio compared to seedlings treated with corresponding concentrations of AgNO₃. VZA/chlorophyll ratio was significantly decreased after all AgNP treatments compared to the control, especially with the highest concentrations of AgNP (100 and 150 µM), which also provoked a significantly lower VZA/chlorophyll ratio than the corresponding AgNO₃ concentration (Tab. 2). By contrast, only treatment with 50 µM AgNO₃ induced a significantly low VZA/chlorophyll ratio compared to the control (Tab. 2).

Ag content

Ag content in tobacco seedlings exponentially increased with the increasing concentrations of both AgNPs and AgNO₃ treatments, being significantly the highest after exposure to 150 µM AgNPs and AgNO₃ compared to control (Tab. 1). Comparison of AgNP and AgNO₃ treatments of the corresponding concentrations revealed that in general Ag uptake in seedlings was higher after exposure to AgNPs compared to ionic Ag. In control seedlings, Ag content was below the instrument detection limit LOQ (<0.1 µg g⁻¹).

Discussion

Germination rate and index values indicated that germination of tobacco seeds was delayed and slower only in some AgNP-treatments than in the control, while AgNO₃ had no significant effect. Previous experiments performed with nanoparticles on germination of different plant species showed negative (Geisler-Lee et al. 2014, Tripathi et al. 2017), positive (Parveen and Rao 2015, Almutairi and Alharbi 2015) as well as neutral effects (El-Temsah and Joner 2012, Yin et al. 2012), depending on physico-chemical char-
Strength MS medium showed high stability of the applied nanoparticles. AgNP stability analyses in the solid half to nanoparticle form of Ag and not Ag+ ions released from implying that the observed AgNP toxicity could be ascribed reduction was recorded. Our results indicate that effects induced by AgNPs and AgNO3 are similar but not identical implying that the observed AgNP toxicity could be ascribed to nanoparticle form of Ag and not Ag+ ions released from nanoparticles. AgNP stability analyses in the solid half strength MS medium showed high stability of the applied AgNPs, with minor aggregation and/or dissociation of Ag+ ions. As previously reported by Peharec Štefanić et al. (2018), this is probably a result of AgNPs stabilization with Phytagel, a natural polymer used for solidification of nutrient media. It is known that some polymer ligands can control access of molecular oxygen to the nanoparticle surface, which would decrease the oxidative dissolution rate of AgNPs (Gunsolus et al. 2015). Therefore, in our treatments with AgNPs added in the solid nutrient medium for germination and growth experiment, Ag was accessible to seeds and developing seedlings in the form of nanoparticles, although the presence of Ag+ ions due to release from the AgNP surface cannot be completely excluded.

The mechanisms explaining the impact of AgNPs on germination and plant biomass are still not completely elucidated. Positive effects can be ascribed to AgNPs ability to generate new pores on the seed coat during penetration, which may help in the influx of nutrients inside the seed or else AgNPs may carry the nutrients along with them, leading to accelerated germination and increased growth rate (Tkalec et al. 2019). On the other hand, Zuverza-Mena et al. (2016) demonstrated that AgNP-treatment of radish sprouts induced a decrease in water content in a dose-dependent manner, which is in accordance with increased dry matter content after AgNP-exposure of tobacco seedlings recorded in our study. These results together with findings that AgNPs affect the balance of water by changing the transpiration of aquaporin genes (Qian et al. 2013), imply that AgNPs can negatively affect plant growth by reducing water uptake.

Chlorophyll fluorescence measurement showed that exposure of tobacco seedlings to AgNPs resulted in reduced values of $\Phi_{PSII}$ and qP, implying an inhibition of the electron transport chain. Significantly reduced qP was previously reported in mustard (Vishwakarma et al. 2017) and pea seedlings (Tripathi et al. 2017) after exposure to AgNPs. Furthermore, a severe disruption of photosynthesis and CO2 assimilation efficiency as well as decrease in photosynthesis rate was found in tomato seedlings treated with AgNPs (Das et al. 2018). Moreover, in these studies a decline in chlorophyll fluorescence parameters was accompanied with a decrease in chlorophyll content, suggesting severe negative effects of AgNPs on photosynthesis. To the contrary, in our study, a significant increase of total chlorophyll was obtained at almost all tested concentrations of AgNPs. An increase in chlorophyll content along with significantly lower values of $\Phi_{PSII}$ and qP was also recorded in tobacco seedling after almost all AgNO3-treatments. A similar impact of AgNPs and AgNO3 on photosynthesis can be correlated with lower stability of AgNPs in the liquid medium used for plant growth, resulting in AgNP aggregation as well as dissolution of Ag+ ions. Several studies suggested that conditions present in the culture medium such as high ionic strength and neutral pH can cause AgNP dissolution (Sharma et al. 2014, Domazet Jurašin et al. 2016). Therefore, our results suggest that AgNPs exposed to either AgNPs or AgNO3 increased synthesis of chlorophyll pigments to overcome the impaired electron transfer imposed by Ag+ ions. This is because Ag+ ions can competitively replace Cu+ ions and bind to plastocyanin, which results in disturbance or inactivation of the photosynthetic electron transport (Sujak 2005, Jansson and Hansson 2008). Moreover, previous proteomic analysis of tobacco seedlings (Peharec Štefanić et al. 2018) revealed up-regulation of proteins involved in the photosynthesis after AgNP and AgNO3 treatments. However, the reduction of $\Phi_{PSII}$ and qP in tobacco seedlings was significantly more pronounced after exposure to AgNO3 compared to corresponding AgNP treatments, although total Ag content was much higher in seedlings exposed to AgNPs. This could be correlated with prominent oxidative stress found in AgNO3-treated seedlings (Peharec Štefanić et al. 2018), which could additionally damage photosynthetic apparatus. In the study of Pardha-Saradhi et al. (2018) it was shown that exposure of wheat
seedlings to AgNPs had a less toxic effect on light harnessing photosynthetic machinery than AgNO₃, which corroborates our results. Contents of analysed carotenoids were also elevated in tobacco seedlings exposed to lower concentrations of AgNPs and most concentrations of AgNO₃. Increased total carotenoids and xanthophylls might play a role in the regulation of light harvesting and energy flow of chlorophyll-excited states. Also, carotenoids are important antioxidant molecules which may represent a defence against elevated ROS (He et al. 2011). A strong increase of carotenoid content has already been found in plants after AgNP exposure, suggesting that carotenoids are implicated in antioxidant defence responses of plants to AgNPs (Mirzajani et al. 2013, Yan and Chen 2019). It has been suggested that xanthophylls, especially zeaxanthin, are involved in the NPQ of excess energy in the antenna of PSII (Jahns and Holzwarth 2012). However, in our study NPQ was decreased in almost all AgNP and AgNO₃ treatments. Still, the VZA/chlorophyll ratio was mostly lower in AgNP treated tobacco seedlings, so it is possible that AgNPs in interaction with chlorophylls acted as quencher removing the excited electron from the chlorophyll (Queiroz et al. 2016).

Different effects induced by AgNPs and AgNO₃ on germination and growth compared to effects of photosynthesis can be related to the stability of the AgNPs in the media used in the experiments. Since the observed effects of AgNPs compared to AgNO₃ were not identical, some of the toxic effects can be attributed to the nanoparticle form of Ag.

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