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Silver nanoparticles affect germination and photosynthesis in tobacco seedlings

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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 $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_3$ 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

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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 ($AgNO_3$), 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.) seedlings, were compared. The significance of the work we are reporting on derives from interactions that have received little attention to date; the difference of possible phytotoxic effects of silver nanoparticles and ionic silver on germination and early growth as well as on photosynthesis. As object of our study we chose tobacco, not only an economically interesting and important plant but also a frequently used model organism in abiotic stress research (Gichner et al. 2004, Peharec Štefanić et al. 2012, Tkalec et al. 2014).

Materials and methods

AgNPs and $AgNO_3$ 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. $AgNO_3$ (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 $AgNO_3$ 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 $AgNO_3$ on the germination and early growth of tobacco (*Nicotiana tabacum* L. cv. Burley). Nutrient medium was sterilised and supplemented with AgNP or $AgNO_3$ 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 H_2O before being placed on the half strength MS medium. Control seeds were germinated on a medium devoid of AgNPs and $AgNO_3$. 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 $AgNO_3$ (200 seeds in total per treatment).

For analysis of effects of AgNPs and $AgNO_3$ 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 $AgNO_3$ 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.

AgNP stability in solid and liquid culture medium

AgNPs stability in the solid nutrient medium was analysed as previously reported (Peharec Štefanić 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-

est applied AgNP concentration in this study), was prepared in a 1 cm quartz cuvette for spectrophotometric absorbance measurements. The cuvette was sealed with Parafilm M to prevent medium from drying and was kept in the same conditions as plant material during 5 days. Measurements of spectrophotometric absorbance were performed using the UV-visible spectrophotometer (ATI Unicam, Cambridge, UK) in the wavelength range of 300–800 nm. For instrument zeroing, solid half strength MS medium devoid of AgNPs was applied. Stability of AgNPs was monitored regularly during the period of 5 days.

For measurements of AgNP stability in the liquid half strength MS medium a similar procedure for spectrophotometric measurements was applied. The differences were that a liquid half strength MS medium was used instead of a solid and that the cuvette was kept for 7 days in the same conditions as the plant material. Measurements of spectrophotometric absorbance were performed in the abovementioned wavelength range. For instrument zeroing, liquid half strength MS medium devoid of AgNPs was applied. Stability of AgNPs was monitored regularly during the period of 7 days.

To confirm the data obtained by spectrophotometric analysis, the size and charge of nanoparticles in 150 μM AgNP solution in liquid half strength MS medium were measured with the dynamic light scattering (DLS) technique using a Zetasizer Nano ZS (Malvern, UK) equipped with green laser (532 nm). Intensity of scattered light was detected at the angle of 173°. All measurements were conducted at 25 °C. The data processing was done with the use of the Zetasizer software 6.32 (Malvern instruments). Measurements were performed at 0 and 15 min as well as 1, 5 and 24 h after the solution of AgNPs in nutrient medium was prepared. Results are reported as an average value of 10 measurements and the size distributions are reported as volume distributions. The charge of AgNPs was evaluated by measuring electrophoretic mobility of AgNPs and results are reported as an average value of 5 measurements.

In addition, AgNPs were visualized in nutrient media of all tested AgNP concentrations after exposure of tobacco seedlings in a liquid medium using a FEI Morgagni 268D electron microscope. TEM samples were prepared by depositing a drop of the sample suspension on a Formvar®/Carbon copper grid. Samples were air-dried at room temperature.

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:

Germination percentage (%) = final number of germinated seeds/total number of seeds × 100.

To calculate germination index (GI) and germination rate (T_{50}), daily counts of germinated seeds were used by employing the following formulas (Farooq et al. 2005):

$$GI = \sum Nt \text{ (number of germinated seeds on the t day)}/Dt \text{ (germination days)}$$

$$T_{50} = t_i + \{[(N/2)-n_i]*(t_i-t_j)/n_i-n_j\}$$

where N is the final number of germinated seeds, n_i and n_j are cumulative number of seeds germinated by consecutive counts at times t_i and t_j, considering that $n_i<N/2<n_j$.

Growth parameters

Three weeks after exposure to AgNPs and $AgNO_3$ 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):

$$RLPR\ \% = 100 \times [1 - (\text{root length}_{stress}/\text{root length}_{control})].$$

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

$$FWPR\ \% = 100 \times [1 - (\text{fresh weight}_{stress}/\text{fresh weight}_{control})].$$

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:

$$DMC\ \% = 100 \times [\text{dry weight}/(\text{fresh weight} + \text{dry weight})]$$

Chlorophyll fluorescence

Chlorophyll *a* fluorescence measurement was carried out with a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). First the minimal fluorescence (F_0) 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 (F_m). Then the leaflets were illuminated with actinic light (100 μmol photons m^{-2} s^{-1}) to measure steady-state fluorescence (F) and maximum fluorescence (F'_m) in light-adapted state. Maximum photochemical quantum efficiency of PSII (F_v/F_m), effective quantum efficiency of PSII (Φ_{PSII}), 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 Φ_{PSII} 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} $CaCO_3$. 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 $AgNO_3$ in a liquid half strength MS medium were taken. Firstly, they were washed with 0.01 M HNO_3 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 $AgNO_3$, 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 $P \leq 0.05$.

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-

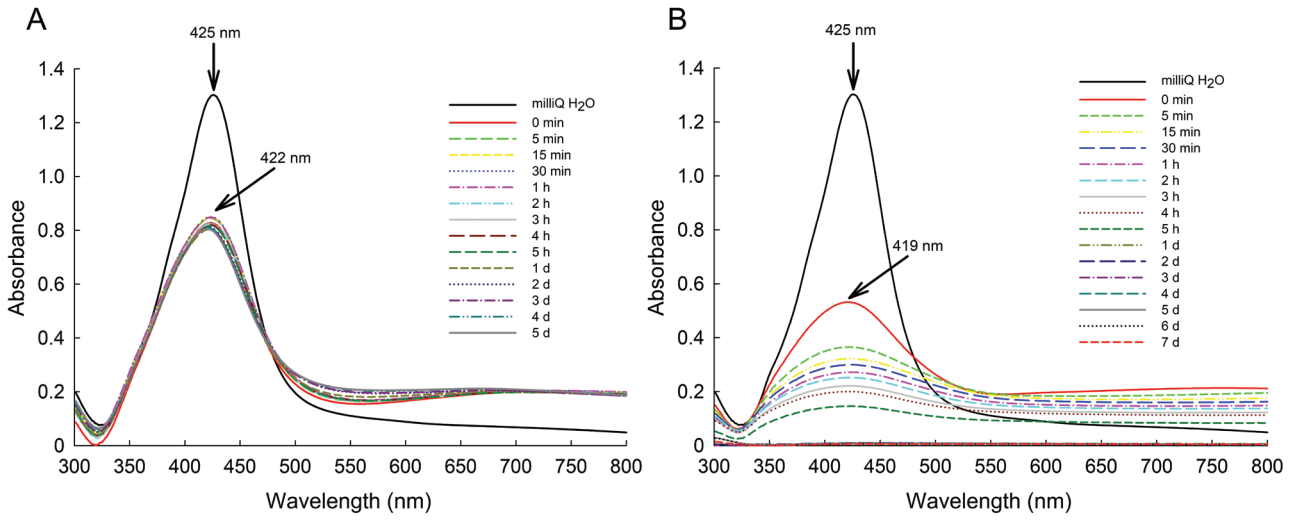

Fig. 1. Stability analyses of AgNPs by UV-Vis absorption spectra in: A – solid half strength MS medium supplemented with 150 µM AgNPs recorded over a period of five days, B – liquid half strength MS medium supplemented with 150 µM AgNPs recorded over a period of seven days. Arrows indicate wavelengths of peak maximums.

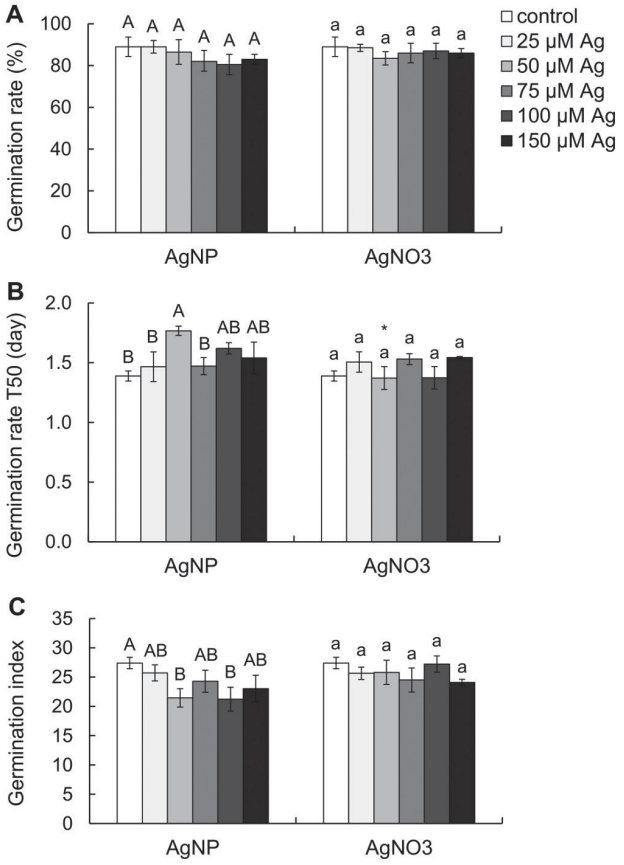

Fig. 2. Germination parameters of tobacco seeds after 5 days of germination on a solid half strength MS medium supplemented with 25, 50, 75, 100 or 150 μM AgNPs or $AgNO_3$. A – germination percentage, B – germination rate (T_{50}), C – germination index. Values are the means ± standard error of three different experiments with 100 seeds per experiment (n = 300). If values are marked with different letters, the treatments are significantly different at $P \leq 0.05$ (one-way ANOVA followed by LSD post-hoc test); capital letters mark the differences among different concentrations of the AgNP treatment as well as control, small letters mark the differences among different concentrations of the $AgNO_3$ as well as control, while asterisks mark the differences among different treatments of the same concentration.

firmed 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 $AgNO_3$ had a significant influence on germination percentage compared to control. Moreover, no significant difference was observed in the treatments with AgNPs and $AgNO_3$ 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 $AgNO_3$ had no significant effect. Comparison of treatments with corresponding concentrations of AgNPs and $AgNO_3$ 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 $AgNO_3$. Corresponding concentrations of AgNPs and $AgNO_3$ were not significantly different (Fig. 2C).

Root growth was significantly enhanced after exposure to lower AgNP concentrations (25 and 50 μM) compared to

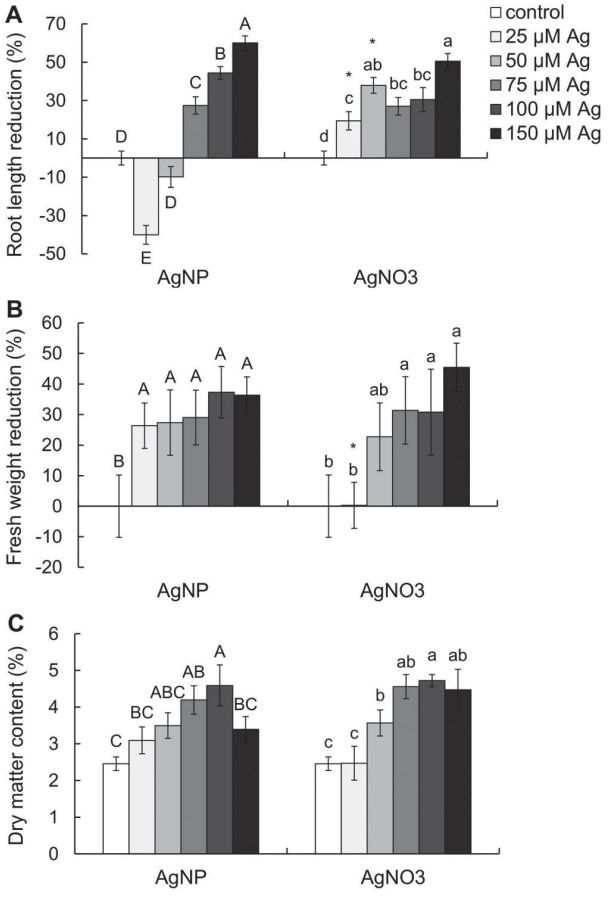

Fig. 3. Growth parameters of three week old tobacco seedlings on a solid half strength MS medium supplemented with 25, 50, 75, 100 or 150 μM AgNPs or $AgNO_3$. A – root length reduction percentage, values are the means ± standard error of three different experiments with 20 seedlings per experiment (n = 60), B – fresh weight reduction percentage, C – dry matter content. For fresh and dry weight, values are the means ± standard errors of three different experiments with 3 replicas per experiment (n = 9). If values are marked with different letters, the treatments are significantly different at $P \leq 0.05$ (LSD test); capital letters mark the differences among different concentrations of the AgNP treatment as well as control, small letters mark the differences among different concentrations of the $AgNO_3$ as well as control, while asterisks mark the differences among different treatments of the same concentration.

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 $AgNO_3$. F_v/F_m – 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 $P \leq 0.05$ (LSD test); capital letters mark the differences among AgNP concentrations as well as control, small letters mark the differences among $AgNO_3$ 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	$0^{d,\#}$	100.00 ± 0.82	$100.00 \pm 4.46^{A,a}$	$100.0 \pm 5.17^{A,a}$	$100.0 \pm 4.33^{A,a}$
AgNP	25	180.03 ± 28.67^{C}	98.57 ± 0.64^{ns}	83.04 ± 7.44^{AB}	89.30 ± 6.68^{AB}	86.84 ± 8.9^{AB}
	50	214.85 ± 30.88^{BC}	98.33 ± 0.46^{ns}	60.76 ± 5.85^{B}	64.78 ± 6.99^{BC}	77.62 ± 5.21^{B}
	75	270.26 ± 18.93^{B}	98.13 ± 0.73^{ns}	57.38 ± 3.37^{B}	58.91 ± 3.49^{C}	66.67 ± 6.33^{B}
	100	358.60 ± 28.02^{A}	98.94 ± 0.73^{ns}	60.76 ± 5.06^{B}	61.46 ± 5.47^{BC}	62.03 ± 5.80^{B}
	150	404.98 ± 19.20^{A}	100.15 ± 0.97^{ns}	65.82 ± 9.69^{B}	63.04 ± 9.39^{BC}	68.45 ± 7.41^{B}
$AgNO_3$	25	$76.92 \pm 16.52^{cd,*}$	99.26 ± 0.67^{ns}	76.66 ± 4.46^{b}	75.81 ± 4.7^{8b}	75.17 ± 7.31^{b}
	50	$152.19 \pm 23.33^{bc,*}$	98.43 ± 1.24^{ns}	$46.72 \pm 7.44^{c,*}$	$44.84 \pm 4.91^{c,*}$	64.02 ± 6.58^{b}
	75	$204.72 \pm 15.11^{b,*}$	99.17 ± 0.84^{ns}	$42.14 \pm 5.85^{c,*}$	$41.56 \pm 4.28^{c,*}$	63.33 ± 4.03^{b}
	100	$230.87 \pm 27.13^{ab,*}$	99.64 ± 1.32^{ns}	$38.75 \pm 3.37^{c,*}$	$39.69 \pm 7.93^{c,*}$	57.34 ± 5.29^{b}
	150	$296.03 \pm 15.24^{a,*}$	99.17 ± 0.98^{ns}	$36.89 \pm 5.06^{c,*}$	$35.17 \pm 6.12^{c,*}$	62.92 ± 3.95^{b}

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 $AgNO_3$ concentrations induced a significant reduction in root growth in comparison to control (Fig. 4). Comparison of treatments with AgNPs and $AgNO_3$ 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 $AgNO_3$-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 $AgNO_3$-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 $AgNO_3$. No significant difference was noticed between the corresponding treatments with AgNPs and $AgNO_3$ (Fig. 3C).

Effects on photosynthesis and photosynthetic pigments

Measurements of chlorophyll *a* fluorescence showed that none of the applied concentrations of either AgNPs or $AgNO_3$ had a significant influence on F_v/F_m compared to control (Tab. 1). On the other hand, relative electron transport rate and qP decreased markedly after exposure to all AgNP and $AgNO_3$ treatments, except for the lowest AgNP concentration (25 μM), where the observed decrease was not statistically different from control value (Tab. 1). Moreover, $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_3$ treatments of corresponding concentrations (Tab. 1).

The majority of the applied concentrations of both AgNPs and $AgNO_3$ 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 $AgNO_3$, 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 $AgNO_3$ (Tab. 2). On the other hand, higher concentrations of AgNPs (100 and 150 μM) resulted in lower pigment content than the corresponding concentrations of $AgNO_3$. 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 $AgNO_3$ (Tab. 2).

Regarding total carotenoids, exposure to 25, 50 and 75 μM AgNPs significantly increased their content compared to control, while among treatments with $AgNO_3$, the 50, 75 and 100 μM concentrations resulted in significant augmentation (Tab. 2). Comparison of corresponding AgNP and $AgNO_3$ concentrations revealed that significantly lower values were measured in seedlings exposed to 100 and 150 μM AgNPs than in the corresponding concentrations of $AgNO_3$,

Tab. 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 $AgNO_3$ in a liquid half strength MS medium. VZA - violaxanthin, zeaxanthin and antheraxantin (xanthophyll cycle pool), chl *a*/*b* - chlorophyll *a*/*b*, 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 $P \leq 0.05$ (LSD test); capital letters mark the differences among AgNP concentrations as well as control, small letters mark the differences among $AgNO_3$ 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 *a*/*b*	Car/Chl	VZA/Chl
control	0	$100 \pm 3.29^{B,c}$	$100 \pm 2.82^{D,d}$	$100 \pm 3.74^{BC,\ b}$	$100 \pm 3.90^{B,d}$	$100 \pm 4.13^{B,d}$	100 ± 4.46^{B}	$100 \pm 1.30^{A,a}$	100 ± 2.79^{A}	$100 \pm 3.27^{A,a}$
AgNP	25	127.86 ± 3.96^{A}	130.77 ± 2.25^{B}	115.41 ± 8.96^{AB}	148.87 ± 2.13^{A}	139.52 ± 4.29^{A}	113.64 ± 5.55^{AB}	94.46 ± 1.32^{BC}	97.67 ± 2.39^{A}	90.27 ± 3.30^{B}
	50	130.51 ± 2.52^{A}	146.02 ± 2.97^{A}	121.27 ± 2.08^{A}	155.66 ± 1.84^{A}	147.83 ± 2.86^{A}	108.34 ± 3.69^{AB}	93.17 ± 0.93^{C}	89.32 ± 3.75^{AB}	84.33 ± 1.87^{BC}
	75	132.54 ± 3.93^{A}	134.14 ± 3.15^{B}	114.79 ± 5.87^{AB}	152.00 ± 4.33^{A}	144.28 ± 5.35^{A}	121.28 ± 2.77^{A}	96.14 ± 1.76^{BC}	95.74 ± 2.46^{A}	88.44 ± 3.42^{BC}
	100	94.03 ± 2.40^{B}	116.10 ± 1.24^{C}	82.57 ± 2.02^{C}	111.80 ± 3.47^{B}	108.89 ± 2.43^{B}	78.79 ± 1.68^{C}	97.51 ± 4.15^{AB}	80.94 ± 1.58^{B}	71.04 ± 3.12^{D}
	150	90.69 ± 1.88^{B}	102.97 ± 1.63^{D}	80.71 ± 2.37^{C}	108.58 ± 5.76^{B}	101.66 ± 1.96^{B}	79.92 ± 2.19^{C}	100.65 ± 0.98^{A}	88.01 ± 2.75^{AB}	78.30 ± 5.20^{CD}
$AgNO_3$	25	$99.96 \pm 4.35^{c,\star}$	$101.31 \pm 1.97^{d,\star}$	95.50 ± 2.93^{b}	$103.87 \pm 2.53^{d,\star}$	$106.55 \pm 1.79^{d,\star}$	$94.76 \pm 4.02^{\star}$	95.70 ± 4.64^{ab}	98.66 ± 4.57	94.22 ± 2.38^{ab}
	50	122.92 ± 5.15^{a}	$132.78 \pm 2.11^{a,\star}$	116.49 ± 2.74^{a}	151.46 ± 2.29^{a}	153.7 ± 3.99^{a}	103.60 ± 9.90	92.47 ± 1.78^{b}	96.30 ± 3.11	87.68 ± 2.22^{b}
	75	$119.16 \pm 2.91^{ab,\star}$	123.74 ± 1.91^{b}	114.63 ± 2.59^{a}	$135.78 \pm 3.87^{bc,\star}$	141.49 ± 3.31^{ab}	97.07 ± 5.07	97.12 ± 3.72^{ab}	96.52 ± 1.90	91.79 ± 1.52^{ab}
	100	$120.31 \pm 2.16^{ab,\star}$	$121.78 \pm 1.87^{b,\star}$	$115.49 \pm 2.85^{a,\star}$	$140.95 \pm 2.67^{ab,\star}$	$134.21 \pm 2.81^{bc,\star}$	$110.13 \pm 1.73^{\star}$	95.46 ± 1.35^{ab}	$100.44 \pm 3.36^{\star}$	$94.85 \pm 2.77^{ab,\star}$
	150	$111.79 \pm 3.23^{bc,\star}$	$113.61 \pm 1.13^{c,\star}$	$103.31 \pm 3.35^{ab,\star}$	$125.54 \pm 3.42^{c,\star}$	$120.97 \pm 1.38^{c,\star}$	$105.85 \pm 2.73^{\star}$	96.90 ± 1.42^{ab}	$98.42 \pm 1.70^{\star}$	$90.99 \pm 3.85^{ab,\star}$

while the opposite result was observed for treatments with 25 and 75 μM AgNPs and $AgNO_3$ (Tab. 2).

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 $AgNO_3$ 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 $AgNO_3$ resulted in a significant increase with all tested concentrations, except the lowest one (25 μM) (Tab. 2). Comparison of corresponding AgNP and $AgNO_3$ treatments revealed that the two highest AgNP concentrations resulted in significanlty lower pigment content than treatments with 100 and 150 μM $AgNO_3$. In contrast, β caroten content was increased only after the treatment with 75 μM AgNPs, while the two highest concentrations significanly 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 $AgNO_3$. 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 $AgNO_3$ concentration (Tab. 2). By contrast, only treatment with 50 μM $AgNO_3$ 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 $AgNO_3$ treatments, being significantly the highest after exposure to 150 μM AgNPs and $AgNO_3$ compared to control (Tab. 1). Comparison of AgNP and $AgNO_3$ 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 $AgNO_3$ 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-

acteristics of the particles, plant species and the applied concentration of AgNPs.

Root growth was significantly enhanced after exposure to lower AgNP concentrations (25 and 50 μM), but significantly reduced with higher concentrations in a dose-dependent manner, which is in accordance with the effects of AgNPs on corn and common bean plantlets (Salama 2012) as well as on wheat seedlings (Asanova et al. 2019). Moreover, higher concentrations of AgNPs had effects on root growth of tobacco seedlings that were as retarding as $AgNO_3$, which inhibited root growth from the lowest applied concentration. Prominent toxic effects of $AgNO_3$ on root elongation have already been reported for mustard (Vishwakarma et al. 2017), barley seedlings (Fayez et al. 2017) and he castor bean (Yasur and Rani 2013). However, in this study the lowest AgNP concentration, which stimulated tobacco root growth, induced prominent reduction in fresh weight compared to corresponding concentration of $AgNO_3$, where no reduction was recorded. Our results indicate that effects induced by AgNPs and $AgNO_3$ 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 transcription 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 Φ_{PSII} and qP, implying an inhibition of the electron transport chain. Significantly reduced qP was previously

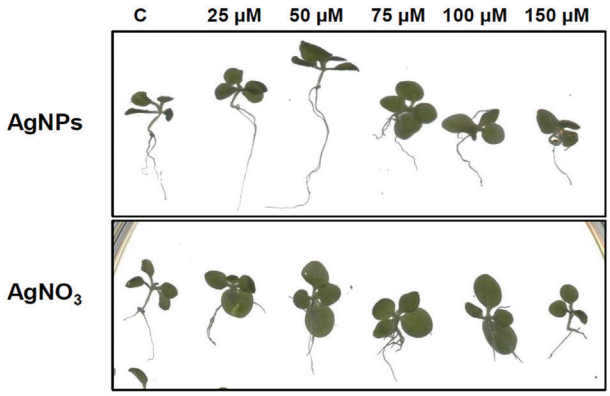

Fig. 4. Three week old tobacco seedlings after growing on a solid half strength MS medium supplemented with 25, 50, 75, 100 or 150 μM AgNPs or $AgNO_3$. C – control (solid half strength MS).

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 CO_2 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 Φ_{PSII} and qP was also recorded in tobacco seedling after almost all $AgNO_3$-treatments. A similar impact of AgNPs and $AgNO_3$ 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 tobacco seedlings exposed to either AgNPs or $AgNO_3$ 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 $AgNO_3$ treatments. However, the reduction of Φ_{PSII} and qP in tobacco seedlings was significantly more pronounced after exposure to $AgNO_3$ 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 $AgNO_3$-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 $AgNO_3$, which corroborates our results. Contents of analysed carotenoids were also elevated in tobacco seedlings exposed to lower concentrations of AgNPs and most concentrations of $AgNO_3$. 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 $AgNO_3$ 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 $AgNO_3$ 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 $AgNO_3$ were not identical, some of the toxic effects can be attributed to the nanoparticle form of Ag.

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