

Effects of exposure of callus cells of two wheat varieties to silver nanoparticles and silver salt (AgNO₃)

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Abstract Metal nanoparticles significantly affect the physiological properties of plants, e.g., seed germination, growth and metabolism. In the present study, the toxic effects of silver nanoparticles (AgNPs) and silver ions were studied on callus cells of two varieties of wheat (*Triticum aestivum* L.): stress tolerant—Parabola; stress sensitive—Raweta. Stress induced by silver particles or ions (0, 20, 40, 60 ppm) was investigated using different parameters such as morphological characteristics, lipid peroxidation and mobilization of defense system which was determined by analyzing the activity of antioxidant enzymes, glutathione (GSH) and proline contents. Microscopic observations revealed deformation of cells after treatment by sol of higher silver concentrations. An increase in malondialdehyde content in both studied varieties was observed. Tested varieties showed an increased proline content in the silver-treated cells. There was no effect of silver on the superoxide dismutases activity, while the activity of catalase was significantly decreased. The changes in the activity of peroxidases in both varieties were opposite. The highest content of intracellular GSH was noticed at a concentration of 20 ppm of both AgNPs and silver ions. The presented results demonstrate a significant similarity of the effects caused by the studied stressors: silver nanoparticles and silver ions. The results characterized the

mechanism of action of nanosilver on wheat callus: morphology disorder, damage to cell membranes, severe oxidative stress and in consequence intensification of production of non-enzymatic antioxidants.

Keywords Wheat · Callus · Silver nanoparticles · Silver ions · Oxidative stress

Introduction

For several years, nanotechnology has increasingly boldly entered various areas of life, from industry and food to cosmetics and medicine (Kokura et al. 2010; Singh et al. 2008; Tiede et al. 2008). Nanoparticles are considered to be particles of size smaller than 100 nm, which have new properties that are different compared to the particles of the same substance of macro size. The production of metal nanoparticles consists of chemical or physical processes. A controlled process allows for the synthesis of monodisperse nanoparticles of similar shape. The synthesis of NPs often requires the use of toxic substances, which necessitate very accurate machining of the remains of the post-reaction sols and use of additional substances to prevent, inter alia, aggregation of particles (Huang and Yang 2005). A lot of attention is being paid to “green synthesis”, a process which is much more environmentally friendly. In such a process, bacteria, yeast, fungi, algae and vascular plants (mainly metallophytes) are exploited (Iravani 2011; Raveendran et al. 2003). Metal nanoparticles may be formed in living organisms by reduction of metal ions absorbed as a soluble salt (Harris and Bali 2008; Bali and Harris 2010). The physical and chemical properties of metal nanoparticles determine their way of interacting with living organisms. They are known to have antimicrobial,

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antifungal and therapeutic properties. Nowadays, silver nanoparticles (AgNPs) have gained great popularity in many areas of life. They are used primarily as antibacterial agents (Marambio-Jones and Hoek 2010) as well as components of drug delivery system or tissue imaging (Godin et al. 2010; Kohl et al. 2011; Kreuter and Gelperina 2008; Meng et al. 2010). The widespread use of nanoparticles exposes all organisms on contact with them. The mechanism that allows transfer of nanoparticles through the cell wall and cell membrane is still not well understood. Nevertheless, it is known that metal nanoparticles can significantly affect cell components. Studies of the impact of AgNPs on the cells of higher organisms showed a reduction of mitochondrial function, cell membrane damage and oxidative stress causing cell injury (Arora et al. 2008; Barbasz et al. 2015; Gorczyca et al. 2015).

The metabolism of plants under stress conditions is biochemically characterized as an increase in the formation of reactive oxygen species (ROS) (Elstner and Osswald 1994; Foyer and Noctor 2000). ROS produced in excess under stress conditions can react with proteins, lipids and DNA molecules, resulting in a number of metabolic disorders, destruction of cell membranes and in consequence cell death (Miller et al. 2010; Lushchak 2011). The defense system against ROS is based on the ascorbate, glutathione, alpha-tocopherol or enzymatic scavengers such as superoxide dismutase (SOD), peroxidases (POX) and catalase (CAT) (Asada 1992; Noctor and Foyer 1998). The toxicity of the nanoparticles may be associated with the possible release of toxicants from their surface, such as metal ions or residues after synthesis (Franklin et al. 2007; Navarro et al. 2008).

In the current work, we aimed at studying the influence of AgNPs on wheat cells. Wheat is a widely cultivated plant exposed to a variety of pathogens. Crop plant protection is an important part of agriculture. So far, the risk of AgNPs has not been fully characterized. Several studies have shown that exposure to metal nanoparticles significantly affected plants physiology, for example seed germination, growth, and metabolism (Table 1). Most of the studies have focused on identifying the effect of metal nanoparticles on the seedlings or mature plants; however, the mechanism of nanoparticle action at the cellular level has still not been thoroughly elucidated.

AgNPs used for the presented experiments were produced by the chemical reduction of silver ions by the natural polyphenolic reducer: tannic acid. Tannic acid contains glucose esterified by gallic acid in the central core, and because of this specific structure it has a stabilizing effect on metal nanoparticles (Dadosh 2009; Dutta and Dolui 2011; Sivaraman et al. 2009, 2010). AgNPs synthesized using tannic acid have unique properties, as presented in our prior studies about its antibacterial activity

against selected strains of *Escherichia coli* and its cytotoxic activity against human cells (Kujda et al. 2015; Barbasz et al. 2015). It is still not known whether the toxicity to living organisms resulted from AgNPs themselves or associated with the release of silver ions from the particles' surface. AgNPs were found to reduce plant growth, photosynthesis and chlorophyll production (Miao et al. 2009). Silver ions released from the surface of silver nanoparticles may inhibit respiratory enzymes and induce oxidative stress by ROS generation (Kim et al. 2007; Pal et al. 2007). Therefore, to determine "what is more cytotoxic—silver nanoparticles or silver ions" here, similarly as in other papers, AgNO₃ was chosen for experiments (Foldbjerg et al. 2011; Kim et al. 2009; Navarro et al. 2008).

As a plant material the callus cells were chosen. This type of cells, thanks to their unique properties, allows determining the direct interaction between the test agent and plant cell defense mechanisms. Two varieties of spring wheat (*Triticum aestivum* L.), tolerant to oxidative stress (Parabola) and a sensitive one (Raweta), were used for callus cell culturing. The differences between the effects of highly purified, stable and well-characterized AgNPs and silver ions on the metabolism of wheat callus cells were discussed.

Materials and methods

Preparation of silver nanoparticles

Silver nanoparticles sols were prepared according to the modified Sivaraman's method using tannic acid as a reducing agent (Sivaraman et al. 2009). Silver nitrate (AgNO₃) was dissolved in distilled water at room temperature to get a silver ion concentration of 1 mmol dm⁻³. 10 cm³ of 0.6 mmol dm⁻³ aqueous solution of tannic acid (previously filtered through 0.22 μm Millipore filters) was added to 350 cm³ stirred silver precursor. The pH of the solution was adjusted with ammonia to a value of 9. The reaction mixture immediately became yellow, but stirring was continued for 60 min. To remove unreacted ions, the obtained sol was washed with deionized water using a stirred membrane filtration cell (Millipore, model 8400) with a cellulose membrane (Millipore, NMWL:100 kDa). The washing procedure was repeated until the conductivity of the filtrate stabilized at 8–10 μS cm⁻¹ and pH at 5.5–5.8.

Techniques of nanoparticles characterization

The silver nanoparticles concentration in the suspension was determined using a densiometer Anton Paar, type DMA5000M, according to the procedure described

Table 1 Toxicity of metal nanoparticles to higher plants

Nanoparticle types	Plant	Effects	References
Silver nanoparticles AgNPs	<i>Allium cepa</i>	Increasing mitotic index in root tip cells, cell wall disintegration	(Kumari et al. 2009)
	<i>Lolium multiflorum</i>	Inhibition of seedling growth	(Yin et al. 2011)
	<i>Phaseolus radiates, Sorghum bicolor</i>		(Lee et al. 2012)
	<i>Triticum aestivum</i>		(Vannini et al. 2014)
		Inhibition of the development of roots	(Gorczyca et al. 2013)
Aluminum oxide (nAl ₂ O ₃), silicon dioxide (nSiO ₂), magnetite (nFe ₃ O ₄), and zinc oxide (nZnO)	<i>Arabidopsis thaliana</i>	Toxic effect on seed germination, root elongation, and number of leaves dependent on the kind of nanoparticles and their size	(Lee et al. 2010)
Fe ₃ O ₄ , TiO ₂ and carbon nanoparticles	<i>Cucumis sativus</i>	Reduction in root growth, seed germination, root elongation	(Mushtaq 2011)
Cu nanoparticles	<i>Eloдея densa</i>	Enhanced lipid peroxidation, Increasing of SOD and CAT activity	(Nekrasova et al. 2011)
CuO nanoparticles	<i>Landolita punctata</i>	Decrease of chlorophyll in comparison to soluble Cu ²⁺	(Shi et al. 2011)
CuO nanoparticles	<i>Raphanus sativus, Lolium perenne, Lolium rigidum</i>	DNA damage, plant growth inhibition	(Atha et al. 2012)
CuO nanoparticles, ZnO nanoparticles	<i>Cucumis sativus</i>	Reduction of plant biomass, increase of SOD, CAT and POD activity in plant root tissue	(Kim et al. 2012)

previously (Oćwieja et al. 2011). UV–Vis spectra of silver nanoparticle sol and tannic acid solutions were recorded with Shimadzu UV-1800 spectrometer (data not shown).

The sizes and morphology of silver particles were determined by scanning electron microscopy (JEOL JSM-7500F) working in transmission mode. Samples for this examination were prepared by dispersing a drop of the silver colloid on a copper grid and then covering by carbon film. Independently, AFM pictures of silver nanoparticles deposited on polyelectrolyte (PAH)-covered mica (Oćwieja and Adamczyk 2014) were registered by the NT-MDT Solver Pro instrument equipped with the SMEN SFC050L scanning head. Imaging was done in semicontact mode using a composite probe possessing a silicon body, polysilicon levers and silicon high-resolution tips. The average values of the particle size, polydispersity and stability of suspensions were determined by dynamic light scattering (DLS) measurements (ZetaSizerNano ZS apparatus from Malvern Instruments). Electrophoretic mobility of nanoparticles at controlled conditions obtained from ZetaSizerNano ZS allowed to determine their zeta potential.

Cell culture

To obtain calli, immature embryos isolated from the seeds of spring wheat (*T. aestivum* L.), stress-tolerant Parabola

and stress-sensitive Raweta, were used. The stress tolerance of these genotypes was confirmed in previous studies (Grzesiak et al. 2013; Filek et al. 2012). Embryos were sterilized in 70 % ethanol for 1 min, and then in 10 % solution of bleaching agent “Domestos” for 10 min. They were rinsed in sterile water and placed on Petri dishes containing Murashige and Skoog (MS) medium (1962) supplemented with 2,4-D (2 mg dm⁻³) to receive undifferentiated calli (Filek et al. 2009). Then 1 g of calli was transferred into 20 cm³ MS media containing AgNPs or AgNO₃ at concentrations (20, 40, 60 ppm) and into the medium without additional supplementation and adopted as a control. The silver (particles and ions) at concentrations used in the experiments was also toxic to human cells cultured in vitro (Barbasz et al. 2015). The culture flasks were placed in a circular shaker in a growth chamber, kept at 28 °C in the dark. After 24 h of in vitro culturing in such conditions, cytological, biochemical analyses and microscopic observations were made. The microphotographs were made with an inverted microscope set Delta Optical IB 100 with a digital camera HDCE-X5.

Determination of lipid peroxidation

The degree of lipid peroxidation was determined according to Dhindsa et al. (1981). About 1 g of calli was

homogenized in 0.5 % trichloroacetic acid (TCA) and, after centrifugation at $19,000\times g$, 0.5 cm^3 of the supernatant was mixed with 2.5 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA. The mixture was boiled for 30 min and malondialdehyde (MDA) concentration, as the indicator of lipid peroxidation, was determined spectrophotometrically at $\lambda = 532\text{ nm}$ at room temperature, corrected for non-specific background by subtracting the absorbance at $\lambda = 600\text{ nm}$. The MDA concentration was calculated using the molar extinction coefficient of $155\text{ mM}^{-1}/\text{cm}^{-1}$.

Proline concentration

The content of proline was determined spectrophotometrically according to the method described by Bates et al. (1973). 1 g of calli was homogenized in 3 cm^3 of 3 % aqueous sulfosalicylic acid and centrifuged at $1000\times g$. 0.5 cm^3 of supernatant was added to 1 cm^3 of 1 % ninhydrin in 60 % acetic acid and the samples were incubated for 20 min at $100\text{ }^\circ\text{C}$. After cooling, the reaction mixture was extracted with 3 cm^3 toluene and vortex shaken. The absorbance was measured at $\lambda = 525\text{ nm}$ using toluene as a blank sample. The proline content was calculated from the calibration curve.

Enzyme assays

Approximately, 1 g of calli was homogenized in a 0.05 M phosphate buffer (pH 7.2) containing 0.1 mmol dm^{-3} EDTA and 0.1 % bovine serum albumin. The homogenate was centrifuged for 10 min at $10,000\times g$. To remove low molecular weight compounds, the supernatant was dialyzed for 8–12 h in dialysis bags (SIGMA) immersed in phosphate buffer (0.05 mol dm^{-3} , pH 7.0) at $4\text{ }^\circ\text{C}$.

Total superoxide dismutases activity [SOD; EC 1.15.11] was assayed according to spectrophotometric cytochrome method by McCord and Fridovich (1969). The reaction mixture consisted of phosphate buffer (pH 7.2), 0.1 mmol dm^{-3} EDTA, 0.1 mmol dm^{-3} cytochrome C and 0.1 mmol dm^{-3} xanthine. Subsequently, xanthine oxidase and the supernatant were added. Absorbance measurements were conducted for 2 min at $\lambda = 550\text{ nm}$. It is assumed that the unit of activity (1 U, 1 U of cytochrome) corresponds to the amount of enzyme which causes 50 % inhibition of the reduction of cytochrome C at $25\text{ }^\circ\text{C}$.

The activity of the catalase [CAT; EC 1.11.16] was quantified spectrophotometrically by measuring the kinetics of H_2O_2 decomposition (Aebi 1984). The supernatant was added to 0.5 cm^3 of 0.054 mol dm^{-3} H_2O_2 and 1.8 cm^3 of 0.05 mol dm^{-3} phosphate buffer (pH 7.0). The absorbance was measured for 2 min at $\lambda = 240\text{ nm}$ at room temperature. For the unit of enzyme activity, a decrease in absorbance within 1 min was adopted.

Measurements of total peroxidase activity [POX; EC 1.11.19] were performed spectrophotometrically according to the modified method of Lück (1962). The amount of products of oxidation of p-phenylenediamine (pPD) in the presence of H_2O_2 was determined. The assay mixture contained 0.05 cm^3 of 1 % solution of pPD, 2 cm^3 of phosphate buffer (0.05 mol dm^{-3}) and 0.05 cm^3 of enzyme extract. To initiate the reaction, 0.05 cm^3 of 0.03 mol dm^{-3} H_2O_2 was added and the absorbance at wavelength of $\lambda = 460\text{ nm}$ was measured for 2 min.

All measurements were carried out using a Thermoscientific UV-Vis spectrophotometer.

The activity of the studied enzymes was expressed relative to the protein content in the supernatant. Protein assessment was done by the method of Bradford (1976) using BSA as the standard.

GSH analysis

The reduced glutathione was determined using the Ellman method (1958). Approximately, 0.75 g of samples was homogenized in 1.5 cm^3 of 0.1 mmol dm^{-3} phosphate buffer (pH 7.4) containing 10 mmol dm^{-3} EDTA. The supernatants were deprotonized by adding 0.075 cm^3 of 50 % trichloroacetic acid (TCA). Subsequently, the samples were centrifuged at $12,000\times g$ for 10 min at $4\text{ }^\circ\text{C}$. Then, 0.1 cm^3 of supernatant was mixed with 0.8 cm^3 of phosphate buffer (pH 8.2) and 0.1 cm^3 of 6 mmol dm^{-3} DTNB. After 2 min absorbance at $\lambda = 412\text{ nm}$ was measured and the level of GSH was determined from the calibration curve. Values were expressed as μmoles of GSH per mg of protein. The total protein concentration was detected according to the method of Bradford (1976) using BSA as the standard.

Statistical analysis

All biochemical analyses were repeated three times and averaged ($\pm\text{SE}$). All the data were subjected to analysis of variance. The significances between the means were assessed using the SAS ANOVA procedure. The data were analyzed by Duncan's multiple range test and *t* test at $p < 0.05$ using PC SAS 8.0. The statistical tests were run using STATISTICA 10.0. software.

Results

The procedure for obtaining nanoparticles included a very thorough purification of sol from the post-reaction remains. To achieve quantitative information about the cytotoxicity of silver nanoparticles, the obtained colloidal suspension was characterized using various physicochemical techniques

(Barbasz et al. 2015). The concentration of AgNPs in purified sol amounted to 330 ppm. The size, distribution and shape of nanoparticles were determined by atomic force microscopy (AFM) and transmission electron microscopy (TEM) (Fig. 1). Both techniques revealed a nearly spherical shape and a similar size of AgNPs. The size of single nanoparticle was defined as an average of two perpendicular maximal particle axes. The distribution of particle sizes was presented on the histogram (number of particles in the diameter range). The average size of AgNPs equals 17 ± 5 nm.

Observations of cells by optical microscopy showed morphological changes of treated cells. It has been found that with increasing concentration of nanoparticles or silver ions, the cells became shorter and “swollen”. This effect was observed after treatment with AgNPs at concentrations greater than or equal to 40 ppm and with silver salt at the highest concentration (60 ppm) (Fig. 2).

Treatment with AgNPs or silver ions caused a significant increase in MDA content in both tested varieties. It was found that at the highest concentration of AgNPs (60 ppm), the level of MDA in Parabola increased 1.5-fold, while after treatment with 60 ppm of AgNO_3 it increased about twice, as compared to the control sample (Fig. 3a). Lipid peroxidation in a sensitive variety (Raweta) was not statistically different after treatment with AgNPs and silver ions at the highest concentrations. In this case, the MDA level increased about 1.9 times relative to the not treated cells (Fig. 3b).

An increase in proline (a marker of osmotic stress) content in silver-treated cells was observed for both varieties (Fig. 4). Nanoparticles or silver ion application at the highest tested concentration resulted in nearly threefold increase in the proline level. However, this increase was smaller for sensitive wheat variety (Raweta).

The activity of antioxidant enzymes (expressed by protein content) for the Parabola variety was similar for cells treated with AgNPs and silver ions. There was no

effect of the addition of silver on SOD activity, while the activity of POX and CAT decreased (Table 2). For cells of the sensitive wheat variety (Raweta), an increase in SOD activity was recorded regardless of the stressor type (Table 2). After treatment of cells with silver ions at a concentration of 60 ppm, the POX activity increased by nearly 50 % (relative to the control). It was found that CAT activity significantly decreased (by about 95 %) in both tested varieties after AgNP or silver ion application.

To explore the relationship between oxidative stress and cytotoxicity of AgNPs, the content of intracellular antioxidant GSH was also determined. In both cases, it was found that low concentrations of AgNPs (20 ppm) caused a significant increase in the level of intracellular GSH (Fig. 5). Then, with the increase of the dose (both AgNPs and silver ions), a slight decrease in GSH content in the cells was observed. A large difference in the concentration of GSH after treatment of the cells by AgNPs or silver ions at activating concentration, i.e., 20 ppm was found in the case of the sensitive variety (Raweta).

Discussion

In recent years, an increase in the use of nanoparticles as pesticides has been found; therefore, it is of crucial importance to clarify whether and how the nanoparticles are toxic to plant cells. The presented experiments were focused on a study of the direct interaction of silver (applied in two forms, i.e., as ions and nanoparticles) with cells of wheat—one of the most popular cereals (Table 3).

Microscopic observations of *in vitro* wheat cells incubated with nanoparticles (AgNPs) or silver ions showed morphological changes dependent on the concentration of stressors. Naturally elongated callus cells in the presence of nanoparticles or silver ions underwent swelling and significant reduction of length; however, no differences

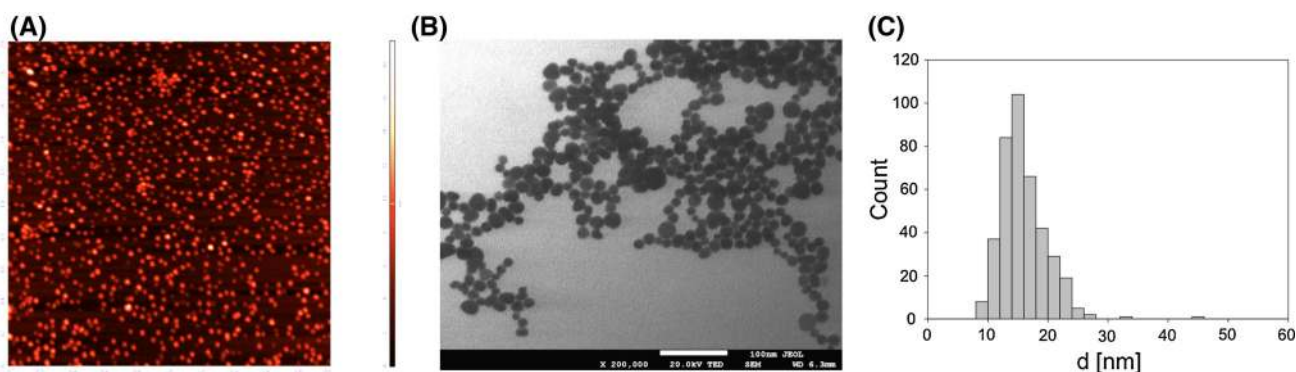
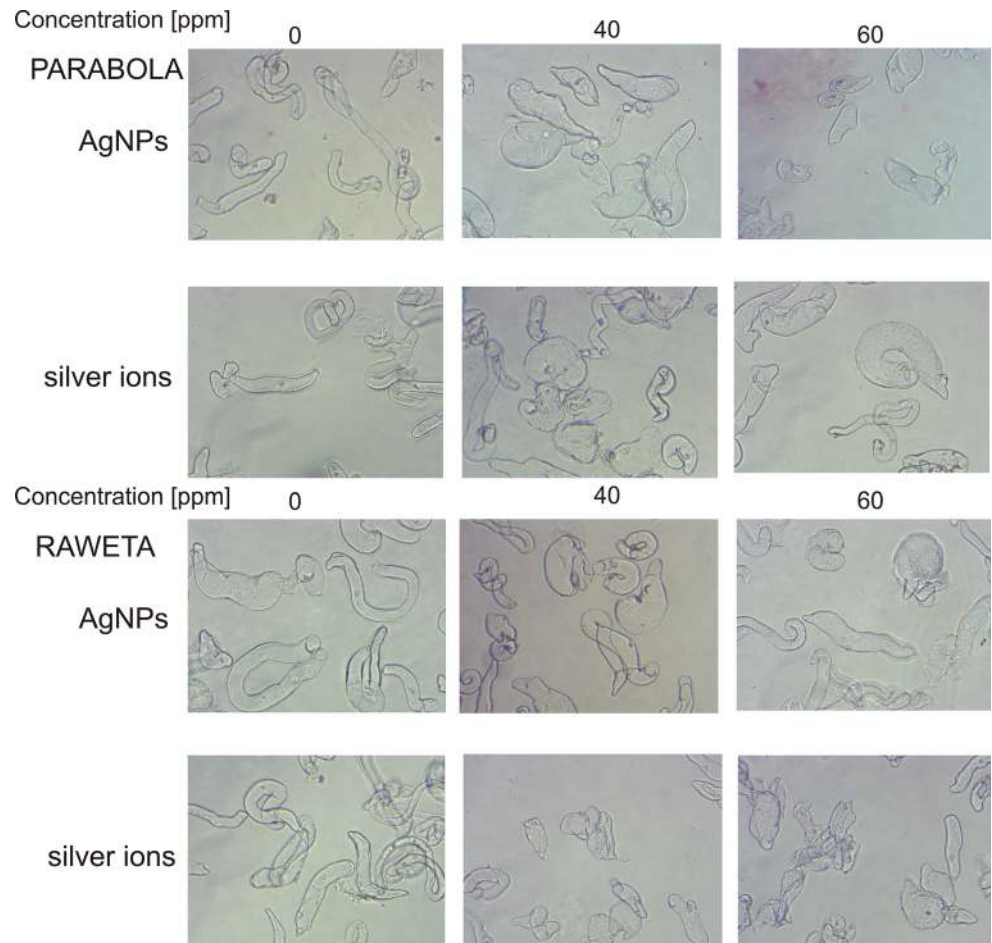


Fig. 1 Images of the obtained nanoparticles: **a** AFM image of monolayer of AgNPs deposited on PHA-covered mica ($2 \mu\text{m} \times 2 \mu\text{m}$), **b** TEM microphotography of AgN deposited on copper grid (*bar* corresponds to 100 nm), **c** the size distribution of AgNPs obtained from TEM

Fig. 2 Images of callus cells (Parabola and Raweta) cultured on control media (0) and supplemented with AgNPs or AgNO₃ for 24 h (40, 60 ppm); observation in *white light*, magnification $\times 200$



between wheat varieties were noticed. Such visible structural deformations indicate that silver used in both forms may act as the stress factor. Changes in the cell structure induced by AgNPs and silver ions, added at similar concentrations into the culture solution, were found earlier by Yin et al. (2011) in *Lolium multiflorum* seedlings.

Changes of MDA concentration as the effect of a modification of membrane structures indicate a stress action. MDA content is generally the accepted indicator of the degree of membrane lipid peroxidation under ROS action, and it is assumed that the magnitude of MDA changes may indicate about plant resistance to stressors (Møller et al. 2007; Selote and Khanna-Chopra 2006). Regardless of the stressor, a greater change in the MDA level was found for the sensitive Raweta variety in comparison to Parabola, which confirms its sensitivity. An increase of MDA level in plant tissues under the influence of metal nanoparticles was found earlier inter alia in rice (*Oryza sativa* L.) (Shaw and Hossain 2013), mung bean (*Vigna radiata* L.) (Nair and Chung 2015) and wheat (*T. aestivum* L.) (Dimkpa et al. 2012). The application of both studied silver forms resulted in an increase of MDA

concentration in the examined wheat varieties. A greater increase of MDA content was noticed for sensitive Raweta that was similar for both applied stressors. In cells of tolerant Parabola, a rise of MDA level was smaller (in comparison to Raweta) with larger effects caused by silver ions (at concentrations of 40 and 60 ppm). This may suggest a structural/chemical relationship between the membranes of plant cells and nanoparticles adsorbed on their surface.

As it was found for Parabola and Raweta seedlings cultured in control conditions, the membranes of the tolerant variety contained a larger fraction of unsaturated fatty acids in comparison to the sensitive one (Filek et al. 2012). Similar differences in membrane saturation between investigated varieties developed under control in vitro conditions were inferred indirectly from variations of MDA content: greater in Raweta than in Parabola cells.

Changes in the activity of antioxidative enzymes caused by silver applied during calli culture point to the occurrence of oxidative stress and ROS action resulting in lipid peroxidation. Superoxide dismutases (SOD) are responsible for catalyzing dismutation of superoxide radical to O₂ and H₂O₂ (Bowler et al. 1992). An increased activity of

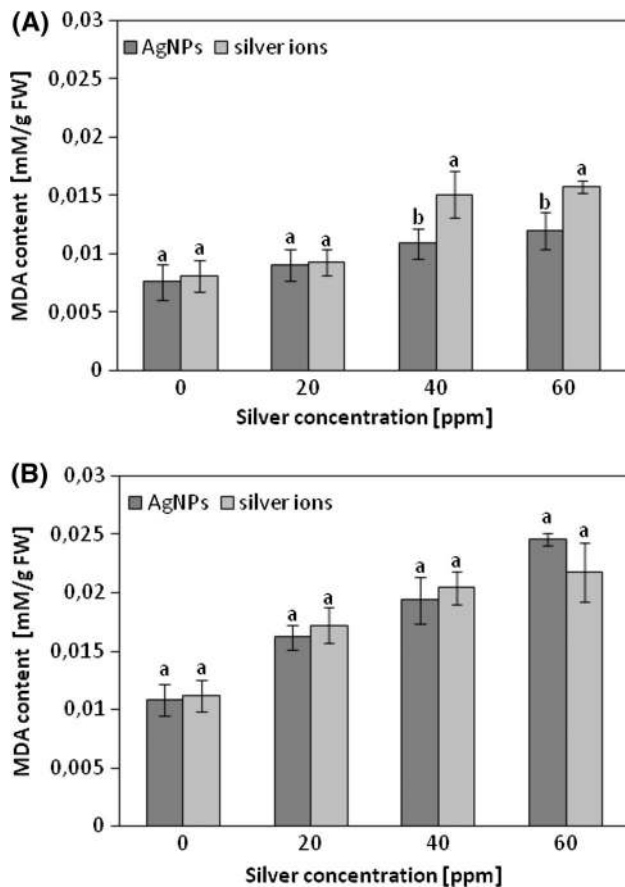


Fig. 3 Content of malondialdehyde (MDA) in callus cells of Parabola (a) and Raweta (b) after culture on control media (0) and after 24 h exposure to silver nanoparticles (AgNPs) and silver ions (AgNO_3) of different concentrations. Values are mean \pm SE. Different letters indicate significant ($p < 0.05$) differences between treatments

superoxide dismutase was registered in cucumber (*Cucumis sativus* L.) (Kim et al. 2012) and rice (*O. sativa* L.) (Shaw and Hossain 2013) under the influence of metal oxide nanoparticles. The results presented here showed that treatment of plant cells with both AgNPs and Ag^+ did not cause a statistically significant change in SODs activity in the tolerant variety. This may suggest that in the tolerant variety, other antioxidants (i.e., non-enzymatic, such as glutathione) may be involved in cell protection, and/or, nanoparticle concentrations used in the present study were not stressful for this variety. On the other hand, in sensitive Raweta, changes in SOD activity were dependent on silver concentration. The increased SODs activity at lower doses of both types of silver forms indicates a mobilization of enzymatic antioxidant systems under these conditions. A decreased activity observed at the highest tested Ag^+ concentration implies a possibility of damage of the SOD protein structure by excessive level of generated ROS (Kang et al. 2007).

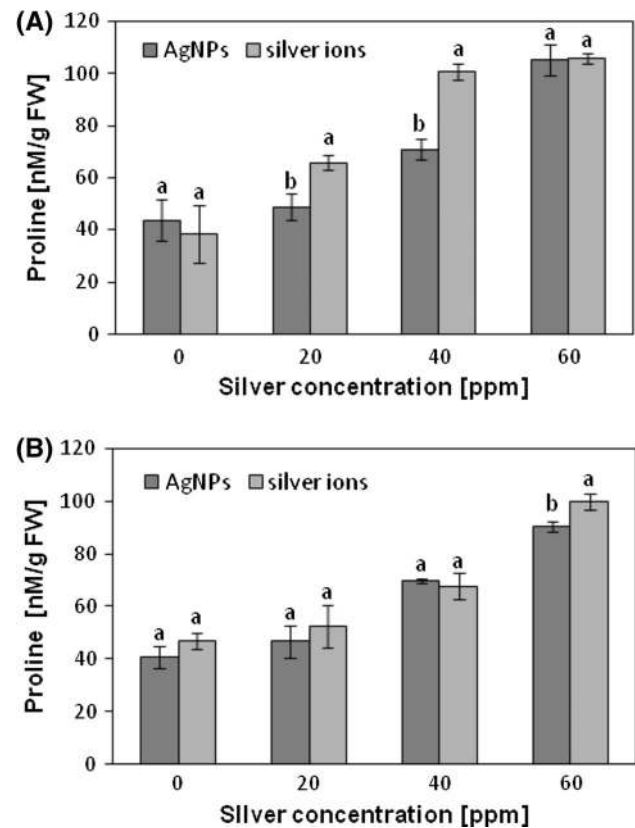


Fig. 4 Proline concentrations in callus cells of Parabola (a) and Raweta (b) after culture on control media (0) and after 24 h exposure to silver nanoparticles (AgNPs) and silver ions (AgNO_3) of different concentrations. Values are mean \pm SE. Different letters indicate significant ($p < 0.05$) differences between treatments

Both, CAT and POX, are arrays responsible for the decomposition of hydrogen peroxide to water and oxygen. The largest reduction of CAT activity (about 95 % in comparison to control) observed at the highest concentration of nanoparticles and silver ions confirms that at this concentration (thus at corresponding amount of generated ROS), the synthesis of this group of enzymes is inhibited (de Carvalho 2008; Lee et al. 2012). The research conducted by Dimkpa et al. (2012) showed that treatment of wheat cells with ZnO resulted in changes in the activity of catalase similar to those found in the present study. The activity of POX distinguished the tested varieties. The increased POX activity in the sensitive variety shows that at higher concentrations of AgNPs and AgNO_3 , a group of these enzymes was activated. The rise of POX activity after treatment with metal oxide nanoparticles (CuO) was also observed in *C. sativus* (Kim et al. 2012). In the case of the tolerant variety, we found that POX activity decreased; however these changes were larger under stress caused by silver ions. The observed variations in the activity of antioxidant enzymes in tested wheat varieties allow to conclude that the system deactivation of reactive oxygen

Table 2 Antioxidant activities (*SOD* superoxide dismutase, *POX* peroxidase, *CAT* catalase) in callus cells of parabola after culture on control media (0) and after 24 h exposure to silver nanoparticles (AgN) and silver ions (AgNO₃) of different concentrations

Concentration (ppm)	SOD (U/mg of proteins)	POX (U/mg of proteins)	CAT (U/mg of proteins)
AgN			
0	0.9716 ± 0.0408 ^a	2.1206 ± 0.0563 ^a	0.00219 ± 0.00001 ^a
20	0.9050 ± 0.0544 ^a	2.0210 ± 0.0378 ^a	0.00032 ± 0.00007 ^b
40	0.8981 ± 0.0136 ^a	1.5163 ± 0.0559 ^b	0.00014 ± 0.00001 ^b
60	0.8931 ± 0.0408 ^a	1.7606 ± 0.0171 ^b	0.00029 ± 0.00014 ^b
AgNO ₃			
0	0.8408 ± 0.0816 ^a	2.3775 ± 0.1358 ^a	0.00223 ± 0.00003 ^a
20	0.8306 ± 0.0272 ^a	1.9271 ± 0.0954 ^b	0.00021 ± 0.00003 ^b
40	1.0031 ± 0.1224 ^a	1.5539 ± 0.1176 ^c	0.00026 ± 0.00012 ^b
60	0.9087 ± 0.0408 ^a	1.4639 ± 0.1319 ^c	0.00013 ± 0.00011 ^b

Values are mean ± SE. Different letters indicate significant ($p < 0.05$) differences between treatments

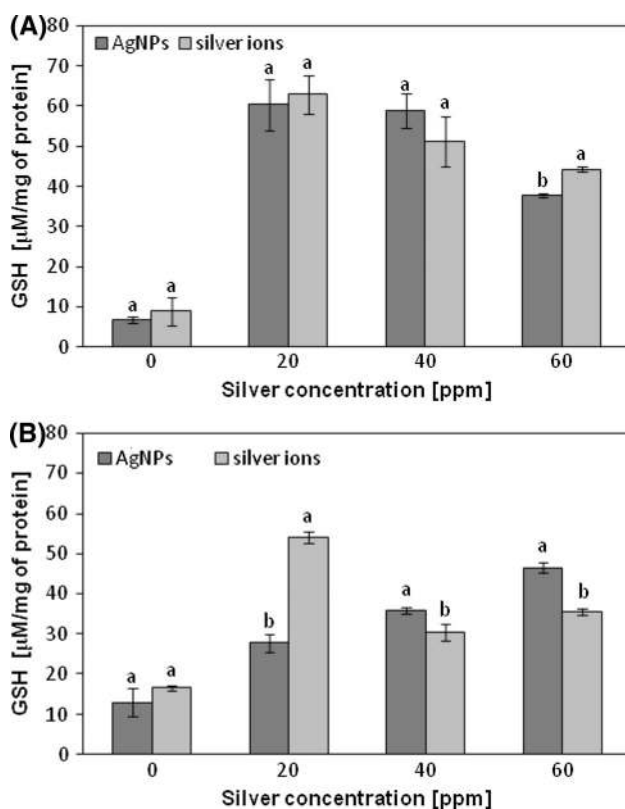


Fig. 5 Concentration of intracellular GSH in calli of Parabola (a) and Raweta (b) treated for 24 h with AgNPs or AgNO₃. Values are mean ± SE. Different letters indicate significant ($p < 0.05$) differences between treatments

species was more activated in the sensitive than in the tolerant variety.

The level of intracellular glutathione (GSH) is accepted as an universal marker of oxidative stress in plants (Apel and Hirt 2004), but this nonenzymatic antioxidant is also responsible for resistance to xenobiotics, sulfur signaling status, tolerance to heavy metals or defense against pathogens (Tausz 2001). The role of GSH as a protectant

against ROS was confirmed here by an increase of GSH levels in callus cells of both genotypes exposed to AgNPs and AgNO₃ at all used concentrations. An increased content of reduced glutathione after treatment with metal ions (CuO) was also recorded by Shaw and Hossain (2013). Greater changes of GSH content in the tolerant variety in comparison to the sensitive one showed that this nonenzymatic compound was the main antioxidant, the synthesis of which was activated in the tolerant variety in conditions of oxidative stress induced by the presence of silver in both forms. However, in the sensitive variety, a smaller increase in GSH level with simultaneous activation of antioxidant enzymes (especially of SOD and POX) can suggest that in the defense mechanism, both enzymatic and nonenzymatic antioxidants are operating.

The interesting observation is the rise of proline content in wheat cells treated by both silver forms. Proline is one of the osmoprotectants synthesized in plants in response to stress conditions. So far, it has been shown that under such stresses as drought (Grzesiak et al. 2013), salt (Kumar et al. 2008), high and low temperatures (Babu and Devraj 2008), heavy metals (Theriappan et al. 2011), mineral deficiency, UV (Saradhi et al. 1995) and biotic stress (Haudecoeur et al. 2009), the proline concentration increased often to values several times higher than that observed in control conditions. Independently of the osmoprotective role of proline, some studies have shown that this molecule can help maintain redox balance in cells (Mohammadkhani and Heidari 2008). Involving of proline in antioxidant defense and its participation in the deactivation of the hydroxyl radical ($\cdot\text{OH}$) was indicated by Chen and Dickman (2005) and Sankar et al. (2007) as well as in stabilization of singlet oxygen ($^1\text{O}_2$) (Matysik et al. 2002). However, the results of the recent studies by Signorelli et al. (2013) suggest that this amino acid plays no significant role in antioxidant processes.

The experimental results performed on in vitro wheat cells revealed a correlation between the increased

Table 3 Antioxidant activities (*SOD* superoxide dismutase, *POX* peroxidase, *CAT* catalase) in callus cells of raweta after culture on control media (0) and after 24 h exposure to silver nanoparticles (AgN) and silver ions (AgNO₃) of different concentrations

Concentration (ppm)	SOD (U/mg of proteins)	POX (U/mg of proteins)	CAT (U/mg of proteins)
AgN			
0	0.8475 ± 0.0679 ^b	1.1426 ± 0.0807 ^b	0.00198 ± 0.00007 ^a
20	0.8646 ± 0.0689 ^b	1.2056 ± 0.0041 ^b	0.00032 ± 0.00013 ^b
40	1.1320 ± 0.0519 ^a	1.0572 ± 0.0559 ^b	0.00028 ± 0.00012 ^c
60	1.1382 ± 0.0272 ^a	1.3632 ± 0.1216 ^a	0.00084 ± 0.00008 ^d
AgNO ₃			
0	0.7625 ± 0.0100 ^c	1.1468 ± 0.1266 ^b	0.00190 ± 0.00001 ^a
20	1.0054 ± 0.0952 ^b	1.2514 ± 0.1355 ^b	0.00023 ± 0.00004 ^b
40	1.2303 ± 0.0136 ^a	1.5927 ± 0.1536 ^a	0.00007 ± 0.00002 ^b
60	0.9110 ± 0.0952 ^b	1.7002 ± 0.0462 ^a	0.00010 ± 0.00007 ^b

Values are mean ± SE. Different letters indicate significant ($p < 0.05$) differences between treatments

proline content and concentration of silver (in both forms) introduced into the culture media for both studied varieties. Taking into account the morphological changes of stressed cells (swelling), it can be supposed that the enhanced proline level is involved in osmoregulation under increased metal accumulation in cells. Greater changes of proline synthesis in tolerant Parabola cells in comparison to sensitive Raweta may indicate a significant contribution of this compound in the cell protection.

Conclusions

One could state that both tested stressors may induce oxidative stress in plant cells as a consequence of the osmotic stress action. The increase of the volume of cells (observed microscopically) was connected with the rise of proline level. The differences in membrane peroxidation confirm the important role of lipid composition in the distinction of both types of wheat cells: sensitive and tolerant in terms of susceptibility to silver stress. Sensitive and resistant varieties can also be distinguished on the basis of activation of antioxidative defense mechanisms. In calli of the tolerant variety (Parabola) only the non-enzymatic antioxidant system was activated, while in Raweta (sensitive), both enzymatic and non-enzymatic pathways were activated in the defense against ROS. The obtained results have demonstrated that exposure of callus cells to silver nanoparticles or silver ions may cause similar results.

Author contribution statement AB and BK designed the research; AB, BK and MO conducted the research; AB and BK analyzed the data; AB and BK wrote the paper; AB and BK had primary responsibility for the final content. All authors have read and approved the final manuscript.

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