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Research Report

Silver Nanoparticles and Silver Ions: Oxidative Stress Responses and Toxicity in Potato (*Solanum tuberosum* L) Grown *in vitro*

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Abstract. Under *in vitro* conditions, we examined the effects of silver nanoparticles (AgNPs) and silver (Ag) ions on potato (*Solanum tuberosum* L.) in terms of silver accumulation, production of reactive oxygen species (ROS), oxidative stress responses, and antioxidative defense systems. At all concentrations (except at 2 $mg \cdot L^{-1}$), the amount of Ag in the shoots and roots of Ag ion-treated plantlets was significantly higher than in plantlets treated with AgNPs. In both treatments, total ROS and superoxide anions were increased at concentrations greater than 2 $mg \cdot L^{-1}$. Damage caused by oxidative stress, such as ion leakage and cell death, was significantly higher in plantlets treated with AgNPs than those treated with Ag ions. Significant increases in the activities of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase (GR),were found in both AgNP-treated, and Ag ion-treated plantlets compared to the control. However, in AgNP-treated plantlets, GR activity was significantly decreased at 20 $mg \cdot L^{-1}$. A significant reduction in glutathione (GSH), ascorbate (ASA), and the ratios of GSH to oxidized glutathione (GSSG) and ASA to oxidized ascorbate (DHA)were observed in plantlets treated with both AgNPs and Ag ions at concentrations higher than 2 $mg \cdot L^{-1}$. Moreover, a greater decrease in GSH and ASA contents was seen in plantlets treated with AgNPs compared to those treated with Ag ions. The present study indicates that both AgNPs and Ag ion treatments impose oxidative stress on potato plantlets under *in vitro* conditions. Furthermore, based on plantlets' responses to oxidative damage, the observed alteration in the activities of radical scavenging enzymes and the depletion of GSH and ASA, AgNPs seem to have higher toxicity than the equivalent mass of Ag ions.

***Additional key words*:** cellular viability, Gluthatione-ascorbate cycle, ion leakage, ROS

Introduction

Particles ranging in size from1–100 nm are classified as nanoparticles (NPs).Thanks to their unique properties, NPs have been incorporated into many everyday products (Navarro et al., 2008). Recently, NPs have attracted much attention for their potential uses in plant growth enhancement, controlled release of agrochemicals, and crop protection (Nair et al., 2010; Khot et al., 2012).

Silver nanoparticles (AgNPs), which have antimicrobial properties, are one of the most commonly used NPs. In plants, they have been shown to considerably alleviate adverse effects of the pathogen *Gibberella fujicuroi* when applied to infected rice seeds (Jo et al., 2015); increase the vaselife of gerbera cut flowers (Solgi et al., 2009);improve the growth and development of *in vitro*-grown potatoes (Bagherzadeh Homaee and Ehsanpour, 2015); induce the growth and antioxidant status of *Brassica juncea* L. seedlings (Sharma et al., 2012); and a low-dose of AgNPs (1 $mg \cdot L^{-1}$) applied to *Brassica rapa* L. significantly increased biomass, despite higher levels induced oxidative stress and DNA damage (Thiruvengadam et al., 2015). The positive or negative effects of AgNPs on plants depends on the size, shape, surface coating, duration of exposure, plant species, and developmental stage (Remédios et al., 2012).

Inhibition of plant growth, reduction in seed germination rate, increased chromosomal aberrations and oxidative damage, have been reported in many plants when exposed to AgNPs (Lee et al., 2012; Patlolla et al., 2012; Oukarroum et al., 2013; Cui et al., 2014). However, it is unclear whether the toxicity of AgNPs is related to specific effects caused by the nanoparticles, Ag ions, or both. Work by Cui et al. (2014) indicated that cysteine, as an Ag^{+} chelator, reduced the toxicity of AgNPs in cucumber and wheat seedlings, and suggested that the toxicity of AgNPs is mediated by Ag ions. Another study on *Spirodela punctuta* treated with

AgNPs showed that particle-specific effects are the main cause of toxicity in this aquatic plant (Thwala et al., 2013). The 'Trojan Horse' phenomenon, in which high amounts of Ag ions are released inside cells, has been suggested to explain the toxicity of AgNPs in living cells (Hsiao et al., 2015). Clearly, there are stills gaps in our knowledge regarding the mode of action of AgNPs within plants.

One consequence of AgNP toxicity in plants is an over-production of reactive oxygen species (ROS). Furthermore, *less* attention has been given to the role of the glutathione-ascorbate cycle in plants treated with AgNPs. Low molecular weight antioxidants such as ascorbate (ASA) and glutathione (GSH), can directly interact with ROS and contribute to the detoxification of plant cells and tissues(Polle, 2001).Here, we attempted to elucidate the relationship between silver uptake and the toxicity of AgNPs and silver ions, with an emphasis on oxidative damage responses and the antioxidant defense system. The aim of this research was to compare the toxicity of AgNPs with the same mass of silver ions based on silver uptake, ROS production, cellular viability, and antioxidative defense mechanisms to gain insights into the action of AgNPs on potato (*Solanum tuberosum* L.) under *in vitro* culture conditions.

Materials and Methods

Silver Suspension and Characterization

Spherical AgNPs with diameters of 20 nm were purchased from US Research Nanomaterials Inc. (Houston, TX, USA). A stock suspension (1 mg/mL) was made by dispersing AgNP powder in deionized water, followed by sonication for10 min. All dilutions were freshly prepared when required.

Plant Materials and Treatments

Potato plantlets (*Solanum tuberosum* L. cv. 'White Desiree') were obtained from the Center of Excellence in Plant Abiotic Stress, Iran. Stem sections with one node were cultured on MS medium (Murashige and Skoog, 1962) containing 0, 2, 10, and 20 $mg \cdot L^{-1}$AgNPs or the same mass of $AgNO_3$. Plants were grown in a growth room at 25±2ºC,under a photoperiod of 16/8 h (light/dark),with a light intensity of 50 μ $mol \cdot m^{-2} \cdot s^{-1}$. All experiments were conducted in triplicate. After four weeks, *in vitro*-grown plantlets were collected for the measurement of various parameters.

Measurement of Ag Content

Plantlets exposed to AgNPs and Ag ion treatments were harvested and washed with deionized water to remove any Ag adhering to the cell surface. Shoot and root samples were dried at 70°C for 48 h. Dried samples were ground, then 50 mg of each dried powder was digested in a mixture of HNO_3 (65%), H_2O_2 (30%) (3:1, *v/v*) and heated at 120°C for 1 h. After cooling, digests were filtered through filter paper (Whatman grade 42, Sigma–Aldrich, St Louis, MS, USA), made up to 10 mL with deionized water, and analyzed using an atomic absorption spectrophotometer (AA-6200, Shimadzu). Ag concentration was estimated using a standard curve, prepared from known concentrations of Ag.

Quantification of Total ROS

Total ROS content was measured using a 2′,7′-dichloro-dihydrofluorescein diacetate (H_2DCFDA) assay (Mahalingam et al., 2006). Fresh leaf samples (100 mg) were ground in a chilled mortar on ice using 10 mM Tris-HCl buffer (pH7.2). Extracts were centrifuged at 14 000 × *g* at 4ºC for 20 min, then 0.9 mL of this extract was mixed with 0.1 mL of 1 mM H_2DCFDA (Sigma–Aldrich, St Louis, MS, USA), and incubated in the dark for 10 min at room temperature. The fluorescence value of each sample was then measured using a fluorometer at 520 nm emission after excitation at 480 nm. Total protein was quantified based on the method of Bradford (1976). Fluorescence intensity was expressed as relative fluorescence units (RFU) per mg of protein.

Quantification of Cell Death and Ion Leakage

To evaluate cellular viability, fresh leaves (0.1 g) were collected, and submerged in 0.25% (*w/v*) Evans blue (Jacyn Baker and Mock, 1994). Leaves were washed with distilled water and incubated in 1 mL of 50% (*v/v*) ethanol containing 1% (*w/v*) sodium dodecyl sulfate (SDS).The absorbance of the extracts was measured (AE-UV1600) at 600 nm. Cellular viability was expressed as Evans blue uptake, A_{600}.

Ion leakage was measured using the method reported by Faisal and Anis (2009). Fresh leaf samples (0.1 g) were collected and rinsed with distilled water. Distilled water (10 mL) was added to each samples, incubated on a shaker for 24 h, and the electrical conductivity (EC_1) of the solutions were measured with a conductivity meter. Samples were then autoclaved for 20 min, and the electrical conductivity (EC_2) of the autoclaved solution was also measured. Electrolyte leakage was calculated as follows: electrolyte leakage (%) = $(EC_1/EC_2) \times 100$.

Histochemical Detection and Quantification of $O_2^{\cdot-}$ and H_2O_2

To visually assess superoxide anion levels, leaves were excised and immersed in50 mM potassium phosphate buffer at pH 6.4 containing 0.1% (*w/v*) nitroblue tetrazolium (NBT) and 10 mM sodium azide, for 12 h in the dark (Fryer et al., 2002; Ramel et al., 2009). Leaves were then incubated in acetic acid–glycerol–ethanol (1/1/3) (*v/v/v*) solution at 100°C for 5 min. After cooling, leaves were placed on a white

background and photographed. To quantify superoxide anion levels, NBT-stained leaves were ground and solubilized with 1 mL of 2 M KOH-DMSO (1/1) (*v/v*) and then centrifuged for 10 min at 12 000 × g (Rook et al., 1985). Formazan content of the supernatant was measured at 630 nm using a spectrophotometer (AE-UV1600) and expressed as A_{630} g^{-1} fresh weight.

Detection of $H_2O_2$was carried out according to the method of Thordal-Christensen et al. (1997). Leaves detached from plantlets under control and treatment condition were incubated in a solution containing 1 $mg \cdot mL^{-1}$ 3,3′-diaminobenzidine (DAB) at pH 3.8 for 12 h in darkness. After removal of chlorophyll from leaves with acetic acid-glycerol-ethanol (1/1/3) (*v/v/v*) solution, H_2O_2 was visualized and photographed.

Antioxidative Enzyme Activities

Fresh leaf samples (0.1 g) were homogenized in 1 mL of phosphate buffer (100 mM, pH7.8) containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and 2% (*w/v*) polyvinylpyrrolidone (PVP) using a chilled mortar and pestle. The homogenates were centrifuged at 13 000 × g for 20 min at 4°C. A UV-visible spectrophotometer (AE-UV1600) was used to determine the activities of antioxidative enzymes in the supernatant. Protein content was measured based on the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Catalase (CAT) activity was measured using the method of Aebi (1984). A total volume of 1 mL was made up using 0.9 mL phosphate buffer (50 mM, pH7.0), 10 mM H_2O_2 and 0.1 mL of enzyme extract. The absorbance was recorded at 240 nm for1 min. Enzyme activity was calculated using an extinction coefficient of 39.4 $mM^{-1} \cdot cm^{-1}$ and expressed as μmol decomposition of H_2O_2 per min per mg protein.

Ascorbate peroxidase (APX) activity was determined according to the method of Nakano and Asada (1981). The reaction mixture consisted of 0.9 mL of sodium phosphate buffer (50 mM ,pH 7.0), ascorbate (0.5 mM), EDTA (0.2 mM), H_2O_2 (0.2 mM) and 0.1 mL of enzyme extract.The decrease in absorbance at 290 nm was monitored for1 min. Enzyme activity was calculated using an extinction coefficient of 2.8 $mM^{-1} \cdot cm^{-1}$. One unit of APX was defined as the amount necessary to break down 1 μmoL of ascorbate per min per mg protein.

Superoxide dismutase (SOD) activity was measured using the method described by Beauchamp and Fridovich (1971). The reaction mixture (1.5 mL) contained sodium phosphate buffer (50 mM, pH 7.8), EDTA(0.1 mM), methionine (13 mM), NBT(75 μM), riboflavin(2 μM), and 50 μL of enzyme extract in a test tube. The reaction was initiated by putting the test tubes under fluorescent lights for 15 min. The reaction was terminated by switching off the light, and the absorbance at 560 nm was read against the blank (non-illuminated) with a spectrophotometer (AE-UV1600). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% NBT photochemical reduction, and was expressed as units per mg protein.

Glutathione reductase (GR) activity was assayed by monitoring the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to dithiobis (2-nitrobenzoic acid) (TNB) by reduced glutathione (GSH), as described by Smith et al. (1988). The reaction mixture (1.0 mL) consisted of 0.98 mL of 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 0.75 mM DTNB, 0.1 mMNADPH,1 mM oxidized glutathione (GSSG)and 0.01 mL the enzyme extract. The increase in absorbance at 412 nm was recorded for 1 min. An extinction coefficient of 14.15 $M^{-1} \cdot cm^{-1}$ was used to calculate enzyme activity, which was expressed as μmol of TNB per min per mg protein.

Determination of Ascorbate and Glutathione Content

The content of reduced (ASA) and oxidized (DHA) ascorbate was determined following the method of Law et al. (1983). Fresh leaves (0.1 g) were homogenized in 1 mL of 6% (*w/v*) trichloroacetic acid (TCA) using a mortar and pestle chilled on ice. Homogenates were centrifuged at 15 000 × g for 15 min at 4°C. The supernatant was used to determine total ascorbate (ASA + DHA) content and ASA content. To estimate the total ascorbate, 200 μL sodium phosphate buffer (150 mM, pH7.4) and 100 μL DTT (10 mM)were added to 200 μl of the supernatant. Samples were incubated for 10 min at room temperature to reduce DHA to ASA. Excess DTT was removed by the addition of 100 μL of 0.5% N-ethylmaleimide (NEM). The same procedure was followed to measure ascorbate except 200 μL of water was substituted for the DDT and NEM. Then, 400 μL of 10% (w/v) TCA, 400 μL of 44% (v/v) H_3PO_4, 400 μL of 4% (*w/v*) α-α'-bipyridyl in 70% (*v/v*) ethanol, and 200 mL of 3% $FeCl_3$ were added to all assay mixtures. After vortex-mixing, samples were incubated at 37°C for 60 min. The absorbance of the samples was recorded at525 nm using a spectrophotometer (AE-UV1600). The concentrations of total ascorbate and reduced ascorbate were calculated from the standard curve, prepared from known concentrations of ASA. The concentration of DHA was calculated by subtracting reduced ascorbate values from those of total ascorbate.

Reduced (GSH), oxidized (GSSG) and total glutathione contents were measured based on the method of Anderson (1985). Fresh leaves (0.1 g) were ground in 1 mL of 5% (*w/v*) sulfosalicylic acid using a chilled mortar and pestle. The homogenate was centrifuged at 10 000 ×g for 10 min at 4°C. The supernatant obtained was used to estimate total glutathione (GSH+GSSG) and GSSG. For total glutathione, 150 μL of the supernatant was added to 700 μL of 143 mM phosphate buffer (pH 7.5) containing 6.3 mM EDTA and 0.3 mM NADPH, followed by adding 100 μL of DTNB, prepared

in143 mM sodium phosphate buffer (pH7.5) and 5 units of GR. After 5 min, the absorbance at 412 nm was measured. GSSG content was estimated in a similar way after derivatization of GSH by adding 10 μL 2-vinylpyridine and 10 μL triethanolamine to150 μL of the supernatant. The amounts of total glutathione and GSSG were determined from a standard curve prepared with GSH. The amount of GSH was calculated by subtracting GSSG from total glutathione concentrations.

Statistical Analysis

Results are the mean of three independent biological experiments ± standard deviations (SD).Data were analyzed using *one-way analysis* of variance (*ANOVA*), and significant differences were compared based on the Duncan post hoc test.Differences were considered significant at $p < 0.05$.

Results

Ag Content of Plantlets Treated with AgNPs and Ag ions

Potato plantlets treated with AgNPs and Ag ions significantly accumulated Ag. After 4 weeks of exposure to AgNPs or Ag ions, no difference was seen between treatment groups in terms of root Ag content at the 2 mg·L^{-1}concentration. However, a remarkable difference was observed at higher concentrations (Fig. 1A).The maximum accumulation of root Ag was observed in plantlets treated with 20 mg·L^{-1} of Ag ions. Moreover, shoot Ag content was not significantly different between the AgNP and Ag ion treatment groups, except at 20 mg·L^{-1} (Fig. 1B). In shoots, the maximum amount of Ag was found in plantlets treated with 20 mg·L^{-1} of Ag ions.

Effect of AgNPs and Ag Ions on Total ROS and Superoxide Anion Production

Results of experiments measuring ROS generation in leaves of control and treated plantlets are presented in Figures 2A and 2B. At the AgNP and Ag ions concentrations of 2 mg·L^{-1}, production of total ROS and superoxide anions were not significantly different compared to the control, while a significant increase in ROS was observed at higher levels of both AgNP and Ag ion treatments. Maximum production of total ROS and superoxide anions was found in plantlets treated with AgNPs at 20 mg·L^{-1}. Following 4 weeks of exposure to AgNPs, total ROS and superoxide in plantlets treated with AgNPs at this level were 55% and 33.2% higher than plantlets treated with Ag ions, respectively ($p < 0.05$).

Histochemical Visualization of $O_2^{\cdot-}$ and H_2O_2

In leaves from plantlets subjected to AgNPs and Ag ions, $O_2^{\cdot-}$ accumulation was detected as insoluble blue formazan precipitates (Fig. 3A, 3B). The intensity of formazan precipitates increased with increasing in Ag concentration. However, the color intensity in plantlets treated with AgNPs was greater than that observed in Ag ion treatments of the same concentration. Formazan spots were detected in plantlets treated with 2 mg·L^{-1} of AgNPs, but not in the equivalent Ag ion treatment.

Accumulation of $H_2O_2$was visualized in the leaves of plantlets treated with AgNPs and Ag ions using DAB staining (Fig. 3C, 3D). Whereas accumulation of H_2O_2 was not detected in plantlets treated with AgNPs or Ag ions at 2 mg·L^{-1}, it was observed as dark brown spots in plantlets treated with higher levels. Furthermore, the color intensity in plantlets treated with AgNPs appeared to be greater than that in Ag ion treatments.

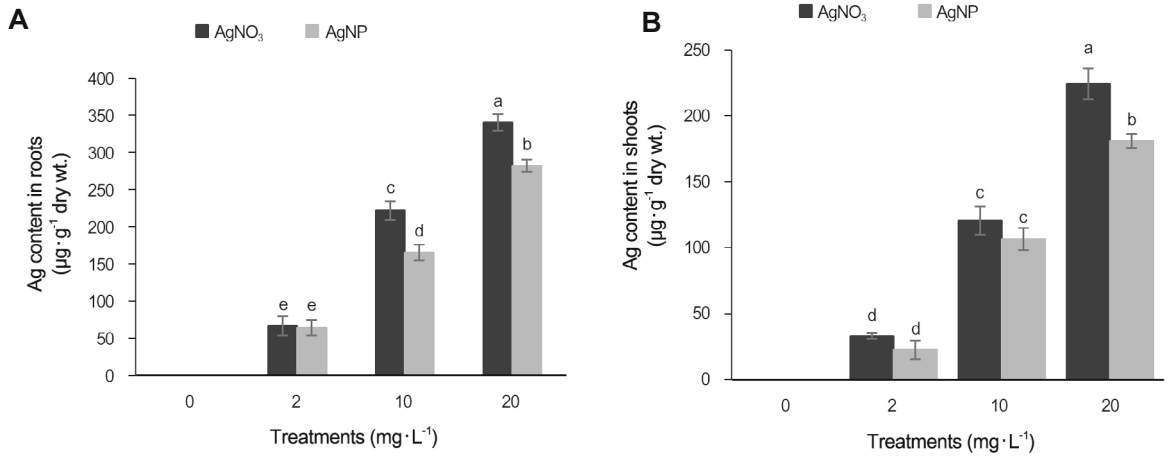

Fig. 1. The effect of silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) at different concentrations (2, 10 and 20 mg·L^{-1}) on the silver (Ag) content of roots (A) and shoots (B) of potato (*Solanum tuberosum* L.). Values are representative of *three independent experiments* and error bars represent standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$)

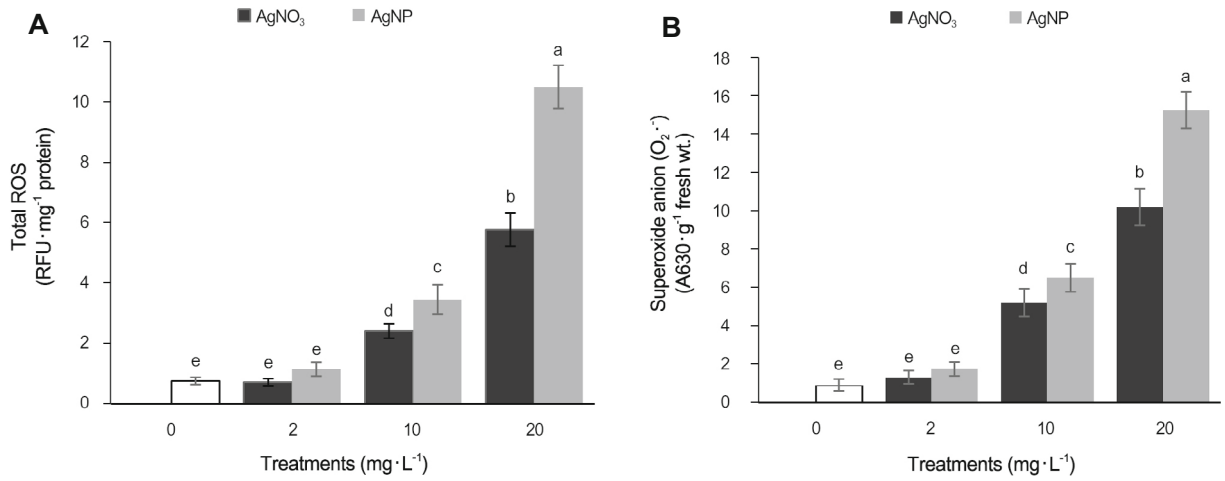

Fig. 2. Effects of silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) at different concentrations (2, 10 and 20 mg·L^{-1}) on total reactive oxygen species (ROS) production (A), and superoxide anion generation (B) in leaves of potato (*Solanum tuberosum* L.). Values are representative of three independent experiments and error bars represent standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$).

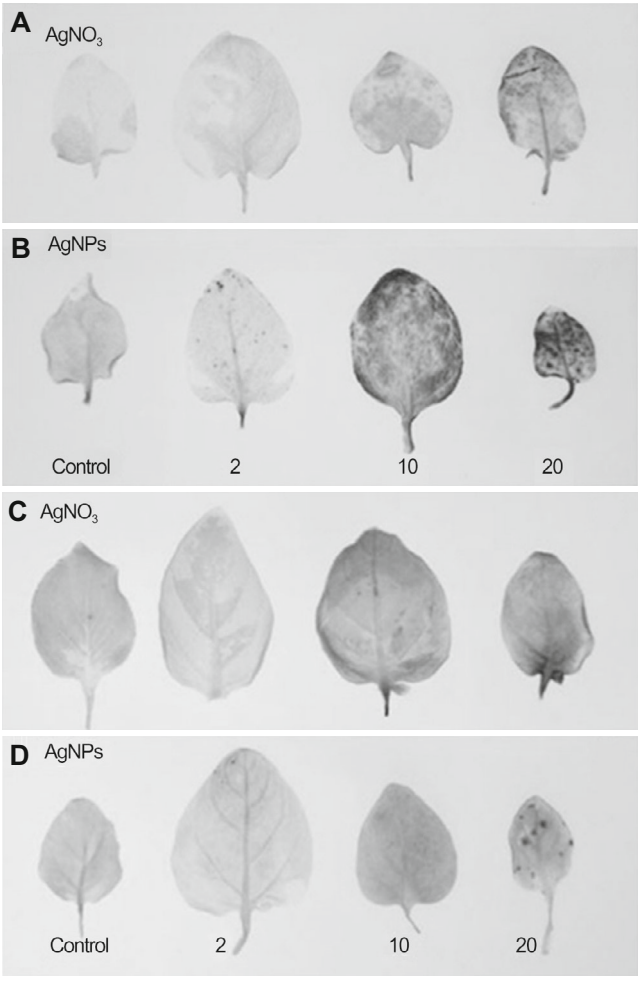

Fig. 3. Effects of silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) at different concentrations (2, 10 and 20 mg·L^{-1}) on the histochemical localization of $O_2^{\cdot-}$ (A, B) and H_2O_2 (C, D) in potato (*Solanum tuberosum* L.). Dark blue precipitates show the reaction of nitroblue tetrazolium (NBT) with $O_2^{\cdot-}$, and brown spots indicate H_2O_2.

Effect of AgNPs and Ag Ions on Electrolyte Leakage and Cell Death

Ion leakage and cellular viability were not significantly different ($p < 0.05$) between untreated plantlets and plantlets subjected to 2 mg·L^{-1} of AgNPs or Ag ions (Fig. 4A, 4B). However, at higher levels, a significant increase was observed in both treatment groups. Maximum ion leakage and cell death were found in plantlets treated with 20 mg·L^{-1}AgNPs. After 4weeks of exposure to AgNPs and Ag ions, the percentage of ion leakage and cell death values recorded inAgNP-treated plantlets were 31.2% and 20% higher ($p < 0.05$), respectively, than those treated with Ag ions at 20 mg·L^{-1}.

Effect of AgNPs and Ag ions on Antioxidative Enzymes

Compared to controls, significant increases in the activities of SOD, CAT, APX, and GR were observed in both AgNP, and Ag ion-treated plantlets (Fig. 5A, 5B, 5C, 5D). SOD activity was not significantly different between AgNP and Ag ion-treated plantlets at the 2 mg·L^{-1}concentration ($p < 0.05$). However, a significant ($p < 0.05$) change in SOD activity was seen in AgNP-treated plantlets at concentrations between 10 and 20 mg·L^{-1}. In plantlets treated with 20 mg·L^{-1} AgNPs, SOD activity was decreased by 17.6% compared to that of plantlets treated with the equivalent concentration of Ag ions.

CAT activity was significantly different between plantlets treated with AgNPs and Ag ions at all concentration levels except 2 mg·L^{-1} (Fig. 5B). Maximum CAT activity was observed in plantlets treated with 10 mg·L^{-1} AgNPs. In plantlets treated with 20 mg·L^{-1} AgNPs, CAT activity declined by 34.4% compared to those treated with Ag ions at the same

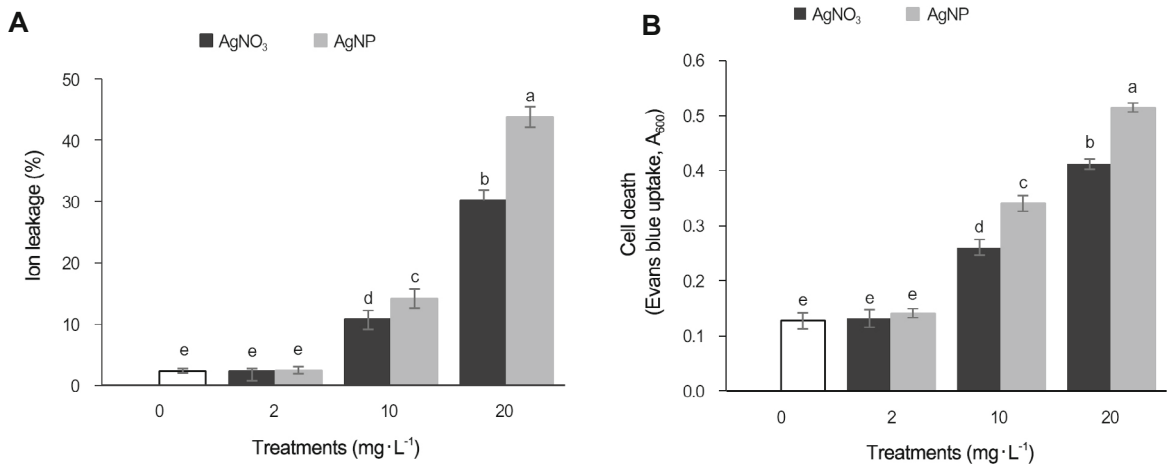

Fig. 4. Effects of silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) at different concentrations (2, 10 and 20 mg·L^{-1}) on ion leakage (A) and cell death (B) in potato (*Solanum tuberosum* L.). Values are representative of three independent experiments and error bars represent standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$).

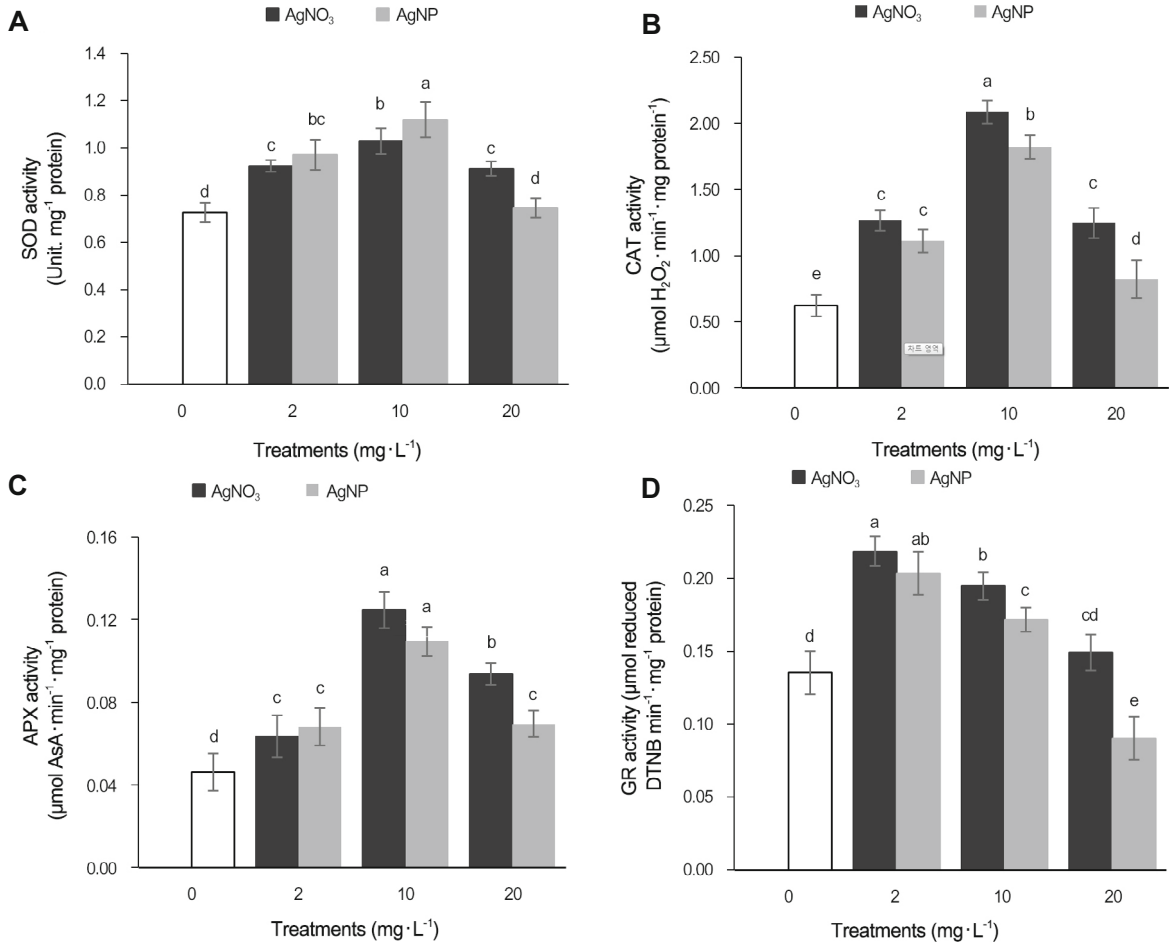

Fig. 5. Effects of silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) at different concentrations (2, 10 and 20 mg·L^{-1}) on activities of SOD(A), CAT (B), APX (C), and GR (D) in potato (*Solanum tuberosum* L.).Values are representative of three independent experiments and error bars represent standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$).

level, but remained significantly ($p < 0.05$) higher than that in the control.

In both AgNP and Ag ion-treated plantlets, APX activity (Fig. 5C) was significantly ($p < 0.05$) increased compared to the control, but compared to each other, APX activity was not statistically different except at 20 mg·L^{-1} where it sig-

nificantly ($p < 0.05$) decreased. Moreover, the reduction in APX activity in AgNP-treated plantlets was 25% greater than in plantlets treated with the equivalent Ag ion concentration.

In leaves from AgNP and Ag ion-treated plantlets, GR activity considerably increased with increasing Ag concentration compared to controls (Fig. 5D), except at 20 mg·L^{-1}, where GR activity decreased in plantlets treated with AgNPs by 38% relative to the equivalent Ag ion treatment.

Effect of AgNPs and Ag Ions on Ascorbate and Glutathione Contents

The contents of GSH and ASA in leaves treated with 2 mg·L^{-1} of AgNPs or Ag ions were not significantly different, relative to the control (Fig. 6A, 6B). However, a significant reduction in GSH, ASA, and the ratios of GSH/GSSG and ASA/DHA were observed in plantlets treated with both AgNPs and Ag ions at concentrations higher than 2 mg·L^{-1} (Fig. 6A, 6B, 6E, 6F). Moreover, the decrease in GSH and ASA contents in plantlets treated with AgNPs was greater than that in plantlets treated with Ag ions. Concomitant with the decrease in GSH and ASA content in plantlets treated with both AgNPs and Ag ions, a significant increase in DHA and GSSG levels was also observed (Fig. 6C, 6D).

Identification of the Most Sensitive Biochemical Response to Ag Treatment

To identify the most sensitive biochemical indicator for potato plantlets treated with either AgNPs or Ag ions, we standardized the data obtained from different biochemical measurements (Table 1 and Table 2).Based on the Z-score of the variables, SOD activity showed the highest value in both AgNP and Ag ion treatments at 2 mg·L^{-1}. The maximum value for Ag ions and AgNP treatments at 10 mg·L^{-1} were CAT and SOD, respectively. Relative to the control, total ROS and GSH showed the highest and the second highest alterations in AgNP treatments at 20 mg·L^{-1}, respectively. However, the cell death value for plantlets treated with Ag ions at 20 mg·L^{-1} was greater than other indicators.

Discussion

In the present research, we compared the toxicities of AgNPs and Ag ions on potato (*Solanum tuberosum* L.) using biochemical indicators of oxidative stress, under *in vitro* conditions. Though plants under both treatment conditions (AgNPs and Ag ions) both accumulated Ag, the quantity of accumulated Ag was significantly lower in plantlets grown with AgNPs compared to those grown with Ag ions. Surprisingly, measuring biochemical indicators of oxidative stress revealed higher toxicity in plantlets treated with AgNPs compared to Ag ion treatments. Indeed, plantlets treated with AgNPs exhibited lower accumulation of Ag and higher ROS generation compared to Ag ion treatments. Given that the amount of silver in plantlets treated with AgNPs was less than the plantlets treated with Ag ions, the toxicity of AgNPs can not exceed that of Ag ions. These peculiar results might be attributed to the mechanism of AgNP toxicity. The 'Trojan Horse' mechanism and particle-specific effects have been proposed to contribute to the higher toxicity of AgNPs relative to Ag ions (Yang et al., 2012; Vannini et al., 2013; Hsiao et al., 2015).

Higher generation of ROS in AgNP-treated plantlets was confirmed by histochemical visualization of H_2O_2 and $O_2^{\cdot-}$ at equal amounts of both Ag treatments. Loss of membrane integrity and induction of cell death have been considered as common consequences of oxidative stress in plants (Demidchik et al., 2014). The values of ion leakage and cellular viability biomarkers were significantly higher in plantlets under AgNP treatments than those in Ag ion-treated plantlets, indicating that AgNP treatments have higher toxicity. A reduction in plant cellular viability and production of intracellular ROS in *Lemna gibba* treated with AgNPs has been reported, which is inconsistent with our results (Oukarroum et al., 2013).

Under stressful conditions, including the presence of heavy metals, the imbalance between the generation and elimination of ROS results in oxidative stress (Das and Roychoudhury, 2014). Plants have developed antioxidant defense systems, including enzymatic and non-enzymatic ROS scavenging systems, to maintain ROS as low as possible(Gill and Tuteja, 2010). SOD, CAT, APX, and GR are enzymatic components of the antioxidant machinery. SOD catalyzes the disproportionation of $O_2^{\cdot-}$ to H_2O_2 and O_2. CAT and APX are responsible for scavenging H_2O_2. GR catalyzes the reduction of GSSG to GSH. Results from anti oxidative enzyme assays indicated that the activities of SOD, CAT, APX and GR were significantly increased compared to the control. These enzymes were at their maximum activity levels at 10 mg·L^{-1} in both Ag treatments, while they showed a significant reduction at 20 mg·L^{-1}. Elevated levels of ROS accumulated in plantlets treated with10 mg·L^{-1} of AgNPs, where the highest values of CAT, APX, and SOD were recorded. This suggests that ROS generation overwhelmed the *scavenging* action of the antioxidative *system.* Moreover, elevation and reduction of antioxidative enzymes can both be related to oxidative stress. Similar to our observations, alterations in antioxidative enzyme levels have been previously reported in *Spirodela polyrhiza* and *Lycopersicon esculentum* treated with AgNPs (Song et al., 2013; Jiang et al., 2014). A significant reduction in the activities of SOD, CAT, APX and GR were observed in plantlets treated with AgNPs or Ag ions at the 20 mg·L^{-1} concentration levels. However, the reduction was greater in plantlets treated with AgNPs. SOD, CAT, APX and GR activities were inhibited by 17.6%, 34.4%, 25%,and 38%, respectively, in AgNP-treated plantlets at 20 mg·L^{-1}, compared

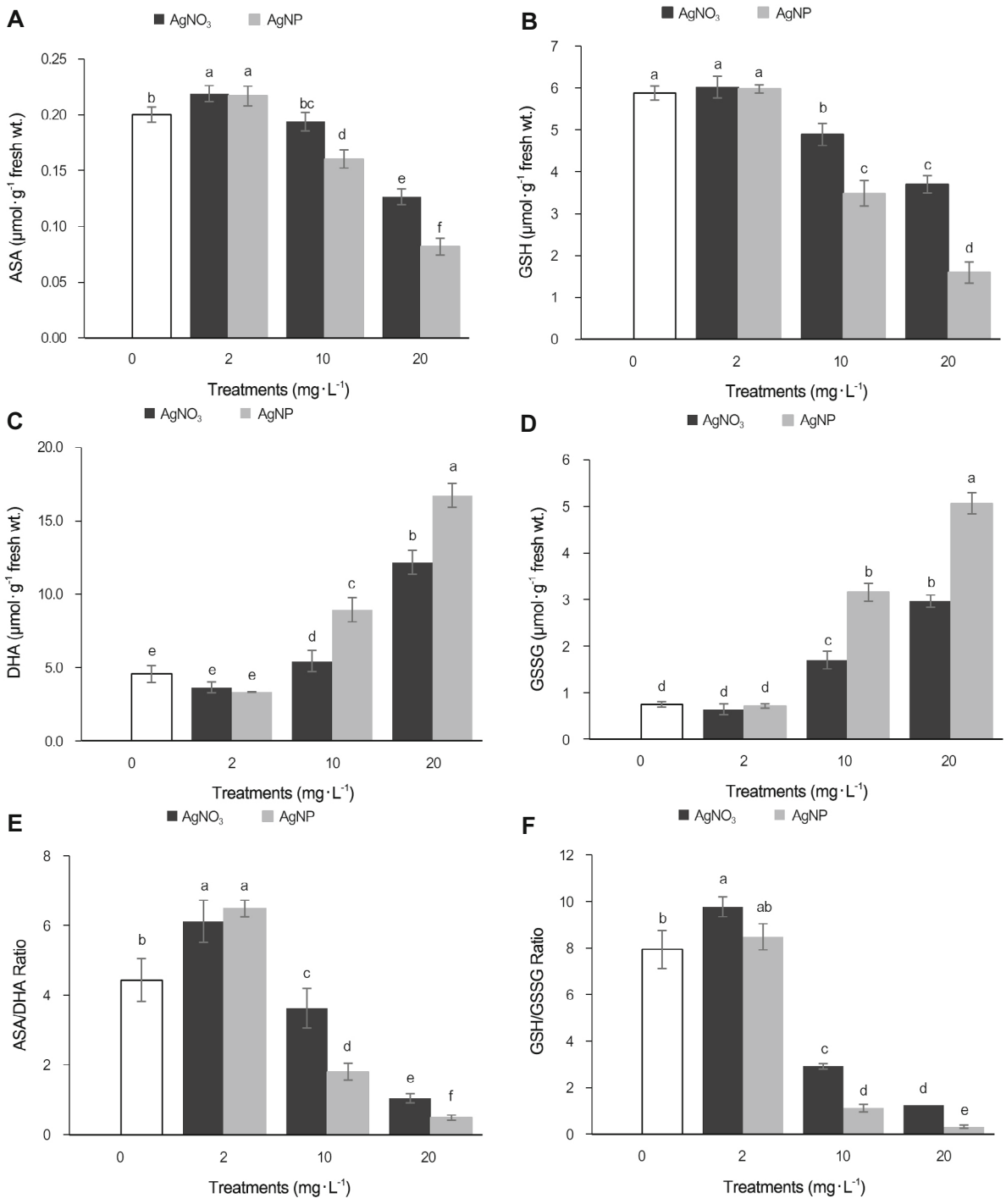

Fig. 6. Effects of silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) at different concentrations(2, 10 and 20 mg·L^{-1}) on the contents of ascorbate (ASA, A), reduced glutathione (GSH, B), dehydroascorbate (DHA, C), oxidized glutathione (GSSG, D), and the ratios of ASA/DHA (E) and GSH/GSSG (F) in potato (Solanum tuberosum L.). Values are representative of three independent experiments and error bars represent standard deviation. Means sharing the same letters are not significantly different ($p < 0.05$).

to plants treated with the equivalent Ag ion levels. This could be linked with higher ROS production in AgNP-treated plantlets. Inactivation of SOD under severe oxidative stress has been reported (Casano et al., 1997).

ASA and GSH are non-enzymatic components of the antioxidant system, which are involved in quenching ROS directly or indirectly via ascorbate–glutathione cycle (Foyer and Noctor, 2011). Compared to the control, no alteration was observed in GSH or ASA contents at 2 mg·L^{-1} in either type of Ag treatment. However, a significant reduction in GSH, ASA, and the ratios of GSH/GSSG and ASA/DHA were seen in plantlets exposed to both AgNPs and Ag ions at concentrations

Table 1. Effects of silver nanoparticles (AgNPs: 2, 10 and 20 mg·L^{-1}) on total reactive oxygen species (T.ROS), cell death (CD), ion leakage (I.L), superoxide anion ($O_2^{\cdot-}$), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), glutathione (GSH), ascorbate (ASA), glutathione disulfide (GSSG), and dehydroascorbic acid levels, based on the Z-score (derived from standardization of data) of the variables. Positive values indicate data above, and negative values indicate data below the variable average.Values in boxes indicate biochemical indicators with remarkable alterations

Treatments	T. ROS	CD	I. L	$O2^{\cdot-}$	CAT	APX	SOD	GR	GSH	ASA	GSSG	DHA
Control	−0.817	−1.010	−0.836	−0.978	−1.320	−1.323	−1.373	−0.565	0.867	0.648	−0.876	−0.738
2	−0.703	−0.918	−0.828	−0.814	−0.340	−0.527	0.364	−0.061	0.928	0.9125	−0.901	−0.912
10	−0.023	0.447	−0.065	0.126	1.080	1.022	1.459	0.545	−0.641	−0.227	0 .640	0.238
20	2.050	1.640	1.870	1.849	−0.913	−0.459	−1.231	−1.777	−1.834	−1.818	1.842	1.842

Table 2. Effects of silver nitrate ($AgNO_3$: 2, 10 and 20 mg·L^{-1}) on total reactive oxygen species (T.ROS), cell death (CD), ion leakage (I.L), superoxide anion ($O_2^{\cdot-}$), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), glutathione (GSH), ascorbate (ASA), glutathione disulfide (GSSG), and dehydroascorbic acid (DHA) levels, based on the Z-score (derived from standardization of data) of the variables. Positive values indicate data above, and negative values indicate data below the variable average. Values in boxes indicate biochemical indicators with remarkable alterations

Treatments	T. ROS	CD	I. L	$O2^{\cdot-}$	CAT	APX	SOD	GR	GSH	ASA	GSSG	DHA
Control	−0.817	−1.010	−0.836	−0.978	−1.320	−1.323	−1.373	−0.565	0.867	0.648	−0.876	−0.738
2	−0.830	−0.984	−0.836	−0.899	−0.026	−0.582	0.031	0.747	0.956	0.957	−0.947	−0.851
10	−0.333	−0.098	−0.285	−0.130	1.620	1.516	0.792	1.252	0.245	0.445	−0.276	−0.483
20	0.658	0.941	0.981	0.849	−0.066	0.405	−0.040	−0.060	−0.507	−0.917	0.521	0.904

higher than 2 mg·L^{-1}. The reduction in GSH and GSH/GSSG levels might be caused by a decrease in GR activity, or direct consumption of GSH through interactions with ROS. GR is a key enzyme of the ascorbate–glutathione cycle that protects cells against oxidative damage by sustaining a high GSH/GSSG ratio (Noctor and Foyer, 1998). Because Ag ions have a high affinity to the sulfhydryl groups of biomolecules, GSH might be targeted by Ag ions and thus drained from the cells. Since the impairment in the ascorbate–glutathione cycle is triggered by GSH depletion, the reduction in the ASA/DHA ratio might be attributed to the decrease in GSH content.

In the present study, data were standardized to identify the most susceptible biochemical indicator implicated in the toxicity of AgNPs or Ag ions. Results indicated that SOD was the most important indicator; an enzyme that acts early in the detoxification process in AgNP treatments. However, in plantlets treated with Ag ions, the activities of both SOD and CAT were positively affected. Furthermore, total ROS and GSH content showed the highest and the second highest variations under the 20 mg·L^{-1}AgNP treatment condition, indicative of higher damage to plantlets treated with AgNPs than those treated with Ag ions.

The present study indicated that both AgNPs and Ag ion treatments exert oxidative stress on potato plantlets in *in vitro* conditions. Plantlets treated with AgNPs produced more ROS than those treated with Ag ions. Oxidative stress damage to plantlets treated with AgNPs was markedly greater than in Ag ion treatments, as evidenced by cellular viability and ion leakage values. Alterations in the activities of scavenging enzymes (SOD, CAT, APX, and GR), and depletion of non-enzymatic antioxidants (GSH and ASA) indicated that AgNPs are possibly more toxic to plants than the equivalent mass of Ag ions. Based on the present results we also conclude that the toxicity of AgNPs can not be attributed simply to the release of the silver ion within the cells; particle-specific effects might contribute to their toxicity.

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