



Physiological and biochemical responses of potato (*Solanum tuberosum*) to silver nanoparticles and silver nitrate treatments under in vitro conditions

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Abstract A study was carried out to investigate the effects of nano-silver particles (AgNPs) or silver nitrate (AgNO₃) supplementation to in vitro culture of potato plant. Results indicated that 2 mg l⁻¹ of AgNPs or AgNO₃ treatment resulted in improvement in some growth parameters such as dry weight, root length and leaf area. However, shoot length was significantly reduced. Total chlorophyll and carotenoids in treated explants were significantly increased. Anthocyanin content increased only in AgNO₃ treatments in a dose-dependent manner, while AgNPs treatments at 2 mg l⁻¹ and higher levels increased and decreased, respectively. Compared with untreated explants, treated explants exhibited no alteration in proline content at 2 mg l⁻¹ but increased at 10 and 20 mg l⁻¹ treatments. Flavonoids content increased only in explants treated with 2 mg l⁻¹ of AgNPs. Total phenolics were significantly increased under all treatments compared with untreated explants. AgNPs and AgNO₃ treatments showed an increase in H₂O₂ content and lipid peroxidation under 10 and 20 mg l⁻¹. Lipid peroxidation and H₂O₂ contents in explants treated with AgNPs were remarkably higher than in case of AgNO₃. Results showed that AgNPs treatment enhanced growth of potato explants, which could possibly be due to inhibitory effects of silver ion on ethylene perception. Furthermore, AgNPs exhibited stronger toxicity than AgNO₃ at 10 and 20 mg l⁻¹ concentrations.

Keywords AgNPs · In vitro · Oxidative stress · Potato

Introduction

Silver has been used by human beings for centuries. In recent years nano-sized particles of silver have found applications in various fields, mainly its application in antibacterial and antifungal activities (Cho et al. 2005; Wright et al. 1999). AgNPs have been applied to control some fungal diseases in plants (Kim et al. 2009; Min et al. 2009), and has been utilized for removal of bacterial contaminants in valerian (*Valeriana Officinalis* L.) grown under in vitro conditions (Abdi et al. 2008).

Anthropogenic release of AgNPs into the environment is inevitable due to overuse in commercial products. Reuse of waste water and sewage for agricultural lands will increase exposure of crop plants to AgNPs released from consumer products. Thus, the major concern about using AgNPs on plants is its phytotoxicity. Publications in this context have been contradictory so far. It has been reported AgNPs treatments enhanced growth in *Brassica juncea* and improved some morphological indices in *Cucumis sativus* (Shams et al. 2013; Sharma et al. 2012). However, negative effects of AgNPs have also been reported on plants. For instance, it has been shown that AgNPs has negative effects on growth of *Oryza sativa* and *Triticum aestivum* (Nair and Chung 2014; Vannini et al. 2014). Results of studies conducted on some wetland plants have indicated seedlings growth were more sensitive to AgNPs than seed germination (Yin et al. 2012). In addition, toxic symptoms were stronger in roots rather than shoots.

Ethylene, a gaseous plant hormone, negatively affect plant regeneration and propagation in vitro. Ethylene induces some abnormalities such as slow growth, small leaf size, weak stems, and root formation on shoots (Ehsanpour and Jones 2001). To cope with this problem silver nitrate has been successfully applied in plant tissue culture to

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improve regeneration and somatic embryogenesis in various crop plants (Purnhauser et al. 1987; Sridevi and Giridhar 2014). Blocking of ethylene action by AgNO₃ might cause improvement in plant growth and regeneration (Beyer 1976). The mechanism by which silver ions block ethylene action is related to the fact that binding of ethylene to its receptor require copper as a cofactor (Rodríguez et al. 1999) and silver ion can substitute copper ion leading to inhibition of ethylene response.

In vitro culture of potato explants are relatively sensitive to ethylene accumulation. Therefore, the aim of this research was to evaluate the application of AgNPs with potential of inhibitory effects on ethylene action, to improve the growth and development of potato explants. To understand the possible toxic effects of AgNPs, oxidative stress in terms of hydrogen peroxide (H₂O₂) content and levels of lipid peroxidation, and non-enzymatic antioxidants, viz., phenolics, flavonoids, anthocyanins, carotenoids and proline were estimated.

Materials and methods

Silver suspension and characterization

Polyvinylpyrrolidone (PVP) coated AgNPs were purchased from US Research Nanomaterials, Inc. (Houston, TX). The average size of the particles was 20 nm, spherical in shape and specific surface area of 18–22 m² g⁻¹. The stock suspension were made by dissolving nano-powder in double distilled water, followed by sonication for 10 min in a water bath sonicator, and all dilutions were freshly prepared before use.

Plant materials and treatments

The virus free, potato (*Solanum tuberosum* L.) cv. White Desiree was obtained from stock shoot culture, Plant Stress Center of Excellence, University of Isfahan. Isfahan, Iran. Single node stem sections were sub cultured on MS (Murashige and Skoog 1962) basal medium without any plant growth regulators. Uniformly selected explants (1 cm long stem cutting) were taken from 4 weeks old in vitro explants and transferred on MS medium containing 0, 2, 10 and 20 mg l⁻¹ AgNPs (20 nm). Similar concentrations were used for silver nitrate. Plants were grown in the culture room at 25 ± 1 °C with 16 h photoperiod under 2500 lux light derived from ordinary white fluorescent lamps. All experiments were carried out with three replications. After 4 weeks, the explants were harvested for analysis of different physiological and biochemical parameters.

Growth measurement

Growth parameters, such as dry weight of shoots and roots, leaf area, root and shoot lengths were measured immediately after harvesting. The dry weight was measured by drying the explants at 70 °C for 48 h. Leaf area was measured by a digital leaf area meter.

Photosynthetic pigment measurement

Photosynthetic pigments were extracted from 100 mg fresh leaves using 80 % (v/v) acetone. Total chlorophyll and carotenoid contents were determined spectrophotometrically at 663, 646 and 470 nm according to the method of Lichtenthaler (1987).

Anthocyanin content

For anthocyanin estimation, 100 mg fresh leaves were extracted overnight at room temperature in 10 ml acidified methanol (methanol:HCl::99:1 v/v). Samples were then centrifuged for 10 min at 3000 rpm and the supernatants were used for anthocyanin measurement as described (Laby et al. 2000). The absorbance was recorded at 530 and 657 nm for each sample, and relative anthocyanin levels determined by the following equation:

$$\begin{aligned} &\text{Relative unit of anthocyanin. g}^{-1} \text{ fr. wt} \\ &= A_{530} - (0.25 \times A_{657}) \times \text{volume (ml) of extract} \\ &\quad \times 1/\text{weight of sample (g)}. \end{aligned}$$

Proline, phenolics and flavonoid content

Proline content was determined according to the method described by Bates et al. (1973). Fresh leaves (100 mg) were homogenized with 2 ml of 3 % (v/v) sulfosalicylic acid and the homogenate centrifuged at 13,000 rpm. The supernatant (2 ml) was treated with 2 ml of freshly prepared glacial acetic acid and acid ninhydrin solution, and then incubated in a water bath at 100 °C for 1 h. The reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml of toluene and vortexed for 15 s. The toluene fraction containing chromophore was allowed to stand for 30 min at room temperature, then the absorbance was read at 520 nm. Proline concentration was determined from a standard curve prepared using analytical grade proline.

Total phenolics in leaf samples were determined using Folin and Ciocalteu's reagent (Singleton et al. 1999). Fresh leaves (100 mg) were homogenized in 5 ml of 80 % methanol and extracted overnight in the dark. The homogenates were centrifuged at 3000 rpm for 10 min. The

supernatant was used for both phenolics and flavonoid contents. The reaction mixture for total phenolics content was prepared by mixing 0.1 ml of methanolic solution of extract, 0.3 ml of 50 % Folin and Ciocalteu's reagent dissolved in water and 0.3 ml 7.5 % Na_2CO_3 . The samples were then incubated at room temperature for 30 min. The absorbance was determined at 765 nm. Standard curve was prepared using gallic acid and total phenolics contents were expressed in terms of gallic acid equivalent (GAE) (μg of GAE g^{-1} fr. wt). Total flavonoid contents in fresh leaves were determined according to aluminum chloride colorimetric method described by Chang et al. (2002). Methanolic solution of extract (0.1 ml) was mixed with 0.2 ml 10 % AlCl_3 , 0.2 ml 1 M potassium acetate, 0.2 ml 80 % methanol and 0.2 ml double distilled water. The mixture was incubated for 30 min at room temperature and then absorbance was measured at 415 nm. Standard curve was made with analytical grade quercetin. Total flavonoid content was expressed as μg of quercetin equivalent (μg of QE.g^{-1} fr. wt).

Estimation of lipid peroxidation and hydrogen peroxide content

Lipid peroxidation was estimated in terms of malondialdehyde (MDA) content according to thiobarbituric acid (TBA) assay described by Heath and Packer (1968). Fresh leaf samples (200 mg) were homogenized in 5 ml of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000g for 15 min at 4 °C. An aliquot of 1 ml from the supernatant was added to 2 ml of 20 % TCA and 2 ml of 0.5 % TBA. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. The absorbance was recorded at 532 nm, and nonspecific absorbance at 600 nm was subtracted. The MDA content was determined by its molar extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$) and the results expressed as nmol MDA g^{-1} fr. wt.

Hydrogen peroxide content was determined according to Velikova et al. (2000). Fresh leaves (500 mg) were ground with 5 ml of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min, and 0.5 ml of the supernatant was mixed with 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The mixture was incubated at room temperature for 15 min, and then absorbance was recorded at 390 nm. H_2O_2 content was calculated using a standard curve and expressed as $\mu\text{mol g}^{-1}$ fr. wt.

Statistical analysis

All results were presented as means \pm standard deviations (SD) from at least three independent series of experiments. Differences between treatments for the different variables

were tested by one-way analysis of variance (ANOVA), followed by Duncan post hoc test. Differences were considered significant at the $p < 0.05$ level.

Results and discussion

Effect of AgNPs and AgNO_3 on growth parameters

AgNPs and AgNO_3 treatments decreased shoot length at all concentrations (Fig. 1A), but significantly increased root length (Fig. 1B), leaf area and dry weight of shoot and root at 2 mg l^{-1} (Figs. 1C, 2A, B). However, these parameters were markedly reduced at the higher levels of AgNPs and

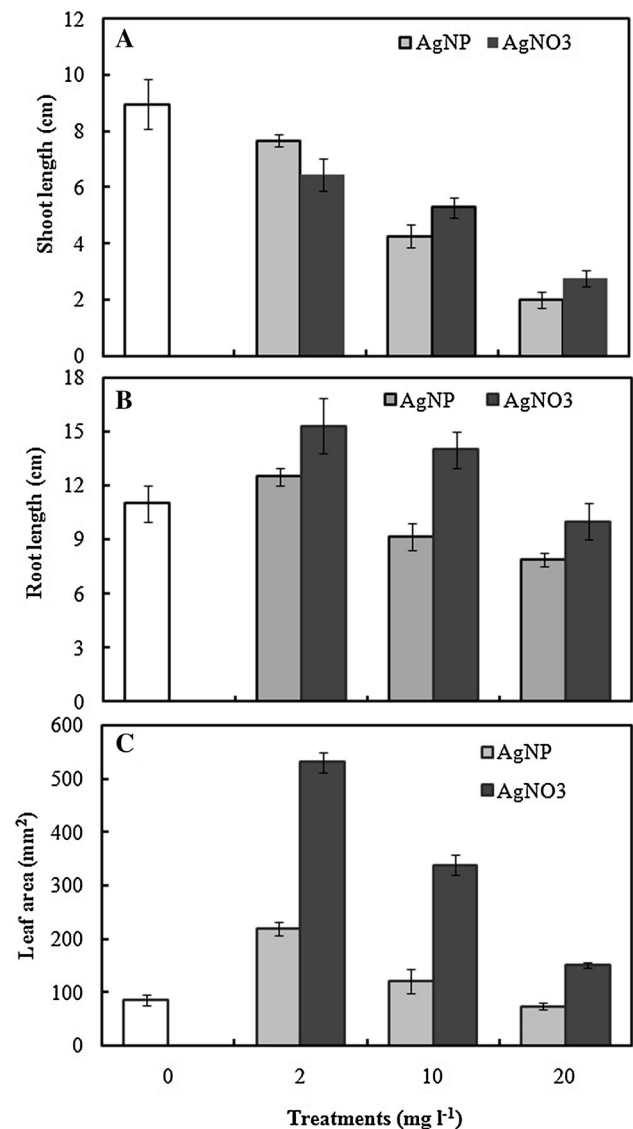


Fig. 1 Effect of AgNPs and AgNO_3 treatments on shoot length (A), root length (B), and leaf area (C) of potato (*Solanum tuberosum*) cv. White Desiree. Values are expressed as means of three independent experiments, \pm standard deviation

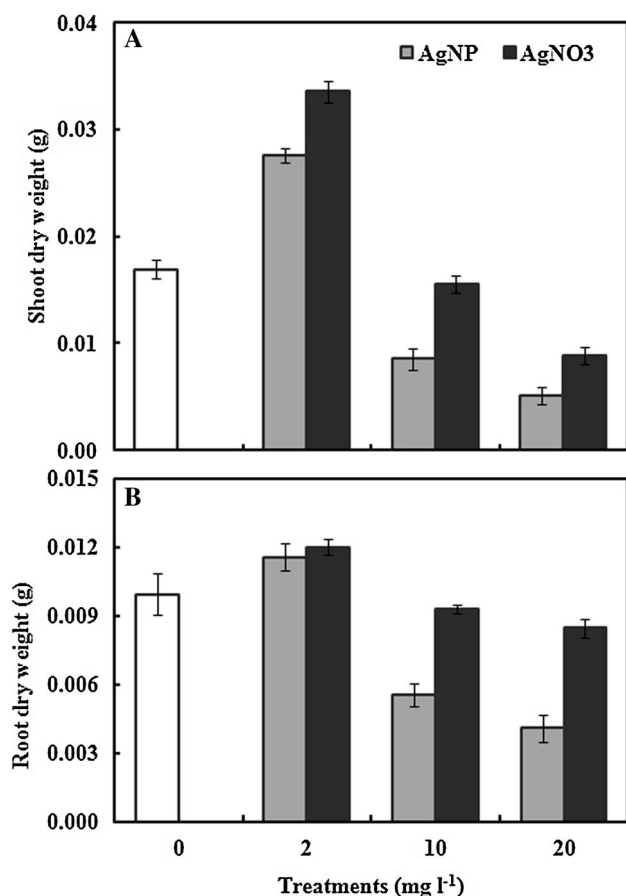


Fig. 2 Effect of AgNPs and AgNO₃ treatments on shoot dry weight (A), and root dry weight (B) of potato (*Solanum tuberosum*) cv. White Desiree. Values are expressed as means of three independent experiments, \pm standard deviation

AgNO₃. Enhancement of dry weight at 2 mg l⁻¹ of AgNPs and AgNO₃ treatments was likely related to increase of leaf area and consequently, resulted in more light capture and more biomass production. It seemed that efficacy of AgNO₃ in improvement of growth indices was better than that of AgNPs. In consistence with our results, root elongation has been observed in *Eruca sativa* exposed to 10 mg l⁻¹ AgNPs (Vannini et al. 2013). Silver ion is a potent inhibitor of ethylene perception and signaling (Beyer 1976), and dissolution of silver ions from AgNPs has previously been reported by Hsiao et al. (2015), therefore, AgNPs action on potato explants might be linked with blocking of ethylene perception after releasing of silver ions inside the cells.

Effect of AgNPs and AgNO₃ on photosynthetic pigments

Total chlorophyll content significantly increased at 2 mg l⁻¹ of AgNO₃ and AgNPs treatments compared to control (Fig. 3A) and decreased at higher levels (10 and

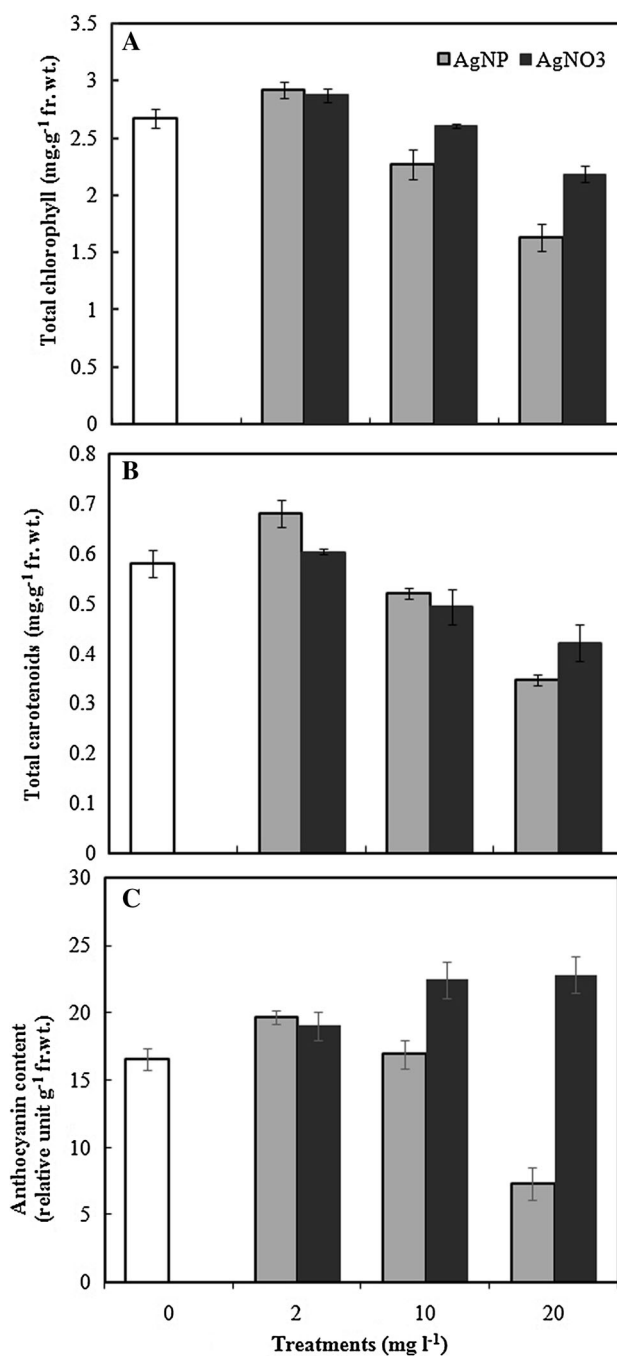


Fig. 3 Effect of AgNPs and AgNO₃ treatments on total chlorophyll (A), carotenoids (B), and anthocyanin (C) contents of potato (*Solanum tuberosum*) cv. White Desiree. Values are expressed as means of three independent experiments, \pm standard deviation

20 mg l⁻¹). However, the reduction in total chlorophyll was greater in AgNPs treatment than that in AgNO₃. Total carotenoids content also significantly increased at 2 mg l⁻¹ of AgNPs treatment, while no difference was observed at this level in AgNO₃ treatment (Fig. 3B). A remarkable reduction in total carotenoids was observed at higher concentrations of both AgNO₃ and AgNPs treatments.

Photosynthetic pigments are considered as potential biomarkers of heavy metal stress (Kumar et al. 2012; Monteiro et al. 2009). The negative effects of AgNPs on total chlorophyll and carotenoids was greater than AgNO₃, suggesting possible higher toxicity of AgNPs than AgNO₃.

Effect of AgNPs and AgNO₃ on anthocyanin content

Explants treated with AgNO₃ showed dose-dependent increase in anthocyanin accumulation in comparison with control (Fig. 3C). Anthocyanin content in explants treated with AgNPs was significantly elevated at 2 mg l⁻¹. However, it decreased at higher levels (10 and 20 mg l⁻¹). These observations suggest that explants treated with AgNO₃ exhibit a better adaptive mechanism by way of enhanced anthocyanin level to cope with silver toxicity than explants treated with AgNPs. It is hypothesized that anthocyanins alleviate stress responses by scavenging free radicals and restricting of H₂O₂ movement in plant cells (Hatier and Gould 2008).

Effect of AgNPs and AgNO₃ on proline, flavonoid and phenolics content

Proline content did not alter at 2 mg l⁻¹ of both AgNPs and AgNO₃ treatments compared to the control (Fig. 4A). An increase in proline accumulation was observed at higher levels (10 and 20 mg l⁻¹). Free proline accumulates in a variety of plants in response to environmental stresses including heavy metals and oxidative stress (Schat et al. 1997; Wang et al. 2009). Proline can serve as a compatible solute, scavenger of free radicals, stabilizer of macromolecules, metal chelator, and a source of reduced nitrogen and carbon leading to alleviation of stressful conditions (Ben Rejeb et al. 2014; Farago and Mullen 1979; Siripornadulsil et al. 2002).

Slight increase in flavonoid content was observed in explants treated with AgNPs and AgNO₃ at 2 mg l⁻¹, while reductions were observed at higher levels (Fig. 4B). Total phenolics were significantly increased by both AgNPs and AgNO₃ treatments compared to the control (Fig. 4C), also the increase was dose-dependent. Phenolic compounds are a diverse and large group of secondary metabolites comprise phenolic acids, flavonoids, anthocyanins, tannins and their derivatives. Phenolics have been considered as antioxidant due to free hydroxyl groups in their structure. Free hydroxyl groups of phenolics are responsible for free radical scavenging activity (Kowalska et al. 2014). Our results showed increase in total phenolics both by AgNPs and AgNO₃ and anthocyanin by AgNO₃ suggesting beneficial effect of treatments in augmenting scavenging capacity of explants.

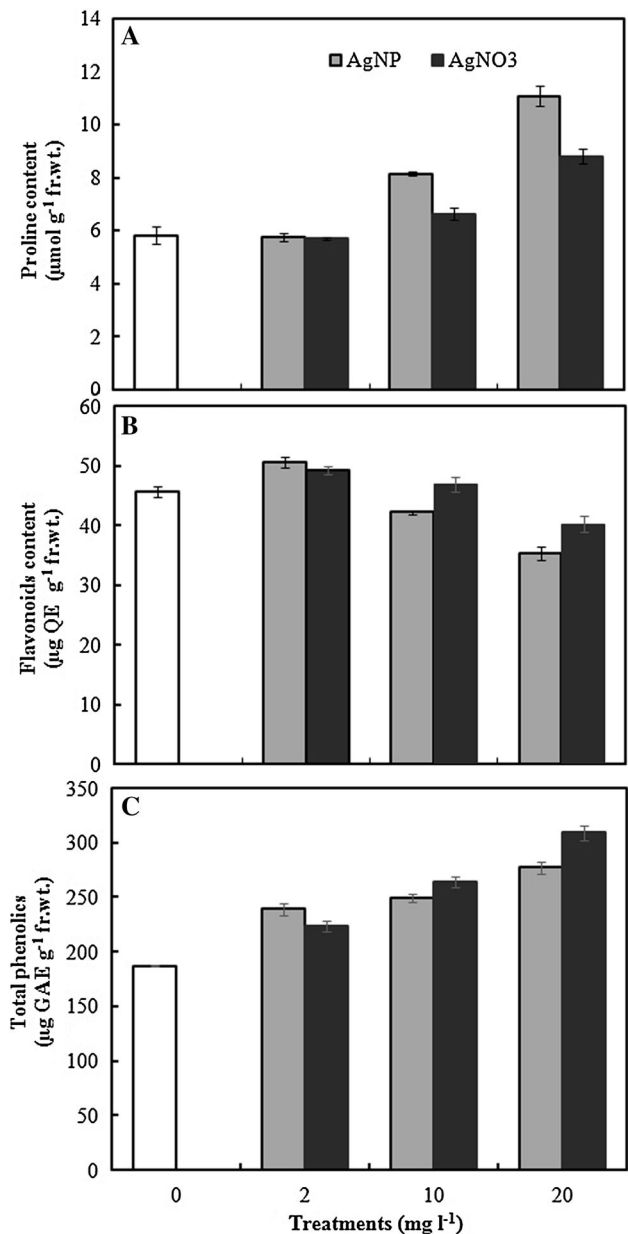


Fig. 4 Effect of AgNPs and AgNO₃ treatments on proline (A), flavonoids (B), and total phenolics (C) contents of potato (*Solanum tuberosum*) cv. White Desiree. Values are expressed as means of three independent experiments, ±standard deviation

Effect of AgNPs and AgNO₃ on hydrogen peroxide (H₂O₂) content and lipid peroxidation

There were little differences in H₂O₂ content in explants treated with 2 mg l⁻¹ AgNO₃ compared to control (Fig. 5A), while it slightly increased in explants treated with AgNPs at the same level. H₂O₂ content was noticeably enhanced in both AgNPs and AgNO₃ treatments at the higher levels (10 and 20 mg l⁻¹). Concentration of H₂O₂ in explants treated with AgNPs was

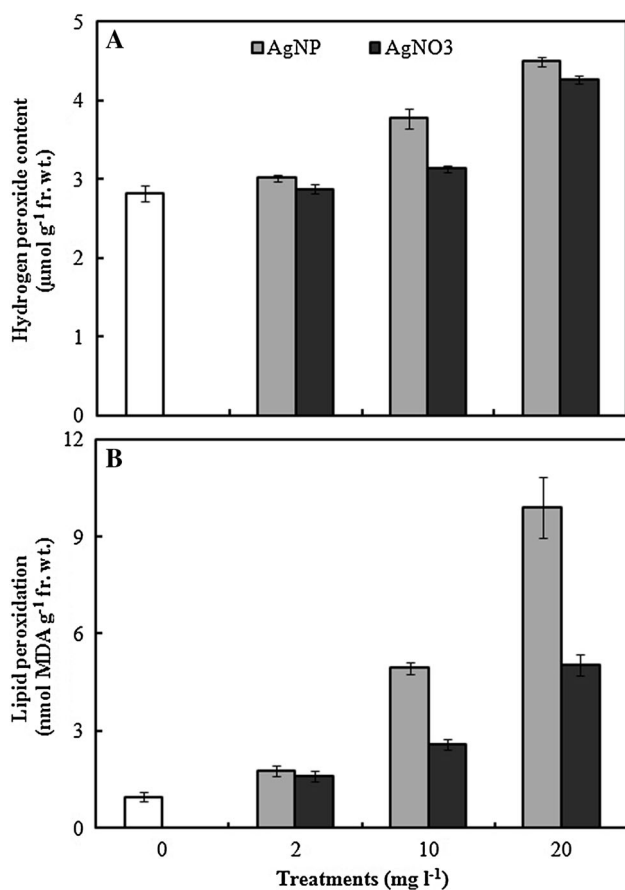


Fig. 5 Effect of AgNPs and AgNO₃ treatments on hydrogen peroxide (A), and MDA content (B) of potato (*Solanum tuberosum*) cv. White Desiree. Values are expressed as means of three independent experiments, \pm standard deviation

higher than that observed with AgNO₃. Lipid peroxidation estimated in terms of malondialdehyde content slightly increased in AgNPs and AgNO₃ treated at 2 mg l⁻¹. However, there was little difference between AgNPs and AgNO₃ treated explants at this level (Fig. 5B). At higher concentration of AgNPs or AgNO₃ treatments, MDA content was significantly increased, and the concentration of MDA in explants treated with AgNPs was more than that of AgNO₃. H₂O₂ accumulation in plant cells lead to lipid peroxidation, DNA damage and it may inactivate enzymes by oxidizing their sulfidryl groups. In the present investigation, the amount of lipid peroxidation and H₂O₂ content in explants treated with AgNPs were noticeably higher than those of AgNO₃ suggesting that phytotoxicity of AgNPs for potato explants grown in vitro is much more than AgNO₃. In agreement with our results, toxicity of AgNPs in rice seedlings and tomato have been previously reported (Nair and Chung 2014; Karami Mehrian et al. 2015).

Conclusion

From the results it can be concluded that except for shoot length, AgNPs and AgNO₃ at 2 mg l⁻¹ improved the growth and development of potato explants under in vitro culture condition. However, the efficacy of AgNPs to improve growth parameters in potato explants was relatively weaker than that observed with AgNO₃ treatments. Based on biochemical responses of treated explants on MDA and H₂O₂ contents, it can be considered that phytotoxicity of AgNPs was stronger than AgNO₃ and the toxicity is likely due to induction of oxidative stress.

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