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RESEARCH ARTICLE

Citric acid and hydrogen sulfide application reduce silver nanoparticles damage in green bean (*Phaseolus vulgaris*) plants

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ABSTRACT: Silver nanoparticles are being extensively used in a broad range of applications in our daily routine life. In the present study, it was investigated if citric acid (CA) and hydrogen sulfide (H_2S) can mitigate adverse effects of silver nanoparticles (AgNPs) in green bean plants. Green bean seedlings were applied with AgNPs either through soil drenching or foliar spray and were then treated with different concentrations of citric acid and NaHS, as H_2S donor. Results indicated that AgNPs induced several stresses in green bean plants. Concomitant foliar and soil appliaction of nanoparticles caused adverse effects on photosynthetic pigments and reduced carotenoid and protein contents, while increasing H_2O_2 content and superoxide dismutase (SOD), and ascorbate peroxidase (APX) activities. It was revealed that citric acid and H_2S application significantly alleviated adverse effects of AgNPs. In the plants challenged with AgNPs, the highest rates of catalase (CAT), glutathione S-transferase (GSTs), and malondialdehyde (MDA) activities were recorded, while these parameters were reduced when plants were also treated with H_2S. Application of 1.5 g/L of citric acid caused sharp decreases in CAT, GST and MDA activities. Among the treatments, the highest levels of APX, SOD, and anthocyanin were observed in the plants treated with AgNPs trough both foliar and soil drench method without citric acid and H_2S treatment. The findings of the present study would increase our knowledge of the interaction of plants with heavy metals and would be useful for designing sophisticated methods for reducing the damages in the stressed plants.

KEYWORDS: Abiotic stress, Antioxidant enzymes, Green bean, Plant response, Silver nanoparticles.

INTRODUCTION

Nanoparticles are extensively used in different fields and synthesis and application of various nanoparticles have gained much attention during the past decades. As a consequence, ever-increasing accumulation of these nanoparticles would have detrimental effects on human health and on environment. Some studies have shown that nanoparticles, similar to other abiotic stresses, induce reactive oxygen species (ROS) accumulation in plant tissues which are harmful to the cells at high concentrations [30]. The production of these compounds, such as hydroxyl radicals, causes lipid peroxidation, inactivation of enzymes, and destruction of cell membranes [7]. Peroxidation of membrane lipids by ROS leads to disruption of normal membrane activity, increased membrane permeability, and decreased membrane stability index [18]. Various studies have shown that nanoparticles may accumulate in plants at high concentrations which reduces their growth and

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metabolism. It has been reported that compared to other living organisms, plants can absorb higher amounts of different nanoparticles [19].

Among the products offered by nanotechnology, silver nanoparticles (Ag NPs) have a special place in studies on the field of plant sciences, as well as in the garment industry due to their antibacterial properties [22]. Due to the effect of Ag NPs on beneficial bacteria and other microorganisms, its excess accumulation in water and soil is associated with harmful environmental effects [65]. Numerous researchers have pointed out the positive and negative effects of Ag NPs on plant growth, and that the use of Ag NPs increases the growth and yield of plants [63]. On the other hand, some studies have shown that Ag NPs can have harmful effects on plants. Vishwakarma et al. [73] reported reduced growth and biomass of rapeseed with the application of Ag NPs. A number of researchers presented evidence that the toxic effects of Ag NPs reduced germination in various plants [41, 1]. In addition, root development was affected by the toxicity of Ag NPs, leading to a reduction in root length and dry weight [1]. Sah et al. [63] also found that silver nitrate at a concentration of 100 ppm increased the number of leaves, while increasing the concentration to 300 ppm reduced the dry weight of plants and flowers.

On the other hand, the toxicity of Ag NPs has detrimental effects on other physiological processes of the plant. Damages due to the toxicity of Ag NPs include reduced photosynthesis, chlorophyll degradation, and reduced membrane stability [73]. The toxicity of Ag NPs was shown to alter the activity of antioxidant enzymes [41]. In a study, a treatment with Ag NPs could significantly reduce photosynthetic pigments, photosynthetic performance, and such antioxidants as glutathione reductase, ascorbate peroxidase (APX), as well as glutathione content [72]. Studies show that AgNPs enter groundwater and effluents and eventually enter the irrigation system of farmlands and contaminate plants [25]. Since plants are the basis of the food chain, the penetration of Ag NPs into the ecosystem has caused concern in agriculture, food security, and human health [36].

Plants have evolved several adaptation mechanisms to combat harsh environments and survive different biotic and abiotic stresses [10]. Thanks to these mechanisms, plants can survive several growth inhibitors, e.g., drought [2], salinity [34, 46, 47], cold [68] and tolerate against biotic stresses including herbivory, insect pests [53] and fungal and bacterial pathogens [48]. This inspired various research groups to seek methods to exploit plant natural defense systems to mitigate adverse effects of biotic and abiotic stresses and maintain normal yield. These methods have been extensively used for plant molecular breeding using transgenic technology [17, 20] and for developing plant inducers [35; 45]. Plant inducers are a group of biological, chemical and physical factors that increase the plants tolerance against various biotic and abiotic stresses. These compounds alter several aspects of plant biology including strengthening cell and tissues [8, 10] higher accumulation of secondary metabolites [58], altered photosynthesis [57] and excited defense responses [59-61]. Different plant inducers have been successfully used to alleviate negative effects of various environmental stresses including cold [68], drought [2], flooding [37] and heavy metal stress [17].

Sulfur-containing defense compounds, including sulfur, hydrogen sulfide, glutathione, phytochelatins, sulfur-containing proteins, and many secondary metabolites, are critical to plant survival during periods of exposure to various stresses. The formation of these compounds in plants is highly correlated with the availability, demand, uptake, and assimilation of sulfur [15]. Sulfur assimilation begins with the uptake of external sulfate by the activity of sulfate transporters in the roots. On the other hand, plants are capable of foliar uptake of hydrogen sulfide (H_2S) as a source of sulfur for growth, in particular under certain conditions of limited access to sulfur in the root medium. H_2S is a colorless, flammable gas with a rotten egg odor that has long been thought to be a toxic gas, but it has been shown to play an important role in plants as a regulator of plant physiological processes such as seed germination, root morphogenesis, photosynthesis, and aging of flowers [76]. H_2S has also been found to be one of the most important defense messengers in plants against abiotic stresses [32]. Numerous studies have shown that the use of H_2S increases the biomass and the length of the growth period in the roots and leaves of stressed plants [78]. Foliar application with H_2S donor resulted in dramatically increased activity of superoxide dismutase (SOD) and catalase (CAT) [76]. H_2S is a key factor in the tolerance to oxidative stress in cells induced by a wide range of abiotic conditions, such as heavy metal toxicity, drought and osmotic stresses, heat stress, flood stress, and other stresses, and plays a role in the plant [32].

Another strategy for plants to mitigate the harmful effects of stresses, e.g., those of heavy metals, is the use of organic acids. The involvement of organic acids in the tolerance, transport, and storage of heavy metals, as well

as a key role in the preservation of cellular homeostasis were reported previously [31]. Organic acids play an important role in the detoxification of heavy metals in plants. Most studies on the moderating effect of organic acids under stress conditions have demonstrated that organic acids in the form of plant secretions cause intercellular deposition of heavy metals. On the other hand, organic acids, such as phytochelatins and amino acids, play a key role in the chelation and separation of heavy metals in vacuoles [62]. An example is citric acid as a weak acid organic with the chemical formula $C_6H_7O_8$ and plays a very important role in chelating heavy metals. The use of citric acid was observed to have inhibitory effects on lipid peroxidation and H_2O_2 content [23]. Foliar application of citric acid significantly increased the concentration of endogenous citric acid in plant tissues and improved plant growth and stress tolerance during the stress period [70]. It was found that the use of citric acid stimulated plant defense mechanisms by increasing the activity of antioxidant enzymes. Moreover, citric acid spraying increased the chlorophyll content of iris leaves compared to the control [28]. Foliar application of thyme with citric acid and salicylic acid elevated the contents of chlorophyll pigments and carotenoids as well as essential oil compounds. Citric acid treatments, particularly at a concentration of 10 mmol, reduced the content of malondialdehyde [44].

The best response of the plant to heavy metals is to prevent the uptake of these elements or at least to limit their uptake to the roots to prevent their transfer to aerial organs of the plant. Although there are several active mechanisms in plants in this regard, it is still commonly observed that these harmful substances enter the aerial parts of the plant, accumulate in the vacuoles of vegetative cells, and disrupt several processes in the plant. In this case, researchers typically follow two perspectives. One is how to reduce the destructive effects of heavy metals entered the plant, and the second is how to prevent these elements from entering the human food chain and harming humans. The second concern is more considered about the plants whose vegetative parts are on use. Both cases were considered in this research. Therefore, since no detailed study has so far investigated the effect of external application of citric acid and H_2S on oxidative damage caused by nanoparticles, including Ag NPs, in green bean plant, the present study was conducted to investigate this issue and find a solution to mitigate the possible negative effects of Ag NPs.

MATERIALS AND METHODS

The present study was conducted on green bean plant, the Sunray cultivar, in Tabarestan Agricultural Genetics and Biotechnology Research Institute, Sari Agricultural Sciences and Natural Resources University in 2018.

AgNPs application and CA and NAHS treatments

Pot factorial experiments were performed based on a randomized complete block design with three replications. Ag nanoparticles at four levels (control, foliar and soil application each at 2500 ppm, and combined application of foliar and soil application with the mentioned concentrations), foliar application of citric acid at three levels (zero, 0.75, and 1.5 g/l), and NaHS foliar application as H_2S donor at two levels (without and with consumption at a concentration of 0.6 mmol). Pots with a mouth diameter of 22 cm and a height of 25 cm were selected and filled with a mixture of field soil and sand in a ratio of 2:1. Seven seeds were planted in each pot. After the establishment of seedlings, four seedlings remained in each pot. In the treatments meant for the soil application of Ag NPs, pot soils were mixed with Ag NPs. Mixed soils were then irrigated to saturated moisture and left for 3 weeks to make the necessary interaction. Foliar application of solutions began 30 days after planting (before flowering) in one step in the evening in calm weather and the traits of interest were measured 2 weeks later.

Protein extraction

To extract the enzyme extract, 1 g of the leaf sample was homogenated using completely chilled porcelain mortar and liquid nitrogen, and then 5 ml of phosphate buffer (pH = 7.5) containing 0.5 mM EDTA was added to the homogenate. The homogenates were then transferred to the microtubes, centrifuged at 15,000 g and 4 ℃ for 15 min, and kept frozen at -80 until further measurements [64].

SOD measurement

SOD activity was measured based on [11]. The reaction solution for measuring SOD activity comprised 835 µof 50 mM sodium phosphate buffer with an acidity of 8, 33 µof riboflavin, and 33 µof enzyme extract. Changes in the absorbance of the reaction solution against a blank were measured by a spectrophotometer (Analytic jena, SPEKOL 1300) at 560 nm.

CAT measurement

CAT activity was measured according to Abi [3], which is based on the breakdown of hydrogen peroxide (H_2O_2) by CAT. The reaction mixture consisted of 3 mL of 50 mM phosphate buffer, 10 mL of 15 mM H_2O_2, and 50 µof the extract. After adding the extract, decreased absorbance was measured at 240 nm using a spectrophotometer for 1 min.

APX measurement

APX was measured as described by Nakano and Asada [52]. The reaction mixture consisting of 2.5 ml of 50 mM phosphate buffer with an acidity of 7.8, 30 mM of ascorbic acid, 0.3 ml of 0.1 mM H_2O_2, and 0.1 ml of the extract. APX activity was read at 290 nm by the spectrophotometer.

Glutathione S-transferase measurement

Glutathione S-transferase (GSTs) activity was measured by the method of Habig [27]. The reaction complex consisted of 1600 µof 100 mM phosphate buffer with 6.25 acidity, 200 µof 30 mM glutathione, 100 µof 0.75 mM 1-chloro, 2 and 4-dinitrobenzene, and 100 µof the extracted enzyme solution. The absorbance was read at 340 nm.

Measurement of chlorophyll and carotenoids

To measure the chlorophyll and carotenoid contents in the leaves, six prepared punches of leaves were immersed in 8 ml of methanol, left at room temperature in the dark for 24 h, and then the absorbed light by the solution was recorded at wavelengths of 665.2, 652.2, and 470 nm [56].

Measurement of total soluble proteins

Soluble protein was measured by the method of Bradford [13]. The reaction complex consisted of 100 µ of the extracted enzyme solution, 200 µof Bradford reagent, and 700 µof deionized water. Finally, the absorbance of the samples was read at 595 nm.

Malondialdehyde measurement

Malondialdehyde (MDA) content was determined as a measure of lipid peroxidation according to Stewart [69]. Leaf samples (0.5 g) were homogenized in 0.1% trichloroacetic acid solution and then centrifuged at 15,000 g for 10 min. Two ml of the supernatant was mixed with 4 ml of 20% trichloroacetic acid solution containing 0.5% of thiobarbituric acid. The obtained complex was incubated at 95˚C for 30 min and then transferred to a cold water bath. The samples were re-centrifuged at 100 g for 100 min. The absorbance of samples was recorded at 532 and 600 nm and lipid peroxidation rate was obtained using the difference between the absorbed wavelengths and an extinction coefficient of 1155 $mmol^{-1}$ cm^{-1}.

H_2O_2content

H_2O_2 levels were determined based on the H_2O_2 reaction with KI. In this method, 0.05 g of fresh leaf tissue was crushed in 5 ml of 0.1% TCA. The resulting extract was centrifuged at 12000 g for 15 min, and then 0.5 ml of 10 mM KH_2PO_4 buffer with an acidity of 7.5, 1 ml of 1 mM KI was added to the supernatant. The reaction mixture was placed in the dark at room temperature for 1 h and then the absorbance of samples was measured at 390 nm [5].

Statistical analysis

Data were analyzed using SAS software version 9.1 and graphs were drawn with Excel software.

RESULTS AND DISCUSSION

The simple effect of Ag NPs was significant on all studied traits at a probability level of 1%. Except soluble protein, all the studied traits were significantly affected by citric acid foliar application. The simple effect of H_2S foliar application showed that the studied traits were significantly influenced by this treatment, except chlorophyll b (Chl. b). The ANOVA results also revealed that the interactions of Ag NPs, citric acid, and H_2S were significant on APX, SOD, and Chl. b traits. Besides, the interactions of Ag NPs and citric acid were significant on SOD and Chl. b traits. According to the results of ANOVA, there were significant differences in the interaction of Ag NPs and H_2S for Chl. b, SOD, APX, catalase, GSTs, and MDA traits. However, the two-way interaction of citric acid and H_2S was significant only on Chl. b.

The results of comparing mean simple effects of the studied factors are shown in Table 1. Soil application and simultaneous soil and foliar application of Ag NPs significantly reduced leaf soluble protein content, but the reduction observed in this trait due to the foliar application of Ag NPs was not statistically significant and was placed in a statistical group with the control level. The lowest soluble protein content (12.60 mg/g wet weight) was recorded in foliar application level + soil

Table 1. Comparison of average effects of Ag NPs, citric acid, and H_2S on some traits studied in green bean plant

Treatment	Levels	H_2O_2 (nmol/g FW)	Protein (mg/g FW)	Carotenoid (µg/ml)	Chlorophyll a (µg/ml)
Silver nanoparticles	control	1.96 ± 0.08 d	15.49± 0.31 a	3.01±0.09 a	7.87±0.18 a
	foliar spraying	2.73± 0.08 c	14.92± 0.21 a	2.57±0.12 b	7.11±0.26 b
	soil application	3.02± 0.05 b	14.15± 0.15 b	2.54±0.17 b	6.77±0.29 b
	foliar spraying + soil application	3.77± 0.10 a	12.60± 0.14 c	2.52±0.12 b	6.67±0.24 b
Citric acid (g/l)	0	2.98±0.16 a	14.48±0.25 a	2.32±0.08 c	6.52±0.18 b
	0.75	2.89±0.15 a	14.23±0.35 a	2.57±0.10 b	7.15±0.25 a
	1.5	2.73±0.15 b	14.16±0.27 a	3.09±0.11 a	7.65±0.20 a
hydrogen sulfide (mmol)	0	3.04±0.12 a	1400±0.22 b	2.45±0.08 b	6.82±0.17 b
	0.6	2.70±0.12 b	14.58±0.25 a	2.90±0.09 a	7.39±0.19 a

Means with similar letters in each column have a significant difference at a level of 5% (LSD test). The numbers in the table represent mean ± SE.

consumption, which was 18.7% less than the control (Table 1). Vishwakarma et al. [73] showed that the stress by Ag NPs reduced total protein content in rapeseed. One of the reasons for the decreased protein content under environmental stresses is their oxidation, which is closely related to elevated free radical content [6].

Examination of leaf pigments (Table 1) indicated that carotenoid and Chl. a traits were affected by Ag NPs treatment and their levels diminished significantly at all levels. However, there was no difference between the application method of Ag NPs in terms of influencing this trait and all were at a single statistical level. Vishwakarma et al. [73] also reported reductions of these traits in rapeseed by the effect of silver heavy metal. The contents of photosynthetic pigments are of special importance as one of the important indicators in the production of photosynthetic products and also because of their decisive roles in resistance to stress. Studies on rapeseed demonstrated that Ag NPs reduced chlorophyll and carotenoid contents and, consequently, researchers highlighted the role of chlorophyll degrading enzymes (e.g., chlorophyllase) as a determining factor in the reduction of plant pigments [71, 73, 80].

According to Table 1, the effect of citric acid foliar application on the measured traits showed that this treatment only at a level of 1.5 g/l could have a significant effect on reducing H_2O_2 content by 9.15% compared to the control, and a level of 0.75 g/l was placed in the same statistical group as the control level. The decreased H_2O_2 level in this study corresponds to that of Maqbool et al. [40]. Citric acid plays an important role in reducing electrolyte leakage and free radical activity by activating scavengers and improving the photosynthetic process [2]. Leaf carotenoid content increased with the use of citric acid, so that the highest and lowest levels were observed for a level of 1.5 g/l and the control, respectively. Chl. a content also improved under the treatment with citric acid, however, no significant differences were observed between the levels of 0.75 and 1.5 g/l. Increases in photosynthetic pigments are directly affected by citric acid, because citrate activates the iron-reductase enzyme in the leaf plasma membrane. Additionally, citric acid, as a natural chelator, plays an important role in intracellular processes. Citric acid increases the active iron content in leaves by lowering intracellular pH [23, 24, 28, 33, 43].

In the present experiment, it was observed that H_2O_2 trait was significantly changed by applying H_2S treatment, so that H_2O_2 decreased by 12.59% with the application of 0.6 mM of H_2S. Elevated H_2O_2 levels in plants induces the activity of a series of antioxidant enzymes at the expense of the plant, but the plant recovers from stress and spends its energy on growth due to the reduced amount of H_2O_2 by H_2S [54]. In addition, spraying H_2O_2 solution significantly increased the soluble protein, carotenoids, and Chl. a by 4.14, 19.83, and 8.35%, respectively. Based on previous findings, H_2S plays an important role in the regulation of plant processes including photosynthesis, root growth, and aging of flowers [14,16,77] it also has an

Table 2. Comparison of mean effects of Ag NPs, citric acid, and H_2S on the activities of APX, SOD, and Chl. *b* content in green bean leaves

silver nanoparticles	citric acid (g/l)	hydrogen sulfide (mmol)	SOD activity (U/mg protien.min)	APX activity (U/mg protien.min)	Chlorophyll b (µg/ml)
Control	0	0	1.58 ±0.19 d-g	39.43 ±1.90 m	0.60 ± 0.03 de
		0.6	1.41 ±0.19 f-h	30.67 ±2.58 m	0.34 ±0.20 fg
	0.75	0	1.04 ±0.14 i-l	40.11 ±2.71 k-m	0.63 ±0.08 de
		0.6	1.38 ±0.03 f-i	18.75 ±2.40 n	0.59 ±0.03 de
	1.5	0	1.47 ±0.03 e-g	38.94 ±3.22 l-m	0.98 ±0.03 b
		0.6	0.86 ±0.13 l	18.05 ±2.79 n	0.77 ±0.04 cd
Foliar spraying	0	0	1.31 ±0.11 g-k	48.62 ±5.08 i-l	0.17 ±0.09 gh
		0.6	1.43 ±0.14 fg	45.25 ±3.62 j-l	0.16 ±0.02 gh
	0.75	0	1.36 ±0.19 f-j	48.27 ±7.99 i-l	0.63 ±0.06 de
		0.6	1.07 ±0.07 i-l	42.46 ±6.13 j-l	0.66 ±0.06 c-e
	1.5	0	1.91 ±0.03 bc	47.85 ±2.30 i-l	0.73 ±0.01 cd
		0.6	1.79 ±0.06 c-e	40.32 ±1.69 m	1.31 ±0.15 a
Soil application	0	0	1.76 ±0.11 c-e	62.16 ±1.97 d-g	0.17 ±0.04 gh
		0.6	1.82 ±0.09 b-d	52.75 ±5.79 g-j	0.23 ±0.01 gh
	0.75	0	1.86 ±0.11 b-d	59.74 ±2.84 e-h	0.53 ±0.09 ef
		0.6	1.09 ±0.13 h-l	50.66 ±2.79 h-k	0.68 ±0.18 c-e
	1.5	0	1.37 ±0.12 f-j	55.94 ±3.64 f-i	0.58 ±0.04 de
		0.6	1.01 ±0.07 kl	49.28 ±5.07 h-l	0.84 ±0.02 bc
Foliar spraying + soil application	0	0	2.42 ±0.04 a	103.64 ±2.01 a	0.18 ±0.02 gh
		0.6	1.83 ±0.07 b-d	70.62 ±3.46 cd	0.09 ±0.01 h
	0.75	0	2.12 ±0.15 ab	81.78 ±3.89 b	0.51 ±0.04 ef
		0.6	1.49 ±0.09 e-g	69.32 ±2.55 c-e	0.50 ±0.09 ef
	1.5	0	1.68 ±0.13 c-e	75.41 ±2.57 bc	0.59 ±0.03 de
		0.6	1.28 ±0.08 g-k	64.91 ±2.50 c-f	0.58 ±0.04 de

Means with similar letters have a significant difference at a level of 5% (LSD test). Numbers in the table indicate mean ± SE.

important function in terms of signaling in plants [32]. In the present experiment, it seems that the positive effects of H_2S on such processes as photosynthesis and stomatal regulation increased the relative water content (RWC) by creating favorable conditions for the plant. Also, the signaling role of H_2S activated the plant antioxidant system, resulting in a significant reduction in H_2O_2 content and electrolyte leakage.

The results of comparison of means showed that CAT activity was significantly lower in the leaves of plants sprayed with citric acid. Citric acid at levels of 0.75 and 1.5 g/l decreased CAT activity by 10.52 and 19.31%, respectively, compared with the control treatment, but no significant difference was observed between these two levels (Fig. 1A). Decreased CAT activity in these conditions may be due to the inhibition of H_2O_2 by Citric

acid, because the deposition of H_2O_2 in an environment stimulates the activity of CAT for its neutralization.

On the other hand, the application of citric acid treatment led to a decrease in the activity of GSTs. The highest and lowest activity of this enzyme were recorded in the control and 1.5 g/l of citric acid treatments, respectively, which were 15.38% lower than the control (Fig. 1B). MDA as a measure of lipid peroxidation revealed that, although the application of citric acid at a low concentration (0.75 g/l) had no significant effects on MDA levels, its doubled concentration (1.5 g/l) could significantly reduce MDA levels by 18.43% compared with the control (Fig. 1C). The production of POS occurs in natural conditions in the absence of biotic and abiotic stresses in plants. Thus, it seems that the use of citric acid induced a positive effect on improving the photosynthetic process and expression of certain proteins, as well as an effect on the Krebs cycle, which provided the plant with favorable conditions and reduced or inhibited the production of ROS, thereby reducing the activity of antioxidant enzymes such as CAT and GSTs [39]. Reductions in antioxidant enzymes and MDA levels by the use of citric acid were shown in several studies [4, 21, 66].

Figure 2 compares the mean effect of Ag NPs and H2S on the studied traits. The highest level of CAT activity was recorded in the leaves of plants that received Ag NPs treatment as foliar application + soil application, but were not sprayed with H_2S. This value was 293% higher than the control. The use of H_2S at this and other levels of Ag NPs reduced CAT activity, with the lowest value in the treatment with no use of Ag NPs using 0.6 mM of H_2S (Fig. 2A).

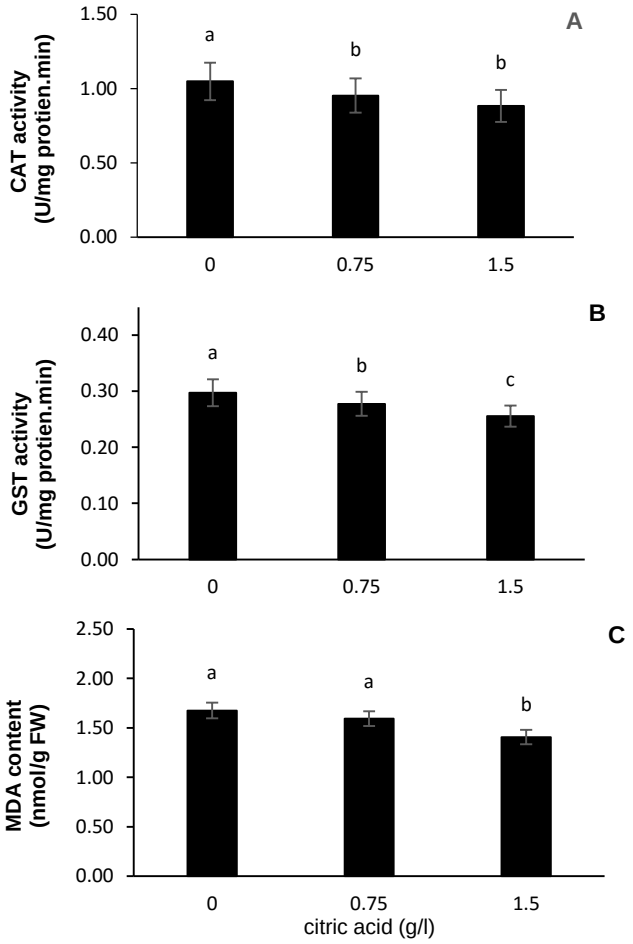

Figure 1. Comparison of the mean effect of citric acid on the activity of CAT (A) and GSTs (B) enzymes and MDA levels (C) in green bean leaves. Means with similar letters have a significant difference at a level of 5% (LSD test). Error bars indicate ±SE.

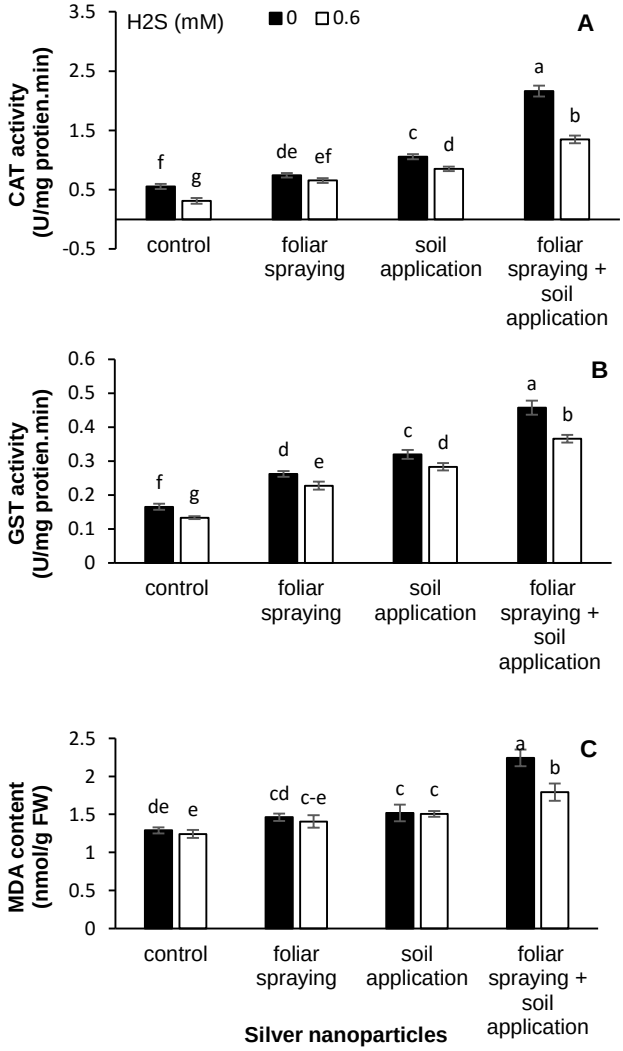

Figure 2. Comparison of the mean interaction of Ag NPs and H_2S on the activity of CAT (A), GSTs (B) and MDA levels (C) on the leaf. Means with similar letters have a significant difference at a level of 5% (LSD test). Error bars indicate ± SE.

The response of GSTs activity to these treatments was similar to that of CAT. The activity of GSTs was also increased by the application of Ag NPs at all levels, in particular the combined soil and foliar application, but spraying with H_2S significantly reduced the activity of this enzyme at all levels of Ag NPs. The lowest activity of GSTs was observed in the treatment without the use of Ag NPs using 0.6 mmol of H_2S, which was 24% lower than that of the control. In the other treatments, the activity of GSTs was significantly higher and from 37 to 176% more than the control (Fig. 2B.). GSTs is involved in the transport of heavy metals to the vacuoles and reduces the harmful effects of Ag by lowering its concentration in the cytoplasm [26]. Since the plant had more contact with Ag NPs in the spraying + soil application treatment, the scavenging activity was intensified in the plant by increasing the amount of GSTs. It also seems that the use of H_2S reduced the effects of stress by Ag NPs, resulting in decreased activity of antioxidant enzymes.

The results of Ag NPs and H_2S interaction on MDA trait were somewhat different from the other studied traits. The application of H_2S led to no significant effects on MDA levels in the control, spraying levels, and soil-Ag NPs application. In Ag NPs foliar application + soil application treatment, however, the use of H_2S caused a significant reduction of 25% in MDA levels compared to no use of H_2S at the same level of Ag NPs, which was still more than that of the control (Fig. 2C). Peroxidation of cell membrane lipids results from the negative effect of ROS such as H_2O_2, leading to disrupted selective permeability of the membrane and the production of toxic compounds. Several studies presented evidence that elevated activities of CAT and GSTs in stress conditions could reduce lipid peroxidation, which is attributed to the breakdown of toxic compounds as one of the important reasons [29]. Mostofa et al. [49] found that H_2S participation in the signaling phenomenon improved the photosynthetic process by increasing chloroplast biogenesis and CO_2 fixation, leading to reduced levels of antioxidants and MDA by mitigating the negative effects of stress.

Table 2 compares the means of SOD, APX, and Chl. b traits affected by the three-way interaction of Ag NPs, citric acid, and H_2S. The results for SOD trait revealed the highest activity in the treatment of Ag NPs as foliar application + soil application, but the difference was not very significant between the treatments. The highest SOD activity (2.42 enzymatic unit mg+ of protein min+) was detected in the treatment with no uses of citric acid and H_2S by the application of Ag NPs as foliar application + soil application, which showed a 53% increase compared to the control. Citric acid at 1.5 g/l significantly increased SOD activity with Ag NPs foliar application compared to other levels of citric acid. However, no significant difference in SOD activity was observed between the use and no use of H_2S. In soil application + foliar application conditions, citric acid at 1.5 g/l significantly reduced SOD activity, but there was not a significant difference in the use and no use of H_2S. The comparison of mean three-way interaction showed that the use of 0.6 mM of H_2S reduced APX activity without the application of Ag NPs, while non-significant differences were found between different levels of citric acid. Based on the results of this experiment, the highest effects of citric acid and H_2S were recorded with the application of Ag NPs as soil application + foliar application, which resulted in a significant reduction of APX activity compared to the control.

According to the comparison of means, Chl. b content was affected by the application of Ag NPs with a significant decrease. On the other hand, the simultaneous application of H_2S and citric acid led to elevated Chl. b content under stressful conditions of Ag NPs. The use of citric acid and H_2S with Ag NPs foliar application increased Chl. b content significantly in comparison to foliar application + soil application of Ag NPs. The treatment with 1.5 g/l citric acid and 0.6 mM of H_2S with Ag NPs spraying resulted in a Chl. b content of 1.31 g/ml, with a two-fold elevation compared to the control. The simultaneous use of citric acid and H_2S apparently had a synergistic effect on this trait and could significantly moderate the harmful effects of stress by Ag NPs.

The analysis results of simple correlations among the studied traits (Table 3) showed that Chl. a was positively and significantly correlated with Chl. b (0.68), protein (0.46), and carotenoids (0.76), while it had negative and significant correlations with SOD (-0.41), APX (-0.65), CAT (-0.55), GSTs (-0.64), H2O2 (-0.61), and MDA (0.60-). In unfavorable conditions, many disruptions occur in the photosynthetic capacity and photosynthetic pigment system [38; 75]. A negative and significant relationship between H_2O_2 and Chl. a and carotenoid levels indicates the destructive role of this oxygenated active species in biological processes, particularly photosynthesis. ROS cause a decrease in the quantity and quality of photosynthesis as a very important and vital phenomenon in plants[55, 74, 79]. As expected, a positive

Table 3. Simple correlation coefficients between all traits of green bean plant studied in this experiment

Variables	Chl a	Chl b	Carotenoid	SOD	APX	CAT	GST	Protein	H2O2	MDA
Chl a	1									
Chl b	0.68**	1								
Carotenoid(3)	0.76**	0.65**	1							
SOD	-0.41*	-0.30	-0.59**	1						
APX	-0.65**	-0.41*	-0.56**	0.67**	1					
CAT	-0.55**	-0.36	-0.48*	0.68**	0.97**	1				
GST	-0.64**	-0.44*	-0.56**	0.64**	0.97**	0.96**	1			
Protein	0.46*	0.26	0.32	-0.49*	-0.90**	-0.92**	-0.91**	1		
H2O2	-0.61**	-0.40	-0.54**	0.63**	0.97**	0.94**	0.99**	-0.91**	1	
MDA	-0.60**	-0.51**	-0.55**	0.66**	0.88**	0.93**	0.90**	-0.81**	0.89**	1

* and ** are significant at 5% and 1% probability levels, respectively.

and significant relationship was observed between H_2O_2 and MDA levels due to the activity of antioxidant enzymes. There were also negative and sometimes significant relationships between pigments (chlorophylls and especially carotenoids) and the activity of enzymes. This result indicates the high sensitivity of antenna molecules to stresses, which are damaged even with the activation of enzymes.

CONCLUSION

According to the results of this study, Ag NPs showed detrimental effects on different aspects of green bean plant and several physiological and biochemical activities have been affected. Based on our findings, simultaneous foliar and soil application of Ag NPs exhibited the highest negative effects compared with the time Ag NPs was solely applied in soil or foliar. On the other hand, H_2S and citric acid spraying could positively alleviate the stress caused by Ag NPs. Among the different levels of citric acid, a level of 1.5 g/l was better than 0.75 g/l. The simultaneous application of the studied factorsaffected Chl. b, SOD, and APX traits, all of which play a positive and important role in plant physiological and biochemical processes. Application of 1.5 g/l of citric acid and 0.6mM of H_2S under simultaneous foliar and soilapplication of Ag NPs reduced the activity of SOD and APX by 89% and 59%, respectively, hence this combination was selected as the best treatment under severe stress conditions. In general, it can be concluded that foliar application of H_2S and citric acid on green bean plants mitigates the adverse effects resulted by Ag NPs application and ultimately helps to improve plant growth.

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کاهش خسارت تنش نانو ذرات نقره در گیاه لوبیا سبز (*Phaseolus vulgaris*) با کاربرد اسید سیتریک و سولفید هیدروژن

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چکیده

نانوذرات نقره به طور گسترده ای در زندگی روزمره و در طیف وسیعی از کاربردها مورد استفاده قرار می‌گیرند. در مطالعه حاضر اثر اسید سیتریک و سولفید هیدروژن در کاهش اثرات منفی نانوذرات نقره در گیاه لوبیا سبز مورد بررسی قرار گرفت. گیاهچه‌های لوبیای سبز از طریق خاک پاشی یا محلول‌پاشی با نانوذرات نقره تیمار شده و سپس با غلظت‌های مختلف اسید سیتریک و NaHS به عنوان اهدا کننده سولفید هیدروژن محلول‌پاشی شدند. نتایج نشان داد که نانو ذرات نقره موجب تنش‌های متعددی در گیاهان لوبیا سبز می‌شود. کاربرد همزمان محلول‌پاشی و خاکی نانوذرات باعث اثرات منفی بر رنگدانه‌های فتوسنتزی و کاهش کاروتنوئید و پروتئین شد، در حالی که فعالیت H_2O_2 و سوپراکسید دیسموتاز (SOD) و آسکوربات پراکسیداز (APX) افزایش یافت. نتایج نشان داد که تیمارهای اسید سیتریک و H_2S به طور قابل توجهی اثرات تخریبی نانو ذرات نقره را کاهش می‌دهد. بیشترین میزان فعالیت کاتالاز (CAT)، گلوتاتیون-S ترانسفراز (GST) و مالون دی آلدئید (MDA) در گیاهان تحت تنش نانونقره ثبت شد، در حالی که وقتی گیاهان با H_2S تیمار شدند، این پارامترها کاهش یافت. استفاده از ۱/۵ گرم در لیتر اسید سیتریک سبب کاهش شدید فعالیت‌های CAT، GST و MDA می‌شود. در میان تیمارها، بالاترین سطح APX، SOD و آنتوسیانین در گیاهان تیمار شده با نانونقره از طریق تیمار توأم محلول‌پاشی و خاک و بدون محلول‌پاشی اسید سیتریک و H_2S مشاهده شد. یافته‌های تحقیق حاضر دانش ما را در مورد تعامل گیاهان با فلزات سنگین افزایش می‌دهد و برای طراحی روش های هدفمند به منظور کاهش خسارات در گیاهان تحت تنش مفید خواهد بود.

کلمات کلیدی: تنش غیرزنده، آنزیم‌های آنتی اکسیدانی، لوبیا سبز، واکنش گیاهی، نانوذرات نقره.