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ORIGINAL ARTICLE

Oxidative Stress Responses and Toxicity of Green Synthesized Silver Nanoparticles (AgNPs) on Basil (*Ocimum basilicum*) Seedlings

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KEYWORDS

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ABSTRACT: This study aimed at investigating how treatment of basil seedlings with green synthesized AgNPs affects their Ag content, oxidative damage and antioxidant enzymes activity. This research was studied as a completely randomized design in four replications. Four levels of silver nanoparticles (0, 4, 10 and 40 mg L^{-1}) were used. After germination, the seedlings were treated for 7 days and then seedlings were harvested for analysis. Findings showed that AgNP treatment increased Ag content $O^{2\bullet-}$, H_2O_2, MDA, and ion leakage in basil seedlings. The use of AgNPs caused a significant increase in the activities of SOD, APX, CAT, and GR enzymes in plants. However, at high levels (40 mg L^{-1}) of AgNPs, enzymes activity decreased significantly. These findings suggest that the application of green synthesized AgNPs to basil seedlings led to oxidative stress. Moreover, the observed changes in radical scavenging enzyme activity indicate that synthetic green nanoparticles have a harmful effect on basil seedlings. This toxicity is more pronounced at higher concentrations.

INTRODUCTION

The production of Ag nanoparticles (AgNPs), versus the use of Ag and silver salts, which dates back to human civilization, has recently become known. Particles ranging from 1 to 100 nm are considered as nanoparticles. They are used in many everyday products due to their unique properties [1]. NPs are specifically used in medicine and agriculture as antifungal, antibacterial and antioxidant [2]. At present, there is a lot of interest in nanoparticles due to their potential to improve plant growth, regulate the release of agrochemicals, and protect crops [3].

The utilization of biological techniques and resources to create AgNPs has significantly risen due to enhanced practicality and great biocompatibility [4-7]. Various biological pathways depending on microorganisms or plant extracts have been widely studied due to their compatibility with the environment and cost-effectiveness for the production of silver nanoparticles, which are very useful. Additionally, the synthesis of AgNPs through plant-based extracts is more beneficial than other biological methods since it doesn't need strict aseptic conditions and strict control over cell culture [5, 8-11].

Silver nanoparticles are commonly used because of their known ability to fight against bacteria and fungi, as well as their properties related to light and electricity [12]. Studies have shown that using the right amount of silver nanoparticles can help with seed germination and plant growth [13-15], leading to better photosynthesis and chlorophyll levels [16, 17], as well as improved efficiency in water and fertilizer usage [18].

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According to some reports, the impact of silver nanoparticles on plants can be negative or positive depending on various factors such as size, shape, duration of exposure, plant species and growth stage [19, 20]. However, increasing the use of silver nanoparticles is likely to have harmful effects on both the environment and human health [19-22]. Studies conducted on different organisms suggest that silver nanoparticles can be toxic due to various mechanisms such as disruption of cell membrane integrity [23], destruction and binding of proteins and/or DNA [14], and generation ROS [24]. Further evaluations have shown that oxidative stress could play a significant role in the phytotoxicity of silver nanoparticles by causing damage to lipids, proteins, DNA molecules and changes in plant hormones or antioxidant enzymes [21, 25].

When plants are exposed to AgNP toxicity, they may produce an excess of free radicals, as ROS, inclusive of superoxide, hydroxyl, proxy, and H_2O_2 [26]. The accumulation of these ROS can be harmful to the development of plantlet [25]. However, the presence of an antioxidant such as an enzyme or a nonenzymatic component can delay this process by scavenging the free radicals [27]. Oxidative stress caused by AgNP toxicity can also alter antioxidant enzymes activity like SOD, CAT, APX, GPX, GPOX, and affect non-enzymatic antioxidants contents in plants [28, 29].

We studied in the present research the potential phytotoxicity of *Satureja khuzistanica* Jamzad AgNPs at the physiological levels in an important crop plant, basil (*Ocimum basilicum*), which is also a commonly used in the food, medicinal and cosmetic-hygiene uses. Ag content, reactive oxygen species (ROS) generation, Ion leakage percent and antioxidant enzyme activity (SOD, CAT, APX and GR) were studied in this research.

MATERIALS AND METHODS

Silver nanoparticles

Silver nanoparticles ranging in size from 10 to 50 nanometers were produced by decreasing the silver ions in a *Satureja khuzistanica* extract. The AgNPs were diluted from high concentration to one mg L^{-1}, allowing for a more even dispersal when placed into water; this was achieved by subjecting it to ultrasonic waves of between 35-40 Watts for a period of ten minutes. This made for an even mixture that formed a homogenous dispersion.

Scanning electron microscopy characterization of Ag-NPs

The dimensions and shape of AgNPs were determined by conducting Scanning Electron Microscopy (Vega 5135 MM, Tescan, Czech Republic). The particles were attached to a microscope holder with a carbon strip in order for them to be examined.

Plant material and AgNPs exposure

To prepare basil seeds for germination, they were treated with a 2% solution of sodium hypochlorite for 20 minutes and then rinsed three times with sterilized distilled water to remove any remaining disinfectant. The seeds were then placed in a growth chamber at 28 ± 1°C in the dark for two days until they had uniformly germinated. Once germinated, the seeds were transferred to petri dishes containing different concentrations (0, 4, 10 and 40 mg L^{-1}) of AgNPs and allowed to grow for one week under controlled conditions of temperature (28 ± 1°C), light/dark cycles (16 ± 8 h) and aeration. After one week of exposure, the seedlings were harvested and stored at -20°C for further analysis.

Ag content measurement

The seedlings that were exposed to AgNPs underwent a process of being washed with deionized water to eliminate any Ag that was sticking to the surface of the plant. The root samples were then dried for 48 hours at a temperature of 70°C. The dried samples were pulverized and 50 mg of each powder was treated with a mixture of HNO_3 (65%) and H_2O_2 (30%) in a 3:1 (v/v) ratio. The mixture was heated at 120°C for an hour, then filtered and diluted to 10 mL with deionized water. An atomic absorption spectrophotometer (AA-240, Agilent) was used to analyze the samples, and the concentration of Ag was determined using a standard curve based on known concentrations of Ag.

Oxidative damage

The damage to the shoot membranes was ascertained by assessing ion leakage [30] and lipid peroxidation. The malondialdehyde (MDA) content of the fresh shoots were extracted using a trichloroacetic acid and thiobarbituric acid solution mixture (0.6% and 5%, respectively). Subsequently, the concentration of MDA was determined spectrophotometrically at 532, 600, and 450 nm (UV 2700, DAOJIN) [31]. Moreover, the levels of superoxide anion ($O^{2\bullet-}$) and hydrogen peroxide (H_2O_2) in the plant material were measured according to the method stated in [32].

Antioxidant enzyme activities

The UV/VIS spectrophotometer was used to determine the antioxidant characteristics of SOD, APX, GR, and CAT. The enzymatic activities of SOD, APX, and GR were measured in U/mg protein, while CAT activity was measured in U/g protein. To determine the SOD activities, we followed the method described in reference [33]. Additionally, an enzyme-linked immunosorbent assay (ELISA) kit from Shanghai Enzyme Linked Biotechnology Co., Ltd. was utilized to measure the activities of SOD in basil leaves. The ml902210 kit was used according to the user manuals found on www.mlbio.cn.

The enzyme activity of ascorbate peroxidase (APX; E.C. 1.1.11.1) was assessed using the method described in [34]. The reaction mix for this included 50 mM sodium phosphate buffer (pH 7.0), ascorbate (0.5 mM), EDTA (0.2 mM), and hydrogen peroxide (0.2 mM). These components were combined with 0.1 mL of the enzyme extract, followed by a decrease in absorbance of 290 nm over one minute being measured. Ascorbate peroxidase activity was estimated utilizing an extinction coefficient at 2.8 $mM^{-1}cm^{-1}$ then converted to unit/mg protein, wherein one unit represented the breakdown of 1 µmoL ascorbate in every minute per unit of mg protein specified in the assay mix.

The activity of CAT found in leaves (EC 1.11.1.6) was measured according to [35]. The process of incubation was initiated by mixing 0.3 grams of basil leaf with 0.1 millimoles per liter of phosphate buffer (pH 7.8) at a temperature of 4°C, followed by centrifugation at 4000 xg for a duration of 15 minutes. The resulting supernatant was then combined with phosphate buffer (pH 7.8) and distilled water, and allowed to rest for three minutes at a temperature of 25°C before the addition of 0.3 milliliters of 0.1 mole per liter H2O2, after which four readings were taken from the absorbance recorded at a wavelength of 240 nm every minute. Each unit of enzyme activity was defined as a decrease in absorbance per minute per 0.01 at a wavelength of 240 nm.

The enzymatic activity of GR (EC 1.6.4.2) was measured according to the method described in [36]. This experiment was completed on a spectrophotometer (UV 2700, DAOJIN); reductions in absorbance at 340 nm were monitored for two minutes. The extinction coefficient of 0.12 mM NADPH at 6.2 mM^{-1} cm^{-1} was used to calculate the GR enzyme activity.

Statistical analysis

The data gathered was examined with the aid of SPSS Ver. 22 statistical software (SPSS Inc., Chicago, IL, USA) using ANOVA. LSD's Test was employed to identify the smallest significant difference between the means acquired from the characteristics in four repetitions ± standard error, with a probability level of $p \leq 0.05$.

RESULTS

We utilized SEM to analyze the size and shape of AgNPs that were synthesized. The results, presented in Figure 1, indicated that most of the AgNPs were spherical and not clumped together. The particle sizes ranged from 10 to 50 nm with an average size of 30 nm. When basil plants were treated with AgNPs, there was a significant increase in the accumulation of Ag. After seven days of exposure to AgNPs, there was a noticeable difference in root Ag content between treatment groups. However, the highest accumulation of root Ag was observed in plants treated with 40 mg L^{-1} of AgNPs as shown in Figure 2.

The study showed that exposure to AgNPs at concentrations of 10 and 40 mg L^{-1} caused oxidative stress in the plants, as evidenced by increases in $O_2^{\bullet-}$, H_2O_2, MDA, and Ion leakage (Figure 3, A-D). Specifically, after 7 days of exposure to 40 mg L^{-1}

AgNPs, there was a significant increase in the levels of superoxide anion (18.2 fold), hydrogen peroxide (5.2 fold), lipid peroxidation (3.4 fold), and Ion leakage (9.2 fold) compared to the control group. However, when exposed to a lower concentration of AgNPs (4 mg L^{-1}), there was no significant difference in the production of superoxide anions, H_2O_2, MDA, and Ion leakage compared to the control group.

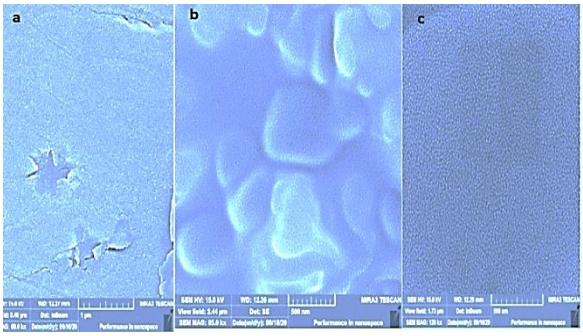

Figure 1. The characterization of Ag NPs was performed using SEM. The Ag NPs suspensions were deposited onto Cu grids coated with carbon film and subsequently dried. A: 60X magnification, b: 85X magnification, c: 120X magnification

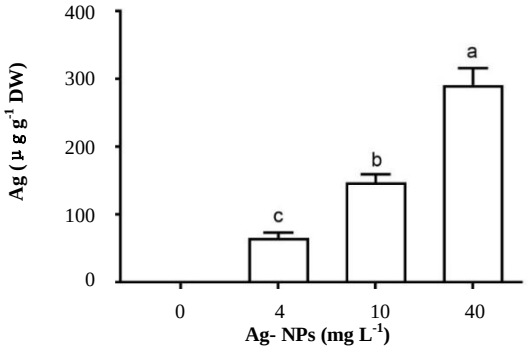

Figure 2. The effect of AgNPs at different concentrations (0, 4, 10 and 40 mg L^{-1}) on the Ag content of roots of basil. The values presented are from three separate experiments, and the error bars indicate the standard deviation. If two means share the same letters, it means they are not significantly different ($p < 0.05$).

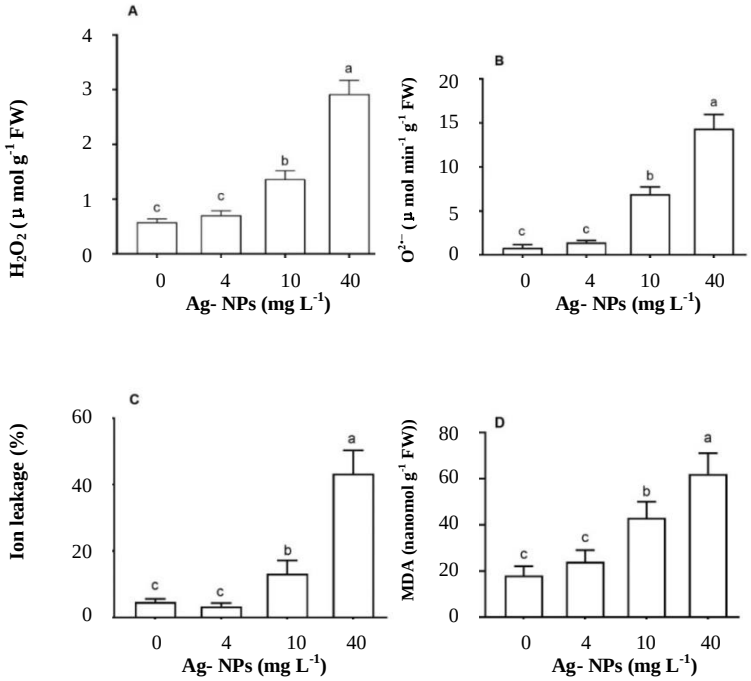

Figure 3. Effects of AgNPs at different concentrations (0, 4, 10 and 40 mg L^{-1}) on hydrogen peroxide (A), superoxide anion (B), Ion leakage (C), and lipid peroxidation (D) of basil plantlets. The values presented are from three separate experiments, and the error bars indicate the standard deviation. If two means share the same letters, it means they are not significantly different ($p < 0.05$).

The results of the study on basil plants treated with AgNPs are shown in Figure 4 (A-D). The administration of AgNPs resulted in a noteworthy rise in the operations of SOD, CAT, and APX up to 10 mg L^{-1}. Nevertheless, exposure to 40 mg L^{-1} AgNPs caused a decline of 31% in SOD activity compared to the treatment with 10 mg L^{-1}. The activities of CAT and APX also decreased after treatment with 40 mg L^{-1} AgNPs compared to 10 mg L^{-1}, but were still higher than the control. Additionally, GR activity increased after treatment with 4 mg L^{-1} AgNPs but decreased gradually at higher concentrations. At 40 mg L^{-1} AgNPs, GR activity reduced by 62.2% compared to 4 mg L^{-1} and was significantly less than that in the control.

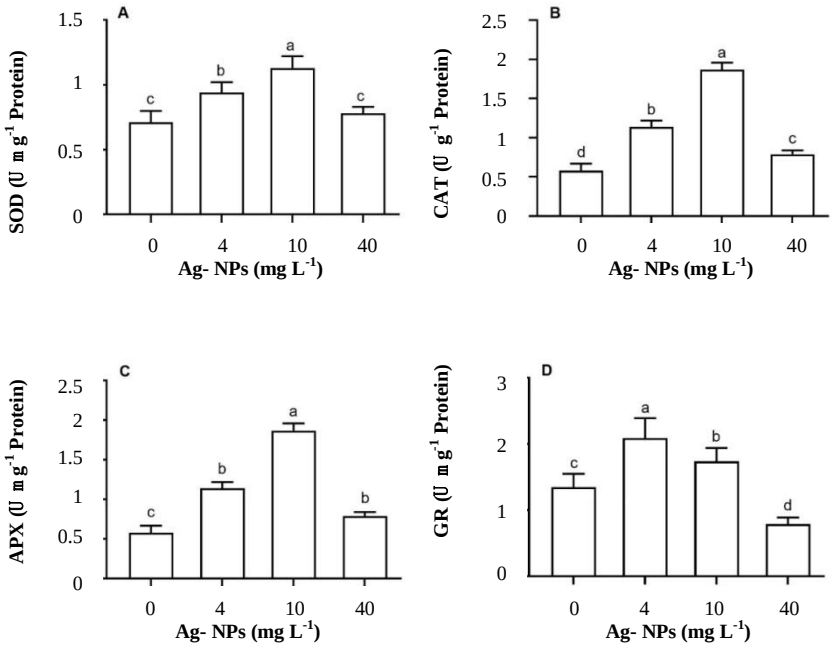

Figure 4. The activities of antioxidant enzymes in basil plantlets treated with different concentrations of AgNPs (0, 4, 10 and 40 mg L^{-1}). SOD (A), CAT (B), APX(C), and GR (D) enzymes. The values presented are from three separate experiments, and the error bars indicate the standard deviation. If two means share the same letters, it means they are not significantly different ($p < 0.05$).

DISCUSSION

In our experiment, we compared the toxicities of silver nanoparticles on basil (*Ocimum basilicum*) by biochemical factors of oxidative stress in greenhouse environment. In the current research, silver accumulation increased in basil plant roots after AgNPs treatment. Several studies conducted on various plant species have demonstrated that treatments with AgNPs often lead to the accumulation of Ag [37-39]. However, the mechanism of silver nanoparticle uptake in higher plants (vascular plants) is not yet fully understood. Some researchers suggest that the high silver accumulation in plants exposed to AgNP is probably due to the direct absorption of Ag nanoparticles. Another possible reason is that silver nanoparticles can be oxidized to Ag^{+} in the root, which can directly enter the root without being dissolved in the solution. [23]. Previous studies have documented that AgNPs were detected within the vacuoles of rice [40] and roots of turnip plant [41], in addition in plasmodesmata, cell wall and middle blade of Arabidopsis roots [42]. These findings are in agreement with our own research.

The presence of Ag NPs was discovered to cause an increase in oxidative stress in basil plants. Seedlings that were exposed to AgNPs had significantly higher levels of ion leakage, MDA, and ROS (H_2O_2 and super oxide onion) compared to the control group. When plants have too much ROS, it can lead to the breakdown of lipids in their biological membranes. Scientists often use the

amount of malondialdehyde (MDA) present as a way to measure this breakdown and study how oxidative stress and redox signaling affect the plant [13]. ROS production, which leads to cell membrane damage through lipid peroxidation, is the main cause of ion leakage and subsequent cell death. We hypothesize that silver nanoparticles can increase lipid peroxidation, thereby increasing ion leakage and membrane physical changes. The higher content of MDA and ion leakage is consistent with the studies of other researchers such as Moldavian mummy [28], wheat [14] and maize [10]. Rico et al. did not observe an increase in lipid peroxidation in rice treated with CeO_2 nanoparticles, but reported ion leakage at higher concentrations [43].

In addition, the level of H_2O_2, one of the most harmful reactive oxygen species, and superoxide anion also increased in seedlings treated with silver nanoparticles, especially at higher concentrations. Abiotic stresses lead to high production of ROS, and as a result, electron transfer causes high oxidative damage to plant biomolecules [44]. Hydrogen peroxide further leads to the initiation of the Fenton reaction, which produces a type of ROS called $HO^{\bullet}$, which is very reactive and no enzyme can neutralize it [12]. These ROS produced in plants cause undesirable and irremeable destruction to DNA, lipids, and proteins [45]. Higher levels of H_2O_2 and $O_2^{-\bullet}$ in onions exposed to silver nanoparticles were reported by Panda et al [4]. The increase in the toxicity of ROS due to the increase in the production of superoxide anion and H_2O_2 has been reported by other researchers [37, 39]. The relationship between plant toxicity caused by nanoparticles and the production of $O_2^{\bullet-}$ and H_2O_2 is not clear, but plants are able to convert species with high toxicity to less toxicity to defend against oxidative stress [45].

Heavy metals, which cause a loss of balance between the generation and quenching of ROS, are another stressful factor that causes oxidative stress [27]. To combat this, plants have developed enzymatic and non-enzymatic fighting complexes to overcome the damage caused by ROS [41]. SOD, CAT, APX and glutathione reductase (GR) enzyme systems play an effective role in ROS detoxification [42]. SOD, which plays an important role in neutralizing superoxide radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and preventing damage to important biological molecules, is the first barrier to deal with ROS [17]. In situations where the amount of H_2O_2 is overproduced due to the defense of SOD or alternately from the induction of other abiotic stress, other additional pathways may play a role for such detoxification [3].

H_2O_2 is detoxified into H_2O and O_2 by catalase, which is one of the common antioxidant enzymes. Another possible second reaction for H2O2 detoxification is the enzyme ascorbate peroxidase, which is accompanied by the oxidation of ascorbate to monodehydroascorbate (MDA) and dehydroascorbate (DHA) [13]. GR enzyme catalyzes the reduction of GSSG to GSH. In this research, we studied the antioxidant stress responses in basil seedlings exposed to AgNPs by investigating the activity of the antioxidant enzyme involved.

The activity of antioxidant enzymes SOD, CAT, APX and GR was significantly increased compared to the control and the maximum activity level was in 10 mg/liter treatments, but they showed a significant decrease in 40 mg/liter.

The study found that seedlings treated with 10 mg L^{-1} silver nanoparticles had high levels of ROS, which led to an accumulation of CAT, APX, and SOD. This explains that the antioxidant system's ability to scavenge ROS is overwhelmed by their production. The research also showed that oxidative stress can cause changes in antioxidant enzyme levels, both increasing and decreasing them. Previous studies have reported similar changes in antioxidant enzyme levels under AgNP treatment [19, 21 and 39]. The amount of SOD, CAT and APX in plants treated with nanoparticles at the level of 40 and GR at concentration levels of 10 and 40 mg L^{-1} were significantly reduced. It is assumed that in high concentrations of silver nanoparticles, the inactivation of the antioxidant enzyme has occurred, which is similar to these previously reported results [19, 37]. The mechanism of inactivation of antioxidant enzymes has not been identified yet, but it seems that it causes their inactivation through the binding of nanoparticles to the active site of the enzyme.

According to the current research, the use of AgNPs on basil seedlings causes oxidative stress, which results in higher levels of MDA and H_2O_2. The rise in ROS levels, along with changes in the activity of neutralizing

enzymes (SOD, CAT, APX, and GR), indicates that oxidative stress is an important reason for the toxicity of silver nanoparticles in basil plants. In conclusion, our findings indicate that higher concentrations of AgNPs can have harmful effects on basil plants.

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Conflict of interests

The authors declare that they have no conflict of interest.

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