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RESEARCH PAPER

Response of Lettuce to Silver Nanoparticles Under Drought Conditions

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Abstract
Nano-fertilization is an emerging technology to improve agricultural productivity under diverse ecosystems. Our study was conducted to investigate the effects of foliar fertilization of silver nanoparticles (AgNPs) on the growth, enzyme activities and water use efficiency (WUE) of lettuce (*Lactuca sativa*, L.) under drought conditions. A pot culture study with 4 AgNPs rates × 2 stress levels in a factorial combination of randomized complete block (RCB) design was conducted. The AgNPs levels were 0 (control), 25, 50, 75 and 100 mg/L) were imposed on lettuce seedlings under ambient and simulated drought (~50% field moisture capacity of soil) conditions. Lettuce plant height, total yields, leaf relative water content (RWC), chlorophyll content, membrane injury (MII), glutathione S-transferase (GST), glutathione reductase (GR), carboxylesterase (CaE), total phenolics and flavonoids and WUE were determined and/or calculated. The results showed that GST activity reduced in treatments of AgNPs + stress compared to stressed plants. In treatment of 100 mg L^{-1} AgNPs + stress, GR activity increased in treatments of 25, 50 and 75 mg L^{-1} AgNPs, but showed a significant decrease in 100 mg L^{-1} AgNPs + stress. CaE activity enhanced in 100 mg L^{-1} AgNPs + stress (about 1.55-fold) compared to stressed plants. Total flavonoid and phenolic contents were the highest in 50 mg L^{-1} AgNPs + stress. It was not obtained significant effects in the WUE rates. In 100 mg L^{-1} AgNPs, MII rates were the highest, and RWC rates were the lowest. Leaf width, plant height and total yield decreased at doses of AgNPs.

Keywords Plant growth · Chlorophyll · Relative water content · Membrane injury · Enzyme activities · Water use efficiency

1 Introduction

Plants face unfavorable conditions due to fluctuations of environmental conditions. These changes cause abiotic stresses such as nutrient imbalance, high or low temperature, salinity and drought, affecting plant growth and development (Due et al. 2008). Among these, drought stress was studied extensively on many plant varieties by many researchers (Laxa et al. 2019; Dasgan et al. 2018; Farooq et al. 2009). Drought stress causes biochemical, physiological and morphological alterations in plants (Ortiz et al. 2015). Although plants have developed a defense and resistance mechanisms against these adverse conditions, they are sometimes insufficient (Cruz de Carvalho 2008).

In recent years, nanotechnology has a growing attention owing to the successful applications in electrical, medical, food and agricultural. Nanoparticles (NPs) are materials in sizes between 1 and 100 nm (Graf et al. 2003) have unique chemical and physical properties due to their nanoscale size and high surface area are the industrial nanomaterials and used in a broad range of different areas (Dakal et al. 2016).

Various techniques are applied to alleviate the negative effects of drought stress on plants. In addition to its beneficial uses in many areas, nanoparticle application is thought to be an effective and promising application in alleviating the effects of drought stress (Shehab et al. 2010; Linh et al. 2020). Nanoparticle application improves plant responses to drought stress. The tolerance of plants under drought conditions was supported by increasing antioxidant enzyme activities and relative water content with

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applications of copper and zinc nanoparticles (Taran et al. 2017), by stabilizing chlorophyll and plant biomass content with treatment of iron nanoparticles (Kim et al. 2015) and by increasing seed germination with application of zinc oxide nanoparticles (Helaly et al. 2014). Silver (Ag) and silver nitrate ($AgNO_3$NPs) stimulate plant growth and these compounds have antimicrobial effects in soils and hydrophonic systems in agriculture (Cho et al. 2005).

AgNPs compounds have various effects depending on the plant species and age, also the concentrations and size of AgNPs (Qian et al. 2013; McShan et al. 2014). In several previous researches, high rates of AgNPs were reported having significant effects on chlorophyll content, leaf area, carbohydrate content and inhibition (Navarro et al. 2008; Miao et al. 2009; Salama 2012). AgNPs causes a decrease in the shoot length and seed germination and an increase in the root elongation. Nanoparticles are one of the most important factors in the formation of oxidative stress caused by reactive oxygen species. (Nel et al. 2006; Xia et al. 2006). Plants induce the synthesis of a series of antioxidant enzymes, including glutathione reductase (GR), carboxylesterase (CaE) and glutathione S-transferase (GST) to eliminate oxidative stress. In addition, another change during oxidative stress is the stimulation of the synthesis of phenolic and flavonoid compounds, which are important elements of the antioxidant mechanism. (Usha and Jyothsna 2010; Jyothsna et al. 2009).

L.sativa is an annual plant species in the *Asteraceae* (*Compositae*) and cultivated for fresh leaves. These vegetables are plants widely consumed, have great importance in the human nutrition and can be found in markets throughout the year (De Vries 1997).

We hypothesize that Ag nanoparticles will exert beneficial effects to improve the biochemical, morphological and physiological properties/processes of lettuce to tolerate and sustain under drought conditions. The objective of our pot culture study was to investigate the effects of different rates of Ag nanoparticles on the growth, yield and enzyme activities of lettuce under ambient and simulated drought conditions.

2 Material and Methods

2.1 Plant Material and AgNPs Treatments

The drought stress and AgNPs applications were carried out in the laboratory of the Horticulture Department, Faculty of Agriculture, Şırnak University, Turkey, in 2018.

A pot culture study with four AgNPs (25, 50, 75 and 100 mgL^{-1}) rates x drought stress levels in a RCB design was conducted. Two factorial experiment designs were used in the study (AgNPs and drought applications). The drought stress was applied according to the method developed by Schröder and Lieth (2002). Two different irrigation systems, ambient (% 100-watering) and moderate drought (50%-watering) conditions, were used to the lettuce plants in the pots. The experiments were conducted as randomized blocks, in triplicate, and it was used three plants in each replicate. Lettuce seedlings were transplanted in 2-L pots containing peat and perlite (3:1) and watered with Hoagland Nutrient Solution (Hoagland 1950). The Hoagland nutrient solution contained macronutrients (N 160, P 53, K 160, Mg 54, Ca 160 mg L^{-1}) and micronutrients (Fe 5, Mn 0.5, B 0.5, Zn 0.05, Cu 0.03, Mo 0.02 mg L^{-1}) (Resh 1991). The nutrient solution used in the experiment was determined as 20 and 40% of the nutrients needed for the lettuce plants. The plants were sown in pots and incubated in the growth chamber in the photoperiod 16/8 (light/dark), at 25 ± 2 °C. The seedlings were planted on September 25 and harvested on December 5.

The silver nanoparticles (AgNPs) used in the experiment were 80 nm in size, 99.99% purity and metal-based (Sigma). The solution containing AgNPs was sonicated for 10 min and then filtered through a 0.45-mm nylon filter membrane (Merck Millipore® syringe filter) prior to foliar application.

Four doses of AgNPs (0, 25, 50, 75 and 100 mg L^{-1}) were applied as a foliar spray, every 3 days. AgNPs treatments were carried out for 20 days after planting and repeated three times.

2.2 Determination of Antioxidant Enzyme Activities

The antioxidant activity tests were carried out in the Biology Department of Science Faculty, Dicle University. The frozen plant tissues were homogenized in 3 v/w 0.05 M Tris–HCl, pH 7.5, containing 2 mM EDTA, 1 mM Dithiothreitol (DTT), and polyvinylpolypyrrolidone (5% wv^{-1}) (Andrews et al. 2005).

The enzyme activities of GST, GR and CaE were measured by a microplate reader system activity using a UV spectrophotometer. The GTS activity was determined by the procedure defined by Habig et al. (1974).The reaction solution for measuring GST activity included 1-chloro, 2-4dinitrobenzene (CDNB) as a substrate and reduced glutathione as a cofactor. 0.1 M potassium phosphate buffer (pH 6.5), 1 mM GSH, 1 mM CDNB and 10 µL sample volume made up the reaction solution. Changes in absorbance at 340 nm, which are proportional to the rate of CDNB conjugation, were used to determine enzyme activity.

GR activity was measured using the method developed by Cribb et al. (1989) In a total volume of 190 µL, the reaction solution contained 0.1 mM 5,5′-dithiobis (2-

nitrobenzoic acid) (DTNB) as substrate, 1.2 mM NADPH and 10 µL of homogenate supernatant. 20 µL of 3.25 mM glutathione oxidized (GSSG) was added once the process was started, and glutathione reduced (GSH) was formed from GSSG. At 405 nm, DTNB decrease was measured.

The CaE activity was analyzed using the method modified by Kumar and Shivanandappa (1999). For 3 min, 5 µL of supernatant and 250 l of 0.05 mM Trizma (pH 7.4) were incubated. The reaction was initiated by adding 5 µL of PNPA (26 mM) as the substrate. For 2 min, the released p-nitrophenol was monitored at 405 nm.

The Bradford reagent (Bradford 1976) was used to measure total protein concentrations in each supernatant, and bovine serum albumin (BSA) was utilized as a standard protein source. 5 L of diluted (1:4) supernatant and 250 L of Bradford reagent were used as protein sources. The protein concentration was determined using the calibration curve created by providing serial dilutions of BSA standard (0–1.4 mg BSA mL^{-1}) and the absorbance was read at λ = 595 nm. The specific activity (nmol min1 mg protein 1) of each enzyme was determined using the total protein levels in each sample.

2.3 Determination of Total Phenolic Content

The contents of total phenolic were determined as described by Singleton et al. (1999). One gram of air-dried samples was powdered using laboratory mill, added of 10 ml of % 80 ethanol and then subjected to ultrasonication. This process was performed for three times. The extracts obtained from three processes were collected, and the solvent was removed in the evaporator. The residue was dissolved in 100 ml of 80% ethanol.

40 µL of ethanolic extract or gallic acid solution (1 $mgml^{-1}$) were mixed with 200 µL Folin–Ciocalteu reagent (FCR) (Sigma-Aldrich, Steinheim, Germany), and 1160 µL distilled water. The mixture was shaken and incubated for 3 min at room temperature. Then, 600 µL of 20% sodium carbonate (Na_2CO_3) solution was added to the mixture. The mixture was shaken for 2 h at room temperature followed the absorbance of solution was measured at 765 nm. The gallic acid was used as the standard. The total concentration of phenolic compounds was determined as µg gallic acid equivalents/mg extract using the equation obtained from a standard gallic acid graph (Fig. 1). To generate standard curve, it was used five points, 20, 40, 60, 80 and 100 μgmL^{-1} of gallic acid (Sigma-Aldrich, Steinheim, Germany) (Fig. 1).

2.4 Determination of Total Flavonoid Content

The method described by Park et al. (1997) was slightly modified to determine the flavonoid concentration of the extracts. The results for flavonoid concentration were expressed as quercetin equivalents. Analiquot of the 1 mL of the extract containing 1 mg of extract in methanol was added to the tubes which contained 0.1 mL of 10% aluminum nitrate in methanol, 0.1 mL of 1 M potassium acetate and 3.8 mL of methanol. The solution was incubated at room temperature for 40 min. The absorbance was measured at 415 nm using a spectrophotometer (Thermo Scientific Multiskan G0). The quercetin was used as the standard, and the flavonoid content was expressed as milligram quercetin equivalent per gram weight of the plant samples. Spectrophotometric analysis was carried out using a five-point calibration curve created with pure quercetin. Quercetin standard compound was obtained from Sigma (Milan, Italy).

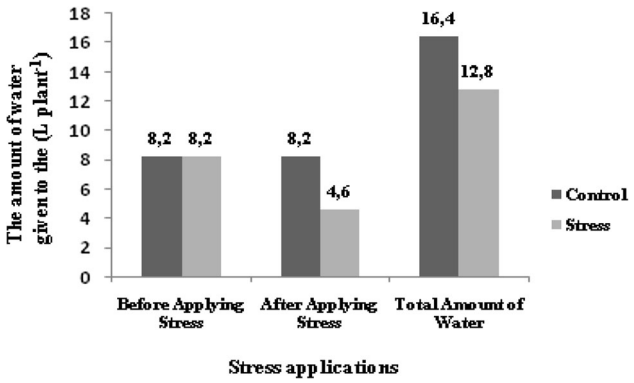

Fig. 1 Calibration curve for total phenolic acid according to gallic acid standard, absorbance at 765 nm

2.5 Growth Parameters

Leaf width, plant length and total yield were used to determine the growth parameters. The length of lettuce plants was measured from the root to the growth tip, and the leaf width was determined in the third leaf as cm from the outside of the leaf. Total yield was evaluated according to the calculations of fresh (FW) and dry weight (DW) of lettuce plants.

2.6 Plant–Water Relations

The data related to relative water contents and water use efficiency were used to determine the plant–water parameters. The fresh weights of the third or fourth leaves collected from each of the three plants were determined. The leaves were kept in pure water for 4 h and turgor weights were determined at the end of this period. The leaf samples were dried in an oven at 65 °C for 48 h, when a constant weight attained, the dry weight was recorded. The relative water content of the leaf samples was calculated using the equation given by Sánchez et al. (2004).

$$\text{Relative Water Content } (\%) = (\text{Fresh Weight} - \text{Dry Weight})/(\text{Turgor Weight} - \text{Dry Weight})\text{x}100$$

The amount of water used in different irrigation applications was recorded. The total amount of water per plant was determined during the production. Total yield was determined by calculating the "water use efficiency" in drought stress and different AgNPs applications, relative to the total amount of water used in control. The water use efficiency was calculated using the following equation;

$$\text{Water Use Effectiveness } \left(\text{gL}^{-1}\right) = \text{Yield } \left(\text{gplant}^{-1}\right)/\text{Water Delivered } \left(\text{Lplant}^{-1}\right)$$

2.7 Membrane Injury Index and Chlorophyll Content

The membrane injury index was calculated by measuring the electrolyte expelled from the cell (Dlugokecka and Kacperska-Palacz 1978; Fan and Blake 1994); after the disks were taken from the outer or the second leaves of the stress and control plants were kept in deionized water for 4 h. The electrical conductivity (EC) was measured, then the same disks were kept at 100 °C for 10 min, the EC value of the solution was measured once more. The membrane damage index (%) in the leaf cells was determined using the following equation (Kusvuran 2010);

$$\text{Membrane Damage Index} = (\text{Lt}-\text{Lc}/1 - \text{Lc})\text{x}100$$

In the equation, Lt is the EC of the leaf at drought stress before autoclaving/EC after autoclaving, Lc is the EC before autoclaving the control leaf disk samples/the EC of control leaf disk samples after autoclaving.

Chlorophyll content of the lettuce plants was measured at the outside of two leaves. The measurements were carried out between 10:00 and 11:00 a.m. using a SPAD meter (Minolta 502).

2.8 Statistical Analysis

Multivariate statistical analyses were performed to evaluate the effects of Ag and stress levels on lettuce using two-way analysis of variance by Jump-13®. The mean and interactive effects of Ag nanoparticle and stress on lettuce were separated by the least significant difference (LSD) test at $p < 0.05$, unless otherwise mentioned. Perform regression and correlation analyses to find relationships between independent and dependent variables.

3 Results and Discussion

3.1 Antioxidant Enzyme Activities

It was obtained a decrease in GST activity in plants treated with 25 (4.84 nmol min^{-1} mg^{-1} protein), 50 (4.56 nmol min^{-1} mg^{-1} protein) and 75 mg L^{-1} (5.58 nmol min^{-1} mg^{-1} protein) of AgNPs, but treatment with 100 mg L^{-1} (7.74 nmol min^{-1} mg^{-1} protein) increased the GST activity, compared with control group (6.56 nmol min^{-1} mg^{-1} protein) (Table 1). GST activity (12.12 nmol min^{-1} mg^{-1} protein) in plants was significantly enhanced with treatment of drought stress. Treatments with 25 (4.03 nmol min^{-1} mg^{-1} protein), 50 (4.82 nmol min^{-1} mg^{-1} protein) and 75 mg L^{-1} (4.73 nmol min^{-1} mg^{-1} protein) of AgNPs under drought stress conditions decreased the GST activity. While GST activity (7.52 nmol min^{-1} mg^{-1} protein) in 100 mg L^{-1} AgNPs under drought conditions was slightly enhanced according to the control plants, it was significantly reduced, compared to the stressed plants (Table 1). Different doses of AgNPs and drought treatments indicated significant differences in GST enzyme activity. AgNPs applications, both alone and in combination with drought, generally inhibited the GST enzyme activity, except for 100 mg L^{-1}.

GST enzymes are stimulated by various abiotic stresses such as heavy metals (Kumar et al. 2013; Yasur and Rani 2013) and drought (Yang et al. 2014) in some plants (Ji et al. 2010). It was reported that GST enzyme activity was enhanced in rice plants exposed to metal (Cr VI) and drought stress (Seppänen et al. 2000). Yılmaz and işcan (2014) obtained an increase in *Pinus brutia* Ten, in drought stress conditions, about twofold of unstressed ones. In another study, it was reported that AgNPs treatments in various doses (ent study underlines the effect of silver metal nanoparticles (at 0, 25, 50, 100, 200 and 400 mg L^{-1}) induced the antioxidant enzyme activities in *Brassica junce* and the maximum antioxidant enzyme activities were found in the highest dose of AgNPs (400 mg L^{-1}) (Sharma et al. 2012a).

It was found that a decrease GR activity in plants treated with 50 (0.29 nmol min^{-1} mg^{-1} protein) and 75 mg L^{-1} (0.42 nmol min^{-1} mg^{-1} protein) of AgNPs, but treatment with 100 mg L^{-1} (0.80 nmol min^{-1} mg^{-1} protein) increased the GR activity, compared with control group (0.74 nmol min^{-1} mg^{-1} protein) (Table 1).The change of GR activity (0.77 nmol min^{-1} mg^{-1} protein) was unimportant as statistically in stressed plants. Increasing rates of AgNPs applications under stressed conditions enhanced the GR activities, 0.94, 1.08 and 1.39 nmol min^{-1} mg^{-1} protein, respectively, but reduced in 100 mg L^{-1} AgNPS +

Table 1 Effect of different doses of AgNPs, in normal and under stress conditions, on GST, GR and CaE enzyme activities (nmol min^{-1} mg^{-1} protein), phenolic and flavonoid (mg g^{-1})

Treatments	GST	GR	CaE	Total phenolic (mg g^{-1})	Total flavonoid (mg g^{-1})
Control	6.56^{bc}	0.74^{c}	13.66^{de}	$28.14^{b–d}$	6.23^{de}
AgNPs 25 mg L^{-1}	4.84^{d}	0.74^{c}	9.14^{e}	39.89^{a}	7.59^{ac}
AgNPs 50 mg L^{-1}	4.56^{d}	0.29^{d}	9.66^{de}	26.43^{cd}	8.40^{ab}
AgNPs 75 mg L^{-1}	5.58^{cd}	0.42^{d}	15.47^{de}	20.35^{de}	5.91^{e}
AgNPs 100 mg L^{-1}	7.74^{b}	0.80^{c}	17.61^{d}	25.14^{cd}	$7.86^{a–c}$
Stressed plants	12.12^{a}	0.77^{c}	40.99^{b}	24.29^{cd}	5.93^{e}
AgNPs 25 mg L^{-1} + stress	4.03^{d}	0.94^{b}	11.13^{de}	30.39^{bc}	$6.93^{c–e}$
AgNPs 50 mg L^{-1} + stress	4.82^{d}	1.08^{b}	15.66^{de}	34.89^{ab}	8.71^{a}
AgNPs 75 mg L^{-1} + stress	4.73^{d}	1.39^{a}	30.16^{c}	24.47^{cd}	8.47^{ab}
AgNPs 100 mg L^{-1} + stress	7.52^{b}	0.37^{d}	63.73^{a}	14.21^{e}	$7.36^{b–d}$
LSD $P < 0.05$	1.68	0.13	8.24	8.00	1.31
Dose	**	**	**	**	**
Stress	**	**	**	**	**
Dose x Stress	**	**	**	**	**

*, **The difference is significant at $p < 0.05$, at $p < 0.01$; *ns* not significant

Stress (0.37 mg L^{-1}), about 50%, compared to the stressed and untreatment (control) plants (Table 1).

Ratnayaka et al. (2003) reported that GR enzyme activity was enhanced cotton plants grown under the mild drought conditions. Labudda and Azam (2014) found that GR activity was significantly increased in *Populus przewalskii* Maximowicz exposed to drought stress. While GR activity was enhanced in some plants subjected to drought stress, such as rice (Srivalli et al. 2003), wheat (Chen et al. 2004), cucumber (Liu et al. 2009), *Lotus japonicus* (Signorelli et al. 2013) and *Vigna radiata* (Nahar et al. 2015), high doses of AgNPs significantly reduced the antioxidant enzyme activities (Hatami and Ghorbanpour 2014).

It was obtained a decrease CaE activity in plants treated with 25 (9.14 nmol min^{-1} mg^{-1} protein) and 50 mg L^{-1} (9.66 nmol min^{-1} mg^{-1} protein) of AgNPs, but treatment with 75 (15.47 nmol min^{-1} mg^{-1} protein) and 100 mg L^{-1} (17.61 nmol min^{-1} mg^{-1} protein) increased the CaE activity, compared with control group (13.66 mg L^{-1}) (Table 1). Exposure to drought stress significantly enhanced the CaE activity (40.99 mg L^{-1}), compared to the control plants (13.66 mg L^{-1}). While AgNPs treatments under stress conditions decreased the CaE activity in 25, 50 and 75 mg L^{-1}, treatment of 100 mg L^{-1}- AgNPs + stress had the highest rate of CaE activity (63.73 mg L^{-1}), compared to both stressed and untreated (control) plants (Table 1).

Although the functions of CaE enzymes in insects, fungi, microorganisms and mammals have been studied in detail, little is known about the functions of these enzymes in plants. Cao et al. (2019) reported that CaE enzymes role in plant defense system and metabolic processes and these enzymes activated signal molecules such as methyl salicylate and methyl jasmonate. Our present study showed that CaE enzyme activity was enhanced in the lettuce plants exposed to moderate drought stress. CaE activity reached maximum rate in treatment of 100 mg L^{-1} AgNPs + stress.

3.2 Total Phenolic Content

Phenolics are the largest group of secondary metabolites that act as free radical scavengers. Accumulation of phenolic compounds in various plants provides them primary antioxidant protection (Kumaran and Karunakaran 2007). The contents of total phenolic compounds are given in Table 1. The results showed that total phenolic content showed a decrease in the treatments of all doses of AgNPS, except for treatment with 25 mg L^{-1} AgNPs. While it was obtained the highest decrease in the content of total phenolic in plants exposed to AgNPs 75 mg L^{-1}, about 1.38-fold, the highest increase in the total phenolic content was found in plants treated with 25 mg L^{-1} AgNPs (39.89 mg g^{-1}), about 1.42-fold of control (28.14 mg g^{-1}).

Application of AgNPs to plants under drought conditions showed different effects. While the applications of AgNPs 50 mg L^{-1} + stress (34.89 mg g^{-1}) and AgNPs 25 mg L^{-1} + stress (30.39 mg g^{-1}) enhanced the total phenolic content, AgNPs 75 mg L^{-1} + stress and AgNPs 100 mg L^{-1} + stress decreased the total phenolic content, compared to the plants under drought stress but untreated

AgNPs. It was found the highest decrease in the content of total phenolic in plant exposed to AgNPs 100 mg L^{-1} + stress (14.21 mg g^{-1}), about 1.98-fold of control (Table 1).

The previous researches showed that the drought stress caused an increase in the total phenolic in lettuce plants. Homaee and Ehsanpour (2015) reported that AgNPs applications (2, 10 and 20 mg L^{-1}) increased significantly the total phenolic content in the *Solanum tuberosum* cv., in *in-vitro* conditions and Yasur and Rani (2013) found that phenolic content in castor seedlings was enhanced by AgNPs applications (500, 1000 and 4000 mg L^{-1}), compared with control.

3.3 Total Flavonoid Content

Flavonoids are the biggest group of phenolic compounds. The total flavonoid contents are given in Table 1. The results showed that total flavonoid content showed an increase in the treatments of all doses of AgNPs, except for treatment with 75 mg L^{-1} AgNPs. While it was found the highest increase in the content of total phenolic in plants exposed to AgNPs 50 mg L^{-1} (8.40 mg g^{-1}), about 1.35-fold, the highest decrease was obtained in the total phenolic content in plants treated with 75 mg L^{-1} AgNPs (5.91 mg g^{-1}), about 1.05-fold of control (6.23 mg g^{-1}) (Table 1).

Application of AgNPs to plants under drought conditions showed different effects on *L. sativa*. Applications of all doses of AgNPs to *L. sativa* under drought conditions enhanced total flavonoid content. While the highest content of total flavonoid was found the application of AgNPs 50 mg L^{-1} + stress (8.71 mg g^{-1}), it was found the lowest content of flavonoid in application of AgNPs 100 mg L^{-1} + stress (7.36 mg g^{-1}), plants under drought stress but untreated AgNPs (5.93 mg g^{-1}) (Table 1).

Changes in the flavonoid content of plants in response to AgNPs applications have been previously reported. Krishnaraj et al. (2012) indicated an increase in total phenolics of *B. monnieri* seedlings with the AgNPs applications. In general, changes in the production of secondary metabolites in plants are associated with oxidative stress resulting from the production of excessive ROS following the exposure to nanoparticles. The ROS can also serve as a signal for other messengers such as secondary modulating salicylic acid (Baxter et al. 2014), jasmonic acid (Wu and Ge 2004) and ethylene (Zhang et al. 2016). Several antioxidant defense mechanisms are activated in plant cells to avoid direct or indirect harmful effects of ROS. Treatment with AgNPs reported increasing the production of phenolics which may act as antioxidants to scavenge ROS (Franklin et al. 2009; Comotto et al. 2014). It was reported that the application of AgNPs induced phenolic and flavonoid production and increased the antioxidant enzymes in *Arabidopsis thaliana*, *spirodela polyrhiza* (Thwala et al. 2013), *pisum sativum* (Tripathi et al. 2017) and *brassica juncea* (Sharma et al. 2012b).

3.4 Growth Parameters

The leaf width and plant height were significantly affected by all doses of AgNPs and drought stress. Leaf width and plant height were decreased in increasing doses of AgNPs. Both leaf width and plant height had the lowest rates in applications of 100 mg L^{-1}AgNPs, respectively, 9.28 and 16.03 cm, compared to control plants (Table 2). The effect of Ag + applications in reducing photosynthesis was higher compared to AgNPs applications, and this effect was widely reported under stress conditions (Winkel 2002; Solfanelli et al. 2006, Ushahra et al. 2014). AgNPs had a significant effect on photosynthetic parts of plants (Cui et al. 2012; Oukarroum et al. 2014). It was reported that AgNPs applications to some plants reduced the total yield (Kumari et al. 2009; Lee et al. 2012; Levardet al. 2012; Qian et al. 2013).

3.5 Plant–Water Relations

The relative water content (RWC) of leaves was generally reduced in increasing doses of AgNPs, for both alone and stressed plants, but the highest decrease was obtained in applications of 100 mg L^{-1}AgNPs both untreated and stressed plants, respectively, 47.16 and 35.52% compared to the control plants (81.52%) (Table 3). The RWC value is one of the most effective parameters used in determining the drought tolerance of plant and indicating the balance between the transpiration rate and the water supplied to the leaf. Therefore, a high RWC value enables the plant to gain resistance to stress conditions. (Dixit et al. 2001; Okunlola et al. 2017).

No significant change in water use efficiency was observed in the applications of AgNPs and drought stress, but it was determined a slight decrease in applications of 100 mg L^{-1} AgNPs both untreated and stressed plants, respectively, 39.55 and 41.0% compared to the control plants (43.74%). The WUE values were very close to each other (Table 3).

3.6 Membrane Injury Index and Chlorophyll Content

The MMI showed that decrease up to 100 mg L^{-1}AgNPs in unstressed conditions. While it was the highest in 100 mg L^{-1} AgNPs (28.84%), it was the lowest in treated with 50 mg L^{-1} (18.71%), in unstressed conditions (24.53%). The biggest changes in MMI were obtained in

Table 2 Effect of different doses of AgNPs, in normal and under stress conditions, on leaf width, plant height and total yield of lettuce plants

Treatments	Leaf width (cm leaf^{-1})	Plant height (cm plant^{-1})	Total yield (g plant^{-1})
Control	15.44^{a}	20.67^{a}	708.55^{ab}
AgNPs 25 mg L^{-1}	13.53^{ab}	20.13^{a}	726.35^{a}
AgNPs 50 mg L^{-1}	11.6^{bd}	18.45^{ab}	724.48^{a}
AgNPs 75 mg L^{-1}	12.54^{bc}	17.72^{ab}	677.36^{c}
AgNPs 100 mg L^{-1}	11.61^{bd}	17.57^{ab}	640.66^{de}
Control + stress	11.06^{bd}	16.53^{b}	565.75^{cd}
AgNPs 25 mg L^{-1} + stress	12.75^{ac}	17.99^{ab}	582.75^{bc}
AgNPs 50 mg L^{-1} + stress	10.62^{cd}	17.89^{ab}	569.40^{c}
AgNPs 75 mg L^{-1} + stress	10.58^{cd}	16.36^{b}	557.09^{cd}
AgNPs 100 mg L^{-1} + stress	9.28^{d}	16.03^{b}	524.82^{e}
LSD $P < 0.05$	2.79	3.31	27.99
Dose	**	*	**
Stress	**	*	*
Dose x Stress	ns	ns	ns

*, **The difference is significant at $p < 0.05$, at $p < 0.01$; *ns* not significant

Table 3 Effect of different doses of AgNPs, in normal and under stress conditions, on relative water potential (RWC) of leaves, water use efficiency (WUE), membrane injury index (MII) and chlorophyll content

Treatments	RWC (%)	WUE (g L^{-1})	MII (%)	Chlorophyll content (nmol chlorophyll mg FW^{-1})
Control	81.52a	43.74c	24.53cd	33.09^{cd}
AgNPs 25 mg L^{-1}	78.99^{ab}	44.84^{a}	21.76^{df}	33.63^{c}
AgNPs 50 mg L^{-1}	75.03^{b}	44.72^{ab}	18.71^{fg}	38.62^{a}
AgNPs 75 mg L^{-1}	62.81^{c}	41.81^{e}	21.05^{eg}	31.49^{de}
AgNPs 100 mg L^{-1}	47.16^{e}	39.55^{h}	28.84^{b}	24.16^{g}
Control + stress	55.07^{d}	44.20^{bc}	24.52^{cd}	31.78^{de}
AgNPs 25 mg L^{-1} + stress	43.95^{e}	45.53^{a}	21.86^{de}	31.32^{e}
AgNPs 50 mg L^{-1} + stress	51.24^{de}	44.48^{ab}	18.40^{g}	36.37^{b}
AgNPs 75 mg L^{-1} + stress	42.85^{f}	43.52^{cd}	27.03^{bc}	26.28^{f}
AgNPs 100 mg L^{-1} + stress	35.52^{g}	41.00^{d}	35.10^{a}	30.50^{e}
LSD $P < 0.05$	3.95	0.72	3.10	1.61
Dose	**	**	**	**
Stress	**	**	**	**
Dose x Stress	ns	ns	ns	ns

*, **The difference is significant at $p < 0.05$, at $p < 0.01$; *ns* not significant

plants grown drought stress conditions alone. MMI was the lowest in plants exposed to 50 mg L^{-1}AgNPs + drought stress (18.40%), but it was the highest in application of AgNPs 100 mg L^{-1} + drought stress (35.10%), followed AgNPs 100 mg L^{-1} + drought stress (27.03%). It was reported that plants exposed to the AgNPs face the water imbalances, cell damage, and decrease the photosynthesis (Kumari et al. 2009; Qian et al. 2013).

The chlorophyll content of lettuce plants exposed to AgNPs ranged from 24.1 to 38.62 nmol chlorophyll mg FW^{-1} according to the SPAD analysis. While the highest rate of chlorophyll was obtained in treatment of AgNPs 50 mg L^{-1} (38.62 nmol chlorophyll mg FW^{-1}), it was the lowest in AgNPs 100 mg L^{-1} (24.16 nmol chlorophyll mg FW^{-1}) when compared with control (33.09 nmol chlorophyll mg FW^{-1}). In plants exposed to the AgNPs under drought stress, while the chlorophyll content enhanced in treatment of AgNPs 50 + stress (36.37 nmol chlorophyll mg FW^{-1}), it was decreased in AgNPs 75 + stress (26.28 nmol chlorophyll mg FW^{-1}) compared to the control plants in drought stress (31.78 nmol chlorophyll mg FW^{-1}) (Table 3). The chlorophyll content in AgNPs

25 mg L^{-1} treatment under stress conditions was close (31.32 nmol chlorophyll mg FW^{-1}), under drought stress alone.

In previous studies, it was showed the decrease in chlorophyll content in *Arabidopsis thaliana* (Qian et al. 2013) *Lycopersicon esculentum* (Song et al. 2013), *Oryza sativa* (Nair and Chung 2014) and *Vigna radiata* (Nair and Chung 2015) grown in drought stress.

4 Conclusions

The results revealed that AgNPs application, both alone and in combination with the drought stress, had different effects on the responses of the lettuce plant. Although GST and GR enzyme activities were increased in drought stress, AgNPs treatments (except for 100 mg L^{-1} AgNPs) were reduced both enzyme activities. CaE activity was enhanced in drought stress and AgNPs treatments, but CaE activity was reached the maximum level in 100 mg L^{-1} AgNPs + stress. Total phenolic and flavonoid contents increased in AgNPs treatments. While AgNPs treatments at high dose increased MII rates, it was reduced RWC, leaf width, plant height and total yield.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest related to this article.

Ethical Approval This study is the authors' own original work, which has not been previously published elsewhere, not currently being considered for publication elsewhere and all authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content. The paper reflects the authors' own research and analysis in a truthful and complete manner.

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