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Full Length Article

Influence of Silver Nano-particles on the Salt Resistance of Tomato (*Solanum lycopersicum*) during Germination

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Abstract

Silver nanoparticles (AgNPs) have been implicated to enhance seed germination and plant growth, improve photosynthetic quantum efficiency and act as antimicrobial agents to manage plant diseases. The role of nanoparticles in the improvement of plant tolerance to environmental stresses such as drought and salinity remains unclear. In this study, we examined the effects of AgNP dose on the salt tolerance of tomato (*Solanum lycopersicum* L.) plants during germination. Tomato seeds were treated with different AgNP doses and germinated under salinity stress. Five concentrations of AgNPs (0.05, 0.5, 1.5, 2 and 2.5 mg L^{-1}) and two levels of NaCl (150 and 100 mM) were tested. Seed germination and seedling growth of tomato plants were markedly inhibited by salt stress, and this effect was alleviated by exposure to AgNPs. The germination percentage, germination rate, root length and seedling fresh and dry weight of tomato were improved after exposure to AgNPs under NaCl stress. The expression of salt stress genes was investigated by semi-quantitative RT-PCR. Of the examined salt stress genes, four genes, AREB, MAPK2, P5CS and CRK1, were up-regulated by AgNPs under salt stress, and three genes, TAS14, DDF2 and ZFHD1, were down-regulated in response to AgNPs. The gene expression patterns associated with AgNP exposure also suggest the potential involvement of AgNPs in response to stress, indicating that they might be useful for improving plant tolerance to salinity.

Keywords: *Solanum lycopersicum* L; Silver nanoparticles; Seed germination; Seedling growth; Salinity; Stress genes
Abbreviations; ABA: abscisic acid, AgNPs: silver nanoparticles, AGP-S1: ADP-glucose pyrophosphorylase large subunit, AIM1: abscisic acid-induced MYB transcription factor, APX2: cytosolic ascorbate peroxidase 2, AREB: abscisic acid response element-binding protein, CAC: clathrin adapter complex, CRK1: cysteine-rich receptor-like protein kinase 42-like, DDF2: dwarf and delayed flowering 2, LOX1: linoleate 9S-lipoxygenase B, MAPK2: mitogen-activated protein kinase 2, NaCl: sodium chloride, NCED3: 9-cis-epoxycarotenoid dioxygenase, NHX6: Na^+/H^+ antiporter 6, N-Si: nano-silicon, P5CS: delta-1-pyrroline-5-carboxylate synthetase, RBOH1: respiratory burst oxidase, RT-PCR: reverse transcription polymerase chain reaction, SGN: Sol Genomics Network, SOS2: salt overly sensitive 2, TAS14: abscisic acid and environmental stress-inducible protein, ZFHD1: zinc finger homeodomain transcription factor

Introduction

Salinity stress is a menace to agriculture and a major environmental factor that affects crops. The increased salinization of arable land is expected to have global effects on crop plant production (Massoud, 1977). Therefore, increasing the tolerance of crop plants to salinity stress must take priority in agricultural studies. In plants, various genes are responsible for salinity resistance and are involved in the salt tolerance process. These genes can limit the rate of salt uptake from the soil and the transport of salt throughout plants, adjusting the ionic and osmotic balance of cells in plant bodies (Munns, 1993). Germination is important for determining the final plant density if planted seeds completely and vigorously germinate (Baalbaki *et al.*, 1990). Interactions between environmental factors and the internal mechanisms of seeds control the ability of plant to germinate successfully (Ni and Bradford, 1992). The first phase of the growth response results from the effects of salt outside the plants. Salt in soil solutions reduce leaf growth and, to a lesser extent, root growth as well (Munns, 1993; Farooq *et al*., 2015). Molecular control mechanisms for salt stress tolerance depend on the regulation of the expression of certain stress genes (Wang *et al.*, 2003).

AgNPs are currently the most produced engineered nanomaterials found in a wide range of commercial products (Davies, 2009). AgNPs have been implicated in agriculture for improving crops. There are many reports indicating that appropriate concentrations of AgNPs play an important role in enhancing seed germination (Barrena *et*

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al., 2009; Shelar and Chavan, 2015), plant growth (Salama, 2012; Sharma *et al.*, 2012; Kaveh *et al*., 2013; Vannini *et al*., 2013) and improving photosynthetic quantum efficiency and chlorophyll content (Sharma *et al.*, 2012). AgNPs are also used as antimicrobial agents to manage plant diseases (Lamsal *et al.*, 2011).

Application of AgNPs has been found quite effective in improving resistance against salinity during germination of *Foeniculum vulgare* Mill. (Ekhtiyari *et al.*, 2011) and *Cuminum cyminum* L. (Ekhtiyari and Moraghebi, 2011). Nano-silicon (N-Si) is a nanoparticle capable of curing the negative effects of salt stress (Haghighi *et al.*, 2012; Kalteh *et al.*, 2014). Likewise, nano zinc oxide also helps in improving salt resistance (Sedghi *et al.*, 2013).

In response to stress, plants activate a number of defense mechanisms that function to increase tolerance to adverse conditions. Furthermore, a large array of genes is activated by stress conditions; thus, a number of proteins are produced to combine in pathways leading to the synergistic enhancement of stress tolerance (Wang *et al.*, 2003). Salinity resistance genes in tomato were identified in many studies and published in the Sol Genomics Network (SGN) database (SGN, 2015). Some of these genes encode transcription factors, which regulate many genes in response to stress, such as zinc finger homeodomain transcription factor family (ZFHD1) (Aoki *et al.*, 2010) and abscisic acid response element-binding protein (AREB) (Orellana *et al.*, 2010; Bastías *et al.*, 2014). Osmolytes are among the genes induced under salinity stress. These genes play an important role in osmotic adjustment under high salinity conditions and include delta-1-pyrroline-5-carboxylate synthetase (P5CS) (Hong *et al.*, 2000) and abscisic acid and environmental stress-inducible protein (TAS14) (Muñoz-Mayor *et al.*, 2012). Genes responsible for controlling K^+ or Na^+ uptake from soil and transport within plants have been identified in transgenic tomato under salinity as ion channels and transporters, such as salt overly sensitive 2 (SOS2) (Olías *et al.*, 2009; Candar-Çakir *et al.*, 2014) and NA^+/H^+ antiporter 6 (NHX6) (Zhang and Blumwald, 2001). The antioxidative defense system in plants under salt stress is related to antioxidative enzymes such as cytosolic ascorbate peroxidase 2 (APX2), which is expressed in tomato under salinity (Zou *et al.*, 2005). Signaling pathways in response to salt stress in tomato include many signaling molecules such as mitogen-activated protein kinase 2 (MAPK2) and MAPK3 (Stulemeijer *et al.*, 2007). Some genes involved in energy metabolism are expressed in tomato under salt stress, including ADP-glucose pyrophosphorylase large subunit (AGP-S1) (Park and Chung, 1998; Yin *et al.*, 2010) and respiratory burst oxidase (RBOH1), which is NADPH oxidase (Zhou *et al.*, 2014).

Although potential of AgNPs in improving salinity resistance has been reported in several plant species (Ekhtiyari *et al.*, 2011; Ekhtiyari and Moraghebi, 2011), its role in alleviation of salinity effect and related mechanisms is still unknown. This study was conducted to reveal the molecular mechanisms that mediate salinity tolerance in tomato.

Materials and Methods

Seed Germination Experiment

To evaluate the effects of AgNPs on the germination traits of tomato seeds under salinity stress, a factorial experiment was conducted based in a completely randomized design with three replications. The seed germination of tomato plants under salt stress was tested in response to AgNPs by planting seeds in the presence of increasing concentrations of AgNPs (0.05, 0.5, 1.5, 2 and 2.5 mg L^{-1}) and two concentrations of NaCl (150 and 200 mM). AgNPs (silver nanopowder, 99.99%, 20 nM) were purchased from U.S. Research Nanomaterials (Houston, TX, USA).

Seeds were immersed in a 5% sodium hypochlorite solution for 10 min to ensure surface sterility (USEPA, 1996), they were then soaked in distilled water for 2 h, and then soaked in serial prepared AgNP concentration suspensions for approximately 2 h after being rinsed four times with distilled water. One piece of filter paper was put into each 100 mm × 15 mm Petri dish, and 5 mL of a test solution was added. Seeds were transferred onto filter paper with 30 seeds per dish and a 3 mm or larger distance between each seed. AgNPs and NaCl serial concentrations were added into Petri dishes in which 5 mL of each solution was added to each Petri dish. Pure water was used for control treatment. Petri dishes were covered, sealed with tape, and incubated at room temperature. Germination seeds were counted based on 2 mm radical emergence at 24 h after planting. Counting was continued until the number of germinated seeds was constant in the last three days of the experiment. Germination was halted after 8 days. In each plate, 10 seedlings were randomly selected to measure dry and fresh weight. The seed germination rate and mean germination time were calculated, and the seedling dry and fresh weight and root length were measured.

Seed Germination

Final germination percentage was calculated based on total number of germinated seeds at the end of the experiment. Measurements were performed according to the International Rules for Seed Testing (ISTA, 1996). Germination indices were calculated using the following equations (Ellis and Roberts, 1981; Alvarado *et al.*, 1987; Ruan *et al.*, 2002):

$$\text{Germination percentage (GP \%)} = (Gf/n) \times 100$$

Where Gf is the total number of germinated seeds at the end of the experiment, and n is the total number of seeds used in the test.

$$\text{Mean germination time (MGT)} = \Sigma\, NiDi/n$$

Where Ni is number of germinated seeds until the ith day, Di is the number of days from the start of the experiment until the ith counting and n is total germinated seeds.

Germination rate (GR) = Σ Ni/ΣTiNi

Where Ni is the number of newly germinated seeds at the time Ti.

= (a/1)+(b-a/2)+(c-b/3)+...+(n-n-1/N)

Semi-quantitative RT-PCR Analysis

Gene expression for salinity stress genes was examined using semi-quantitative RT-PCR. Genes were selected based on previous studies of tomato genes that were induced with salinity stress and involved in salt tolerance. ESTs related to tomato genes expressed in salinity stress, which are found in the SGN database (SGN, 2015), were used to design gene-specific primers.

Total RNA was extracted using the TRIzol reagent (Sigma, USA) from tomato seedlings, and then it was reverse transcribed into cDNA using the RT-PCR Superscript™ II reverse-transcriptase kit (Invitrogen, USA) with Oligo(dT) primers. The gene-specific primers were designed from EST sequences available in the SGN database (Table 1). PCR was used to amplify cDNAs for each of the investigated tomato genes using combinations of forward and reverse primers with synthesized cDNA as a template. Five templates were tested for expression analysis of each salt stress gene; the treatment combinations NaCl150 AgNPs1.5 and NaCl200 AgNPs2 with their controls: NaCl150 and NaCl200, in addition to non-treated controls. The PCR reaction volume was 20 µL, which contained 4 µL 5X PCR ready mix containing Taq polymerase (Solis BioDyne, Estonia), 0.4 µM of each primer, 3.5 µL cDNA and sterile distilled water was used to obtain a final volume of 20 µL. Amplifications were performed under the following conditions: an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at temperatures ranging from 52°C to 58°C (varied with every primer pair, Table 1), and an extension at 72°C for 1:30 min. A final extension at 72°C for 7 min was performed after all cycles were complete. The constitutively expressed gene clathrin adapter complex (CAC) was used as a positive control (Expósito-Rodríguez *et al.*, 2008). The PCR products were separated in a 1.2% agarose gel (Sigma, USA). Electrophoresis was then performed for 30 min at 100 volts in tris/borate ethylenediaminetetraacetic acid (EDTA) buffer (TBE) (Sigma, USA), and the gel was stained with 3 µL of 10 mg mL^{-1} ethidium bromide (Sigma, USA) in 120 mL of TBA buffer. The bands stained in the gel were visualized and documented using a gel documentation system (Bio-Rad Laboratories, USA) with UV-light and Gel Doc 2000 Quantity One software. Expression analysis of each gene was performed at least three times as independent PCR reactions and run in an electrophoresis gel, and one of the images was presented as representative data for each gene. The PCR products in the gel were quantified by measuring band intensity using ImageJ software, which generated a peak diagram for each band from which the area under each peak could be determined, representing the intensity of the bands in the gel.

Statistical Analysis

The means and standard deviations were derived from three repeated samples for each treatment and related control for germination and seedling growth measurements and three independent PCR reactions for gene expression analysis. Data obtained from the various treatments were statistically analyzed using the *t*-test at the 0.01 and 0.05 levels.

Results

The Effect of AgNPs on Seed Germination and Seedling Growth of Tomato under Salt Stress

In this study, we examined the role of AgNPs in enhancing tomato tolerance for salinity and highlight molecular mechanisms that mediate salinity tolerance in tomato by investigating the expression profiles of salt tolerance genes at the germination stage. Germination percentage, germination rate and main germination time for NaCl treatment (150 and 200 mM) and AgNP treatments were significantly different compared with non-treated plants (Table 2). A slight decrease in germination percentage was observed for all NaCl and AgNP treatments compared with non-treated plants, particularly under NaCl200 stress. The germination rate was significantly depressed under salt stress, and it was reduced by 29% and 43.3% compared with the non-treated plants under NaCl150 or NaCl200 stress, respectively. The mean germination time was positively affected with all NaCl and AgNP treatments, and it decreased by 6% and 9.3% compared with the non-treated plants for NaCl150 and NaCl200, respectively which means that there was earlier germination compared with non-treated plants. However, the addition of certain AgNP concentrations significantly alleviated the inhibitory effects of NaCl stress. The germination percentage increased from 4.1 to 5.26% with AgNP treatment compared with that of the NaCl150 control, and the best germination percentage value (100%) was observed with exposure to 0.5 mg/L AgNPs under NaCl150 stress. The improvement in the germination percentage with NaCl200 stress was from 1.2% to 4.73% with AgNP treatments of 0.5, 1.5 and 2 mg/L, whereas exposure to 0.05 and 2.5 mg/L AgNPs inhibited the germination percentage compared with the NaCl200 control. The germination rate improved at all AgNP concentrations compared with that of the NaCl150 and NaCl200 controls. The germination rate value at 2.5 mg/L AgNP under

Table 1: Primer combinations used for semi-quantitative RT-PCR analysis for fifteen tomato salt stress genes in addition to the internal control gene CAC

Gene	Forward primers sequences	Reverse primers sequences	EST no. in SGN	Annealing Temp.	Product size (bp)
RBOH1	CGGAGTCGGTATTGGTGGAG	AAGCCGAGTCTACACCGTTG	Solyc08g081690.2.1	58°C	284
AREB	ACAGGAGGGAGTGGTAAGGA	AGTCAAAGAGCCTTGCCTCT	Solyc04g078840.2.1	56°C	159
AGP-S1	ACACTCCCCTTTGTCCACTT	ACTCTCCCCAAATGAACCGT	Solyc01g109790.2.1	54°C	237
AIM1	CGGTGAAGGTCGTTGGAATT	TTGACCACCTATTGCCCCAA	Solyc12g099120.1.1	58°C	176
TAS14	CGTCGGAGGATGATGGAGAA	GTGTTCAATGCATCCCAGGG	Solyc02g084850.2.1	58°C	168
APX2	CCACTTGAGGGACGTGTTTG	CCCTTCCTTTTCCCCACTCA	Solyc06g005150.2.1	56°C	187
LOX1	TGTCTTTGGGTGGAATTGTGG	GGATTGCTCAGTTTCCCTTCC	Solyc01g099190.2.1	56°C	217
MAPK2	ACCACCTCAACGAGAAGCAT	TTGCTAGGCTTCAAGTCCCT	Solyc08g014420.2.1	58°C	192
SOS2	GGGTTGGGAAGAATTGGCGA	AGGCCCAGCAAGTAAAAGCA	Solyc01g005020.2.1	54°C	162
DDF2	GCCGAAATCTTCCGACCTTT	AGCTTCCACATGATCTCCCA	Solyc03g026270.1.1	52°C	192
ZFHD1	GGTCATGCTGTTGATGGGTG	AATAGGTGGCGGTGGAATCA	Solyc02g067310.2.1	52°C	153
NHX6	ACAAACCGCAGAAAAGCCTT	CCATGGCCGATTCTTCCAAG	Solyc04g056600.2.1	54°C	158
NCED3	GTATGGTTCACGCCGTTCAA	GCCTTGCAATTCCAGAGTGA	Solyc07g056570.1.1	58°C	153
P5CS	TCTTTACAGTGGTCCTCCCC	TATACGTTCCCCATGCAGCA	Solyc08g043170.2.1	52°C	229
CRK1	TGTATTCCTGCTCCTGGTGG	CTCCTGCAGCAAAATCCCTC	Solyc03g112730.2.1	58°C	169
CAC	CCTCCGTTGTGATGTAACTGG	ATTGGTGGAAAGTAACATCATCG	Solyc08g006960.2.1	56°C	173

Table 2: Influence of AgNP concentrations on germination percentage (GP), germination rate (GR) and mean germination time (MGT) for tomato plants at three levels of salinity. The values are M (means) ±SD (standard deviations) of three replicates. (^{b}) Indicates significant effects at a 0.05 probability level, and (^{a}) indicates significant effects at a 0.01 probability level, related to the NaCl150 or NaCl200 salinity treatments. (^{d}) Indicates significant effects at a 0.05 probability level, and (^{c}) indicates significant effects at a 0.01 probability level, related to the non-treated control. Blank cells indicates to treatments that not implemented. mg = milligram, L = liter, % = percent and mM = millimolar

AgNP (mg L^{-1})	Salt stress (mM)		
	0	150	200
	Root length (cm)		
Control	11.64±1.11	4.56^{c}±0.71	2.03^{c}±0.56
0.05		5.66^{bc}±0.92	3.71^{ac}±0.47
0.50		5.23^{c}±0.77	3.67^{ac}±0.42
1.50		7.21^{ac}±1.29	3.93^{ac}±0.73
2.00		5.53^{bc}±0.69	3.99^{ac}±0.57
2.50		3.17^{ac}±0.86	3.17^{ac}±0.86
	Seedling fresh weight (g)		
Control	0.20±0.02	0.112^{d}±0.056	0.104^{c}±0.022
0.05		0.079±0.099	0.046^{bc}±0.059
0.50		0.159^{b}±0.049	0.159^{b}±0.054
1.50		0.177^{a}±0.021	0.124^{c}±0.017
2.00		0.121^{c}±0.008	0.093^{c}±0.035
2.50		0.131^{c}±0.016	0.095^{c}±0.010
	Seedling dry weight (g)		
Control	0.119±0.0001	0.014^{d}±0.007	0.012^{d}±0.120
0.05		0.011^{d}±0.074	0.013^{d}±0.019
0.50		0.053^{b}±0.009	0.053^{b}±0.070
1.50		0.083^{a}±0.001	0.024^{d}±0.001
2.00		0.013^{d}±0.121	0.012^{d}±0.0004
2.50		0.013^{d}±0.001	0.012^{d}±0.001

NaCl150 stress (14.11 seed/day) was the best, and it was similar to the non-treated plant value (14.56 seed/day). There was significant improvement in the mean germination time recorded with 2.5 mg/L AgNPs under NaCl150 stress and at 1.5, 2 and 2.5 mg/L AgNPs with NaCl200 stress compared with controls.

The root length and fresh and dry weights of tomato seedlings were significantly reduced under NaCl stress and AgNP treatment compared with non-treated plants (Table 3). Root length was significantly depressed under NaCl stress and reduced by 60.82% and 82.56% compared with non-treated plants under NaCl150 and NaCl200 stress, respectively (Supplementary Fig. 1 and 2). Fresh weight was reduced by 44% and 48% compared with non-treated plants under NaCl150 and NaCl200 stress, respectively. Dry weight also reduced by 88.2% and 89.9% compared with non-treated plants under NaCl150 and NaCl200 stress, respectively. However, treatment with certain AgNP concentrations significantly alleviated the inhibitory effects of NaCl stress on tomato plant root length and fresh weight values. The best root length value (7.21 cm) was observed with exposure to 1.5 mg/L AgNPs under NaCl150 stress, which was a 58.11% improvement in root length compared with the NaCl150 control. Treatment with 2.5 mg/L AgNPs under NaCl150 stress decreased the tomato root length by 30.48% compared with the NaCl150 control. Root length values at all AgNP concentrations were significantly improved from 56.15% to 96.55% under NaCl200 stress. Fresh weight significantly increased by 41.96% and 85.03% under NaCl150 at AgNP concentrations of 0.5 and 1.5 mg/L, respectively, compared with NaCl150 control. Under NaCl200 stress, the fresh weight of tomato plants significantly increased by 52.88% for 0.5 mg/L AgNP treatment. Dry weight significantly increased with AgNP concentrations of 0.5 and 1.5 mg/L under NaCl150 stress compared with the NaCl150 control. Under NaCl200 stress, the dry weight for tomato plants significantly increased with 0.5 mg/L AgNPs compared with the NaCl200 control.

Table 3: Influence of AgNP concentrations on root length (RL), seedling fresh (FW) and dry weight (DW) for tomato plants at three levels of salinity. The values are M (means) ±SD (standard deviations) of three replicates. ([b]) Indicates significant effects at a 0.05 probability level, and ([a]) indicates significant effects at a 0.01 probability level, related to the NaCl150 or NaCl200 salinity treatments. ([d]) Indicates significant effects at a 0.05 probability level, and ([c]) indicates significant effects at a 0.01 probability level, related to the non-treated control. Blank cells indicates to treatments that not implemented. mg = milligram, L = liter, cm = centimeter, % = percent and mM = millimolar

AgNP (mg L^{-1})	Salt stress (mM)		
	0	150	200
	Germination percentage		
Control	100.0±1.50	95.0^{d}±1.00	94.42^{d}±1.00
0.05		98.89^{b}±1.92	92.22±1.92
0.50		100.0^{a}±2.00	98.00^{b}±1.00
1.50		98.89^{b}±1.92	95.56±1.92
2.00		98.89^{b}±1.92	98.89^{b}±1.00
2.50		99.67^{a}±1.53	91.11^{b}±1.92
	Germination rate (seed/days)		
Control	14.557±0.237	10.34^{d}±1.51	8.25^{c}±0.200
0.05		11.78^{bd}±1.21	8.70^{c}±0.188
0.50		11.36^{bc}±0.587	10.24^{bc}±0.691
1.50		11.30^{bc}±0.825	9.29^{bc}±0.225
2.00		10.97^{c}±0.128	10.34^{bc}±0.273
2.50		14.11^{ac}±1.01	9.03^{c}±0.552
	Mean germination time (days)		
Control	31.777±0.133	29.87^{d}±0.964	28.813^{c}±0.650
0.05		29.89±1.40	27.677^{c}±0.517
0.50		29.30^{c}±0.176	28.80^{c}±1.07
1.50		29.95^{d}±1.10	26.980^{bc}±0.201
2.00		29.92^{c}±0.490	26.30^{bc}±0.173
2.50		28.043^{bc}±0.650	27.277^{bc}±0.401

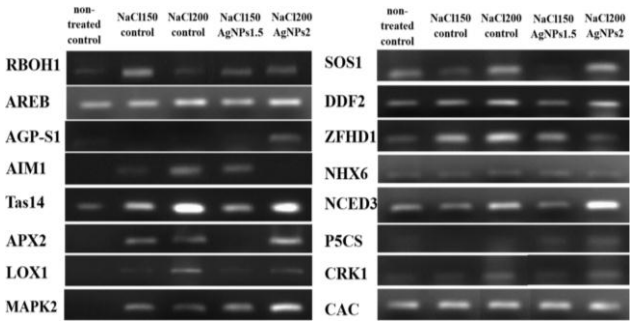

Fig. 1: Semi-quantitative RT-PCR analysis of fifteen salt stress genes expressed in tomato seedlings under two NaCl concentrations, NaCl150 and NaCl200, and two treatment combinations of NaCl and AgNPs, NaCl150 AgNPs1.5 and NaCl200 AgNPs2, in the addition to non-treated plants. CAC was used as a positive control

Expression Pattern of Tomato Salt Stress Genes in Response to AgNPs under Salt Stress

The expression of salt stress genes was investigated by semi-quantitative RT-PCR using cDNA isolated from tomato seedling tissues. Two treatment combinations, NaCl150 AgNPs1.5 and NaCl200 AgNPs2 with their respective controls: NaCl150 and NaCl200, in addition to a non-treated control, were chosen for semi-quantitative RT-PCR analysis. As shown in Fig. 1 and 2 (Supplementary Table 1), mRNA for the CAC gene, as a positive control, was almost uniformly expressed in the tested treatments and controls. Analysis of salt stress gene expression by semi-quantitative RT-PCR revealed different expression patterns in response to AgNPs as evidenced by the relative intensity of their gel bands.

The RBOH1 gene demonstrated an increase in expression with both NaCl stresses and AgNP treatments compared with non-treated control plants. After exposure to AgNPs, RBOH1 gene expression decreased by 2.09-fold with NaCl150 stress, whereas it increased by 4.57-fold with NaCl200 stress compared with NaCl150 and NaCl200 controls. The expression of the AREB gene under NaCl200 stress was higher than under NaCl150 stress and its expression increased by 2.78 and 1.07-fold after treatment with AgNPs compared with its expression in the NaCl150 and NaCl200 controls, respectively. The AGP-S1 gene was only induced with NaCl200 AgNPs2 treatment; it was 16.11-fold higher than the NaCl200 control. Abscisic acid-induced MYB transcription factor (AIM1) was not expressed in non-treated tomato plants, and it had strong expression with NaCl and AgNP treatment combinations with the exception of NaCl200 AgNPs2 treatment, which had the lowest expression level and the level was similar to the non-treated plants. However, AIM1 expression increased by 2.71-fold after exposure to AgNPs under NaCl150. The TAS14 gene was more abundantly expressed after salt stress and AgNP treatments, but its expression was 1.32 and 1.21-fold lower after exposure to AgNPs under NaCl150 and NaCl200 control treatment, respectively. APX2 expression was observed to be the lowest level in non-treated plants and with the NaCl150 AgNPs1.5 treatment, whereas its highest level was with NaCl200 AgNPs2 treatment. By contrast, its expression increased by 2.95-fold after exposure to AgNPs under NaCl200 stress. Linoleate 9S-lipoxygenase B (LOX1) was not expressed in non-treated control plants and had high expression in tomato plants with both NaCl stress and AgNP treatments. Nevertheless, the LOX1 gene had its highest expression level in the NaCl200 control, which was reduced by 1.79-fold after exposure to AgNPs, whereas NaCl150 increased its expression by 1.22-fold. MAPK2 was also not expressed in non-treated control plants and had strong expression in tomato plants with both NaCl stresses and AgNP treatments. Moreover, MAPK2 gene expression increased by 2.22 and 4.38-fold after treatment with AgNPs compared with its expression with the NaCl150 and NaCl200 controls, respectively. Surprisingly, SOS2 gene expression decreased under NaCl150 stress, whereas it increased with NaCl200, and its lowest expression level was observed with NaCl150 AgNPs1.5 treatment compared with the non-treated control.

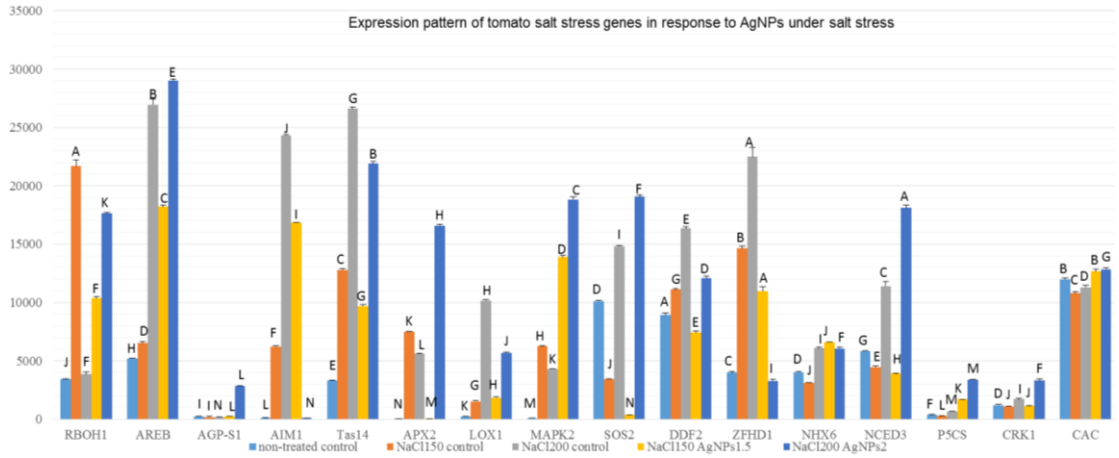

Fig 2: The relative expression level of tomato salt stress genes (related to non-treated control) under two NaCl concentrations, NaCl150 and NaCl200, and two treatment combinations, NaCl150 AgNPs1.5 and NaCl200 AgNPs2. CAC was used as a positive control. The relative intensity values shown (y axis) are an average of three replicates. The intensity of each band was measured with ImageJ software. Bars represent the mean ± SD of three independent experiments. Different letters indicate a significant difference among treatments at the 0.05 levels by *t*-test

Nevertheless, after AgNP treatment, SOS2 was expressed 1.28-fold higher than the NaCl200 control. Dwarf and delayed flowering 2 (DDF2) gene expression increased under both NaCl stress levels, whereas it decreased by 1.49 and 1.35-fold after exposure to AgNPs compared with the NaCl150 and NaCl200 controls, respectively. The ZFHD1 gene had strong expression in tomato plants with NaCl stress and AgNP treatment compared with non-treated plants with the exception of NaCl200 AgNPs2 treatment, which demonstrated the lowest expression level. Nevertheless, ZFHD1 gene expression decreased by 1.33 and 6.81-fold after exposure to AgNPs compared with the NaCl150 and NaCl200 controls, respectively. NHX6 gene expression increased in tomato plants with NaCl stress and AgNP treatments with the exception of the NaCl150 control in which it decreased compared with non-treated plants. However, NHX6 gene expression increased by 2.11-fold with NaCl150 stress after exposure to AgNPs, whereas it was not affected under NaCl200 stress after exposure to AgNPs. 9-cis-epoxycarotenoid dioxygenase (NCED3) gene expression decreased under NaCl150 stress, whereas it increased under NaCl200 stress compared with non-treated plants. After exposure to AgNPs, NCED3 gene expression decreased by 1.14-fold under NaCl150 stress, whereas it increased by 1.58-fold under NaCl200 stress compared with the NaCl150 and NaCl200 controls. P5CS gene expression increased in tomato plants with both NaCl stress and AgNP treatments with the exception of the NaCl150 control, which had the lowest expression level. After exposure to AgNPs, P5CS gene expression increased by 5.74-fold under NaCl150 stress and by 5.16-fold under NaCl200. Similar to the NCED3 and SOS2 genes, cysteine-rich receptor-like protein kinase 42-like (CRK1) gene expression in tomato plants decreased under NaCl150 stress, whereas it increased under NaCl200 stress compared with non-treated control plants. However, the CRK1 gene was expressed after exposure to AgNPs 1.03 and 1.92-fold higher compared with the NaCl150 and NaCl200 controls, respectively.

Discussion

Salinity has known inhibitory effects on water uptake, the germination of seeds and seedling root elongation (Katembe *et al.*, 1998; Debez *et al.*, 2004). Our results suggest that AgNPs play an important role in moderating the inhibition seed germination and plant growth in saline environments by inducing salt tolerance in plants. We found that exposure to AgNPs is capable of increasing of the germination percentage, the germination rate, the root length and the seedling fresh and dry weights of tomato plants under NaCl stress. The mean germination time improved under salt stress and with AgNP treatments. The best dose for AgNPs for tomato seed germination under NaCl150 salinity was 2.5 mg/L, whereas the best seedling growth values were observed at 1.5 mg/L AgNPs at the NaCl150 salinity level. Exposure to 2 mg/L AgNPs appeared to be proper to enhancing tomato seed germination and root length under NaCl200 salinity. The treatment combinations: NaCl150 AgNPs1.5 and NaCl200 AgNPs2 were chosen for molecular analysis. Previous reports have suggested that AgNPs have positive effects on seed germination, seedling growth, yield and physiology and metabolism in different plant species (Siddiqui *et al.*, 2015). These findings suggest that AgNPs may be directly or indirectly involved in morphological changes and physiological processes in plants. Thus, our findings indicate that it is plausible that AgNPs play a protective role in the germination of tomato seeds under salt stress. Similarly, the AgNPs alleviated the

inhibitory effects of salt stress and improved the germination percentage, germination speed, vigor of stems, fresh weight and root length of *cuminum cyminum* L. seed (Ekhtiyari and Moraghebi, 2011) and *Foeniculum vulgare* mill (Ekhtiyari *et al.*, 2011) at the germination stage. In addition, our findings are in agreement with Haghighi *et al.* (2012) who reported that application of N-SiO_2 increases the germination parentage of tomato under saline-stress conditions.

Of the investigated salt stress genes in tomato plants, the AIM1, AGP-S1, APX2, LOX1 and MAPK2 genes were expressed only under NaCl stress, and they were not expressed in non-treated plants. Of investigated genes, twelve genes were expressed under NaCl150 stress at a level lower than that under NaCl200 stress, after exposure to AgNPs. Only three investigated salt stress genes were expressed under NaCl150 stress greater than that under NaCl200 stress. The reduction in the expression of the P5CS gene at the NaCl150 level was similar to that for the P5CS gene from the soybean cultivar Ataem-7, which had expression levels in this cultivar under the NaCl150 stress that was the same as its expression level in control. By contrast, P5CS gene expression in another soybean cultivar, Üstün-1, was 4.44-fold higher than control (Celik and Atak, 2012).

The expression of four genes: AREB, MAPK2, P5CS and CRK1, was up-regulated by AgNPs under both salt levels, NaCl150 and NaCl200. By contrast, the TAS14, DDF2 and ZFHD1 genes were down-regulated with AgNPs under both NaCl levels. These genes, which were differentially regulated by AgNPs in our study, are involved in regulating abscisic acid (ABA) responses, which mediate the expression of a number of salt-responsive genes and in many ways act as a mediator for the whole-plant response to salt stress (Sharma *et al.*, 2005; Mahajan and Tuteja, 2005; Vinocur and Altman, 2005; Tran *et al.*, 2007; Brock *et al.*, 2010; Yaish *et al.*, 2010; Zhang *et al.*, 2013). The MAPK pathway is used by animal and human cell lines to transduce AgNPs signals (Eom and Choi, 2010). In the nematode *Caenorhabditis elegans*, the expression of MAPK increases in response to AgNPs (Lim *et al.*, 2012).

The expression of other genes i.e., AIM1, LOX1 and NHX6, is up-regulated by AgNPs under NaCl150 stress whereas they are down-regulated under NaCl200 stress. AIM1 and LOX1 pathways are involved in controlling gene expression by ABA under abiotic stresses (Ganesan *et al.*, 2012; Vicente *et al.*, 2012). LOX1 also regulates root development by controlling the emergence of lateral roots through the production of (10E, 12Z)-9-hydroperoxy-10, 12-octadecadienoate (Vellosillo *et al.*, 2007). NHX6 gene expression is regulated by SOS2 activity on the vacuolar membrane directly and indirectly (Olías *et al.*, 2009), which participates in ion homeostasis and cell expansion under normal conditions (Venema *et al.*, 2003).

The other investigated genes i.e., RBOH1, SOS2, APX2 and NCED3, were down-regulated by AgNPs under NaCl150 stress and up-regulated by AgNPs under NaCl200 stress. This expression pattern for such genes is due to their role in catalyzing the reduction of H_2O_2 to water (Zou *et al.*, 2005; Verslues *et al.*, 2007; Zhang *et al.*, 2009; Zhou *et al.*, 2014). This result was confirmed by Lei *et al.* (2008) who found that nanoparticles reduced antioxidant stress by reducing H_2O_2 and increasing enzymes such as APX, resulting in improved seed germination in some plant species. Down-regulation of the SOS2 gene by both salinity levels in our study is similar to its expression in *Brassica oleracea*, which demonstrated down-regulation of SOS2 gene expression in shoot tissue (Kumar *et al.*, 2009). NCED3 gene expression was also induced in response to three different AgNP sizes and shapes (Syu *et al.*, 2014). Plant adaptations to salinity are of three distinct types: osmotic stress tolerance, Na^+ or Cl^- exclusion, and the tolerance of tissue to accumulated Na+ or Cl− (Munns and Tester, 2008). The changes in the molecular response to AgNPs among the tested salinity levels suggest that AgNPs can alter their protective mechanisms at higher salinity levels. A recent study by Kohan-Baghkheirati and Geisler-Lee (2015) revealed that AgNPs affect the fewest genes (575) in the *Arabidopsis* genome compared with those affected by Ag+ (1,010), heat (1,374), drought (1,435), salt (4,133) and cold (6,536). Moreover, 111 genes were unique for AgNPs, and they were enriched for three biological functions: response to fungal infection, anion transport, and cell wall/plasma membrane related (Kohan-Baghkheirati and Geisler-Lee, 2015). The expression patterns of genes investigated in this study suggest that they are regulated in response to AgNPs, which represents a novel abiotic stressor for plants (Geisler-Lee *et al.*, 2013; Qian *et al.*, 2013).

Conclusion

Exposure to AgNPs alleviated the adverse effects of salt stress and improved the germination, root length and seedling fresh and dry weight of tomato seeds under NaCl stress. Four salt stress genes *AREB*, *MAPK2*, *P5CS* and *CRK1*, were up-regulated by AgNPs under salt stress, and three genes *TAS14*, *DDF2* and *ZFHD1* were down-regulated. The expression of other salt stress genes varied between the two salinity levels NaCl150 and NaCl200.

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