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Metal Nanoparticles-Mediated Changes on Gene Expressions and Physiological Parameters of *Capsicum annuum* L.

Metal Nanopartiküller Aracılığıyla, *Capsicum annuum* L.'nin Gen Ekspresyonu ve Fizyolojik Parametreleri Üzerindeki Değişiklikler

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ABSTRACT

Objective: The uptake and accumulation of nanoparticles by plants create a potential threat for human health in cases where humans consume the plants. The aim of the study was to analyze the potential beneficial or inhibitory effects of nAl_2O_3 and nZnO on *Capsicum annuum* L. (pepper)'s germination, root growth, and expression levels of aquaporin and dehydrin genes.

Material and Method: Different concentrations (0.5, 2.5, or 5.0 mM) of nAl_2O_3 and nZnO were used for the germination of pepper seeds. ICP-MS analysis was performed to determine ion contents in nanoparticle-treated pepper plants. Levels of aquaporin and dehydrin gene expressions were analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Results: The pepper germination was not affected by nanoparticle applications. While nAl_2O_3 treatments did not change root growth, higher concentrations of nZnO negatively affected root length and root number. In particular, the application of 0.5 mM nZnO significantly upregulated aquaporin and dehydrin gene expressions in roots. Downregulation of dehydrin gene expression occurred in stems and roots after exposure to nAl_2O_3 treatments.

Conclusion: The gene expression alterations and changes of growth parameters showed especially nZnO have potentially phytotoxic for pepper plants. Moreover, expression analysis suggested that the tested genes may play roles in response to the nanoparticle-based abiotic stress.

Keywords: Aquaporin, dehydrin, nanoparticles, nAl_2O_3, nZnO, germination, *Capsicum annuum* L., pepper

ÖZ

Amaç: Nanopartiküllerin bitkiler tarafından alınması ve biriktirilmesi, bu bitkilerin insanlar tarafından tüketilmesi durumunda insan sağlığı için potansiyel bir tehdit oluşturmaktadır. Bu çalışmada, nAl_2O_3 ve nZnO'nun *Capsicum annuum* L. (biber)'in çimlenmesi, kök büyümesi ile aquaporin ve dehidrin genlerinin ekspresyon seviyeleri üzerindeki potansiyel yararlı veya inhibe edici etkilerinin analiz edilmesi amaçlanmıştır.

Gereç ve Yöntem: Biber tohumlarının çimlenmesi için farklı konsantrasyonlarda (0,5, 2,5 veya 5,0 mM) nAl_2O_3 ve nZnO kullanılmıştır. Nanopartikül uygulanmış biber bitkilerinde iyon içeriklerini belirlemek için, ICP-MS analizi yapılmıştır. Aquaporin ve dehidrin gen ekspresyonlarının seviyeleri ise kantitatif ters transkripsiyon polimeraz zincir reaksiyonu (qRT-PCR) ile analiz edilmiştir.

Bulgular: Nanopartikül uygulamasının biber çimlenmesi üzerinde etkisi tespit edilmemiştir. nAl_2O_3 uygulamaları kök gelişimini değiştirmezken, yüksek nZnO konsantrasyonları kök uzunluğunu ve kök sayısını olumsuz yönde etkilemiştir. Köklerdeki aquaporin ve dehidrin gen ekspresyonu, özellikle 0,5 mM nZnO uygulaması ile artmıştır. nAl_2O_3 uygulanan kök ve gövdelerde ise dehidrin gen ekspresyonu azalmıştır.

Sonuç: Gen ekspresyon ve büyüme parametrelerindeki değişiklikler, özellikle nZnO'nun biber bitkileri için potansiyel olarak fitotoksik olduğunu göstermiştir. Ayrıca, ekspresyon analizi, test edilen genlerin nanopartikül bazlı abiyotik strese yanıt olarak rol oynayabileceğini önermektedir.

Anahtar Kelimeler: Aquaporin, dehydrin, nanopartikül, nAl_2O_3, nZnO, çimlenme, *Capsicum annuum* L., biber

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INTRODUCTION

Engineered nanomaterials with their unique technical properties have extensive use in various technology and industrial sectors including mechanical industries, energy applications, environmental remediation, biosensing applications and many others (1).

Metal/metal oxide nanoparticles as engineered nanomaterials are synthesized by the addition of reducing agents to produce metal nanoparticles or oxidizing/precipitating agents for metal oxide nanoparticles (2). Because of the wide variety of applications of these nanoparticles in various industries, their effects on environment and several organisms including plants have been extensively studied (1).

Since the nanoparticles can contaminate plants in different stages of their life cycle (3), detailed studies should have been performed at molecular, cellular, metabolic, and physiological levels to comprehend the actual effects of metal oxide nanoparticles on plants. Analysis of their effects on seed germination and root elongation as a phytotoxicity parameter of these nanoparticles (4) have been studied in many plant species.

Assessment of changes on gene expression levels are another important parameter to obtain nanoparticle-based changes in plant body. Plants contain a large number of aquaporins, which are proteins responsible for controlling water transport and facilitating the transport of uncharged solutes across membranes (5). The importance of aquaporins has been demonstrated in various abiotic stress conditions (5) and alterations in their gene expressions following nanoparticle treatments were also obtained in plants (6). Even though their roles are not yet clearly understood, dehydrins are considered as stress proteins, which play a role in dehydration stress, and it is also suggested that some dehydrins may have critical roles in plant growth (7). It was demonstrated that expression levels of several genes such as aquaporins, oxidative response genes and housekeeping genes were affected by nanoparticle treatments (8,9).

The uptake and accumulation of these nanoparticles by plants create a potential threat for human health in cases where humans consume the plants (10). *Capsicum annuum* L. (peppers) belong to the Solanaceae family and they are extensively consumed as both vegetables and spices across the world. Large areas of land in several countries such as Mexico, China, Korea, USA have been used for pepper cultivation because of their use in many cuisines (11). It suggests that there is possible nanoparticle contamination risk for these large land cultivation areas. In the study, pepper was used as a plant material because of its high consumption and production levels across the world and it was aimed to analyze potential beneficial or inhibitory effects of two metal oxide nanoparticles (nAl_2O_3 and nZnO) on its germination, root growth and expression levels of aquaporin (*CaAqp*) and dehydrin (*CaDhn*) genes.

MATERIAL AND METHOD

Plant Material

The pepper (*Capsicum annuum* L.) hybrid Bafra F1 seeds were used as a plant material. The seeds were gifted from Mehmet Yüksel, Yüksel Tohum (Antalya, Turkey) and kept in the dark at 4°C until use.

Characterization of Nanoparticles

The nAl_2O_3 (ca. 40 nm) and nZnO (<100 nm) nanopowders were purchased from PlasmaChem (Berlin, Germany) and Sigma Aldrich (Saint Louis, MO, USA), respectively. Although commercial manufacturers characterized the nAl_2O_3 and nZnO in detail, scanning electron microscope (SEM) and transmission electron microscope (TEM) were also used to visualize shape and morphology of the nanoparticles.

For SEM analysis, the nanoparticles were placed on a carbon disc and coated with a few nm thick gold-layers by using a Baltec SDC 005 sputter-coater. SEM images were obtained using a Carl Zeiss Evo®40 instrument under high vacuum with an accelerating voltage of 10 kV.

For TEM analysis, FEI Tecnai G2 Spirit BioTwin CTEM instrument operating at an accelerating voltage of 120 kV were used.

Medium Preparation

Murashige and Skoog (MS) basal medium were purchased from Sigma Aldrich (Saint Louis, MO, USA). 0.5, 2.5 and 5 mM of nAl_2O_3 and nZnO were used to test their effects on pepper plants. Firstly, nanopowders were suspended in half strength MS medium (pH: 6.0) by sonication for 30 min in ultrasonic water bath at room temperature, and then the suspensions were homogenized by stirring them for 20 min before use.

Plant germination and seedling establishment

The pepper seeds were imbibed with distilled water for 16 hours before treatments. Then, the seeds were transferred to 900 cm^3 vitrovents (Duchefa, The Netherlands) including 1 layer of sterile damp filter paper moistened with 5 mL of sterile one-half-MS medium (pH: 6.0) containing 0.5, 2.5 and 5 mM nAl_2O_3 or nZnO. For the control group, the seeds were transferred to the vitrovents including 1 layer of sterile damp filter paper moistened with 5 mL of sterile half strength MS medium without any of nanoparticles. Then, the vitrovents were incubated at 24°C for the first 7 days in the dark and then 8 days in the light. Each vitrovent containing 10 seeds per control group and nanoparticles treatments were used and each treatment was replicated three times.

Germination Data Analysis

The germination percentages, root number, length of roots and leaves of the control and nanoparticles-treated plants were obtained at the end of the 15th day of *Capsicum* seeds. SPSS version 25 (SPSS, USA) was used to perform statistical analysis. Germination percentages were analyzed with Chi-square statistics. One-way ANOVA (Analysis of variance) was performed to compare mean differences of leaf length. Root

length data were subjected to ANOVA, followed by Tukey post-hoc test to compare mean values. Because root number values do not have a normal distribution, the data were analyzed with the Kruskal-Wallis test, followed by the Mann-Whitney U test as a post hoc test. $p \leq 0.05$ was used as the level of significance for all the statistical analyses.

Gene expression analysis of *Capsicum annuum* L. genes

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to obtain impacts of nAl_2O_3 and nZnO on the expression levels of genes, *CaDhn* (Accesion no. AY225438.1) and *CaAqp2* (*CaTIP1;1*, Accession no. GU116569). Firstly, total RNA was extracted from the 15-day-old stems and 15-day-old roots of the peppers using the Plant/Fungi Total RNA purification kit (Norgen Biotek) according to the manufacturer's instructions, with DNaseI treatment (DNase I, RNase-free, NEB). The RNA quantification was performed using Nanodrop equipment (Thermo Scientific Nanodrop™ 2000) and RNA samples were visualized by running them in 1.5% agarose gel with EtBr to check their integrity. Secondly, cDNA templates were reverse transcribed from purified RNA samples (3 µg per treatment) using SuperScript™ IV First-Strand Synthesis System (Invitrogen™) according to manufacturer's instructions. The obtained cDNAs were kept at -20°C until use in qRT-PCR. The NCBI database (https://www.ncbi.nlm.nih.gov/) and its BLAST tool (blast.ncbi.nlm.nih.gov) were used to get full-length sequences and verification of mRNA sequences of *CaDhn* and *CaAqp2* genes, respectively. All gene-specific qRT-PCR primers from mRNA sequences were designed by Primer3 v4.1.0 (12).

The housekeeping genes, *Actin* mRNA, *GapdH* (glyceraldehyde 3-phosphate dehydrogenase GapCp) and *EIF5A2* (Eukaryotic translation initiation factor 5A2) were used for normalization and their primer sequences were obtained from Wan et al. (13).

The primers used in the present study are listed below (5' - 3'):

Actin mRNA (F; TGTTATGGTAGGGATGGGTC, R; TTCTCTCTATTTGCCTTGGG),

GapdH (F; ATGATGATGTGAAAGCAGCG, R; TTTCAACTGGTGGCTGCTAC),

EIF5A2 (F; CCTGTTATCGTGCTACTTTG, R; GTTTCATTGCCKTGCCAGAT)

CaAqp2 (F; CGATGGCGTCACTACTCCTC, R; CACCAACGAAAGCACCGA)

CaDhn (F; GGAGAAATTGCCAGGGTATCACTC, R; CAGAACACCACAATCATAACATACC)

qRT-PCR analysis was carried as three replicates. Reactions (10 µL) contain template cDNA (1 µL), Biorad iTaq Universal SYBR Green Supermix (5 µL), reverse (4 µM) and forward (4 µM) primers and nuclease-free water. CFX96 Touch Real Time PCR instrument (Biorad, France) was used for qRT-PCR analysis and the analysis conditions were 3 min at 95°C followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C.

Melt curve analysis was performed following each qRT-PCR amplification by heating the product from 65°C to 95°C, 0.5°C in 5 s increments. The optimal cycle threshold (Ct) was determined using Biorad CFX Manager 3.1 software. The above-mentioned housekeeping genes were used for normalization using GeNorm V3 algorithm (14). The obtained normalized c_q values were processed according to the $\Delta\Delta c_q$ method and presented as relative expression values for each gene.

Determination of ion content

Inductively coupled plasma mass spectrometry (ICP-MS, Thermo ICP MS X Series 2) was used to determine the level of aluminum and zinc ion contents of nAl_2O_3 or nZnO treated pepper plants. The control and nanoparticles-treated plant samples (ca. 0.1 g) were weighted and digested with a 6 mL 65% (v/v) nitric acid. Following incubation in an ultrasonic water bath for 15 min, the samples were kept at room temperature for 24 h to complete the extraction. One-way ANOVA was performed to compare mean differences of ion contents in nanoparticles-treated plants.

Dissolution of Al^{3+} and Zn^{2+} ions in the exposure medium was obtained according to Leclerc and Wilkinson (15):

1. Centrifugation of the medium at 2500×g using centrifugation tubes containing 3 kDa centrifugal ultrafiltration unit (Merck Millipore, Germany) for 15 min at 0., 1., and 7. day.

2. Repetition of the 1st step four times and collecting only the filtrate at each centrifugation step.

3. Addition of 2% nitric acid to the 1 mL of filtrate.

4. ICP-MS analysis was evaluated to measure the release rates of Al^{3+} and Zn^{2+} from nAl_2O_3 or nZnO.

For all ICP-MS analysis, multi-element ICP QC Standard solution (Chem-Lab, Belgium) was used as ICP-MS standard.

RESULTS

Characterization of Nanoparticles

SEM (Figure 1A-1B) and TEM (Figure 1C-1D) analysis were performed to visualize the shape and size of the tested nanopar-

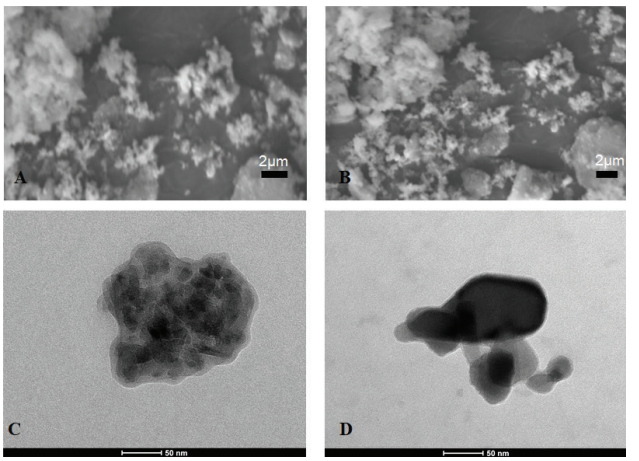

Figure 1. Images of the nAl_2O_3 (A, SEM; C, TEM) and nZnO (B, SEM; D, TEM).

ticles. The images demonstrated the presence of uneven size with irregular, elongated, spherical shaped nanoparticles. While TEM analysis clearly showed that nZnO has different sizes from 40 nm to 100 nm, the nAl_2O_3 images were very low-resolution so it was not possible to obtain their mean sizes.

Effects of nAl_2O_3 and nZnO on the germination parameters of pepper

The effects of different nAl_2O_3 and nZnO concentrations (0.5, 2.5 and 5 mM) on pepper germination were analyzed and the germination percentages are presented in Figure 2. The results showed that the germination percentages of pepper seeds germinated in the MS medium at those nanoparticles concentrations were not statistically different in comparison to the control group seeds, which were germinated in the medium without nanoparticles.

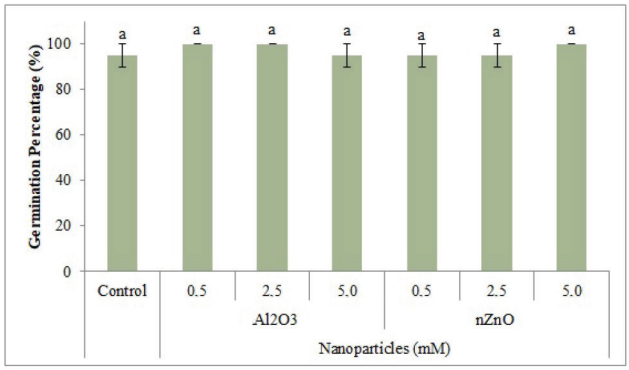

Figure 2. Germination percentages of nAl_2O_3 and ZnO-treated pepper seeds.

The average length of root and leaf of pepper plants were recorded and the results are presented in Table 1. Even though the longest leaves (60.8 mm) were obtained in the control and 2.5 mM nAl_2O_3-treated pepper plants, there were no statistically significant differences among the tested plants.

While the control and nAl_2O_3-treated pepper plants had statistically similar root lengths, the application of nZnO at different concentrations affected root length compared to control plants. The longest roots (36.2 mm) and the shortest roots (14.7 mm) were obtained with the application of the lowest and highest nZnO concentrations, respectively.

Regarding number of roots, similar effects were observed with the application of nZnO at the end of the 15th day (Table 2). Even though a statistically similar number of roots were obtained in the control and nAl_2O_3-treated pepper plants, higher nZnO concentrations had adverse effects on the number of roots. The decreased root numbers (1.11 and 1.00) were obtained with the application of 0.25 and 0.5 mM nZnO concentrations (Table 2).

Determination of ion content

To understand the detailed impacts of applied metal nanoparticles on pepper plants, ICP-MS analysis was performed to obtain Al^{3+} and Zn^{2+} ions in pepper stems and roots following nAl_2O_3 and nZnO treatments. Interestingly, Al^{3+} ions were not detected in nAl_2O_3-treated pepper stems and roots with ICP-MS analysis. ICP-MS analysis showed the presence of Zn^{2+} ions in both pepper stems and roots with the application of nZnO in MS medium (Table 3). An accumulation of Zn^{2+} ions [44.3 and 70.4 mg/kg FW (fresh weight)] was found in 0.5 mM and 2.5 mM nZnO applied stems, respectively. The higher levels of Zn^{2+} ions were determined in the 0.5 mM (312.4 mg/kg FW) and 2.5

Table 1. Root and leaf length of control and nanoparticles-treated pepper plants.

Nanoparticles	Concentration (mM)	Average root length (mm)*	Average leaf length (mm)*
Control	0	29.1±1.59b	60.8±1.57a
Al_2O_3	0.5	26.8±1.48b	59.4±3.03a
	2.5	27.5±1.89b	60.8±1.66a
	5.0	29.4±1.78ab	59.4±2.01a
ZnO	0.5	36.2±1.97a	55.5±2.56a
	2.5	22.7±1.31bc	57.0±2.27a
	5.0	14.7±0.88d	55.2±2.06a
	Significance test	*F=13.830*	*F=1.079*
		p=0.000	*p=0.383*
	Statistical Analysis Test	One-way ANOVA Tukey	One-way ANOVA

* Values were represented as mean±SE.
The different letters following the means in each column show statistical differences at p≤0.05 according to ANOVA Test. The mean differences were analyzed vertically.

Table 2. The impacts of nAl_2O_3 and nZnO on root numbers of pepper plants[a].

		Kruskal-Wallis Test		
Nanoparticles	**Concentration (mM)**	**Number of roots[b]**	**Mean rank**	*Significance test*
Control	0	1.84±0.27	78.53	χ^2=20.037 df=6 p=0.003
Al_2O_3	0.5	1.85±0.24	79.83	
	2.5	2.15±0.33	82.93	
	5.0	1.37±0.17	63.61	
ZnO	0.5	1.58±0.25	68.50	
	2.5	1.11±0.07*	55.74	
	5.0	1.00±0.00*	50.00	

[a]Root number values of the tested groups were individually compared with each other by using the Mann-Whitney U nonparametric test.
[b]Values were represented as mean±SE.
*Values were significantly different (p≤0.05)

mM (933.5 mg/kg FW) nZnO-applied pepper roots. However, no statistical differences were detected in the content of Zn^{2+} ions between control and 5 mM nZnO-applied pepper stems and roots (Table 3).

Table 3. Zn^{2+} content of pepper stems and roots treated with nZnO.

	Zn^{2+} content (mg/kg FW)	
Concentration (mM)	**Stem***	**Root***
0 (Control)	2.21±0.06c	4.27±0.07c
0.5	44.32±0.19b	312.44±1.16b
2.5	70.39±0.60a	933.49±3.61a
5.0	2.81±0.13c	4.68±0.02c

* Values were represented as mean±SE.
The different letters following the means in each column show statistical differences at p≤ 0.05 according to ANOVA Test.

ICP-MS analysis was performed to obtain the release rates of dissolved Al^{3+} and Zn^{2+} ions from nAl_2O_3 and nZnO containing MS media in 1st and 7th day. The results showed that the dissolution of Al^{3+} and Zn^{2+} ions from the applied nanoparticles was very low (Table 4). While Al^{3+} release from nAl_2O_3 was only 0.053%, Zn^{2+} release from nZnO was 0.153% even after 7 days (Table 4).

Table 4. The percentages (%) of Al^{3+} and Zn^{2+} released from 2.5 mM nAl_2O_3 or nZnO containing culture medium.

Duration (days)	**Al^{3+} release (%)**	**Zn^{2+} release (%)**
0	-	-
1	0.028	0.069
7	0.053	0.153

qRT-PCR analysis of *CaAqp2* and *CaDhn* genes

To understand the effects of the tested metal nanoparticles at the molecular level, relative expression levels of two pepper genes (*CaAqp2* and *CaDhn*) were analyzed by qRT-PCR (Figure 3 and

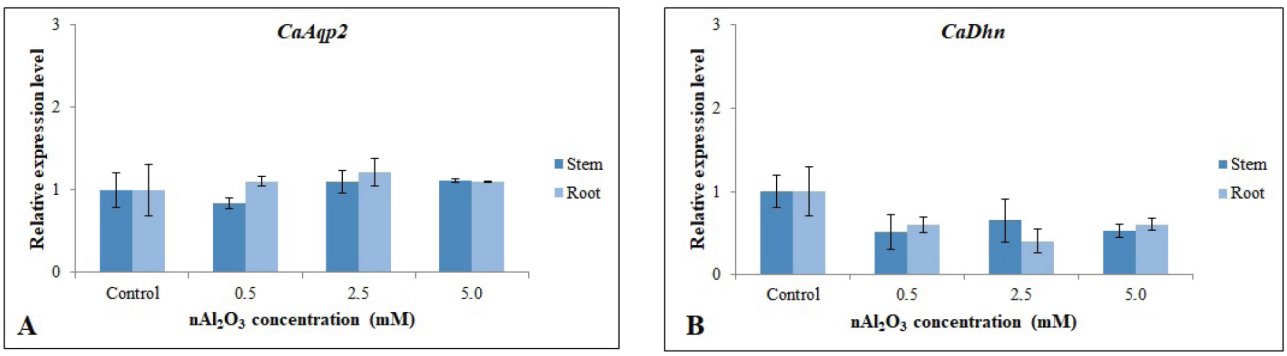

Figure 3. Relative expression levels of *CaAqp2* and *CaDhn* in nAl_2O_3-treated pepper plants.

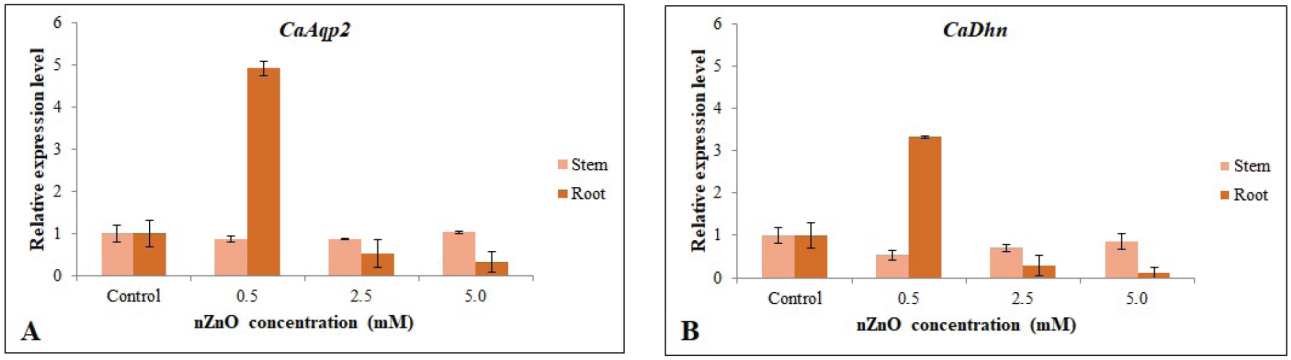

Figure 4. Relative expression levels of *CaAqp2* and *CaDhn* in nZnO-treated pepper plants.

Figure 4). Application of nAl_2O_3 did not have a significant effect on the relative expression of *CaAqp2* in stems and roots (Figure 3A). The only decrease in gene expression levels was obtained in stems of 0.5 mM nAl_2O_3-treated pepper plants. Application of 2.5 and 5 mM nAl_2O_3 led to a slight increase of *CaAqp2* in stems and roots. On the other hand, nAl_2O_3 treatments inhibited *CaDhn* gene expression in stems and roots with respect to the control groups (Figure 3B). 0.5 and 5 mM nAl_2O_3 treatments resulted in statistically similar gene expression levels in stems and roots. 2.5 mM nAl_2O_3 treatments upregulated *CaDhn* gene expression in stems compared to the other applied concentrations.

The *CaAqp2* and *CaDhn* expression levels of pepper plants were strongly affected by nZnO treatments (Figure 4A and 4B). The highest *CaAqp2* expression (almost five times higher expression than that of control) was obtained in the roots of 0.5 mM nZnO-treated pepper plants (Figure 4A). Increased concentrations of nZnO applications resulted in inhibition of *CaAqp2* expression in roots. While *CaAqp2* expression levels showed a slight decrease in lower concentrations of nZnO applications in stems, the highest nZnO concentration (5 mM) resulted in similar gene expression levels when compared to the control group (Figure 4A). *CaDhn* gene expression in roots was significantly affected with the application of 0.5 mM nZnO (Figure 4B). Its expression was three-times higher than that of the control roots. However, higher nZnO treatments dramatically decreased *CaDhn* gene expression levels in roots. nZnO-applied pepper plants had lower gene expression of *CaDhn* in stems with respect to the control group (Figure 4B).

DISCUSSION

An increase in the worldwide use of engineered metal nanoparticles is predicted to result in an elevated transfer of these particles to the environment. The studies and modeling analysis showed that thousands of tons of nanoparticles including Al, Zn, Ag, Fe etc. have been released into the environment due to their extensive production (16). The studies demonstrated their toxic and sometimes promoting effects in various plant growth parameters. Being one of the most consumed vegetables, the contamination of pepper production areas with these nanoparticles is highly possible. Among these nanoparticles, nZnO and nAl_2O_3 are the metal oxides and they have a wide application from electronics to biomedical applications and many others (17,18). In the present study, the impacts of nAl_2O_3 (ca. 40 nm) and nZnO (<100 nm) metal oxide nanoparticles were tested on pepper germination and seedling establishment parameters. The results showed that germination of pepper seeds was not affected with treatments of different concentrations of nZnO and nAl_2O_3. Similarly, *Cucurbita pepo* (zucchini) germination was unaffected by the treatment of 1000 mg/L nZnO (<5 nm and <10 nm) (19). Kumar et al. (20) tested the impacts of nZnO (≤50 nm) on several plant species and reported its inhibitory effects on cucumber germination and on shoot and root growth of wheat, green gram and cucumber. However, germination of rice was not significantly inhibited by the nanoparticles. Lee et al. (21) tested the effects of various metal oxide nanoparticles including nAl_2O_3, $nSiO_2$, nFe_3O_4 and nZnO on seed germination and root elongation of *Arabidopsis thaliana*. The results showed that while nZnO was the most phytotoxic, nAl_2O_3 was not toxic for these plants. Regarding root length and number, none of the concentrations of nAl_2O_3 used affected root growth of peppers. While the lowest concentration of nZnO treatments increased average root length, the highest levels of nZnO decreased root length compared to the control plants in this study. Higher nZnO concentrations also negatively affected the number of roots. Similarly, an increased root length in rice was obtained when the seeds were soaked in <100 mg/L nZnO solution for 1-2 days, but the root growth was dramatically inhibited at concentrations of 500 and 1000 mg/L ZnO (22). Similar to the shortest roots resulting from high nZnO concentrations, the application of higher nZnO concentrations also resulted in reduced root length in barley (23).

The studies show that the size, concentration, composition, physical and chemical properties determine the fate of these nanoparticles on plants (24). The plant cell walls have a size exclusion limit (~5-20 nm) (25). However, some nanoparticles such as silver nanoparticles in *Chlamydomonas reinhardtii* (26) led to the formation of bigger pores in the cell walls and thereby resulted in the entrance of larger particles. Root junction, wounding, endocytosis, and symplastic transport could be other ways to accumulate or transport the nanoparticles in the

plant body (1). Moreover, plants can accumulate nanoparticles directly or as metal ions (27). In the present study, no Al^{3+} ions were detected in nAl_2O_3-treated pepper stems and roots with ICP-MS analysis. The results suggested that the ion content was probably out of detection limits. However, the increased Zn^{2+} contents in pepper stems and especially in the roots suggested that even though the nZnO used in the present study is much bigger than the size limit for the plant cell wall, their uptake from shoots and roots could be acquired by one of above-mentioned mechanisms. The very low rates of dissolution of these ions from exposure to medium demonstrate that any obtained effects in pepper plants could be due to the direct uptake of these nanoparticles instead of their released ions. It can be concluded that the obtained effects on root length and root numbers of nZnO-treated plants were probably due to the high uptake of nanoparticles and accumulation in roots and stems.

Plants have complex response and defense mechanisms to adapt to different stress conditions (28). The effects of nanoparticles could be also obtained at the level of gene expression. In the present study, relative expression levels of *CaAqp (CaTIP1;1)* from the tonoplast intrinsic proteins subfamily and *CaDhn* were investigated in 15-day-old pepper seedlings after exposure to nAl_2O_3 and nZnO.

Dehydrins are considered as stress proteins, and they play role in drought stress tolerance (7). They also have other functions such as binding metal ions, binding DNA, binding phospholipids and they can scavenge free radicals (7). Downregulation of *CaDhn* was observed in stems and roots following nAl_2O_3 and nZnO treatments. Upregulation of *CaDhn* was only observed with lowest levels of nZnO treatments in stems. The altered gene expression in stems and roots profiles indicates that this gene may have a function in metal oxide nanoparticle-based stress tolerance. In addition to their functions in water movement across cell membranes, aquaporins also have roles in response to the biotic and abiotic stress. The adverse effects of changes in aquaporin expression may especially be observed during germination and seedling development. However, nAl_2O_3 treatments did not significantly affect the investigated aquaporin gene expressions in pepper stems and roots. On the other hand, while nZnO treatments at 0.5 mM concentration led to an upregulation of *CaAqp2* in root tissues, higher levels of nZnO resulted in a downregulation of the gene in those tissues. Since roots are the first target for nanoparticles, alterations in gene expression might occur more often than expected in roots. It could be the reason for the critical alterations of gene expression in roots compared to that of stems. Aquaporin genes were significantly activated in tomato roots by carbon nanotubes treatments (29). The same increase was also observed in *Arabidopsis* plants after 1 week of exposure to Ag nanoparticles, but their levels decreased with prolonged exposure times (6). The altered *CaAqp2* expressions in the present study indicate that these nanoparticles affected plant's water and small molecules homeostasis following nZnO treatments. A slight decrease of aquaporin genes was also observed in pepper stems in nZnO applications. Gitto and Fricke (30) showed that the decrease in expression of certain barley aquaporin genes was stronger with lower Zn treatments than higher Zn treatments. It is concluded that plants would have limited Zn transport to the shoot to prevent major toxicity (30).

CONCLUSION

The extensive use of metal nanoparticles worldwide is predicted to result in an elevated transfer of these particles to the environment. In the present study, the impacts of nAl_2O_3 and nZnO on pepper germination, root growth and gene expression levels were analyzed. The results showed that pepper germination was not affected by nanoparticles applications. While nAl_2O_3 treatments did not significantly change root growth, higher concentrations of nZnO negatively affected root length and root number. In particular, the application of 0.5 mM nZnO upregulated aquaporin and dehydrin gene expressions in roots, significantly. Downregulation of dehydrin gene expression was obtained in stems and roots after exposure to nAl_2O_3 treatments. These gene expression alterations and changes of growth parameters showed that nZnO especially is potentially phytotoxic for pepper plants. Moreover, expression analysis suggested that the tested genes may play roles in response to the nanoparticle based abiotic stress.

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