Effects of ZnO nanoparticles in alfalfa, tomato, and cucumber at the germination stage: Root development and X-ray absorption spectroscopy studies*

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Abstract: Past reports indicate that some nanoparticles (NPs) affect seed germination; however, the biotransformation of metal NPs is still not well understood. This study investigated the toxicity on seed germination/root elongation and the uptake of ZnO NPs and Zn²⁺ in alfalfa (*Medicago sativa*), cucumber (*Cucumis sativus*), and tomato (*Solanum lycopersicum*) seedlings. Seeds were treated with ZnO NPs at 0–1600 mg L⁻¹ as well as 0–250 mg L⁻¹ Zn²⁺ for comparison purposes. Results showed that at 1600 mg L⁻¹ ZnO NPs, germination in cucumber increased by 10 %, and alfalfa and tomato germination were reduced by 40 and 20 %, respectively. At 250 mg Zn²⁺ L⁻¹, only tomato germination was reduced with respect to controls. The highest Zn content was of 4700 and 3500 mg kg⁻¹ dry weight (DW), for alfalfa seedlings germinated in 1600 mg L⁻¹ ZnO NPs and 250 mg L⁻¹ Zn²⁺, respectively. Bulk X-ray absorption spectroscopy (XAS) results indicated that ZnO NPs were probably biotransformed by plants. The edge energy positions of NP-treated samples were at the same position as Zn(NO₃)₂, which indicated that Zn in all plant species was as Zn(II).

Keywords: absorption; nanoparticles; optical emission spectroscopy; speciation; X-ray absorption spectroscopy (XAS).

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INTRODUCTION

Nanoparticles (NPs) are unit materials with at least two dimensions, smaller than 100 nm [1]. Nanotechnology, the study and application of NPs, has rapidly grown in the last few years. Applications for NPs are found in a wide variety of disciplines, including medicine, biology, electronics, and engineering. Intensive research, focused on improving and finding new applications for ZnO NPs, has highlighted the importance of these materials. Recent reports indicate that ZnO NPs are potentially applicable in the field of bioimaging [2] and in the production of printable electronic devices [3]. These NPs may also be an option for the production of antimicrobial textiles [4], and they have potential application in nanomedicine for the treatment of cancer (NPs preferentially killed cancerous cells) [5]. However, information on the fate, transport, and toxicology of NPs in the environment and human health is still scarce.

To properly understand the effects of these materials in the environment, an interdisciplinary approach is needed [6]. To solve this issue, new scientific fields such as nanotoxicology and nanoecotoxicology have emerged [7,8]. Studies performed at the cell level in bacteria and human cell cultures have determined the toxicity of several nanomaterials. It has been reported that ZnO provoked a cytotoxic response in macrophage and epithelial cells, while CeO₂ favored cellular resistance [9]. Recently, Wang et al. [10] observed severe damage in liver and lungs of rats exposed by inhalation to Fe₂O₃ and ZnO NPs. Additional reports have described the toxicity of NPs in different living systems, including microalgae [11], nematodes [12], and aquatic organisms [13], among others. With regard to plants, Lin and Xing [14] reported the inhibition of root growth in radish, rape, ryegrass, lettuce, corn, and cucumbers by NPs of aluminum, alumina, zinc, and zinc oxide, and carbon nanotubes. Lee et al. [15] found that Cu NPs reduced growth and produced necrosis in mung bean (*Vigna radiata*) and wheat. The nanophytotoxicity of ZnO (20 nm) has also been investigated in ryegrass, which displayed a high vacuolation and biomass reduction in the presence of this nanomaterial [16]. Boonyanitipong et al. [17] demonstrated that ZnO NPs reduced root development in *Oryza sativa*. However, very little research has been directed to determine the biotransformation of metal NPs in plants. Understanding biotransformation is extremely important to know how NPs change as they are in contact with living organisms and the environment. Mainly with the use of synchrotron techniques, it has been possible to determine that NPs behave differently. Ceria (CeO₂) NPs were found untransformed in alfalfa (*Medicago sativa*), corn (*Zea mays*), cucumber (*Cucumis sativus*), and tomato (*Lycopersicon esculentum*) [18]. On the other hand, NPs were not detected in *Prosopis juliflora-velutina*, *Salsola tragus*, and *Parkinsonia florida* germinated in ZnO NPs [19].

The aim of this research was to determine the phytotoxicity and uptake of commercial ZnO NPs and the Zn²⁺ ions in three edible plant species: alfalfa (*M. sativa*), tomato (*L. esculentum*), and cucumber (*C. sativus*). Bulk X-ray absorption spectroscopy (XAS) experiments were performed in order to determine the possible ZnO and Zn²⁺ biotransformation in plant tissues. NPs with a particle size less than 10 nm were selected for this study. Tomato and cucumber are recommended by the U.S. Environmental Protection Agency (EPA) to perform seed germination/root elongation toxicity tests. Alfalfa was included because it is very important forage in the southwestern United States and northern Mexico and because it is a plant profusely studied by several research groups.

MATERIALS AND METHODS

NP characterization

The ZnO NPs (Meliorum Technologies, Rochester, NY) were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). For purity determination, 100 mg of NPs was digested in a microwave oven using a mixture of plasma pure HNO₃ + HCl (1:1) as per Xie et al. [20]. Subsequently, the concentration of Zn in the digested samples was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Perkin Elmer

Optima 4300 DV, Shelton, CT). X-ray diffraction (XRD) experiments, aimed at phase identification and particle size determination, were carried out using a Siemens D5000 diffractometer equipped with an MBraun position sensitive detector. Powder diffraction patterns corresponding to a 20–60° 2 θ range ($\lambda = 1.5406 \text{ \AA}$) were recorded in the reflectivity geometry. The data collection time for each XRD pattern was approximately 90 min.

Preparation of ZnO suspensions and determination of Zn²⁺ concentration

Suspensions of ZnO NPs at the following concentrations were prepared: 0, 50, 100, 200, 400, 800, and 1600 mg L⁻¹. The suspensions were sonicated for 30 min to avoid aggregation according to Lin and Xing [14]. In order to determine the concentrations of Zn²⁺, the procedure reported by Ling and Xing [14] was followed with only minimum modifications. Briefly, suspensions were centrifuged for 1 h at 4000 rpm (Fisher Scientific 8K, Houston, TX) and filtered through 551 Whatman Schleicher & Schuell filters (Sigma-Aldrich, St. Louis, MO). Concentrations of Zn found in the supernatants were used as reference for the Zn²⁺ treatments (selected concentrations were 0, 0.05, 0.5, 5, 10, 50, and 250 mg Zn²⁺ L⁻¹) that were prepared from Zn(NO₃)₂.

Germination experiments

Seeds of tomato (*L. esculentum*, Roma FV; Western Seeds International, El Centro, CA), cucumber (*C. sativus*, Poinsett 76; Western Seeds International, El Centro, CA), and alfalfa (*M. sativa*, WL535; Del Norte Seed & Feed, El Paso, TX) were used in this study. Seeds were treated for 30 min with a 4 % NaClO solution for disinfection, followed by rinsing with sterilized Millipore water (MPW). Germination paper (Nasco, Fort Atkinson, WI) cut to fit regular Petri dishes was used as inert material. A piece of germination paper was placed on the bottom of the Petri dish, and 5 mL of ZnO or Zn²⁺, at the appropriate concentrations, was added [14]. Thirty seeds of each species were placed in every Petri dish, covered with a second germination paper piece, dampened with 10 drops of an antibiotic–antimycotic solution (Sigma A5955, Sigma, St. Louis, MO), and covered. Aluminum paper was used to protect seeds from light, and the treated seeds were placed in a growth chamber at 20 °C. The germination was evaluated when approximately 65 % of the control roots were at least 5 mm long [21]. To eliminate any surface metal, the seedlings were rinsed first with 0.01 M HNO₃ and later with MPW. Roots and stems of 10 seedlings per replicate were measured and dried in an oven at 70 °C for two days. Average weight was calculated on a 10-seedling basis.

Quantification of Zn in dry plant tissues

Plant tissues were digested in a CEM microwave oven (CEM Corporation, Mathews, NC) following the EPA 3051 method [22] using 3 mL of plasma pure HNO₃. The digested samples were diluted to 25 mL with MPW and read on the ICP-OES. A blank and a standard were read every 10 samples for quality control/quality assurance (QC/QA) purposes.

Statistical analysis and quality control

Three replicates per treatment with 30 seeds per replicate were set up for the experiments following a completely random design. Standard reference material 1570a (spinach leaves) was used to validate the digestion and analytical method. Spikes were used to determine the percent recovery of Zn. Data was reported as mean $\pm$ standard error (SE), and a one-way ANOVA analysis (SPSS 13.0 package) was followed by the Tukey's multiple comparison test.

XAS experiments

A portion of seedlings treated with 1600 mg ZnO NPs L⁻¹ and 250 mg Zn²⁺ L⁻¹ was frozen in liquid nitrogen and lyophilized in a Labconco FreeZone 4.5 freeze-dryer (Kansas City, MO) at -53 °C and 0.140 mBar pressure for three days. Powdered dry tissues were placed on aluminum sample holders and covered with Mylar[®] tape. The XAS spectra of plant samples, ZnO NPs, and Zn nitrate were collected at beam line 7-3 at the Stanford Synchrotron Radiation Laboratory (SSRL). Fluorescence spectra for Zn edge were obtained using a Canberra 29-element array germanium detector (Meriden, CT) by monitoring the Zn K_α. The standard operating conditions of the beam line were 3 GeV beam energy, a 50–100 mA beam current, and a Si(220) ϕ 90 monochromator. Spectra from samples were calibrated with the spectrum from a Zn foil at the time of data collection.

The data analysis was done using the WinXAS software [23]. The edge energy was calibrated using the edge position of an internal Zn foil with edge energy of 9659 eV. The calibration of the sample spectrum was performed using first- and second-degree derivatives of the inflection point of the Zn foil. A polynomial fitting subtraction was used to remove background. A first-degree polynomial was used on the pre-edge region and a fourth-degree polynomial was used on the post-edge region of the spectrum. The conversion into k space was based on the energy of the photoelectrons ejected from the samples. Kinetic energy of the photoelectrons ejected was calculated based on the Zn edge position and converted into wave vector space (k space). The resulting scattering curves were then k weighted to three in order to enhance dampened scattering oscillations and Fourier transformation into interatomic distance space. The coordination numbers and the Debye–Waller factors were obtained by least-squares fitting of the extended X-ray absorption fine structure (EXAFS) data using FEFF800 [24]. The spectra of Zn-seedling samples were fitted to the EXAFS spectra of the model compounds that best matched the samples.

RESULTS AND DISCUSSION

ZnO NP characterization and determination of Zn²⁺ ions in ZnO NP suspensions

XRD analyses showed no impurities in any of the ZnO NP samples used in this study. The average particle size was determined from the full width at half maximum (FWHM) of the (110) Bragg peak using the Scherrer equation [25], where the FWHM was obtained from the best fit of the peak profile to a combination of Gaussian and Lorentzian functions. This analysis yielded an average particle size of 8 nm. The ICP analyses indicated a purity of 100.3 % ± 3. The detection limit for Zn was 0.018 mg L⁻¹ and percent recovery was 98 %. The Zn²⁺ concentrations in 50, 100, 200, 400, 800, and 1600 mg L⁻¹ ZnO NP suspensions were of 3.2, 7.2, 9.6, 17.2, 25.6, and 24.17 mg L⁻¹, respectively. These concentrations were used as reference for experiments aimed to determine the phytotoxicity of Zn ions. The NP solubility and release of metal ions are significant factors in establishing the fate and transport of nanomaterials in living organisms [11,26].

Effect of ZnO NPs and Zn²⁺ on seed germination

Seed germination percentage has been used to evaluate the toxicity of metals and metalloids in several plant species [27]. In this study, seed germination and root elongation were evaluated when 65 % of the total seeds were germinated and root elongation was more than 0.5 mm [21]. The germination percentage for alfalfa, tomato, and cucumber seeds soaked in 0, 50, 100, 200, 400, 800, and 1600 mg L⁻¹ of ZnO NPs is shown in Fig. 1A. With the exception of the controls and treatment of 1600 mg L⁻¹ ZnO, alfalfa seed germination followed a bell-shape trend (Fig. 1Aa). As compared to control, only 800 and 1600 mg L⁻¹ of ZnO NPs caused a significant reduction in alfalfa germination (40 %). However, even when not significantly different from control, 50 mg L⁻¹ ZnO NPs reduced this parameter in a 25 %. It has been proven that different nanomaterials are able to produce dissimilar effects on seed germination.

Fig. 1 Percent germination of (a) alfalfa, (b) tomato, and (c) cucumber seeds treated with (A) 0–1600 mg L⁻¹ ZnO NPs and (B) 0–250 mg L⁻¹ Zn²⁺ supplied as Zn(NO₃)₂. Data are means ± SE of three replicates. Lowercase letters indicate statistically significant differences at *P* < 0.05.

Klaine et al. [28] reported that ZnO NPs can decrease germination percentage in corn seedlings, while TiO₂ NPs can stimulate seed germination in spinach seedlings. Tomato seed germination was evaluated after 13 days of treatment (Fig. 1Ab). No significant differences in percent germination (with respect to controls) were found in tomato seeds exposed to ZnO concentrations between 50 and 400 mg L⁻¹ (Fig. 1Ab). However, approximately a 20 % germination reduction was observed in tomatoes treated with 1600 mg L⁻¹ of ZnO NPs. On the other hand, as shown in Fig. 1Ac, the percent germination of cucumber showed a significant increase in ZnO levels of 400 and 1600 mg L⁻¹, as compared to controls. In this case, germination was determined after 12 days of exposure. Up to now, it is unknown if the ZnO NPs interfere with the production of gibberellins, a phytohormone that promotes seed germination. The results of the present study differ from those reported by Lin and Xing [14], who found that 2000 mg L⁻¹ of 20 nm ZnO NPs did not affect germination of cucumber. This suggests that the difference in NP size plays a role in phytotoxicity; however, varietal differences could also drive the results.

Several reports indicate that NP type and solubility are important factors related to the toxicity in plants [11,29]. In addition, Brunner et al. [30] stated that NP solubility plays an important role in the toxicity of metal oxide nanomaterials in human mesothelioma and in rodent fibroblast cells. They found that both cell cultures (human MSTO-211H and rodent 3T3 cells) died when treated with 15 mg L⁻¹ of ZnO. However, the same cell cultures were not affected by tricalcium phosphate NPs at this same concentration.

In order to determine the role of the dissociated Zn^{2+} ions in the toxicity of ZnO NPs, the quantification of Zn^{2+} was performed in all ZnO NP suspensions. Zn^{2+} concentrations were found in a range between 3.0 and 25 mg L^{-1} . Based on these findings, selected concentrations for seed exposure were of 0, 0.05, 0.5, 5, 10, 50, and 250 mg L^{-1} Zn^{2+} . These were chosen in order to include at least one order of magnitude to the left and right of the minimum and maximum Zn^{2+} concentration value determined in the suspensions.

The results for the germination of the plant species treated with Zn^{2+} ions are shown in Fig. 1B. The pH of $\text{Zn}(\text{NO}_3)_2$ solutions was adjusted to the same pH as that of ZnO NP suspensions ($\text{pH} = 7.0 \pm 0.1$). A 40 % reduction in germination was observed in alfalfa seeds treated with 50 and 250 mg L^{-1} of Zn^{2+} (Fig. 1Ba). However, no statistical difference was observed. A slight increase in alfalfa germination was observed at 0.05 mg L^{-1} of Zn^{2+} ; however, germination was reduced by 34 % at 250 mg L^{-1} . This value was significantly different from that of the 0.05 mg L^{-1} Zn^{2+} . Al-Yemeni and Al-Helal [31] have reported a reduction of 30 % in alfalfa germination with 5 mM ZnCl_2 ($\approx 330 \text{ mg L}^{-1}$ Zn^{2+}), which is higher than the concentration used in the present study. The difference could be attributed to dissimilarities in varietal constitution. No significant differences were observed in tomato germination with Zn^{2+} concentrations between 0 and 50 mg L^{-1} (Fig. 1Bb). However, at 250 mg L^{-1} , a dramatic reduction of 84 % was observed. It has been reported that low Zn^{2+} concentrations promoted tomato germination [32], although reports indicated that, at certain levels, Zn^{2+} inhibited aquaporins involved in seed germination [33]. Further experiments can be performed in order to determine if at this Zn^{2+} concentration, inhibition of germination in tomato was due to the inhibition of aquaporins. Figure 1Bc shows no significant effects on cucumber germination with the exception of the treatment at 10 mg L^{-1} Zn^{2+} . Ozdener et al. [34] demonstrated that Zn^{2+} at concentrations up to 1000 mg L^{-1} did not affect root elongation of *Eruca sativa* germinated in the Zn treatment. According to El-Ghamery et al. [35], the effect of metal treatment on seed germination depends on the time of exposure, metal concentrations in the media, and plant species. Alfalfa belongs to the *Fabaceae* family, while tomato and cucumber correspond to the *Solanaceae* and *Cucurbitaceae* families, respectively. Further biochemical and analytical studies may provide a better insight into the nature of plant resistance and tolerance to ZnO NPs and Zn^{2+} ions.

Effect of ZnO NPs and Zn^{2+} on root seedling growth

The effects of ZnO NPs and Zn^{2+} ions on root growth of alfalfa, tomato, and cucumber seedlings are presented in Table 1. Inhibitory concentrations of 50 % (IC_{50}) were observed in alfalfa and tomato seedlings treated with 800 and 1600 mg L^{-1} of ZnO NPs. However, cucumber response to the NPs was positive as hormesis was observed [36]. Seedlings germinated in 200, 400, and 800 mg L^{-1} of ZnO NPs were 2.7, 1.9, and 1.4 times larger ($P < 0.05$) than control roots, respectively. These results agree with those found by Lin and Xing [14], who reported that 2000 mg L^{-1} of ZnO (20 nm in size) affected root growth in radish, rape, ryegrass, and lettuce seedlings raised from seeds previously soaked in NP suspensions. Lin and Xing [14] have stated that toxicity of nanomaterials is due not only to the presence of ZnO NPs but also to Zn^{2+} ions released in the media. Cell walls in plants contain small pores ranging from 5–20 nm, which allow for transportation of NPs less than 20 nm through the cell wall and the bilayer lipid membrane of the plasma membrane [37]. Hitherto, it is not known if the NPs interact with the production of auxins, phytohormones that promote root elongation. Furthermore, since the NPs used in this investigation are smaller than 20 nm, it is very likely that they were able to cross the cell wall; thus, affecting the normal germination and growth process.

A significant decrease (about 30 %) in alfalfa root length occurred in the treatment of 50 mg L^{-1} Zn^{2+} (Table 1). In addition, at 250 mg L^{-1} of Zn^{2+} , the IC_{50} was observed. *Nigella sativa* and *Triticum aestivum* [35] were more sensitive to Zn^{2+} than the plant species used in the present study, since in those two species 25 mg L^{-1} of Zn^{2+} affected root growth. According to Herren and Feller [38] and Atici et

al. [39], Zn is an essential element for plant growth but at relatively high concentrations can interfere with several metabolic processes and can also modify the mineral root uptake.

Table 1 Average root length (cm) of seedlings germinated with 0–1600 mg L⁻¹ of ZnO NPs and 0–250 mg L⁻¹ of Zn²⁺ supplied as Zn(NO₃)₂.

ZnO (mg L ⁻¹)	Alfalfa (cm)	Tomato (cm)	Cucumber (cm)
0	1.5 ± 0.1a	1.0 ± 0.0a	1.7 ± 0.1de
50	1.2 ± 0.1b	0.9 ± 0.0a	1.9 ± 0.1cd
100	1.5 ± 0.1a	0.72 ± 0.1bc	1.7 ± 0.1de
200	1.3 ± 0.1ab	0.74 ± 0.0b	4.6 ± 0.1a
400	1.2 ± 0.0b	0.7 ± 0.0bcd	3.3 ± 0.1b
800	0.7 ± 0.0c	0.57 ± 0.0d	2.3 ± 0.1c
1600	0.8 ± 0.0c	0.58 ± 0.0cd	1.5 ± 0.0e
Zn²⁺ (mg L⁻¹)			
0	1.6 ± 0.1a	0.9 ± 0.0a	1.7 ± 1.0b
0.05	1.6 ± 0.1a	0.9 ± 0.0a	1.8 ± 1.0ab
0.5	1.6 ± 0.1a	0.8 ± 0.0ab	2.1 ± 1.0a
5	1.4 ± 0.1a	0.7 ± 0.0b	1.9 ± 1.0ab
10	1.5 ± 0.1a	0.9 ± 0.1a	0.9 ± 1.0d
50	1.1 ± 0.0b	0.7 ± 0.0ab	1.3 ± 1.0c
250	0.8 ± 0.0b	0.4 ± 0.0c	1.2 ± 0.0cd

*One-way ANOVA and Tukey's test were used to determine statistical significance of the differences between treatment means. The level of significance was at $p \leq 0.05$.

The IC₅₀ in cucumbers was observed in seedlings treated with 10 mg L⁻¹ of Zn²⁺. However, at higher concentrations (50 and 250 mg L⁻¹ Zn²⁺), the root length reduction was less conspicuous since a decrease of 24 and 29 %, respectively, was observed. Differences in plant species is the reason for dissimilar Zn uptake, tolerance, and toxicity [40].

Effect of ZnO NPs and Zn²⁺ on biomass production

The DW of 10 seedlings of alfalfa, tomato, and cucumber germinated in ZnO NP concentrations varying from 0 to 1600 mg L⁻¹, and 0–250 mg L⁻¹ Zn²⁺ is shown in Table 2. It was observed that alfalfa biomass production was not significantly affected by the ZnO NP levels used in this study, although this species accumulated the most Zn. In tomatoes, seedlings increased the biomass production (35 % with respect to controls) when seeds were germinated with 800 mg L⁻¹ ZnO NPs. Cucumber biomass yield displayed a U-shaped response, since concentrations between 100 and 400 mg L⁻¹ of ZnO NPs decreased biomass by about 10 %. Lower and higher concentrations tended to increase seedling weight. The increase in biomass and root growth of plants exposed to NPs has also been observed by Gao et al. [41], who reported that single fresh weight and DW of soil-grown spinach plants treated with nanonutrient TiO₂ NPs increased by 60.21 and 70.32 %, respectively.

For comparison purposes, the biomass production with Zn²⁺ ions was recorded. As seen in Table 2, a significant increase in biomass weight was obtained by alfalfa and tomato seedlings treated with 250 mg L⁻¹ Zn²⁺ (59 and 390 %, respectively), as compared to controls. These results differ from those obtained with ZnO NPs, barring the tomatoes at 800 mg L⁻¹ of NPs. There is no clear explanation

tion for these results, but it is hypothesized that Zn^{2+} ions are more readily available towards activating plant genes involved in biomass production. This hypothesis is supported by results found by Puzio et al. [42]. Biomass production in cucumber seedlings was not affected by the different concentrations of Zn^{2+} . These results were similar to the results observed in seedlings treated with the ZnO NPs, which corroborate the differential tolerance of plant species to Zn and ZnO NPs.

Table 2 Biomass weight (mg) of 10 seedlings germinated with (a) 0–1600 mg L^{-1} of ZnO NPs; and (b) 0–250 mg L^{-1} of Zn^{2+} supplied as $\text{Zn}(\text{NO}_3)_2$.

ZnO (mg L^{-1})	Alfalfa (mg)	Tomato (mg)	Cucumber (mg)
0	13.1 ± 0.3	$14.8 \pm 0.8\text{b}$	$19.3 \pm 0.2\text{a}$
50	10.8 ± 0.9	$16.7 \pm 0.5\text{ab}$	$17.8 \pm 0.4\text{ab}$
100	13.1 ± 0.9	$17.1 \pm 0.5\text{ab}$	$17.1 \pm 0.4\text{b}$
200	15.1 ± 0.9	$16.3 \pm 1.1\text{ab}$	$16.8 \pm 0.2\text{b}$
400	12.4 ± 0.5	$15.0 \pm 0.4\text{b}$	$17.1 \pm 0.1\text{b}$
800	10.3 ± 2.7	$20.0 \pm 1.5\text{a}$	$17.6 \pm 0.7\text{ab}$
1600	11.8 ± 1.6	$16.9 \pm 0.99\text{ab}$	$17.6 \pm 0.4\text{ab}$
Zn^{2+} (mg L^{-1})			
0	$14.4 \pm 1.3\text{b}$	$15.6 \pm 0.2\text{b}$	19.3 ± 0.16
0.05	$15.5 \pm 0.6\text{ab}$	$10.8 \pm 1.7\text{b}$	19.2 ± 0.4
0.5	$15.3 \pm 0.4\text{ab}$	$15.8 \pm 0.6\text{b}$	18.4 ± 2.1
5.0	$15.0 \pm 0.7\text{ab}$	$15.6 \pm 0.6\text{b}$	18.5 ± 1.3
10	$15.3 \pm 0.9\text{ab}$	$18.6 \pm 1.4\text{b}$	15.5 ± 3.5
50	$16.1 \pm 0.2\text{ab}$	$16.2 \pm 0.7\text{b}$	13.6 ± 3.6
250	$22.9 \pm 4.0\text{a}$	$76.7 \pm 14.5\text{a}$	19.8 ± 0.0

*One-way ANOVA and Tukey's test were used to determine statistical significance of the differences between treatment means. The level of significance was at $p \leq 0.05$.

Zn uptake by seedlings germinated in ZnO NPs and Zn^{2+}

Concentrations of Zn in seedlings grown with ZnO NPs are shown Fig. 2a. As seen in Fig. 2a, the concentration of Zn in tissues increased as the external concentration of NPs increased. At 1600 mg L^{-1} of NPs, the concentration of Zn (mg kg^{-1} DW) was: alfalfa (4700) > tomato (2100) > cucumber (1600). The tolerance of diverse species to metal stress causes variations in metal accumulation. As described previously, some of the ZnO NPs were dissolved in the media to form hydrated Zn^{2+} cations available for seedling uptake [9]. However, in the present study, the amount of Zn^{2+} ions released at 1600 mg L^{-1} of NPs was only 25 mg Zn L^{-1} . Thus, the most Zn accumulation came from the NPs. This was corroborated in Fig. 2b, which shows the concentration of Zn in seedlings germinated with 0–250 mg L^{-1} of Zn^{2+} . Similar to the results found with the NPs, as the external Zn^{2+} increased, Zn levels in tissues increased, thus presenting a concentration order as the one observed in seedlings grown with the NPs. At 250 mg L^{-1} Zn^{2+} treatment, the highest Zn concentration was found in alfalfa (3500 mg Zn kg^{-1} DW) followed by tomato (1100 mg Zn kg^{-1} DW). Differences in cell wall composition from diverse plant species and genotypes can result in higher or lower uptake of metal ions by plant roots, especially in non-metal hyperaccumulators [43].

Fig. 2 Zn concentration in ■ alfalfa, □ tomato, and ■ cucumber seedlings germinated with (a) ZnO NP suspensions (at 0–1600 mg L⁻¹); and (b) 0–250 mg L⁻¹ Zn²⁺ supplied as Zn(NO₃)₂. Data are means of three replicates ± SE. Lowercase letters indicate statistically significant differences in Zn content in each plant species at $P \leq 0.05$.

Bulk X-ray absorption near-edge spectroscopy (XANES) results

Figure 3 shows the characteristic Zn K-edge of XANES spectra from the ZnO NPs, Zn(NO₃)₂, and the XANES spectra of plant samples treated with ZnO NPs and Zn²⁺. Results showed Zn K-edge at 9659 eV, which corresponds to the electronic transition from the Zn 1s orbital to the Zn 4p orbital. In addition, XANES spectra of all ZnO NP-treated seedlings indicated that the pre-edge and edge energy positions of samples are at the same position as that of the Zn(NO₃)₂, which indicates that Zn in all

Fig. 3 XANES K-edge spectra (9659 eV) of ZnO NPs, Zn(NO₃)₂ model compounds and spectra from alfalfa, tomato, and cucumber seedlings treated with 1600 mg L⁻¹ of ZnO NPs and 250 mg L⁻¹ of Zn²⁺ supplied as Zn(NO₃)₂.

plant species was as Zn(II). In addition, it is shown that XANES spectra of Zn in plant tissues are different from that of ZnO NPs, suggesting either an absence or a biotransformation of the NPs.

The Fourier transformed EXAFS for seedlings treated with 250 mg kg⁻¹ of Zn²⁺ and 1600 mg kg⁻¹ of ZnO NPs are shown in Fig. 4, and the FEFF fittings of Zn in seedlings and ZnO NPs are shown in Table 3.

The FEFF fittings from seedling spectra and the model compounds Zn(OH)₂ and ZnO suggested that Zn inside tissues was coordinated to six O and two Zn atoms. The Zn–O interactions in samples were found between 1.86 and 2.06 Å, and Zn–Zn interactions between 3.09 and 3.17 Å. This again suggests that Zn in tissues was not as ZnO NPs. These same findings have been reported for the desert species *P. juliflora-velutina*, *S. tragus*, and *P. florida* when they were germinated in ZnO NPs [19].

Fig. 4 Fourier transformed EXAFS from 2.0 to 12.2 Å of the Zn taken up by seedlings germinated with (I) 250 mg L⁻¹ Zn²⁺, and (II) 1600 mg L⁻¹ ZnO NPs. At the top right of each graph, the experimental k^3 EXAFS (dotted line), fitting (solid line), and back transform (dashed line) are shown. Lowercase letters indicate plant species (a) alfalfa, (b) tomato, and (c) cucumber.

Moreover, mesquite germinated in a free-NP media and transferred to a ZnO NP suspension, presented the same result [44] as Zn spectra resembles that of Zn(NO₃)₂. XAS studies of *Datura innoxia* plants treated with Zn ions have shown that Zn in tissues coordinated mainly to oxygen atoms from carboxylate groups of organic acids [45]. More recently, Salt and et al. [46] have reported that Zn (from Zn²⁺ ions) in the hyperaccumulator *Thlaspi caerulescens* accumulated in roots mainly coordinated with histidine, while in shoot it was found as hydrated cation. The bulk XAS results from whole seedlings of the present study have shown that the three plant species store Zn in a very similar chemical structure regardless of the form (either as NP or an ion) in which Zn is fed. With the use of electron microprobe and confocal microscopy, Zhao et al. [47] have recently demonstrated that ZnO NPs enter into the xylem in corn. Thus, it is very likely that both ZnO NP storage and biotransformation occur in plants. Further microspectroscopy studies will provide additional information on this matter.

Table 3 FEFF fittings of ZnO NPs and Zn in seedlings germinated with (a) 1600 mg L⁻¹ of ZnO NPs; and (b) 250 mg L⁻¹ of Zn²⁺ supplied as Zn(NO₃)₂.

Sample	Interaction	CN	R(Å)	σ ² (Å ²)
ZnO NPs solid	Zn–O	4	1.95	0.0052
	Zn–Zn	12	3.21	0.0100
	Zn–Zn	9	3.76	0.0101
Zn(NO ₃) ₂ -treated alfalfa seedlings	Zn–O	4	1.97	0.0020
	Zn–O	2	1.86	0.0050
	Zn–Zn	2	3.17	0.0071
ZnO NP-treated alfalfa seedlings	Zn–O	4	1.94	0.0074
	Zn–O	2	1.88	0.0020
	Zn–Zn	2	3.10	0.0097
Zn(NO ₃) ₂ -treated tomato seedlings	Zn–O	4	1.94	0.0049
	Zn–O	2	2.09	0.0036
	Zn–Zn	2	3.14	0.0030
ZnO NP-treated tomato seedlings	Zn–O	4	1.90	0.0042
	Zn–O	2	2.11	0.0098
	Zn–Zn	2	3.16	0.0061
Zn(NO ₃) ₂ -treated cucumber seedlings	Zn–O	4	1.88	0.0122
	Zn–O	2	1.87	0.0055
	Zn–Zn	1	3.16	0.0089
ZnO NP-treated cucumber seedlings	Zn–O	4	1.87	0.0052
	Zn–O	2	2.09	0.0082
	Zn–Zn	1	3.15	0.0082

CONCLUSIONS

This study demonstrated that ZnO NPs affected seed germination of alfalfa and tomato. A 40 % reduction in alfalfa germination was observed in seeds soaked in 800 and 1600 mg L⁻¹ of ZnO NPs while a 20 % germination reduction was observed in tomato seeds treated with 1600 mg L⁻¹ of ZnO NPs. On the other hand, the percent germination of cucumber showed a significant increase in ZnO levels of 400 and 1600 mg L⁻¹. Inhibitory concentrations of 50 % (IC₅₀) were observed in alfalfa and tomato seedlings treated with 800 and 1600 mg L⁻¹ of ZnO NPs. Cucumber root seedlings were 170 % larger than control roots when the seeds were soaked in 200 mg L⁻¹ of ZnO NPs. Higher concentrations of NPs resulted in a reduction in biomass production in this plant species. Zn concentration in tissues increased as the external concentration of NPs increased. At 1600 mg L⁻¹ of NPs, the concentration of Zn (mg kg⁻¹ DW) was: alfalfa (4700) > tomato (2100) > cucumber (1600). Differences in plant species is the reason for dissimilar ZnO uptake, tolerance, and NP toxicity. Bulk XAS analysis showed that it is very likely that ZnO NPs are biotransformed in plants. However, it is necessary to determine if the NPs were biotransformed on/in the root surface and to what extent this process occurred in the growth media.

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