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Effect of ZnO nanoparticles on corn seedlings at different temperatures; X-ray absorption spectroscopy and ICP/OES studies

Martha Laura López-Moreno [a,b,*], Guadalupe de la Rosa [c], Gustavo Cruz-Jiménez [d], Laura Castellano [c], Jose R. Peralta-Videa [b,e,f], Jorge L. Gardea-Torresdey [b,e,f]

[a] University of Puerto Rico at Mayagüez, P.O. Box 9019, Mayagüez 00681-901, Puerto Rico
[b] Chemistry Department, The University of Texas at El Paso, El Paso, TX 79968, United States
[c] Division of Science and Engineering, University of Guanajuato, Loma del Bosque, 103, Col. Lomas del Campestre, C.P. 37150, Guanajuato, Mexico
[d] Division of Natural and Exact Sciences, Campus Guanajuato, University of Guanajuato, Col. N. Alta s/n, Guanajuato, C.P. 36050, Mexico
[e] Environmental Science and Engineering PhD Program, the University of Texas at El Paso, 500 West Univ. Ave, El Paso, TX 79968, United States
[f] University of California Center for Environmental Implications of Nanotechnology (UC CEIN), The University of Texas at El Paso, El Paso, TX 79968, United States

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ABSTRACT

Previous studies have shown that ZnO nanoparticles (NPs) affect corn germination and root growth. However, there is a lack of information about the effects of these NPs at different temperatures. In this study, corn seedlings were exposed to ZnO NPs (24 ± 3 nm) at 0–1600 mg L^{-1} and ionic Zn 0–250 mg L^{-1} for 15 days. Germination, root elongation, Zn uptake and oxidation state, enzyme activity and protein expression were analyzed. At 20 and 25 °C, 400 mg ZnO NPs L^{-1} significantly reduced the germination (40 and 53% respectively), while no effect of Zn^{2+} was observed. Temperature and Zn concentration affected root growth. At 20 °C, ZnO at 50, 400, and 1600 mg L^{-1} reduced root growth by 18, 47, and 26% respectively. At 25 °C, 100 and 800 mg L^{-1} increased root growth by 22 and 27%, while at 30 °C, 100 mg L^{-1} reduced root growth by 42%. At 30 °C, 0.1 mg L^{-1} of Zn^{2+} increased the growth by 50%. Zn accumulation in seedlings was significant only at 1600 mg L^{-1} ZnO and 250 mg L^{-1} Zn^{2+}. Ascorbate peroxidase activity increased by 24 and 57% under exposure to ZnO at 400 and 1600 mg L^{-1} at 25 °C. At any temperature the XAS analyses showed presence on NPs in roots. Exposure to ZnO NPs did not show changes in protein expression; however, a protein band with molecular weight of 85 kDa decreased its expression at 30 °C, while a protein of 75 kDa increased its expression at 30 °C. This study suggests that temperature may alter the way the ZnO NPs interact with plants.

1. Introduction

Nanomaterials (NMs) have been fundamental to the development of nanotechnologies. Different NMs have been synthesized in recent years to be used in personal care products, industry, medicine and agriculture [1]. Although the number of nanomaterials and nanoproducts steadily increase in the market, their interactions with living organisms are not well understood. Thu, rules to properly manage and dispose of NMs and nanoproducts after end user application are still missing. Plants are exposed to NMs, either intentional or unintentional. Intentional exposure includes the use of nano-enabled pesticides and fertilizers in agricultural activities [2–5]. Previous reports indicate that nanoparticulate ZnO and MgO have the potential to be used to combat pathogenic fungi including *Alternaria alternate, Fusarium oxysporum, Rhizopus stolonifer and Mucor plumbeus* [6]. Other reports suggest the use of nano-fertilizers to increase nutrient availability to plants, avoiding excessive input of chemical elements into the environment. However, maximum benefits/threats from ions released by nanoparticles (NPs) into plant cells, compared with those from common fertilizers, are not well documented [7]. Zinc and boron nano-fertilizers have been reported to increase fruit yield and quality of pomegranate (*Punica granatum cv. Ardestani*) without affecting the physical characteristics of fruit [8]. ZnO NPs have also been used as fungicide in agriculture. Ghosh et al., [9] evaluated the toxicity of ZnO NPs in *Allium cepa*, *Nicotiana tabacum*, and *Vicia faba*. The authors observed that ZnO NPs promoted cell death in root of *A. cepa* and showed higher toxicity, compared with the Zn bulk form.

Corn (*Zea mays*) is one of the main crops in the world, with a production of 780 metric tons per year. USA, China, Mexico, and Argentina are the top corn producers for staple food and Brazil for ethanol production. According to the USDA, >90 million acres were planted with corn in 2015 [10]. However, crop production is affected by genetic or environmental factors. Ambient temperature, light, water quality and quantity, and contaminants, are among the last. Temperature plays a very

* Corresponding author at: University of Puerto Rico at Mayagüez, P.O. Box 9019, Mayagüez 00681-901, Puerto Rico.
E-mail address: martha.lopez@upr.edu (M.L. López-Moreno).

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important role in plant growth, since warming or cooling conditions affect plant-microbe interactions, nutrient availability and transport inside plant tissues, and flowering time, among others [11].

It is well known that temperature is one of the most critical factors affecting seed germination [12,13]. Kumar et al. [14] reported the optimal germination temperature for Kalmegh (*Andrographis paniculata Wall. Ex Nees*) was 25 °C, in comparison to 20 and 30 °C. If, besides temperature, seeds are exposed to additional abiotic stress such as metals or metal oxide nanoparticles, the emergence of radicle can be prevented [15,16]. Although many reports have described the effects of NMs on seed germination to the best of the authors' knowledge, there are no reports on the effect of temperature and ZnO NPs as abiotic stress elicitors on germination and growth of corn plants. In this study, corn seeds were exposed to ZnO NPs at 0–1600 mg L^{-1} and Zn^{2+} from $Zn(NO_3)_2$ 0–250 mg L^{-1} and set at 20, 25, and 30 °C. In this study, X-ray Absorption Spectroscopy (XAS) and Inductively Plasma Optical Emission Spectroscopy (ICP/OES) techniques were used to evaluate Zn uptake, oxidative stress, as well as the oxidation state of Zn in ZnO NPs exposed to corn plants.

2. Materials and methods

ZnO NPs were obtained from Melliorum technologies (Rochester, NY). Previous characterization of NPs was reported by Keller et al. [17]. Zinc nitrate $Zn(NO_3)_2$ 99% purity was purchased from Alfa Aesar.

2.1. Preparation of ZnO NP suspensions and Zn^{2+} solutions

Suspensions of ZnO NPs were prepared in Millipore water at the following concentrations: 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 [18]. The pH of these solutions was about 7.0 ± 0.1. Solutions of Zn^{2+} were prepared from $Zn(NO_3)_2$ at 0, 0.05, 0.5, 5, 10, 50, and 250 mg Zn^{2+} L^{-1} in Millipore water and the pH was adjusted to 7.0 ± 0.2.

2.2. Germination experiments

Corn (Golden variety) kernels were set in a 4% $NaClO_4$ solution for disinfection for 30 min and rinsed three times with sterilized Millipore water. Filter paper was used as the inert material for germination. The paper was cut to fit the Petri dishes and sterilized to avoid contamination. Ten kernels were placed between two pieces of paper and watered with 5 mL of ZnO or Zn^{2+} suspension/solution [18]. Two milliliters of antimycotic/antibiotic solution (A5955, Sigma Aldrich) were added to the top of the second filter paper. Petri dishes were covered with aluminum paper and placed at 20, 25, and 30 °C. According to USEPA [19], seeds were allowed to germinate until about a 65% of the root controls were at least 5 mm long. Percent of germination was calculated in every treatment and every temperature. Seedlings were rinsed with 0.01 M HNO_3 and Millipore water to eliminate any surface metal. Root and stem length of 10 seedlings per replicate were measured and oven dried at 70 °C for two days. The average weight was calculated on a 10-seedling basis. A second set of treatments at same temperatures and concentrations were placed for oxidative stress experiments.

2.3. Quantification of Zn in corn seedlings

After 15 d of exposure to different treatments and ZnO and Zn^{+2} concentrations, seedlings were digested on a CEM microwave oven (CEM Corporation Mathews, NC; USA) with 3 mL of plasma pure HNO_3 according to USEPA 3051 method [20]. Samples were diluted to 25 mL with Millipore water and Zn content was measured by ICP/OES Perkin Elmer Optima 4300 DV (Perkin-Elmer Optima 4300 DV, Shelton, CT). A blank and a standard were read every ten samples for QC/QA purposes.

2.4. Determination of catalase specific activity

Catalase activity was determined following the procedure previously described by Gallego et al. [21]. Corn seedlings exposed to different treatments for 15 d were homogenized in 0.1 M KH_2PO_4 buffer at pH 7.4 ± 0.1 and then centrifuged at 9000 rpm for 10 min in a refrigerated centrifuge (MWP Med. Instruments, Warsaw, Polland).The supernatant was placed in a quartz cuvette with 10 mM H_2O_2 in phosphate buffer and the absorbance was recorded at 240 nm using a UV/VIS Spectrophotometer (UV–Visible Spectrophotometer, Evolution 60S, Thermo Scientific, China).

2.5. Determination of ascorbate peroxidase activity

Ascorbate peroxidase was determined according to the procedure previously described by Murgia et al. [22], with slight modifications. Seedlings were homogenized in a solution containing 0.1 M KH_2PO_4 (buffer at pH 7.4 ± 0.1), 25 mM ascorbate and 17 mM H_2O_2. The mixture was homogenized and the absorbance was recorded at 265 nm in a UV/VIS Spectrometer (UV–Visible Spectrophotometer, Evolution 60S, Thermo Scientific, China). Bovine serum albumin (BSA) was used as a standard to quantify the protein content in corn seedlings.

2.6. X-ray absorption spectroscopy experiments (XAS)

Corn seedlings exposed to 1600 mg ZnO L^{-1} and 250 mg Zn^{2+} L^{-1} for 15 d were frozen in liquid nitrogen and lyophilized on a Labconco FreeZone 4.5 freeze-dryer at −53 °C and 0.140 mBar pressure for 3 days. Powdered dry tissues were placed on aluminum sample holders and covered with Mylar© Tape.

XAS experiments were done at beam Line 7–3 at the Stanford Synchrotron Radiation Laboratory (SSRL). A Canberra 29-element array germanium detector was used to monitor Zn K_α fluorescence spectra. 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 spectra from Zn foil at the time of data collection. Data analysis was done using Athena software [23]. Zinc edge energy was calibrated using the edge position of an internal zinc foil with edge energy of 9665 eV. AUTOBK algorithm was used in spectra background subtraction according to Newville et al. [24]. Edge-step data normalization was determined by a linear pre-edge subtraction and regression of a quadratic polynomial beyond the edge. Polynomial difference is extrapolated to E_0 and used as normalization constant in the relationship $\chi(E) = [\mu(E) - \mu_0(E)] / \mu_0(E_0)$. Normalized data is obtained after subtraction of the curvature of the regressed quadratic and the difference in slope between the post- and pre-edge polynomials after the edge. Main parameters used for fine tune normalization and background removal were: edge-step = 0.83, normalization range 150–663, k-weight = 2, and E shift = 1.

2.7. SDS-PAGE analysis

Total protein was extracted from corn seedlings exposed to different concentrations of ZnO NPs and Zn^{2+}. Protein concentration of each extract was determined using a Bradford assay. Furthermore, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [25]. Briefly, the corn seedling extracts (15 μg of protein) were mixed with loading buffer, subjected to electrophoresis using 12% polyacrylamide gels at a constant voltage (90 V). Molecular weight standards were obtained from BIO-RAD. Gels were stained with Coomasie Brilliant Blue R-250.

2.8. Statistical analysis

Data from all experiments was reported as mean ± Standard Deviation (SD). A one way ANOVA analysis followed by a Tukey's H.S.D. was