

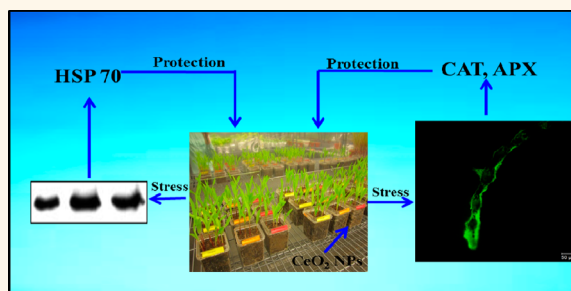
Stress Response and Tolerance of *Zea mays* to CeO₂ Nanoparticles: Cross Talk among H₂O₂, Heat Shock Protein, and Lipid Peroxidation

Lijuan Zhao,^{†,¶} Bo Peng,[§] Jose A. Hernandez-Viezas,^{†,¶} Cyren Rico,^{†,¶} Youping Sun,^{||} Jose R. Peralta-Videa,^{†,¶} Xiaolei Tang,[§] Genhua Niu,^{||} Lixin Jin,[⊥] Armando Varela-Ramirez,[§] Jian-ying Zhang,[§] and Jorge L. Gardea-Torresdey^{†,‡,¶,*}

[†]Chemistry Department, [‡]Environmental Science and Engineering PhD Program, [§]Department of Biological Sciences, Border Biomedical Research Center, and [⊥]Department of Geological Sciences, The University of Texas at El Paso, 500 West University Avenue, El Paso, Texas 79968, United States, ^{||}Texas AgriLife Research Center at El Paso, Texas A&M University System, El Paso, Texas 79927, United States, and [¶]UC Center for Environmental Implications of Nanotechnology (UC CEIN), The University of Texas at El Paso, 500 West University Avenue, El Paso, Texas 79968, United States

ABSTRACT The rapid development of nanotechnology will inevitably release nanoparticles (NPs) into the environment with unidentified consequences. In addition, the potential toxicity of CeO₂ NPs to plants and the possible transfer into the food chain are still unknown. Corn plants (*Zea mays*) were germinated and grown in soil treated with CeO₂ NPs at 400 or 800 mg/kg. Stress-related parameters, such as H₂O₂, catalase (CAT), and ascorbate peroxidase (APX) activity, heat shock protein 70 (HSP70), lipid peroxidation, cell death, and leaf gas exchange were analyzed at 10, 15, and 20 days post-germination. Confocal laser scanning microscopy was used to image H₂O₂

distribution in corn leaves. Results showed that the CeO₂ NP treatments increased accumulation of H₂O₂, up to day 15, in phloem, xylem, bundle sheath cells and epidermal cells of shoots. The CAT and APX activities were also increased in the corn shoot, concomitant with the H₂O₂ levels. Both 400 and 800 mg/kg CeO₂ NPs triggered the up-regulation of the HSP70 in roots, indicating a systemic stress response. None of the CeO₂ NPs increased the level of thiobarbituric acid reacting substances, indicating that no lipid peroxidation occurred. CeO₂ NPs, at both concentrations, did not induce ion leakage in either roots or shoots, suggesting that membrane integrity was not compromised. Leaf net photosynthetic rate, transpiration, and stomatal conductance were not affected by CeO₂ NPs. Our results suggest that the CAT, APX, and HSP70 might help the plants defend against CeO₂ NP-induced oxidative injury and survive NP exposure.



KEYWORDS: CeO₂ NPs · *Zea mays* · H₂O₂ · stress enzymes · cell death · photosynthesis

The field of nanotechnology opens up potential novel applications for agriculture. Large amounts of nanoparticles (NPs) are administered to crops as novel delivery tools for fertilizers, herbicides, and pesticides.¹ However, the toxicity of NPs to plants has to be considered before their wide application in agriculture. Previous studies evaluated the phytotoxicity of various NPs to plants by measuring some growth-related parameters, such as germination rate, root elongation, and biomass production.^{2–8} Lopez *et al.*, for the first time, demonstrated the effects of CeO₂ NPs to soybean (*Glycine max*) at the gene level.⁶ More recently, Khodakovskaya *et al.*⁹ discovered

that multiwalled carbon nanotubes induced up-regulation of the stress-related genes in tomato leaves and roots. Atha *et al.* showed that copper oxide NPs induced DNA damage in radish (*Raphanus sativus*) and ryegrass (*Lolium perenne* and *Lolium rigidum*).¹⁰ There is an extensive interest in investigating whether the NPs can induce stress response in edible plants and how the plants tolerate the stress induced by NPs. Currently, the scientific community is in need of deep insight into the interaction of NPs with terrestrial plants.

CeO₂ NPs are one of the 13 engineered nanomaterials included in the list of priority for immediate testing by the

* Address correspondence to jgardea@utep.edu.

Received for review July 3, 2012 and accepted October 10, 2012.

Published online October 10, 2012
10.1021/nn302975u

© 2012 American Chemical Society

Organization for Economic Cooperation and Development (OECD).^{11,12} CeO₂ NPs are widely used as polishing material, additive in glass and ceramic, fuel cell materials, in agricultural products, and automotive industry.^{13,14} Reports indicate that CeO₂ NPs are toxic to inhabitants of aquatic and terrestrial ecosystems like bacteria, green algae, fish, and soybean plants (*Glycine max*).^{6,13–16} Corn is the most widely grown grain crop in the Americas, with 332 million metric tons harvested annually in the United States.¹⁷ The fate and response of corn plant grown in NP-contaminated soil are unknown. Asli and Neumann¹⁸ reported that cell wall pores in corn primary roots have a mean diameter of 6.6 nm. Thus, particles, with a diameter far greater than the cell wall pore dimensions will not penetrate the root epidermal cell walls. Our previous study also showed that a very small percentage of CeO₂ NPs could be transferred from corn roots to shoots.¹⁹ This suggests that CeO₂ NPs could produce a low impact on the above ground plant parts. However, there is evidence that the attached NPs on root surfaces inhibited the water transpiration to the leaves.¹⁸ This indicates that NPs can cause impact on the upper part tissues, even though the main accumulation is localized in roots. Therefore, it would be very important to determine the stress in the upper plant parts, where there is low direct interaction with the CeO₂ NPs.

Tan *et al.*²⁰ showed that multiwalled carbon nanotubes triggered reactive oxygen species (ROS) generation in rice (*Oryza sativa*) cell lines. Shen *et al.* also showed the over accumulation of H₂O₂ in *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) leaf protoplasts induced by single-walled carbon nanotubes (SWCNTs).²¹ The NP-induced ROS generation and its potential damage to proteins and lipids in tissues have been reported less. In this study, corn plants were germinated and grown for 20 days in soil spiked with CeO₂ NPs at 0, 400, and 800 mg/kg levels. The uptake of Ce in corn root and shoot tissues during 20 days was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). The stress-related responses, such as H₂O₂, antioxidant enzyme, heat shock protein 70 (HSP70), lipid peroxidation, and cell death, were determined every 5 days during growth. Additionally, localization of H₂O₂ was visualized by using a fluorescence probe and confocal microscopy. This study is the first report, to our knowledge, which describes the stress response of edible plants exposed to CeO₂ NPs. Here, we demonstrated that CeO₂ NPs induced oxidative stress, while CAT and APX play important roles to combat the stress. Oxidative damage did not occur due to the defensive system. These findings greatly enhance the understanding of the interaction of CeO₂ NPs with crop plants.

RESULTS AND DISCUSSION

H₂O₂ Production and Localization. CeO₂ NPs have a large hydrodynamic size (2124 ± 59 nm) in soil solution.¹⁹

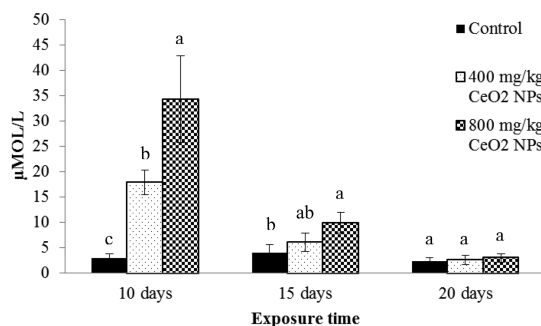


Figure 1. H₂O₂ production in shoots of corn plants germinated and grown in soil treated with CeO₂ NPs at 0, 400, and 800 mg/kg. All data show the means of a total of three replicates (nine plants/replicate). Error bars represent standard deviation. Means with same letters, within each exposure time, are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$).

Large-sized CeO₂ NP aggregates hardly penetrate root epidermis cell walls of corn plants. In addition, negatively charged CeO₂ NPs (−22.8 mV) may be retained by cation exchange sites of root cell walls, restricting their transport to shoots. Our previous study showed a ratio of Ce shoot/Ce root close to 0.1, indicating that most of the CeO₂ NPs accumulated in root tissues.¹⁹ This could indicate low toxicity to the above ground parts. However, over production of H₂O₂ was observed in shoots at the early stage of growth. As shown in Figure 1, both 400 and 800 mg/kg CeO₂ NP treatments induced H₂O₂ over accumulation in shoots at 10 days. The H₂O₂ concentrations in shoots treated with 400 and 800 mg/kg CeO₂ NPs were 5 and 10 times higher compared to control, respectively ($p \leq 0.05$). These results indicate that corn plants suffered from a concentration-dependent oxidative stress at the early growth stage. As the exposure time increased, the generation of H₂O₂ gradually decreased in all CeO₂ NP-treated plants and no over production was observed at 20 days (Figure 1). The H₂O₂ expression pattern suggested that the plants may initiate an adaptive response to tackle the over expressed H₂O₂ that could, otherwise, cause further damage. Although a previous report showed increased ROS production in *Arabidopsis* and in rice following SWCNT treatments,²¹ a time course following the treatment was not performed, which might have missed important adaptive responses. In the present study, it is very likely that an earlier response occurred before the tenth day; however, because the shoots' biomass was too small to be collected at day 5, the earlier response could not be recorded.

The knowledge about the contribution of different cells to the accumulation of H₂O₂ is important in order to gain deeper insights into the cellular response of plants to adverse conditions.²² Confocal laser scanning microscopy was used to image H₂O₂ distribution in corn plant shoots. As previously shown, the production of H₂O₂ at 800 mg/kg was highest at 10 days; thus, only 10 day plants' shoots were collected for H₂O₂ localization.

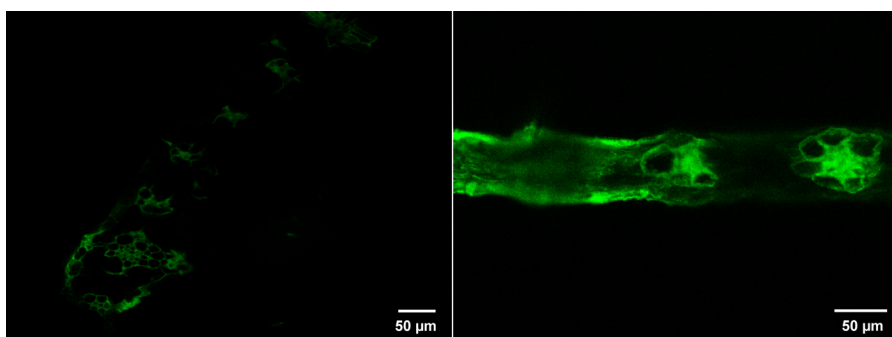


Figure 2. Confocal microscopy imaging of H_2O_2 production in shoot cross sections of corn plants germinated and grown for 10 days in soil treated with CeO_2 NPs at 0 and 800 mg/kg. DCF-DA fluorescence distribution in cross section of (left) control and (right) CeO_2 NP-treated corn plants.

The representative transversal sections of control and CeO_2 NP-treated corn leaves incubated with DCF-DA were imaged by confocal microscope (Figure 2).

The green fluorescence signals, which represent the presence of H_2O_2 , were found to be much brighter in the leaves of CeO_2 NP-treated plants, compared to control. This is consistent with the H_2O_2 concentration data (Figure 1). In control leaves, most of the H_2O_2 was localized at bundle sheaths (Figure 2, left), while in NP-treated plants the H_2O_2 was in epidermis cells, bundle sheath, and parenchyma (Figure 2, right). H_2O_2 in control leaves was associated with cell differentiation and elongation.²³

H_2O_2 Scavenging Enzyme Activity. The enzymes CAT and APX are known to be involved in the detoxification of H_2O_2 by converting the H_2O_2 to water and oxygen.²¹ We determined the dynamic changes of both enzymes at different growth stages. As shown in Figure 3, CAT activity was significantly up-regulated by CeO_2 NPs at 10 days ($p \leq 0.05$). The CAT activity, in shoots of plants treated with 400 and 800 mg/kg CeO_2 NPs, was 39 and 30 times higher than the control, respectively. It was noted that CAT activity was lower at 800 mg/kg CeO_2 NPs than at 400 mg/kg CeO_2 NPs. This may indicate that high levels of NPs lead to inhibition of CAT enzyme activity. As time passed, CAT activity declined. At 15 days, the over production of CAT activity was not observed.

Similar to CAT, the APX activity was also up-regulated at 10 and 15 days. APX activity, in shoots of plants treated with 400 and 800 mg/kg CeO_2 NPs, was 3 and 10 times higher than the control, respectively; the difference at 800 mg/kg was statistically significant compared to control ($p \leq 0.05$). High concentrations of CeO_2 NPs led to high levels of APX activity. CAT and APX activities showed a concomitant decrease in corn shoots with a decrease in H_2O_2 levels, which suggests that corn plants increased the production of both enzymes in order to degrade the excess of H_2O_2 . This explains why at 20 days the over production of H_2O_2 in shoots was not observed. CAT and APX activity in shoots may serve as a biomarker for CeO_2 NP stress in corn plants.

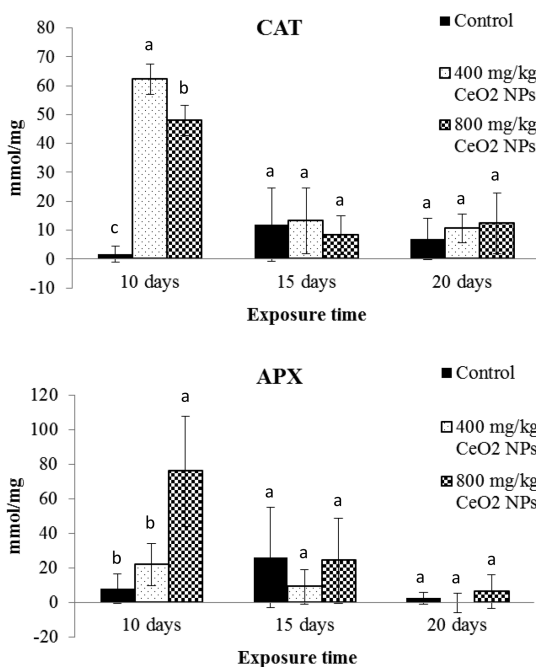


Figure 3. Antioxidant enzyme CAT and APX activity of corn plants germinated and grown in soil treated with CeO_2 NPs at 0, 400, and 800 mg/kg. All data show the means of a total of three replicates (nine plants/replicate). Error bars represent standard deviation. Means with same letters within each exposure time are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$).

Result from ICP-OES showed that the Ce concentration in corn shoots was very low at 10 days (not detected and 4.6 mg/kg, respectively, for 400 and 800 mg/kg treatments) (Figure S1 in Supporting Information), where the highest H_2O_2 production was found (18 and 34 $\mu\text{mol/L}$, respectively). Moreover, at day 20, the concentration of Ce in shoots was the highest (1 and 20 mg/kg, respectively, for 400 and 800 mg/kg treatments), while the H_2O_2 levels were similar to the control levels (2.6 and 3.0 $\mu\text{mol/L}$). This suggests that the oxidative stress observed in shoot tissues was not caused, directly, by the accumulated CeO_2 NPs. The oxidative stress observed in shoots must be related to the interaction of NPs with roots. There is evidence that the attached NPs on root surface inhibited the water

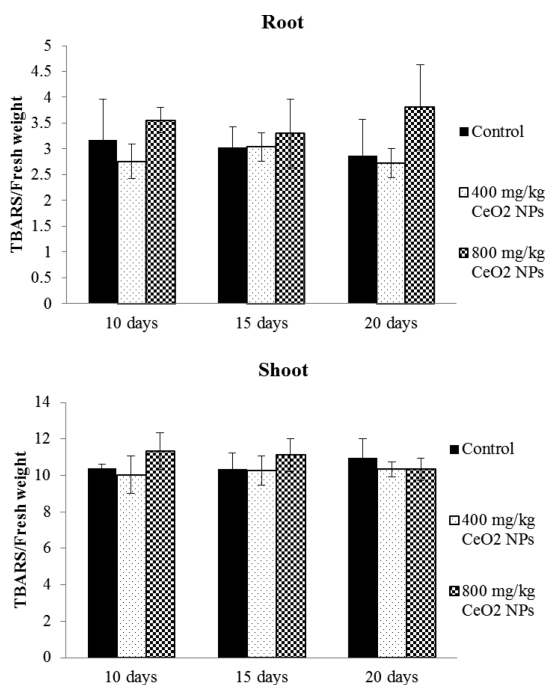


Figure 4. Biochemical detection of lipid peroxidation caused by CeO₂ NPs in roots and shoots of corn plants germinated and grown for 20 days in soil treated with CeO₂ NPs at 0, 400, and 800 mg/kg. All data show the means of a total of three replicates (nine plants/replicate). Error bars represent standard deviation. Means with same letters within each exposure time are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$).

transport, affecting the leaves' responses.¹⁸ There are other possible mechanisms here, as well (other than water transport), such as altered hormone production/distribution and impacts on endophytes. The changes in enzyme activities in shoots suggested that the root–NP interactions also affected the physiology of the shoots. As shown above, the average size of CeO₂ NP aggregates in soil solution was around 2 μm ; thus, the toxicity probably was produced by the interaction between the NP aggregates and the root, not because of the accumulation in shoots.

Lipid Peroxidation. To test if H₂O₂ accumulation causes membrane damage, lipid peroxidation in corn roots and shoots was analyzed. H₂O₂ acts as a signaling molecule and, at the same time, it has high permeability across membranes and can cause membrane damage. Whether H₂O₂ will act as a signaling or damaging molecule depends on the delicate equilibrium between its production and scavenging.²⁴ As shown in Figure 4, none of the treatments produced significant changes in thiobarbituric acid reacting substances (TBARS) concentrations, in both roots and shoots.

It is known that ROS molecules can rapidly attack lipids, leading to irreparable membrane damage. At 10 days, H₂O₂ was over produced in shoots of NP-treated plants; however, lipid peroxidation was not induced. Considering both CAT and APX activity data, it is concluded that both antioxidant enzymes successfully

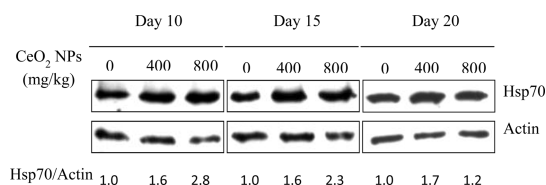


Figure 5. Western blot results of the expression level of HSP70 in roots of corn plant after 10, 15, and 20 days of growth in soil treated with CeO₂ NPs at 0, 400, and 800 mg/kg. Equal amounts of total root proteins (20 μg) were loaded in each lane and resolved by SDS-PAGE, which was subsequently transferred to nitrocellulose membrane. HSP70 was probed with rabbit anticytoplasmic HSP70 antibody and visualized by goat anti-rabbit secondary antibody. Relative abundance of HSP70 was normalized to the expression level of actin. The ratio of HSP70 to actin was measured by densitometry software LABWORKS.

eliminate the excess of H₂O₂, avoiding the potential lipid damage caused by the CeO₂ NPs.

Heat Shock Protein 70. Heat shock proteins act as molecular chaperones in normal protein folding and assembly but may also function in the protection and repair of proteins under stress conditions.^{25,26} There have been numerous reports of an increased HSP expression in plants, in response to heavy metal stress. Recently, there was a report about increased HSP90 gene expression in tomato plants, in response to CNTs stress.⁹

In corn shoots, the expression of HSP70 did not show differences due to the change in concentration of CeO₂ NPs at all exposure times (0 to 20 days) (Figure S2 in the Supporting Information). Interestingly, in roots, the Western blot analysis showed that CeO₂ NPs induced up-regulation of HSP70 (Figure 5). At all exposure times, HSP70 levels in roots treated with either 400 or 800 mg/kg CeO₂ NPs were significantly higher than control ($p \leq 0.05$). Wang *et al.*²⁶ reported that HSPs can play a crucial role in protecting plants against stress by re-establishing normal protein conformation and, thus, cellular homeostasis. This suggests that the plants might initiate an adaptive process to the adverse conditions by up-regulation of HSP70. In addition, it was noted that even on day 20, at a time when the over produced H₂O₂ had been eliminated by the CAT and APX, the production of HSP70 was still higher compared to control. Agarwal *et al.*²⁷ showed that, in rice, HSP101 was produced during the post-stress phase, implying its role in the plant's recovery to normal conditions after stress. Our data suggest that the HSP70 protects the plants from potential damage, which might be caused by excessive deposition of NPs/NP aggregates. The mechanisms underlying the protection wait for further investigation.

Detection of Cell Death (Plasma Membrane Integrity). To test if the H₂O₂ accumulation leads to oxidative damage and cell death, we examined the relative ion leakage (plasma membrane integrity) in 20 day old corn roots and shoots. Relative ion leakage rates are

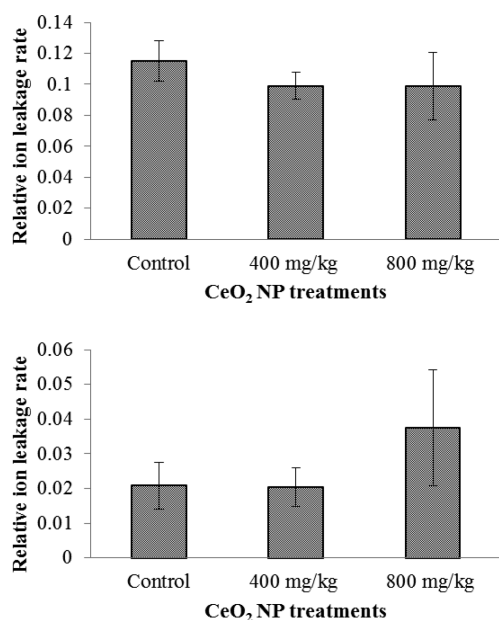


Figure 6. Relative ion leakage of corn roots germinated and grown for 20 days in soil treated with CeO₂ NPs at 0, 400, and 800 mg/kg. Data are means of a total of three replicates (nine plants/replicate). Error bars stand for standard deviation. Means with same letters within each exposure time are not significantly different (Tukey's HSD multiple comparison at $p \leq 0.05$).

shown in Figure 6. The levels of ion leakage in corn plants treated with 400 and 800 mg/kg CeO₂ NPs were similar with control, indicating that no membrane damage was induced, even when the NPs accumulated in the internal part of the cell wall, close to the membranes (see TEM in Figure S3 in the Supporting Information). These data are consistent with lipid peroxidation data. Very likely, CAT and APX protect the membrane from damage by scavenging the over produced H₂O₂. On the other hand, HSP70 may also serve as a protective mechanism for membrane integrity. There have been much evidence that suggests HSP may be associated with a protective role on membranes. In soybean seedlings, the accumulation of HSP15 in membrane fractions coincides with a decrease of solute leakage after heat stress treatment.²⁸ Neumann *et al.* reported that HSP70 could be involved in the protection of membranes against cadmium damage.²⁹ They found that, in soybean treated with CdSO₄ (10⁻³ M), a considerable amount of HSP70 was bound to membranes; perhaps connected with the formation of complexes between heavy metals and proteins and the resulting denaturation.

METHODS

Nanoparticle Characterization. CeO₂ NPs (Meliorum Technologies, primary size of 10 ± 1 nm) were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). The hydrodynamic size of CeO₂

TABLE 1. Leaf Net Photosynthetic Rate (P_n), Transpiration (E), and Stomatal Conductance (g_s) of Corn Plants Germinated and Grown for 20 Days in Soil Treated with CeO₂ Nanoparticles at 0, 400, and 800 mg/kg

CeO ₂ NPs	P_n ($\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) ^y	E ($\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	g_s ($\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)
0	2.9 ± 0.2 a ^z	1.6 ± 0.1 a	93.8 ± 9.8 a
400	3.0 ± 0.1 a	1.4 ± 0.2 a	83.6 ± 12.9 a
800	3.2 ± 0.2 a	1.4 ± 0.1 a	81.5 ± 7.1 a

^yData are means ± standard error of five replicates, two plants per replicate, one leaf per plant. ^zMeans followed by same letter are not significantly different among treatments by Tukey's HSD multiple comparison at $p \leq 0.05$.

There is evidence that carbon NPs induced the loss of membrane integrity in onion epidermis cells.³⁰ The loss of membrane integrity was considered a result of mechanical damage exerted by C₆₀(OH)₂₀ aggregation instead of ROS.³⁰

Photosynthesis. Photosynthesis is one of the physiological processes most sensitive to environmental stresses.³¹ Also, the capacity for the plant to maintain normal photosynthesis rate under stressful conditions can be a good indicator of plant adaptability.³² Gas exchange (net photosynthetic rate, transpiration, and stomatal conductance) of corn leaves was measured 20 days after sowing. Results showed (Table 1) no significant differences in gas exchanges between control and CeO₂ NP-treated plants (Table 1), indicating that at the concentrations tested and the growth stage evaluated corn plants tolerated the CeO₂ NPs.

CONCLUSIONS

The safe use of NPs in agriculture requires a thorough understanding of the interaction of NPs and edible plants. In the work presented herein, we grew corn in soil spiked with CeO₂ NPs. These NPs are some of the most commonly used NPs, and their environmental background level is expected to be continuously increasing. Utilizing multiple stress-related assays, we found over production of H₂O₂ and up-regulated HSP70, which demonstrated that CeO₂ NPs caused stress response in corn plants. Even though we found a stress response, no loss of membrane integrity was observed. In addition, the corn photosynthetic pathways were not affected by CeO₂ NPs. Our results indicate that corn plants have protective mechanisms to adapt to toxicity induced by CeO₂ NPs. A long-term observation, especially, the whole life cycle, is still needed in order to obtain a comprehensive evaluation of the impact of CeO₂ NPs on corn plants.

NPs in soil solution was 2124 ± 59 nm, with a zeta-potential of -22.8 ± 4.5 mV. Size and zeta-potential of the NPs in suspensions were measured using a Malvern Zetasizer (Nano-ZS 90, Malvern). All size and zeta-potential determinations were performed in CeO₂ NP suspensions at 25 mg/L soil solution with a

sonication time of 30 min. Soil solution was extracted as 1:10 soil/deionized water at a pH of 8.2.

Exposure of Corn Plant to Soil Spiked with CeO₂ NPs. Magenta boxes were filled out in triplicate with 250 g of soil and sown with nine corn kernels (Golden variety, Del Norte Seeds Company, El Paso, TX) to have enough biomass for determinations. The nine corn plants from each box were used in each assay. Soil was collected from Fabens, TX, and classified as sandy loam soil, with the following physicochemical properties: clay 3.73%, silt 12.15%, sand 84.10%, organic matter content 0.04%, and pH 7.9. Triplicate CeO₂ NP suspensions were sonicated for 30 min and applied to the soil to make final concentrations of 400 and 800 mg NPs/kg soil. Plants were grown for 20 days in an environmental growth chamber at 25 °C, 16/8 h light/dark cycle, and 65% humidity.

ICP-OES Analysis of Ce in Corn Tissues. The corn shoots were harvested at 10, 15, and 20 days and were analyzed for Ce content by ICP-OES (Perkin-Elmer Optima 4300 DV, Shelton, CT). Prior to ICP analysis, shoot samples were digested with concentrated plasma-pure HNO₃ and H₂O₂ (30%) (1:4) using a microwave acceleration reaction system (CEM Corp.; Mathews, NC). Certified Reference Material (peach leaves, NIST 1547) was processed as samples, and the Ce recovery rate was 100.82 ± 0.48.

H₂O₂ Measurement. H₂O₂ concentration in crude extracts from fresh corn shoots was determined by spectrofluorometry, according to the method described by Gay and Gebicki.³³ Fresh seedlings (500 mg) were ground in liquid nitrogen and suspended in 4 mL of 100 mM potassium phosphate buffer (pH 6.8). The mixture was added with appropriate volumes of reagents to give a final concentration of 25 mM H₂SO₄, 100–150 μM xylenol orange, and 100–250 μM ferrous iron (ferrous ammonium sulfates) in a volume of 2 mL. After 30 min in the dark, the absorbance was measured at 560 nm, with XO/Fe²⁺ as blank.

Visualization of H₂O₂ in Tissues. Fluorescent probes of 2, 7-dichlorofluorescein diacetate (DCF-DA) (Sigma-Aldrich) were used for *in vivo* observation of the distribution of H₂O₂ in different cells of the corn plant tissue.³⁴ DCF-DA is a cell-permeant indicator for ROS that is nonfluorescent until it is oxidized to the brightly fluorescent DCF in the presence of H₂O₂.³⁵ Corn leaf segments (2 cm) were embedded in 10 μM DCF-DA in phosphate-buffered saline (PBS) for 30 min at 37 °C in darkness. Then, the segments were washed three times in the same buffer for 15 min. Three leaf segments, one segment per plant, were sectioned at 10 μm using a Minotome plus cryostat (Triangle Biomedical Science, Durham, NC). DCF-DA green fluorescence (485 nm excitation, 530 nm emission) was observed with a confocal laser scanning microscope (Zeiss, New York, NY), equipped with a 10× objective and assisted with ZEN 2009 software (Zeiss, New York, NY).

Catalase and Ascorbate Peroxidase Assay. The catalase (CAT) enzyme activity was assayed according to Gallego *et al.*,³⁶ with minor modifications. Ascorbate peroxidase (APX) activity was determined according to Murgia *et al.*,³⁷ with minor modifications. All operations were performed in a cold room at 0–4 °C. Specific details regarding the assay of CAT and APX can be found in the Supporting Information.

Estimation of Lipid Peroxidation. The degree of lipid peroxidation in plants was determined following the method of Heath and Packer.³⁸ Fresh root/leaf (300 mg) was ground in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) using mortar and pestle and centrifuged at 10 000g for 20 min. A 1 mL of the supernatant was added with 1 mL of TCA (20%) containing 0.5% (w/v) thiobarbituric acid (TBA) and 100 μL of butylated hydroxytoluene (BHT, 4% in ethanol). The mixture was heated at 95 °C for 30 min, then quickly cooled in an ice bath and centrifuged at 10 000g for 15 min. The absorbance of the supernatant was measured at 532 nm. The unspecific turbidity of the sample was measured at 600 nm and subtracted with the absorbance at 532 nm. A total of 0.25% TBA in 10% TCA served as blank. The concentration of lipid peroxides together with the oxidatively modified proteins of the plants was quantified and expressed as total TBARS, an index of lipid peroxidation, in terms of nmol g⁻¹ fresh weight using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

Protein Extraction and Western Blot Analysis of HSP70. Corn plants were removed from soil at day 10, day 15, and day 20 and were thoroughly washed with tap water followed by deionized water.

The tissues were immediately stored at –80 °C for later use. One gram of frozen root or shoot tissues was ground in liquid N₂ and extracted with 2 mL of QB buffer (Supporting Information). The extracted solutions containing protein samples were centrifuged for 20 min at 16 000g at 4 °C. The supernatants were carefully transferred to 1.5 mL Eppendorf tubes. Protein quantitation was performed by BCA protein assay kit (Biorad).

Protein quantitation was performed according to Nieto-Sotelo *et al.* with some modifications.³⁹ Specifically, 20 μg of protein was separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and wet transferred to nitrocellulose membrane (GE Healthcare Life Sciences) and then blocked for 1 h at room temperature in Tris-buffered saline with Tween (TBS-T) [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] buffer containing 5% nonfat milk. Membranes were then incubated with the primary antibodies rabbit antiheat cytoplasmic shock protein 70 (1:3000), rabbit anti-rubisco (1:10,000), or mouse antiactin (1:1000) (Agrisera antibodies, Vannasa, Sweden) overnight at 4 °C with gentle shaking. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize the stained bands with an enhanced chemiluminescence visualization kit (Santa Cruz Biotechnology).

Detection of Cell Death (Plasma Membrane Integrity). Cell death was estimated by measuring ion leakage from root following the method of Coskun *et al.*, with minor modifications.⁴⁰ Specifically, four root segments (around 4 cm long) were cut and rinsed three times with deionized water to remove the leakage ion related to the immediate injury inflicted on the tissue during cutting. The root segments were incubated in 15 mL vials containing 10 mL of deionized water for 3 h in a rotary shaker at room temperature. Following 3 h incubation, the conductivity of the solution was measured (Meter conductivity 3 star W/PRB) and boiled at 100 °C for 20 min. When the solution was cooled to room temperature, the conductivity was measured again and the results were expressed as relative conductivity [(conductivity after incubation/conductivity before boiling) × 100].

Gas Exchange Measurement. Leaf net photosynthetic rate (*P_n*), transpiration (*E*), and stomatal conductance (*g_s*) were measured by placing the leaf in the cuvette of a portable gas exchange system (CIRAS-2; PP Systems, Amesbury, MA). The environmental conditions in the cuvette were controlled at leaf temperature = 25 °C, PPF = 600 μmol·m⁻²·s⁻¹, and CO₂ concentration = 380 μmol·mol⁻¹. The data were recorded when the environmental conditions and gas exchange parameters in the cuvette were stable.

Statistical Analysis. The plants for Ce concentration, H₂O₂ concentration, lipid peroxidation, antioxidant enzymes, and ion leakage rate analysis were grown separately. At harvest, the tissues from nine plants were collected, mixed, and the mixture was separated into three groups. The data were reported as averages of three replicates. A one-way ANOVA test was performed, and Tukey-HSD multiple comparisons conducted test performed using the statistical package SPSS version 12.0 (SPSS, Chicago, IL). Statistical significance was based on probabilities of *p* ≤ 0.05.

Conflict of Interest: The authors declare no competing financial interest.

Acknowledgment. This material is based upon work supported by the National Science Foundation and the Environmental Protection Agency under Cooperative Agreement Number DBI-0830117. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or the Environmental Protection Agency. This work has not been subjected to EPA review and no official endorsement should be inferred. The authors also acknowledge the USDA Grant No. 2011-38422-30835, the Toxicology Unit of the BBRC (NIH NCRR Grant No. 2G12RR008124-16A1), and the NSF Grant No. CHE-0840525. Also, the authors thank the staff of the Cell Culture and High Throughput Screening (HTS) Core Facility of UTEP for services and facilities provided. This core facility is supported by Grant 5G12RR008124 to the Border

Biomedical Research Center (BBRC), granted to the University of Texas at El Paso from the National Center for Research Resources (NCRR) of the NIH. J.L.G.-T. acknowledges the Dudley family for the Endowed Research Professorship in Chemistry.

Supporting Information Available: CAT and APX activities determination, QB buffer preparation, TEM sample preparation, uptake of Ce in corn plant tissues, Western Blot result of corn leaves, TEM images, and gas exchange results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES AND NOTES

- Perez-de-Luque, A.; Rubiales, D. Nanotechnology for Parasitic Plant Control. *Pest Manage. Sci.* **2009**, *65*, 540–545.
- Yang, L.; Watts, D. J. Particle Surface Characteristics May Play an Important Role in Phytotoxicity of Alumina Nanoparticles. *Toxicol. Lett.* **2005**, *158*, 122–132.
- Lin, D. H.; Xing, B. S. Phytotoxicity of NPs: Inhibition of Seed Germination and Root Growth. *Environ. Pollut.* **2007**, *150*, 243–250.
- Stampoulis, D.; Sinha, S. K.; White, J. C. Assay-Dependent Phytotoxicity of Nanoparticles to Plants. *Environ. Sci. Technol.* **2009**, *43*, 9473–9479.
- Ma, Y.; Kuang, L.; He, X.; Bai, W.; Ding, Y.; Zhang, Z.; Zhao, Y.; Chai, Z. Effect of Rare Earth Oxide Nanoparticles on Root Elongation of Plants. *Chemosphere* **2010**, *78*, 273–279.
- Lopez-Moreno, M. L.; de La Rosa, G.; Hernandez-Viezcas, J. A.; Castillo-Michel, H.; Botez, C. E.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Evidence of the Differential Bio-transformation and Genotoxicity of ZnO and CeO₂ Nanoparticles on Soybean (*Glycine max*) Plants. *Environ. Sci. Technol.* **2010**, *44*, 7315–7320.
- Dietz, K.; Herth, S. Plant Nanotoxicology. *Trends Plant Sci.* **2011**, *16*, 582–589.
- Khodakovskaya, M.; Dervishi, E.; Mahmood, M.; Xu, Y.; Li, Z.; Watanabe, F.; Biris, A. S. Carbon Nanotubes Are Able To Penetrate Plant Seed Coat and Dramatically Affect Seed Germination and Plant Growth. *ACS Nano* **2009**, *3*, 3221–3227.
- Khodakovskaya, M. V.; Silva, K.; Nedosekin, D. A.; Dervishi, E.; Biris, A. S.; Shashkov, E. V.; Galanzha, E. I.; Zharov, V. P. Complex Genetic, Photothermal, and Photoacoustic Analysis of Nanoparticle–Plant Interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 1028–1033.
- Atha, D. H.; Wang, H.; Petersen, E. J.; Cleveland, D.; Holbrook, R. D.; Jaruga, P.; Dizdaroglu, M.; Xing, B.; Nelson, B. C. Copper Oxide Nanoparticle Mediated DNA Damage in Terrestrial Plant Models. *Environ. Sci. Technol.* **2012**, *46*, 1819–1827.
- Anonymous, *Nanotechnology Consumer Product Inventory*; Project on Emerging Nanotechnologies, Woodrow Wilson International Center for Scholars and the Pew Charitable Trusts, Washington, DC, 2011. Available at <http://www.nanotechproject.org/consumerproducts>.
- Organization for Economic Cooperation and Development, *List of Manufactured Nanomaterials and List of Endpoints for Phase One of the OECD Testing Programme*; Series on the Safety of Manufactured Nanomaterials No. 6, Paris, France, 2008; [http://www.oalis.oecd.org/olis/2008doc.nsf/LinkTo/NT000034C6/\\$FILE/JT03248749.PDF](http://www.oalis.oecd.org/olis/2008doc.nsf/LinkTo/NT000034C6/$FILE/JT03248749.PDF).
- Kosynkin, V. D.; Arzgatkina, A. A.; Ivanov, E. N.; Chtoutsas, M. G.; Grabko, A. I.; Kardapolov, A. V.; Sysina, N. A. The Study of Process Production of Polishing Powder Based on Cerium Dioxide. *J. Alloys Compd.* **2000**, *303–304*, 421–425.
- Corma, A.; Atienzar, P.; Garcia, H.; Chane-Ching, J. Y. Hierarchically Mesoporous Doped CeO₂ with Potential for Solar-Cell Use. *Nat. Mater.* **2004**, *3*, 394–397.
- Thill, A.; Zeyons, O.; Spalla, O.; Chauvat, F.; Rose, J.; Auffan, M.; Flank, A. M. Cytotoxicity of CeO₂ Nanoparticles for *Escherichia coli*. Physico-Chemical Insight of the Cytotoxicity Mechanism. *Sci. Total Environ.* **2006**, *40*, 6151–6156.
- Johnston, B. D.; Scown, T. M.; Moger, J.; Cumberland, S. A.; Baalousha, M.; Linge, K.; van Aerle, R.; Jarvis, K.; Lead, J. R.; Tyler, C. R. Bioavailability of Nanoscale Metal Oxides TiO₂, CeO₂, and ZnO to Fish. *Sci. Total Environ.* **2010**, *44*, 1144–1151.
- Maize, Rice and Wheat: Area Harvested, Production Quantity, Yield; Food and Agriculture Organization of the United Nations, Statistics Division, 2009.
- Asli, S.; Neumann, P. M. Colloidal Suspensions of Clay or Titanium Dioxide NPs Can Inhibit Leaf Growth and Transpiration via Physical Effects on Root Water Transport. *Plant Cell Environ.* **2009**, *32*, 577–584.
- Zhao, L.; Peralta-Videa, J. R.; Varela-Ramirez, A.; Castillo-Michel, H.; Li, C.; Zhang, J.; Aguilera, R. J.; Keller, A. A.; Gardea-Torresdey, J. L. Effect of Surface Coating and Organic Matter on the Uptake of CeO₂ NPs by Corn Plants Grown in Soil: Insight into the Uptake Mechanism. *J. Hazard. Mater.* **2012**, *225*, 131–138.
- Tan, X. M.; Lin, C.; Fugetsu, B. Studies on Toxicity of Multi-Walled Carbon Nanotubes on Suspension Rice Cells. *Carbon* **2009**, *47*, 3479–3487.
- Shen, C.; Zhang, Q.; Li, J.; Bi, F.; Yao, N. Induction of Programmed Cell Death in *Arabidopsis* and Rice by Single-Wall Carbon Nanotubes. *Am. J. Bot.* **2010**, *97*, 1602–1609.
- Rodriguez-Serrano, M.; Romero-Puertas, M. C.; Pazmino, D. M.; Testillano, P. S.; Risueno, M. C.; Rio, L. A. D.; Sandalio, L. M. Cellular Response of Pea Plants to Cadmium Toxicity: Cross Talk between Reactive Oxygen Species, Nitric Oxide, and Calcium. *Plant Physiol.* **2009**, *150*, 229–243.
- Rodriguez, A. A.; Grunberg, K. A.; Taleisnik, E. L. Reactive Oxygen Species in the Elongation Zone of Maize Leaves Are Necessary for Leaf Extension. *Plant Physiol.* **2002**, *129*, 1627–1632.
- Sharma, P.; Jha, A. B.; Dubey, R. S.; Pessarakli, M. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *J. Bot.* **2012**, 10.1155/2012/217037.
- Hall, J. L. Cellular Mechanisms for Heavy Metal Detoxification and Tolerance. *J. Exp. Bot.* **2002**, *53*, 1–11.
- Wang, W.; Vinocur, B.; Shoseyov, O.; Altman, A. Role of Plant Heat-Shock Proteins and Molecular Chaperones in the Abiotic Stress Response. *Trends Plant Sci.* **2004**, *9*, 244–252.
- Agarwal, M.; Sahi, C.; Katiyar-Agarwal, S.; Agarwal, S.; Young, T.; Gallie, D. R.; Sharma, V. M.; Ganesan, K.; Grover, A. Molecular Characterization of Rice HSP101: Complementation of Yeast HSP104 Mutation by Disaggregation of Protein Granules and Differential Expression in Indica and Japonica Rice Types. *Plant Mol. Biol.* **2003**, *51*, 543–553.
- Lin, C.-Y.; Chen, Y. M.; Key, J. L. Solute Leakage in Soybean Seedlings under Various Heat Shock Regimes. *Plant Cell Physiol.* **1985**, *26*, 1493–1498.
- Neumann, D.; Lichtenberger, O.; Gunther, D.; Tschiersch, K.; Nover, L. Heat-Shock Proteins Induce Heavy-Metal Tolerance in Higher Plants. *Planta* **1994**, *194*, 360–367.
- Chen, R.; Ratnikova, T. A.; Stone, M. B.; Lin, S.; Lard, M.; Huang, G.; Hudson, J. S.; Ke, P. Differential Uptake of Carbon Nanoparticles by Plant and Mammalian Cells. *Small* **2010**, *6*, 612–617.
- Hsiao, T. C.; Acevedo, E. Plant Response to Water Deficits, Water Use Efficiency, and Drought Resistance. *Agric. For. Meteorol.* **1974**, *14*, 59–84.
- Niu, G.; Rodriguez, D. S. Growth and Physiological Response to Drought Stress in Four Oleander Clones. *J. Am. Soc. Hortic. Sci.* **2008**, *133*, 188–196.
- Gay, C.; Gebicki, J. M. A Critical Evaluation of the Effect of Sorbitol on the Ferric-Xylenol Orange Hydroperoxide Assay. *Anal. Biochem.* **2000**, *284*, 217–220.
- Rodriguez-Serrano, M.; Romero-Puertas, M. C.; Zabalza, A.; Corpas, F. J.; Gómez, M.; del Río, L. A.; Sandalio, L. M. Cadmium Effect on Oxidative Metabolism of Pea (*Pisum sativum* L.) Roots. Imaging of Reactive Oxygen Species and Nitric Oxide Accumulation *In Vivo*. *Plant Cell Environ.* **2006**, *29*, 1532–1544.
- Garza, K. M.; Soto, K. F.; Murr, L. E. Cytotoxicity and Reactive Oxygen Species Generation from Aggregated Carbon and Carbonaceous Nanoparticulate Materials. *Int. J. Nanomed.* **2008**, *3*, 83–94.
- Gallego, S. M.; Benavides, M. P.; Tomaro, M. L. Effect of Heavy Metal Ion Excess on Sunflower Leaves Evidence for

- Involvement of Oxidative Stress. *Plant Sci.* **1996**, *121*, 151–159.
37. Murgia, I.; Tarantino, D.; Vannini, C.; Bracale, M.; Carravieri, S.; Soave, C. *Arabidopsis thaliana* Plants Overexpressing Thylakoidal Ascorbate Peroxidase Show Increased Resistance to Paraquat-Induced Photooxidative Stress and to Nitric Oxide-Induced Cell Death. *Plant J.* **2004**, *38*, 940–953.
 38. Heath, R. L.; Packer, L. Photoperoxidation in Isolated Chloroplasts I. Kinetics and Stoichiometry of Fatty Acid Peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198.
 39. Nieto-Sotelo, J.; Martinez, L. M.; Ponce, G.; Cassab, G. I.; Alagon, A.; Meeley, R. B.; Ribaut, J.; Yang, R. Maize HSP101 Plays Important Roles in Both Induced and Basal Thermotolerance and Primary Root Growth. *Plant Cell.* **2002**, *14*, 1621–1633.
 40. Coskun, D.; Britto, D. T.; Jean, Y.; Schulze, L. M.; Becker, A.; Kronzucker, H. J. Silver Ions Disrupt K^+ Homeostasis and Cellular Integrity in Intact Barley (*Hordeum vulgare* L.) Roots. *J. Exp. Bot.* **2011**, *63*, 151–162.