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XAS Corroboration of the Uptake and Storage of CeO_2 Nanoparticles and Assessment of their Differential Toxicity in Four Edible Plant Species

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Abstract

Fate, transport, and possible toxicity of cerium oxide nanoparticles (nanoceria, CeO_2) are still unknown. In this study, seeds of alfalfa (*Medicago sativa*), corn (*Zea mays*), cucumber (*Cucumis sativus*), and tomato (*Lycopersicon esculentum*) were treated with nanoceria at 0–4000 mg L^{-1}. Cerium uptake and oxidation state within tissues were determined using ICP-OES and XAS, respectively. Germination rate and root elongation were also determined. Results showed that nanoceria significantly reduced corn germination (about 30% at 2000 mg L^{-1}, $p < 0.05$), and at 2000 mg L^{-1} germination of tomato and cucumber was reduced by 30% and 20%, respectively ($p < 0.05$). Root growth was significantly promoted ($p < 0.05$) by nanoceria in cucumber and corn but reduced ($p < 0.05$) in alfalfa and tomato. Almost at all concentrations nanoceria promoted shoot elongation in the four plant species. XAS data clearly showed the nanoceria within tissues of the four plant species. To the authors' knowledge, this is the first report on the presence nanoceria within plants.

Keywords

Nanoceria; Toxicity; Cerium speciation; Cerium absorption; Edible plants

INTRODUCTION

Nanosized materials (NMs) include particles of 100 nm or less. Currently, engineered nanomaterials, widely known as nanoparticles (NPs), are been used in medicine, electronics, catalysis, cosmetics, and pharmaceuticals (1). Dissimilar to bulk materials, NPs have individual physical and chemical properties pertaining to their morphology and composition. Size, shape, purity, and catalytic activity of NPs determine their interaction with the environment and living organisms (2). Nanoceria (CeO_2 NPs) are synthesized for application in engineering processes involving catalysts, polishing agents, fuel additives, and microelectronics (3). Release of this NM and its impact on living organisms, including humans, is practically unknown and in fact a subject of interest among scientists, governments, industries, and ordinary people (4).

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Rare earth elements (REEs) such as Ce, La, Pr, and Nd have been used to improve growth and yield of several crops (5), which in some way has increased their deposition in localized environments (6). Reports indicate that bulk REEs, especially Ce^{+3} compounds, accumulated in plants (7–10); however, to the author's knowledge, few reports exist on the uptake, accumulation, and biotransformation of nanoceria in living organisms. For instance, Thill and collaborators (11) investigated the toxicity of CeO_2 NPs in *Escherichiua coli.* They found that nanoceria can be absorbed by the outer membrane of *E. coli* inducing toxicity through reduction on the surface of bacteria. Auffan et al. (12) affirmed that nanoceria are reduced in biological media because Ce^{+4} absorb UV light and nanoceria are able to storage oxygen due to their antioxidant properties. Despite their physicochemical properties, NP reactivity is size-dependent because of the surface/volume ratio or surface area (13). These researchers found that different sizes of nanoceria can provoke diverse toxicity reactions in aquatic organisms such as the green algae *Pseudokirchneriella subcapitata*, and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. To the authors' knowledge there are no reports on the uptake of nanoceria by terrestrial plants.

In the present study, seeds of the edible plants cucumber (*Cucumis sativus*), tomato (*Lycopersicum esculentum*), alfalfa (*Medicago sativa*), and corn (*Zea mays*) were selected to determine the phytotoxicity and uptake of commercial CeO_2 NPs. These species were selected because of their worldwide importance as crop plants (14) and were treated following the USEPA guidelines for seed germination and root elongation toxicity tests (15). Alfalfa was included in this study because it is very important forage in the southwestern U.S. and it has been extensively studied by several research groups.

In this study, suspensions of 7 nm cubic CeO_2 NPs were prepared at 0, 500, 1000, 2000, and 4000 mg L^{-1}. NP phytotoxicity on seed germination was determined through percentage germination reduction, biomass production, and seedling elongation. In addition, Ce uptake and Ce disposition within tissues were studied by using inductively coupled plasma-optical emission spectroscopy (ICP-OES) and x-ray absorption spectroscopy (XAS), respectively.

MATERIALS AND METHODS

Nanoparticle Characterization

Nanoparticles of CeO_2 were purchased from Meliorum Technologies (Rochester, NY). For characterization, 100 mg of NPs were digested on a microwave oven using a mixture of plasma pure HNO_3 + H_2O_2 (1:4) as per Packer et al. (16). Cerium was quantified by ICP-OES. Particle size and composition for CeO_2 NPs were determined using a Siemens D500 X-ray diffractometer (Roseggerstrasse Leoben, Austria) in the range 20°–60° in 2 at 10 s per step.

Preparation of CeO_2 Suspensions

Suspensions of CeO_2 NPs were prepared at 0, 500, 1000, 2000, and 4000 mg L^{-1} in Millipore water (MPW), stirred by 5 min to avoid aggregation, and sonicated for 30 min. The pH of each suspension was recorded. In this study, treatments with Ce ions were not established because after 15 days in solutions, the nanoceria showed a very low dissolution (1.5, 1.2, 9, and 13.2 mg CeO_2 L^{-1} for the 500, 1000, 2000, and 4000 mg CeO_2 L^{-1}, respectively). In addition, to the authors' knowledge, no data has been reported on the effect of Ce(IV) in plants.

Germination Experiments

Seeds of alfalfa (*Medicago sativa*, Mesa variety), tomato (L*ycopersicum esculentum*, Pomodoro, Western Seed International, El Centro, CA), cucumber (*Cucumis sativus*, Poincett 76, Western Seed International), and corn (*Zea mays*, Del Norte Seed, El Paso, TX) were disinfected using a 4% $NaClO_4$ solution, stirred for 30 min and rinsed with sterilized Millipore

water (MPW). Triplicate samples of thirty (cucumber and corn) and 50 (alfalfa and tomato) seeds were placed in Petri dishes on a piece of germination paper containing 5 mL of NP suspensions. Seeds were covered with a second piece of germination paper and 10 drops of an antimycotic-antibiotic solution (A5955, Sigma, St Louis, MO) were added to each Petri dish. Subsequently, the dishes were covered with aluminum paper to avoid light and set at room temperature (25°C). The germination was recorded when almost 65% of control roots were 5 mm long (15). Soybean germination was recorded after 6 days, corn 8, and alfalfa and cucumber 9 days. Ten seedlings were used to calculate the biomass production and root elongation. In addition, seedlings from all treatments were washed with 0.01M HNO_3, rinsed with MPW and dried in an oven at 70°C for three days.

Quantification of Ce in Dry Tissues

Samples were digested on a CEM microwave oven (CEM Corporation, Mathews, NC) following the USEPA 3051 method using a mixture of plasma pure HNO_3 and H_2O_2 (1:4) after Packer et al. (16) with slight modifications. Samples were diluted to 25 mL and metals were quantified by ICP-OES (Perkin Elmer, Shelton, CT). Blank, spikes, and standard reference material (peach leaves SRM 1547, NIST, Gaithersburg, MD) were analyzed to validate the method. In addition, a 0.05 ppm Ce standard was analyzed every 10 samples for QC/QA purposes.

Statistical Analysis

Treatments were allocated in a completely random design and the data were reported as mean of three replicates ± SE. Data were analyzed using a one-way analysis of variance and the Tukey's test with the statistical package SPSS 15.0.

XAS analysis

For the X-ray Absorption spectroscopy experiments, roots from the 4000 mg CeO_2 L^{-1} treatment were immersed in liquid nitrogen for 45 min and lyophilized on a freeze-dryer at −53°C and 0.140 mBar pressure for 3 days (Labconco FreeZone 4.5, Kansas City, MO). After that, samples were homogenized in a mortar, loaded in aluminum sample holders and covered with Kapton© Tape.

The XAS spectra were collected on beam Line 7–3 at Stanford Synchrotron Radiation Lightsource (SSRL, Palo Alto, CA). During data collection, the synchrotron radiation accelerator had a ring storage energy of 3 GeV and a beam current of 50–100mA. CeL_{III} spectra were collected using a Canberra 29-element germanium detector and Si(220) ϕ 90 monochromator. A Ce foil was used to calibrate samples spectra. Fluorescence and transmission mode were used for collecting all sample spectra and model compounds, respectively at room temperature. Cerium nitrate and cerium oxide NPs were used as model compounds.

The WinXAS software (17) was used to analyze the data. The edge energy from individual spectrum was calibrated using the edge energy from the internal cerium foil (5723 eV). First and second degree derivatives of the inflection point of the metal foil were used to calibrate the sample spectrum and a polynomial fitting substraction was done in order to remove background. A 1^{st} degree polynomial was used on the pre-edge region and a 4^{rd} degree polynomial was used on the post-edge region of the spectrum. Ce speciation was determined based on the XANES spectra of the model compounds.

RESULTS AND DISCUSSION

Effect of Nanoceria on Seed Germination

Germination and root elongation data were collected when 65% of the total seeds were germinated and root growth was equal or larger than 0.5 mm (15). Equation (1) was used to calculate germination percent, while relative germination and germination change were calculated using equations (2) and (3) respectively. Averages of three replicates of each treatment were considered in these equations in order to perform the appropriate statistical analysis.

$$\%G=\frac{\#\text{Germinated seeds}}{\text{Total\# seeds}}\times 100 \quad \text{Eq. (1)}$$

$$RG=\frac{\%\text{ Germination in treatment}}{\%\text{Germination in control}}\times 100 \quad \text{Eq. (2)}$$

$$GC=\text{Relat.germination of treatments}-\text{Relat.germination of controls} \quad \text{Eq. (3)}$$

Where:

%G = Percent germination

RG = Relative germination

GC = Germination change

A positive sign in germination change from this calculations means that this parameter was enhanced while a negative sign indicates a reduction in germination rate. In addition, percent of root growth and reduction in root growth were calculated in order to determine the effect of NP concentrations in root emergence (equations 4 and 5),

$$\%RG=\frac{\text{Rootelongation in treatments(cm)}}{\text{Rootelongation in controls(cm)}}\times 100 \quad \text{Eq. (4)}$$

And the root growth reduction (RGR) was calculated through equation (5)

$$RGR=\%\text{Rootgrowthin treatments}-\%\text{ Rootgrowthin controls} \quad \text{Eq. (5)}$$

Data pertaining to germination are shown in Figure 1. As shown in this figure, alfalfa was slightly reduced only at 1000 and 2000 mg CeO_2 NPs L^{-1}. Tomato germination had a significant reduction (30%) at 2000 mg L^{-1} ($p \leq 0.05$). Cucumber germination was reduced by about 20% at 2000 mg CeO_2 NPs L^{-1} ($p \leq 0.05$). On the other hand, corn germination was significantly reduced at 500, 1000, and 2000 mg CeO_2 NPs L^{-1} (about 30%, $p \leq 0.05$). These results suggest that at the concentrations tested, nanoceria caused relatively low toxicity on seed germination of alfalfa and cucumber, and moderate toxicity on tomato and corn. Barrena

et al. (14) reported no effects on germination but but some perturbations in functions of cucumber and lettuce seedlings treated with Ag and Fe_3O_4 NPs. Biochemical studies as well as tests at higher concentrations are needed to determine the type of toxicity exerted by these NPs on terrestrial plants. As explained above, in this research work only the nanoceria were considered because there are no reports on the effects of Ce(IV) plants. However, further studies need to be done in order to compare the toxicity of NPs versus ceria bulk materials.

Effect of Nanoceria on Root and Stem Elongation

Figure 2 shows the effects of nanoceria on the growth of roots and shoots in cucumber, alfalfa, tomato, and corn seedlings. As seen in Figure 2a, at the concentrations tested, nanoceria significantly ($p < 0.05$) stimulated root growth in cucumber, with maximum expression at 2000 mg L^{-1} and a minimum at 500 mg L^{-1} (140 and 50% over control, respectively). Reports indicate that Ce precipitate as cerium peroxide in cell walls and intercellular spaces of epidermal and cortical cells, but not in meristematic cell (18). This suggests that oxidative stress in the growing zone is reduced allowing an increase in root elongation. There are some reports about root growth stimulation by Ce. For instance, Yuan et al. (5) reported that "Changle", a fertilizer that contains Ce at 50.2% increased root growth in rice seedlings. Figure 2 also shows a slight decreased in corn root growth at 1000 mg CeO_2 L^{-1} and slight increases at 500, 2000, and 4000 mg nanoceria L^{-1}. This seems to be a hormetic effect with a typical inverted U response, but more research is needed to test the hypothesis. Alfalfa root growth decreased significantly ($p < 0.05$) at 2000 and 4000 mg L nanoceria concentrations. In addition, tomato roots showed a significant root growth reduction at 1000 and 4000 mg CeO_2 L^{-1} (Figure 2). More studies, including genotype-environment interactions are needed to explain these results.

The effects of CeO_2 NPs on seedling shoot growth are shown in Figure 2b. As seen in this figure, compared to control, only stems of corn seedlings treated with CeO_2 NPs at 500 and 4000 mg L^{-1} showed a significant ($p < 0.05$) growth reduction (7 and 20%, respectively). Alfalfa stems were significantly longer in plants treated with 500 and 1000 mg L^{-1}, and all treatments increased cucumber stem length. In the case of cucumber, the stem length increased significantly ($p < 0.05$) as the external NP concentrations increased up to 2000 mg L^{-1}. Even though at 4000 mg L^{-1} cucumber stems were significantly longer compared to control and plants treated with 500 mg L^{-1}, the increase at 4000 mg L^{-1} was only 25% while at 2000 mg L^{-1} it was about 100%. This suggests that at 4000 mg L^{-1}, nanoceria start to be toxic to cucumber plants. An explanation for these results could be the superoxide dismutase mimetic activity of nanoceria (19). It is very likely that at 4000 mg L^{-1} nanoceria mimic superoxide dismutase, but interfere with other enzymatic functions of the plant.

Effect of CeO_2 NPs on Biomass Production

Table 1 displays the biomass production (dry weight) of 10 seedlings per plant species treated with nanoceria. As shown in this table, the biomass of alfalfa was significantly ($p < 0.05$) reduced at 500 mg CeO_2 NPs L^{-1} but increased at 4000 mg L^{-1}. Also corn biomass production was significantly ($p < 0.01$) diminished respect to control, by nanoceria at 500 and 4000 mg L^{-1}. Asli and Neuman (20) found that certain nanomaterials can accumulate on root surface of plants causing an inhibition of the root hydraulic conductivity. The decrease in water supply causes inhibition of root growth, higher xylem tension, and lower transpiration, resulting in an eventual desiccation and weight loss. Another study reported that Ce (supplied as cerium nitrate) can reduce the weight of corn shoots (21).

Cerium Uptake by Seedlings

Figure 3 shows the concentration of Ce in cucumber, alfalfa, tomato and corn seedlings treated with nanoceria. As seen in this figure, Ce concentrations in alfalfa and tomato increased

significantly ($p < 0.05$) as the external NP concentration increased. At 4000 mg L^{-1} alfalfa seedlings had about 6000 mg Ce kg^{-1} dry weight and tomato about 3000 mg Ce kg^{-1} dry weight. Cucumber showed similar Ce concentration in tissues for the 1000–4000 mg L^{-1} treatments (about 400 mg Ce kg^{-1} dry weight). And corn showed about 300 mg Ce kg^{-1} dry weight only at 4000 mg L^{-1} treatment. Lin and Xing (22) suggested that root exudates may change NP properties and behavior limiting their absorption. Xu et al. (7) reported that soil Ce present as CeO_2 cannot be absorbed by roots of some plants like corn because of the complexation within the rizosphere. Scarce literature concerning uptake of NPs by plants is available, reason why the present data cannot be compared to other results. As mentioned previously, further studies need to be done with ceria bulk materials to compare uptake and mobility between these materials.

XANES results

XANES spectra from Ce root and CeO_2 NPs as model compound are shown in Figure 4. The L_{III} edge energy for Ce (E_0= 5723 eV) was used to collect the spectra. The spectrum from the Ce NPs shows two distinctive white line features at 5.730 and 5.737 KeV. These white lines correspond to a characteristic mixture of ground-state electronic configurations of the Ce ($4f^0$ and $4f^1$) (23). The CeL_{III}- edge normalized XANES spectrum fromCeO_2 NPs and spectra from root seedlings treated with 4000 mg CeO_2 NPs L^{-1} revealed that all root seedlings were able to uptake and storage Ce as nanoceria. Cerium was found to be in the same oxidation state (+4) inside seedling roots. These results corroborated that Ce did not undergo any chemical transformation inside root tissues and remained unaltered after uptake by roots (as CeO_2 NP). Further studies need to be performed in order to determine the Ce oxidation state in the aerial part of plants.

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ABBREVIATIONS

XAS	x-ray absorption spectroscopy
NMs	nanomaterials
NPs	nanoparticles
REEs	rare earth elements
USEPA	U.S. Environmental Protection Agency
ICP-OES	inductively coupled plasma-optical emission spectroscopy
XANES	x-ray absorption near edge structure

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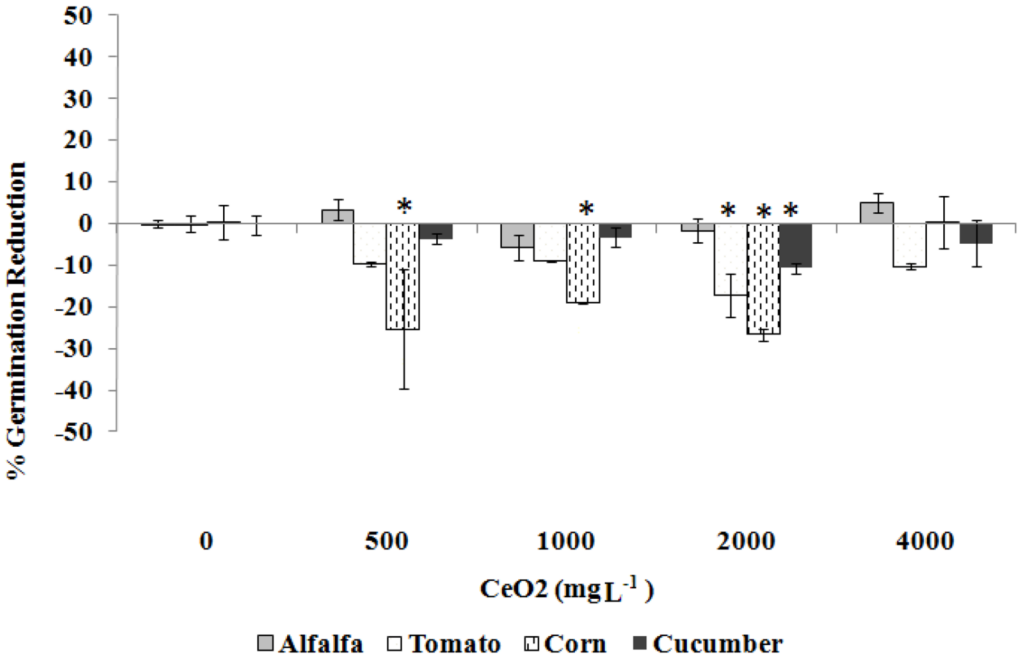

Figure 1.

Percent germination of ▒ alfalfa, □ tomato, ▒ corn, and ■ cucumber seeds treated with CeO_2 NPs solutions at 0–4000 mg/L. Data represent mean ± SE of three replicates. One-way ANOVA and Tukey's test were used to determine statistical significance of the differences between treatment means. *Statistically significant at $p \leq 0.05$.

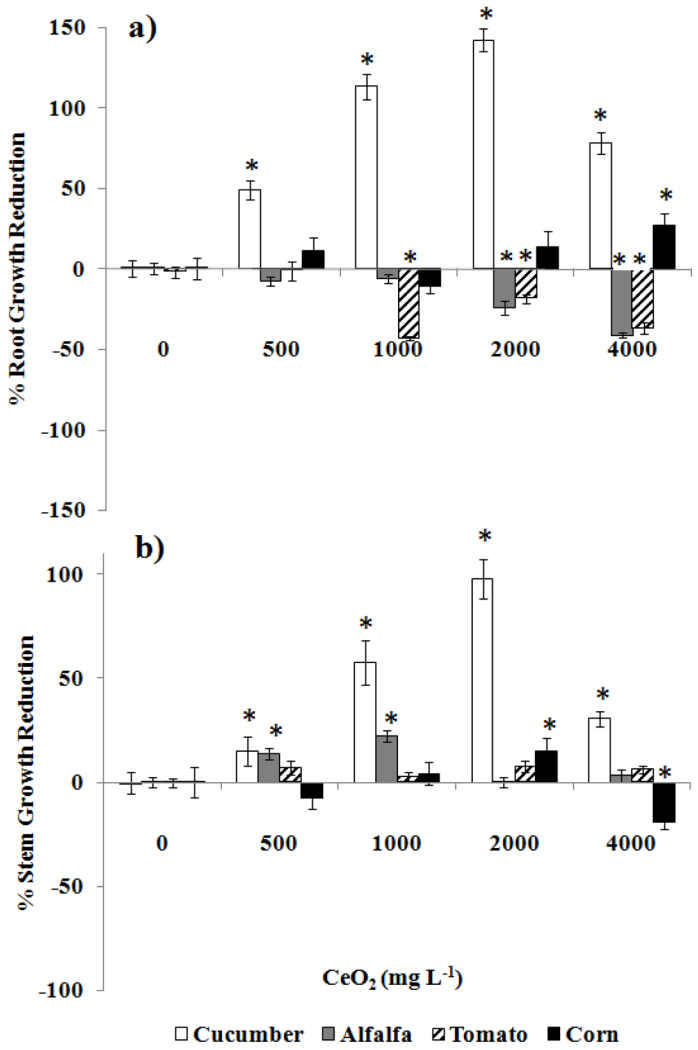

Figure 2.

Percent root (a) and stem (b) growth reduction of ■ cucumber, ▒ alfalfa, □ tomato, and ▨ corn seedlings exposed to 0–4000 mg/L CeO_2 NP suspensions. Data represent mean ± SE of three replicates. One-way ANOVA and Tukey's test were used to determine statistical differences between treatment means. *Statistically significant at $p \leq 0.05$.

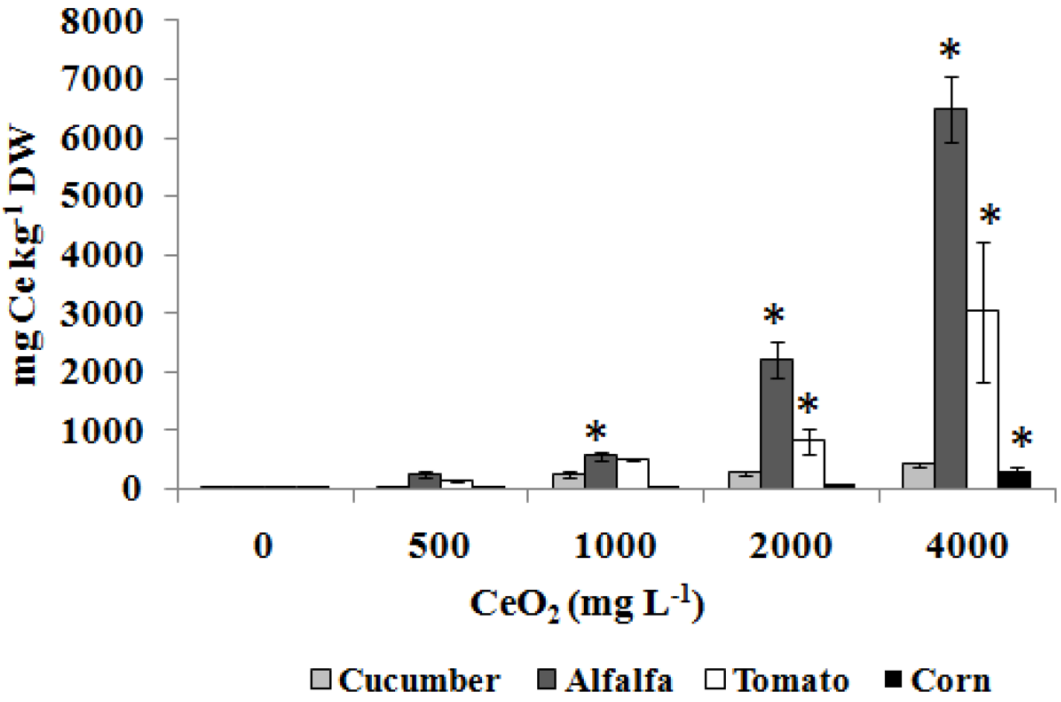

Figure 3.

Cerium concentration in ■ cucumber, ▒ alfalfa, □ tomato, and ▨ corn seedlings treated with CeO_2 NP suspensions at 0–4000 mg/L. Data are mean ± SE of three replicates. One-way ANOVA and Tukey's test were used to determine statistical differences between treatment means. *Statistically significant at $p \leq 0.05$.

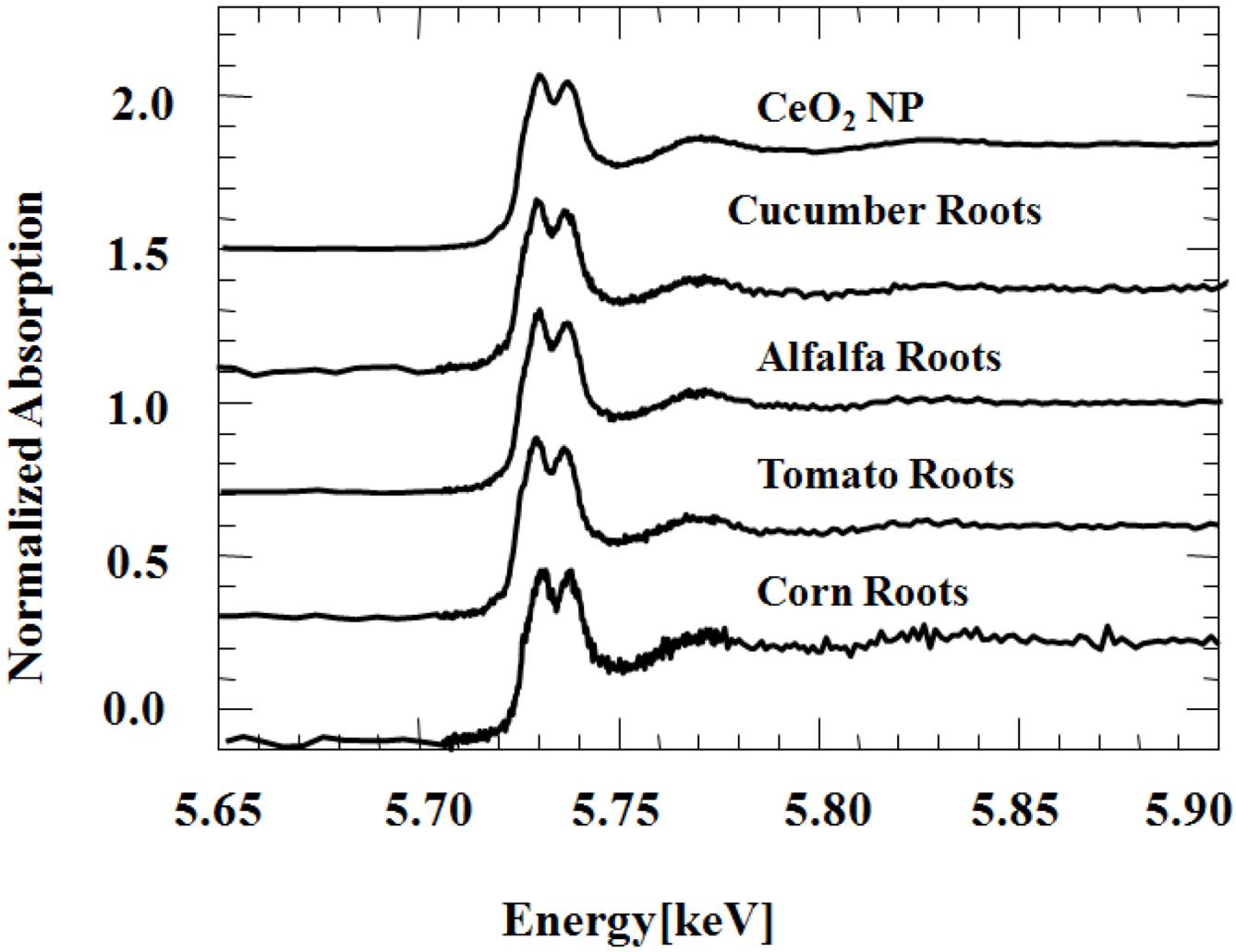

Figure 4.
XANES Ce L_{III}-edge normalized spectra(5723eV) of CeO_2 NPs (model compound) and spectra from cucumber, alfalfa, tomato, and corn roots exposed to 4000 mg/Lof CeO_2 NPs.

Table 1

Biomass weight (mg) of 10 plants germinated with CeO_2 NPs at 0–4000 mg L^{-1}.

CeO_2 (mg/L)	Cucumber (mg)	Alfalfa (mg)	Tomato (mg)	Corn (mg)
0	192.90 ± 4.40	16.7 ± 0.05	15.7 ± 0.07	831.5 ± 106.00**
500	186.20 ± 0.36	15.90 ± 0.04*	14.90 ± 0.03	539.10 ± 49.40*
1000	180.70 ± 7.20	16.90 ± 0.05	16.40 ± 0.03	710.40 ± 36.40
2000	191.00 ± 7.90	17.60 ± 0.06	16.00 ± 0.07	671.40 ± 38.70
4000	182.80 ± 3.80	18.60 ± 0.07*	14.70 ± 0.04	502.20 ± 0.1.70*

One-way ANOVA and Tukey's test were used to determine statistical significance of the differences between treatment means.

* Significant at 0.05 and

** 0.01.