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Nano impact

Cerium oxide nanoparticles are a widely used nanomaterial with many important applications, and their interactions with ecosystems are inevitable. We previously found CeO_2 NPs inhibited root elongation of *Lactuca* plants in aqueous suspensions. This study for the first time evaluates the phytotoxicity of CeO_2 NPs in a plant agar medium, which provides a soil-like environment while avoiding the uncertainties intrinsic to natural ecosystems. Compared to deionized water, the bioavailability of CeO_2 NPs in the agar medium was reduced, but the sensitivity of asparagus lettuce to CeO_2 NPs was increased. This study implies the phytotoxicity of CeO_2 NPs was affected by the exposure medium employed.

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ARTICLE

Effect of Cerium Oxide Nanoparticles on Asparagus Lettuce Cultured in an Agar Medium

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The increasing chances of manufactured nanoparticles (NPs) release to the environment highlight the importance of understanding their interactions with plants, which are basis of the ecosystems. In this study, the phytotoxicity of CeO_2 NPs on asparagus lettuce was assessed. Lettuce seeds were treated with CeO_2 NPs in a plant agar medium at a wide range of concentrations (0-2000 mg/L) for 5 days. At high concentrations (≥500mg/L), CeO_2 NPs altered the activity of superoxide dismutase (SOD), induced lipid peroxidation and cell membrane damage, and inhibited the root growth. The results of X-ray absorption near edge fine structure (XANES) indicate that part of CeO_2 NPs was transformed from Ce(IV) to Ce(III) in the roots. The released Ce^{3+} may account for the phytotoxicity of CeO_2 NPs.

Nano impact

Cerium oxide nanoparticles are a widely used nanomaterial with many important applications, and their interactions with ecosystems are inevitable. We previously found CeO_2 NPs inhibited root elongation of *Lactuca* plants in aqueous suspensions. This study for the first time evaluates the phytotoxicity of CeO_2 NPs in a plant agar medium, which provides a soil-like environment while avoiding the uncertainties intrinsic to natural ecosystems. Compared to deionized water, the bioavailability of CeO_2 NPs in the agar medium was reduced, but the sensitivity of asparagus lettuce to CeO_2 NPs was increased. This study implies the phytotoxicity of CeO_2 NPs was affected by the exposure medium employed.

Introduction

Engineered nanoparticles (NPs) are widely used in cosmetics, electronics, optical devices, medicine, chemical catalysis, etc. The unique properties of these materials might present adverse effects on both organism and the environment. Plants as the basic components of the ecosystem are easily exposed to NPs in atmospheric, terrestrial and aquatic environments. Moreover, NPs that entered into the plants may be transfer to herbivorous consumers and further caused the potential effect on human being. [1] Accordingly, the interactions between NPs and plants are of particular concern.[2]

Cerium dioxide nanoparticles (CeO_2 NPs), as one of the most important manufactured nanoparticles, have extensive commercial applications. [3,4] Previous studies demonstrated that CeO_2 NPs could be taken up by plant roots and transported to the aerial parts including the edible tissues.[5,6] The interactions between CeO_2 NPs and plants are complex. Enhancive, inhibitive and no effects of ENPs on plant growth at different developmental stages have been documented. Ma et al. found that CeO_2 NPs inhibited root elongation of lettuce, but showed no toxicity to rape, radish, wheat, cabbage, tomato and cucumber.[7] The results of Wang et al. demonstrated that low concentrations of CeO_2 NPs (≤10 mg L^{-1}) enhanced plant growth and fruit yield of tomato plants.[8] However, a further study indicated that second generation tomato seedlings grown from seeds collected from treated parent plants with CeO_2 NPs accumulated much smaller biomass and were somewhat weaker than seedlings grown from seeds from untreated parent plants.[9] López-Moreno and colleagues found that CeO_2 NPs reduced corn, tomato and cucumber germination.[5] The root growth was promoted by CeO_2 NPs in cucumber and corn but reduced in alfalfa and tomato. These authors later reported that CeO_2 NPs could compromise the quality of rice, diminished plant growth and yield of soybean and shut down nitrogen fixation.[10] CeO_2 NPs are generally recognized as stable in biological or environmental systems and remain unaltered after uptake by plant roots. [5-6] However, we recently proved that CeO_2 NPs can be transformed to $CePO_4$ and cerium carboxylates in hydroponic plants with the assistance of biogenic reducing substances and organic acids.[11] The high sensitivity of *Lactuca* plants to the released Ce^{3+} results in the species-specific toxicity of CeO_2 NPs mentioned above.[7,12]

Biotransformation of CeO_2 NPs to Ce(III) species in soil cultured plants was also reported recently. [13]

The above mentioned studies on phytotoxicity of CeO_2 NPs were carried out under either hydroponic or soil culture conditions. Due to their small size and surface properties, CeO_2 NPs tend to aggregate and precipitate in aquatic solutions. It is almost impossible to maintain a constant exposure concentration of CeO_2 NPs in nutrient solutions. Soil is a complex system of minerals, organic material, water, gasses, and living organisms. All these components can interact with NPs. It is difficult to carry out mechanism studies on interactions between plants and NPs in real soil environments. Recently, plant agar media are introduced in phytotoxicity studies of NPs to optimize dispersion and prevent aggregation. [14] Moreover, viscous soft gels resemble soil more than H_2O does, and thus allow the interactions between plants and NPs to be studied in a soil-like environment while avoiding the uncertainties intrinsic to natural ecosystems, which can affect both the rhizosphere and the properties of the NPs. [2]

In the present study, asparagus lettuce was used to assess the phytotoxicity of CeO_2 NPs. Representative parameters such as root/shoot lengths, antioxidant enzyme activities, hydrogen peroxide content, lipid peroxidation and ion leakage were investigated to understand the plant's defence and response to abiotic stress caused by CeO_2 NPs. To explore the proximate mechanisms of the observed toxicity, contents and chemical species of Ce in asparagus lettuce were analyzed and the effect of Ce^{3+} on root elongation of asparagus lettuce was measured.

Experimental methods

Synthesis and characterization of CeO_2 NPs.

CeO_2 NPs used in this study were synthesized by a precipitation method.[6] Particle size and morphology were determined by transmission electron microscope (TEM, JEM 200CX, Japan). X-ray diffraction (XRD, X'pert PRO MPD, Holland) was used to determine their crystal forms. A CeO_2 NP suspension (100 mg/L) in deionized water was prepared for measurement of hydrodynamic sizes and zeta potential (Nicomp 380 ZLS Zeta potential/Particle system, Santa Barbara, CA, USA). Chemical purity of the particles was analyzed by inductively coupled plasma-mass spectrometer (ICP-MS, Thermo X7, USA).

Plant culture and nanoparticle application.

Seeds of asparagus lettuce (*Lactuca sativa* Linn. var. *angustata* Irish ex Bremer) were purchased from the Chinese Academy of Agricultural Sciences. The plant assays were conducted in an agar medium. Each test unit contained 30 mL of 0.5% agar along with a specific concentration of NPs. CeO_2 NP stock solutions in deionized water were ultraviolet sterilized and ultrasonically dispersed for 15 min before use. Autoclaved agar medium (120℃ for 2 h) was thoroughly mixed with or without the NPs to obtain a series of CeO_2 suspensions (0, 2, 20, 200, 500, 1000, 2000 mg/L). After an ultrasonic treatment at 60 ℃ for 15 min, the CeO_2 NP suspensions were poured into 90 mm × 18 mm Petri dishes and quick-froze in a 20℃ freezer to avoid aggregation and precipitation. To test the homogeneity of the distribution of CeO_2 NPs, 10 agar samples were taken from 5 different points of the agar medium containing 200 mg/L CeO_2 in a Petri dish (5 from upper parts and 5 from lower parts, as shown in Figure S1). Contents of Ce in the samples were analyzed by ICP-MS.

Uniform asparagus lettuce seeds were selected and sterilized by 10% NaClO solution for 5 min and washed 3 times with deionized water. After being soaked in water for two hours, fourteen seeds were inserted onto the surface of the agar medium. Four replicates were set for each treatment. All the Petri dishes were covered and sealed with parafilm, placed in the dark at 20 ℃ in a climate incubator. After 5 days of treatment, the germination was halted. Germination rates were calculated. Root and shoot lengths were measured with a meter ruler.

Ce content determination.

Shoots and roots were separated from the thoroughly washed seedlings and lyophilized to constant weights in a freeze dryer. The dried tissues were digested on a heating plate using a mixture of concentrated plasma-pure HNO_3 and H_2O_2 (v/v: 4/1). Ce contents were analyzed by ICP-MS. A standard reference material (bush branches and leaves, GBW07602) was also digested to examine the recovery. Indium (20 ng/mL) was used as an internal standard to compensate for matrix suppression and signal drifting. Analytical runs concluded the obtained residual solution, spike recovery samples, and calibration verification samples. Recovery from GBW07602 was 99% and spike recovery averaged 102%. Relative standard deviation was 2.5%. Detection limit is 0.01 ng ml^{-1}.

Stress response of asparagus lettuce to CeO_2 NPs.

After 5 days of treatment, the seedlings were thoroughly washed with flowing tap water then deionized water to remove the agar medium. The roots were excised and homogenized with PBS (50 mM, pH 7.8) in an ice bath. Then the extracts were centrifuged for 10 min at 4 ℃and 10000×g. The supernatants were preserved for SOD, POD activities and MDA contents analyses, using the assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

H_2O_2 contents in the roots were examined by the method previously described by Begum and Ikhtiari[15]. Fresh roots from the control and CeO_2 treated plants were excised and immersed in 0.1 mg/L diaminobenzidine (DAB, pH 3.2) for 35 min in the dark at room temperature. Then the root segments were then washed thoroughly followed by grounding in a mixture of 2 M KOH and DMSO at a ratio of 1:1.167 (v/v). Subsequently, the extracts were centrifuged at 10000×g for 5 min and the absorbance of the supernatants at 700 nm was measured on a Multimode Microplate Reader (SpectraMax M2, USA).

Ion leakage from roots was measured by a conductivity method based on the procedure of Lutts et al. with slight modifications. [16]

The conductivity of deionized water (C_w) was first measured. The 5-day-old root samples were immersed in test tubes containing 2 mL of deionized water after three washes with deionized water to remove external contamination. Test tubes were covered and incubated on a shaker (100 rpm) at room temperature (25℃) for 1 h. The electrolyte conductivity of the solution (C_0) was measured. Samples were then boiled at 100 ℃ for 15 min, and the conductivity of the solution (C_t) was measured after cooled. Electrolyte leakage (EL) was defined as: EL (%) = (C_t- C_w) / (C_0- C_w) × 100%.

Microscopy observations.

After 5 days of treatment, fresh roots from control and the treatment of 2000 mg/L were observed using a light microscopy to examine morphology and cell division state of the root tips. Samples for LM were prepared following standard procedures. [17]

Speciation analysis by X-ray absorption near edge fine structure spectroscopy (XANES).

Seedlings treated with 2000 mg/L CeO_2 NPs were thoroughly washed. Roots were separated, lyophilized, ground into powder and pressed into slices (10 mm × 2 mm) for the XANES analysis. CeL_3-edge (5723eV) spectra were collected in the fluorescence mode. Measurements were performed at the beamline 1W1B at Beijing Synchrotron Radiation Facility. The ring storage energy of the synchrotron radiation accelerator in the data acquisition was 2.5 GeV with current intensity of 50 mA. $CePO_4$, $Ce(CH_3COO)_3$, $Ce_2(C_2O_4)_3$ and CeO_2 NPs were used as standard compounds and analyzed in transmission mode. Normalization and linear combination fitting (LCF) of the XANES spectra was accomplished by the Athena software.

Impact of Ce^{3+} ions on root growth.

A series of $Ce(NO_3)_3$ solutions (0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 mg/L) in 0.5% agar culture media were prepared to test the sensitivity of asparagus lettuce to Ce^{3+} ions. After 5 days of treatment, root lengths were measured by a rule meter.

Statistical analysis.

The data processing was performed on Statistical Packages for the Social Sciences (SPSS) 17.0. One-Way ANOVA followed by Tukey's HSD or Bonferroni test was performed to examine the statistic differences. All errors are expressed as standard deviations (SD). $P< 0.05$ was considered to be a significant difference.

Results

Physicochemical characteristics of CeO_2 NPs.

The TEM image shows that the NPs present as a truncated octahedral shape with an average size of 7.1±0.4 nm (Figure 1). Hydrodynamic size was 99.3±2.0 and the NPs were well dispersed with a positive zeta-potential (47.4 mV). Hydrodynamic size reflects the interaction between initial particles and the solvent. There is a hydration layer capping on the particle surface. Herein, CeO_2 NPs were aggregated in water and capped by hydration layer. Therefore, the hydrodynamic size is larger than the TEM size. XRD spectrum of the ceria NP exhibits the cubic fluorite structure. Chemical purity of the NPs was 99.98%. The distribution of CeO_2 NPs in the agar medium was homogeneous (ESI, Figure S2).

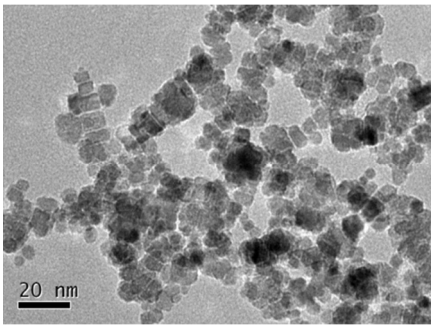

Figure 1.TEM image of CeO_2 NPs.

Ce contents.

As shown in Figure 2, Ce contents in the roots were much higher than that in the shoots and the contents increased with the increasing of the exposure concentrations.

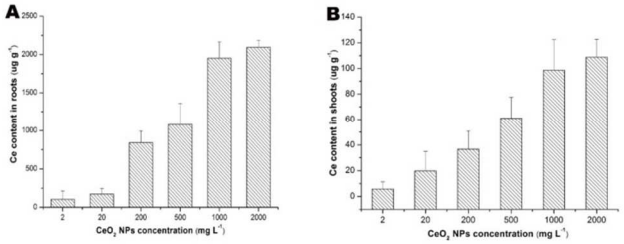

Figure 2. Ce contents in the roots (A) and shoots (B) of asparagus lettuce treated with CeO_2 NPs for 5 days. The means are averaged from 4 replicates of asparagus lettuce. The values were given as mean ± SD (standard deviation). Significant differences versus control were marked with"*" ($p < 0.05$).

Growth analysis.

CeO_2 NPs had no significant effects on seed germination (ESI, Figure S3) and shoot growth (Figure 3B) of asparagus lettuce at all exposure concentrations. But CeO_2 NPs inhibited the root elongation at concentrations higher than 500 mg/L (Figure 3A).

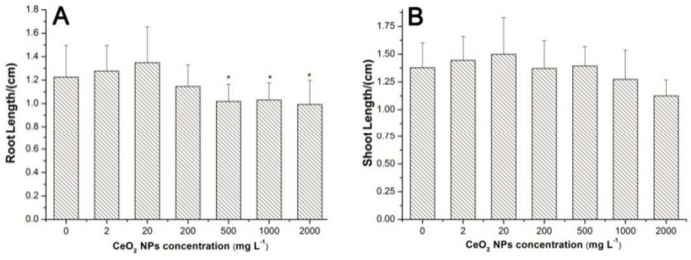

Figure 3. Root lengths (A) and shoot lengths (B) of asparagus lettuce cultured in agar amended with different concentrations of CeO_2 NPs for 5 days. The means

are averaged from 4 replicates with 14 seeds of asparagus lettuce. The values were given as mean ± SD (standard deviation). Significant differences versus control were marked with * ($p < 0.05$).

Stress responses of asparagus lettuce to CeO_2 NPs.

As showed in Figure 4, there was no significant change of H_2O_2 accumulation at all exposure concentrations.

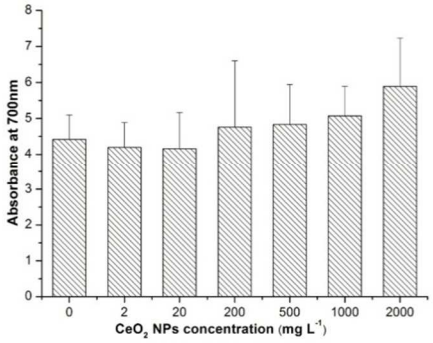

Figure 4. H_2O_2 content in roots of asparagus lettuce treated with CeO_2 NPs for 5 days. The means are averaged from 4 replicates with 14 seeds of asparagus lettuce. The values were given as mean ± SD (standard deviation)

SOD activities were significantly down-regulated at high concentrations (500, 1000 and 2000 mg/L) compared to the control (Figure 5A). However, POD activities were only slightly enhanced at all exposure concentrations except 2000 mg/L (Figure 5B). Significant increases of MDA levels (Figure 5C) and leakage of electrolytes (Figure 5D) were observed at high concentrations (500, 1000, 2000 mg/L) of CeO_2 NPs treatments, which indicate the membrane damage of the root cells.

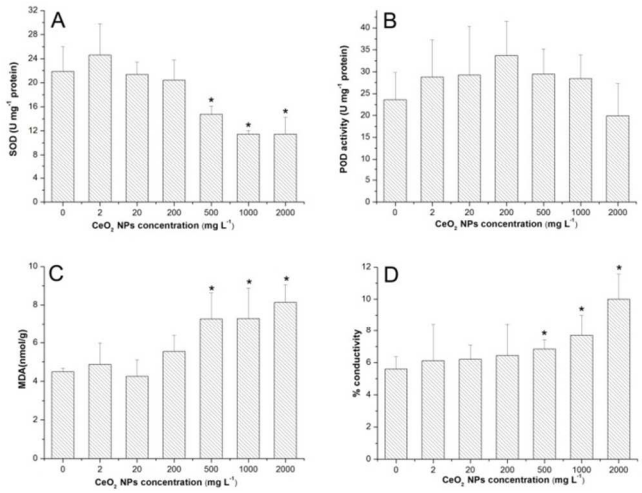

Figure 5. SOD (A), POD (B) activities, MDA contents (C) and ion leakage (D) in roots of asparagus lettuce treated with CeO_2 NPs for 5 days. The means are averaged from 4 replicates of asparagus lettuce. The values were given as mean ± SD (standard deviation).Significant differences versus control were marked with''*'' ($p < 0.05$).

Morphological change of root structure.

At 500 mg/L and higher concentrations, the roots of asparagus lettuce were swollen and twist, along with brownish lesions. As can be seen from the LM photograph of the root tips, the region close to the end of root swelled (Figure 6). Meanwhile, the cell population in meristem zone of treatment group was far less than control.

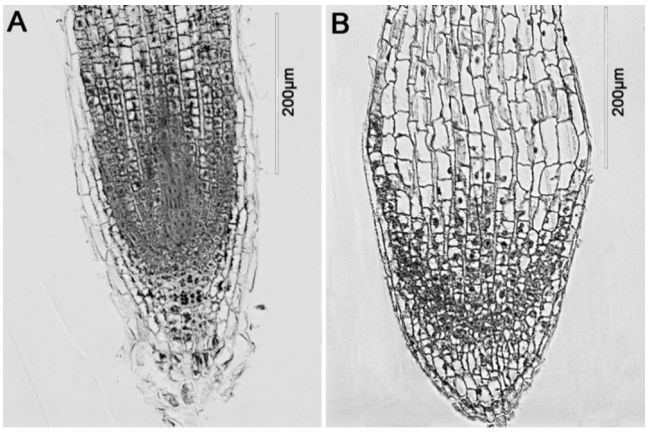

Figure 6. Light microscopic images of roots of asparagus lettuce after 5 days exposure. (A) control, (B) exposure with 2000 mg/L CeO_2 NPs.

Chemical species of Ce in the roots.

Normalized Ce L_{III} XANES spectra of model compounds and roots of asparagus lettuce were shown in Figure 7. Peak A in the spectra represents the characteristic peak of Ce (III), which comes from $CePO_4$, $Ce(CH_3COO)_3$ and $Ce_2(C_2O_4)_3$ here. Double peaks B and C indicate the characteristic peak of Ce (IV), which come from CeO_2 in this study [18]. Compared with spectra of the standard references, the spectra from roots of treated asparagus lettuce obviously showed the mixed feature of peak A, B and C. These indicate that Ce in roots present an mixed oxidation state of Ce(IV) and Ce(III). To obtain the quantitative information of Ce species in the roots, LCF was performed on the normalized spectra of samples using CeO_2, $CePO_4$, $Ce(CH_3COO)_3$ and $Ce_2(C_2O_4)_3$ as the standard compounds. The fitted lines and fitting parameters indicate that the results are satisfying and convincible (ESI, Figure S4). In the root samples, Ce species presented as 78.3% CeO_2 and 21.7% Ce carboxylates.

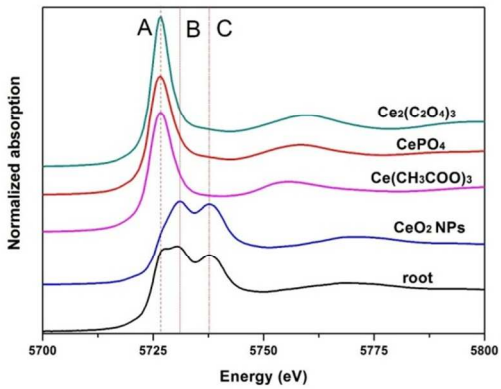

Figure 7. XANES Ce L_{III}-edge spectra (5723 eV) of standard references and rootsof asparagus lettuce treated with 2000 mg/L CeO_2 NPs for 5 days. Vertical dash line and dotted line marked the feature of Ce(III) and Ce(IV) compounds, respectively.

Effects of Ce^{3+} on root elongation.

As shown in Figure 8, root elongation of lettuce was inhibited by Ce^{3+} ions at all exposure concentrations even as low as 0.005 mg/L.

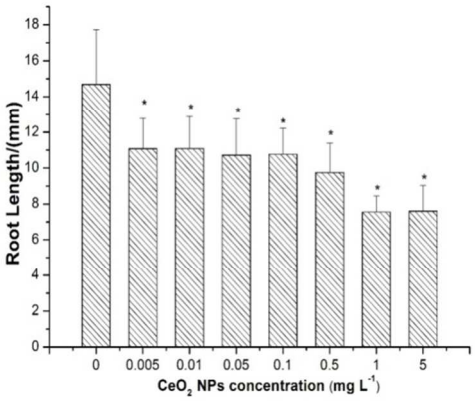

Figure 8. Root lengths of asparagus lettuce treated with Ce^{3+} for 5 days. The means are averaged from 4 replicates of asparagus lettuce. The values were given as mean ± SD (standard deviation). Significant differences versus control were marked with‘‘*’’ ($p < 0.05$).

Discussion

Phytotoxicity of NPs is influenced by various factors such as physicochemical properties of NPs, plant growth stage and properties of culture media. It has been demonstrated that exposure medium has an important effect on phytotoxicity of NPs.[19] Agar is a commonly used culture medium for plants. It is semisolid and more close to a realistic environment for plant growth. Agar media can avoid aggregation of NPs and distribute NPs evenly in test units [20]. But the soft gels restrict NP transport within the polysaccharide structure, and thus hinder NP bioavailability.[2] In the agar medium, Ce contents in both the roots and shoots of asparagus lettuce treated with CeO_2 NPs for 5 days were much lower than those cultured in CeO_2 NP aqueous suspensions. For example, at the same exposure concentration of 2000 mg/L, Ce contents in the roots and shoots of asparagus lettuce treated with CeO_2 NPs in the agar medium were 2090 μg/g and 110 μg/g. While in the aqueous suspension, Ce contents were 36220 μg/g and 1210 μg/g, respectively. [12]This was further confirmed by the TEM images of the root sections (ESI, Figure S5). In deionized water, a large quantity of CeO_2 NPs distributed on the root epidermis. However, in agar medium, no CeO_2 NPs can be found.

Although the bioavailability of CeO_2 NPs was limited, it seems that asparagus lettuce was more sensitive to the toxic effects of CeO_2 NPs in the agar medium than in distilled water (ESI, Figure S6). It is generally accepted that the toxicity of NPs may be caused by excess production of reactive oxygen species (ROS) [21]. ROS are normal products of plant cell functions, including free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (•OH), as well as hydrogen peroxide (H_2O_2), and so forth, and a delicate balance between their generation and removal must be strictly controlled to prevent oxidative stress.[22] Excessive production of ROS may cause lipid peroxidation, further promoting oxidative stress[23]. Malondialdehyde (MDA), which forms during peroxidation of unsaturated fatty acids and is indicative of lipid peroxide, was determined as a function of NP treatments. In the present study, with the increases of CeO_2 NP concentrations, SOD activities exhibited a decrease tendency. Meanwhile, the significant increases of MDA and ionic conductance levels at high concentrations suggest that the production of excess ROS had not been cleared timely. Membrane systems of the root cells were damaged, and ultimately the permeability of cell membrane was enhanced. Further, the changed osmotic pressure induced cell water loss and plasmolysis. The reduced root elongation and increased root diameters at high concentrations of CeO_2 NPs probably resulted from retarded cell division and increased cell width, along with more cortical cell columns in the case of CeO_2 NPs. These symptoms were similar to that of cucumber plants exposure to bulk Yb_2O_3[24] and the thick root syndrome in cucumber induced by ethylene. [25]

Biotransformation determines the ultimate fate and toxicity of manufactured nanoparticles in living organisms. The release of heavy metal ions may play a key role in phytotoxicity of metal-based NPs.[2] Plants can produce reducing substances such as phenols and sugars which will bring about redox reaction with the aid of biogenic organic acids.[11, 24] We previously demonstrated that Ce^{3+} released from CeO_2 NPs could induce species-specific toxicity to Lactuca plants.[12] In aqueous suspensions, about 6% of CeO_2 was reduced to cerium (III) carboxylates in lettuce roots after 5 days treatment. Now in this study, at the same exposure concentration (2000 mg/L), we found more than 20% of total Ce in the roots was transformed to Ce(III). Moreover, Asparagus lettuce that cultured in the agar medium showed a higher sensitivity to Ce^{3+} than those cultured in deionized water.[12] It is reasonable to postulate that the phytotoxicity of CeO_2 NPs observed in the present study was also attributed to the released Ce^{3+}.

As a conclusion, we evaluated the phytotoxicity of CeO_2 NPs in a plant agar medium, which provides a soil-like environment while

avoiding the uncertainties intrinsic to natural ecosystems for the first time. CeO_2 NPs showed a higher toxicity to asparagus lettuce in the agar medium than in aqueous suspensions. The phytotoxicity of CeO_2 NPs was probably attributed to the released Ce^{3+} in the roots.

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Notes and references

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