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Multigenerational exposure to cerium oxide nanoparticles: Physiological and biochemical analysis reveals transmissible changes in rapid cycling *Brassica rapa*

Xingmao Ma [a,*], Qiang Wang [b], Lorenzo Rossi [a], Stephen D. Ebbs [c], Jason C. White [d]

[a] Zachry Department of Civil Engineering, Texas A&M University, College Station, TX 77843, United States
[b] Department of Civil & Environmental Engineering, Southern Illinois University Carbondale, IL 62901, United States
[c] Department of Plant Biology and Center for Ecology, Southern Illinois University Carbondale, IL 62901, United States
[d] Department of Analytical Chemistry, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, CT 06504, United States

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ABSTRACT

The unique redox chemistry on the surface of cerium oxide nanoparticles (CeO_2NPs) and their broad applications in society have caused many concerns over the release and accumulation of these materials in the environment. Many investigations have been conducted with regard to the environmental health and safety effect of CeO_2NPs, including their impact on plant health. However, most previous studies were conducted on the early seedling development stage, with a small number of recent investigations examined the impact of CeO_2NPs throughout the life cycle of plants (e.g. from seed to seed). However, the long term, multigenerational impact of CeO_2NPs on plants remains unclear. The main aim of this study was to assess the physiological and biochemical consequences of multi-generational (three) CeO_2NPs exposure over a range of concentrations (0–1000 mg/L) on *B. rapa*. The results showed that plants in the second and third generation displayed slower plant growth and smaller biomass. The Brassica plants also bore 39%, 59% and 61% less siliques after two generations of exposure to 10, 100 and 1000 mg/L of CeO_2NPs. The numbers of seeds produced per silique were also reduced in the third generation plant by over 50% following the exposure to 100 and 1000 mg/L of CeO_2NPs. In addition, plants in the later generations generally contained higher concentrations of hydrogen peroxide (H_2O_2) in their tissues. Altogether, our results suggest that the second and third generation plants might have experienced higher oxidative stress than the first generation plants. This study provided first evidence that the impact of CeO_2NPs varied across generations and long term evaluation extending several generations of plant growth is necessary to obtain a realistic understanding on the long term impact of engineered nanoparticles.

1. Introduction

Cerium oxide nanoparticles (CeO_2NPs) are widely used in the production of catalysts, sunscreen creams, fuel additives, microelectronics and polishing agents due to their unique catalytic and optic properties (Cassee et al., 2011). The increased applications of CeO_2NPs could lead to greater occurrence and impact of this nanoparticle on the environment (Rico et al., 2013a). This concern has spurred many studies on the environmental health and safety effect of CeO_2NPs, including their impact on plants (Schwab et al., 2015). The general conclusion from the previous studies, many of which were conducted in short term and hydroponic systems, was not surprising; the impact of CeO_2NPs on plant growth depends upon both the plant species and exposure concentration (Ma et al., 2010; Lopez-Moreno et al., 2010).

While these short term studies provided insightful information concerning the extent and mechanisms of CeO_2NP toxicity, it remains unclear how this nanoparticle will affect plant development under more realistic conditions, which will likely involve exposure over a much longer time but at relatively lower concentrations. With the realization of this knowledge gap, several longer term studies utilizing the entire life cycle of plants have emerged. For example, Zhao et al. (2013) reported that exposure to 10 nm CeO_2NPs at 400 and 800 mg/kg soil for 53 days (one generation) did not cause any overt toxicity to cucumber as indicated by inconsequential changes in a series of physiological parameters (e.g. photosynthesis, stomatal conductance). Notably, the plant yield from a higher exposure of 800 mg/kg soil was reduced by 31.6%. A subsequent study from the same group also reported that 800 mg/kg soil CeO_2NPs significantly decreased the phenolic content in cucumber (Zhao et al., 2014). A similar finding was reported for corn that although 800 mg/kg CeO_2NPs did not affect plant photosynthesis, exposure did significantly reduce the corn biomass by 38% (Zhao et al., 2015). Interestingly, the yield of wheat was

* Corresponding author at: Zachry Department of Civil Engineering, Texas A&M University, 3136 TAMU, College Station, TX 77843, United States.
E-mail address: xma@civil.tamu.edu (X. Ma).

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unaffected after the plants were exposed to 400 mg/kg CeO_2NPs under field conditions, but antioxidant enzymes such as superoxide dismutase (SOD) activity and the root and leaf cell microstructures were significantly altered (Du et al., 2015). Wang et al. (2012) irrigated tomato plants from seed germination to fruit ripening (70 days) with low concentrations of CeO_2NPs suspensions (<10 mg/L) and noticed either insignificant or slightly enhanced plant growth and fruit yield. The authors collected seeds from the control and 10 mg/L CeO_2NPs treated plants and then grew them hydroponically in either control or 10 mg/L CeO_2NPs suspensions again (Wang et al., 2013). Interestingly, the tomato seedlings exposed to 10 mg/L of CeO_2NPs for two generations were significantly smaller than the control or the seedlings which were exposed to CeO_2 NPs only once (Wang et al., 2013). This study provided the first evidence of variable generational response of plants to CeO_2NPs. Consistent with this observation, a recent study showed that continuous exposure of Arabidopsis to 75 μg/L of 20 nm silver nanoparticles (AgNPs) significantly reduced the seed germination of the offspring Arabidopsis over three subsequent generations (Geisler-Lee et al., 2014). These studies highlight the need for more comprehensive evaluations on the generational impact of engineered nanoparticles on plants.

This study aims to evaluate the effect of CeO_2NPs on plant growth and the oxidative stress responses of a rapid cycling *Brassica rapa* (*B. rapa*) in soil growing condition across three generations. This variety of *B. rapa* has been specially bred to have a short life cycle, going seed to seed in approximately 35 days (Williams and Hill, 1986). The *B. rapa* plants were irrigated with suspensions containing 0, 10, 100 or 1000 mg/L of CeO_2NPs to maturity so that the seeds could be collected. The seeds were germinated and the growth cycles repeated under the same irrigation conditions for another generation so that another offspring generation of seeds could be obtained. Altogether, three generations of seeds: (first (F_0), second (F_1) and third (F_2) generation) were collected. The first generation was the wild type seeds. All three generations of seeds were then germinated and cultivated together in the same environment at the same time. The key question we aimed to address was: would the multigenerational exposure to CeO_2NPs affect the biological processes, seed yield and reproduction of *B. rapa* plants to an extent that they might further affect the plant growth and oxidative stress responses of subsequent generations? During the exposure, a series of physiological parameters such as the plant biomass, shoot height, chlorophyll content, and biochemical parameters such as root membrane lipid peroxidation, and activities of several important antioxidant enzymes such as SOD and catalase (CAT) were monitored.

2. Materials and methods

2.1. Characterization of CeO_2NPs

The CeO_2NP suspension (10% weight in H_2O or 109,000 mg/L) used in this study were purchased from Sigma Aldrich (St. Louis, MO). The particles were not surface modified. The primary nanoparticle size was 20 ± 1.9 nm (mean ± standard deviation) as revealed by transmission electron microscope (TEM, Hitachi H-7650, Hitachi, Japan) images and was consistent with the values provided by the vendor (<25 nm) (data not shown). Most nanoparticles had a spherical shape. The nanoparticles aggregated after they were suspended in DI water and the extent of aggregation varied with the concentrations of particles. The hydrodynamic diameter and zeta potential of the CeO_2NPs in liquid suspensions were measured with a Zetasizer Nano Z90 (Malvern Instrument Ltd., Worcestershire, UK). The hydrodynamic diameter of CeO_2NPs suspended in DI water at 10, 100 and 1000 mg/L was 267.1 ± 2.9, 252.9 ± 4.5 and 208.4 ± 0.9 nm, respectively. The zeta potential of CeO_2NPs in these solutions was 15.5 ± 1.8, 33.7 ± 0.2 and 46.1 ± 0.2 mV, respectively. The reported values for the hydrodynamic diameter and the zeta potential were mean ± standard deviation (n = 3).

2.2. Plant growth and seed harvest

The plant species used in this study was rapid cycling *B. rapa* (University of Wisconsin-Madison, WI). The original *B. rapa* seeds (F_0) were purchased from Carolina Biological Supply Company (Burlington, NC). The seeds were sterilized in 2.7% commercial Clorox bleach for 10 min, and washed three times with DI water. The F_0 seeds were placed in 100 mm × 15 mm polystyrene petri dishes (10 seeds each) with a thin layer of DI water above the supporting filter paper and three dishes were prepared altogether. The petri dishes were then sealed with parafilm, and wrapped with aluminum foil. The stratification started with cold exposure at 4 °C for three days, after which petri dishes were unwrapped and incubated in a growth cart with 16-h photoperiod. The light intensity was 133 μmol m^{-2} s^{-1}. The growth cart was exposed to ambient temperature (~25 °C) and humidity (10–30%). After cotyledons were fully open, seedlings were gently transferred to pots (one plant per pot) containing 50 g of the mixture of topsoil/vermiculite (1:1 v/v ratio). The topsoil/vermiculite mixture had been saturated with one quarter strength Hoagland solution (Phyto Technology Lab, Shawnee Mission, KS) before transplant. The pots were set in the same growth carts as used for seed germination. A 10 mL aliquot of CeO_2NP suspension at concentrations of 0, 10, 100 or 1000 mg/L were supplied to the corresponding pots daily. During irrigation, CeO_2NPs suspension or DI water was slowly and evenly poured on the soil surface to uniformly distribute NPs in the media. As per the propagation instructions provided with the seeds, in the early floral stage, dried bee sticks were used to facilitate pollination between plants in the treatment scenario. Brassica plants in different treatment groups were placed far enough to prevent outcrossing during pollination. All Brassica plants proceeded through the 35 day lifecycle as well as additional five days for seed maturation. Irrigation was stopped when the siliques were fully mature. Seeds were harvested after siliques were completely dried. The seeds harvested from the F_0 (parental) plants were denoted as F_1 (second generation) seeds. The F_1 seeds were then germinated and treated under the same exposure scenarios as their parental plants to collect the third generation seeds (F_2). At least ten plants were grown for each treatment to ensure that enough seeds were collected. All harvested seeds and the purchased standard seeds were stored at 4 °C in dark.

2.3. Seed germination

To assess initial seed quality, 30 seeds from each of the nine seed sources across three generations (F_0 × 1, F_1 × 4 and F_2 × 4) were weighed; the measurement was repeated three times. Then, a total of one hundred F_0, F_1 and F_2 seeds from each seed source were germinated separately in petri dishes (20 seeds/petri dish, 5 replicates per treatment) in DI water to further assess the influence of generational exposure of the parental plants on seed quality. The germination rate was calculated as the number of seedlings with the cotyledons fully opened out of 20 seeds tested in each petri dish on the third day after stratification.

2.4. Seedling growth

After the cotyledons fully opened, seedlings were transplanted to the soil mixture as described earlier for continued growth. CeO_2NPs exposure started after the seedlings were transplanted. All seedlings were irrigated with either DI water or corresponding CeO_2NPs suspensions at 10, 100 and 1000 mg/L. For F_1 and F_2 seedlings, the irrigation scenario was exactly the same as their parental plants. Plant height (e.g. total of stem or stem plus inflorescence height) was measured (five replicates) every five days. Fresh plant biomass (five replicates) was also measured after gently pulling the seedlings out of the growth media and rinsing the roots three times with DI water on day 10, day 15 and day 20. Out of the five replicates, three plants were randomly chosen