1 **Multigenerational Exposure to Cerium Oxide Nanoparticles: Physiological and**
2 **Biochemical Analysis Reveals Transmissible Changes in Rapid Cycling *Brassica rapa***

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ABSTRACT

The unique redox chemistry on the surface of cerium oxide nanoparticles (CeO₂NPs) 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 CeO₂NPs, 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 CeO₂NPs throughout the life cycle of plants (e.g. from seed to seed). However, the long term, multigenerational impact of CeO₂NPs on plants remains unclear. The main aim of this study was to assess the physiological and biochemical consequences of multi-generational (three) CeO₂NPs 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 CeO₂NPs. 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 CeO₂NPs. In addition, plants in the later generations generally contained higher concentrations of hydrogen peroxide (H₂O₂) 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 CeO₂NPs 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.

Keywords:

Cerium oxide nanoparticle; oxidative stress; ROS; *Brassica rapa*; multigenerational impact

Introduction

Cerium oxide nanoparticles (CeO₂NPs) are widely used in the production of catalysts, sunscreen creams, fuel additives, microelectronics and polishing agents due to their unique catalytic and optic properties¹. The increased applications of CeO₂NPs could lead to greater occurrence and impact of this nanoparticle on the environment². This concern has spurred many studies on the environmental health and safety effect of CeO₂NPs, including their impact on plants³. The general conclusion from the previous studies, many of which were conducted in short term and hydroponic systems, was not surprising; the impact of CeO₂NPs on plant growth depends upon both the plant species and exposure concentration^{4,5}.

While these short term studies provided insightful information concerning the extent and mechanisms of CeO₂NP 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.⁶ reported that exposure to 10 nm CeO₂NPs 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 CeO₂NPs significantly decreased the phenolic content in cucumber⁷. A similar finding was reported for corn that although 800 mg/kg CeO₂NPs did not affect plant photosynthesis, exposure did significantly reduce the corn biomass by 38%⁸. Interestingly, the yield of wheat was unaffected after the plants were exposed to 400 mg/kg CeO₂NPs under field conditions, but antioxidant enzymes

such as superoxide dismutase (SOD) activity and the root and leaf cell microstructures were significantly altered⁹. Wang et al.¹⁰ irrigated tomato plants from seed germination to fruit ripening (70 days) with low concentrations of CeO₂NPs 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 CeO₂NPs treated plants and then grew them hydroponically in either control or 10 mg/L CeO₂NPs suspensions again¹¹. Interestingly, the tomato seedlings exposed to 10 mg/L of CeO₂NPs for two generations were significantly smaller than the control or the seedlings which were exposed to CeO₂ NPs only once¹¹. This study provided the first evidence of variable generational response of plants to CeO₂NPs. 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 generations¹². 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 CeO₂NPs 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 days¹³. The *B. rapa* plants were irrigated with suspensions containing 0, 10, 100 or 1000 mg/L of CeO₂NPs 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 (F₀), second (F₁) and third (F₂) 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 CeO₂NPs 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.

Materials and Methods

Characterization of CeO₂NPs

The CeO₂NP suspension (10% weight in H₂O 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 $\pm$ 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 CeO₂NPs in liquid suspensions were measured with a Zetasizer Nano Z90 (Malvern Instrument Ltd, Worcestershire, UK). The hydrodynamic diameter of CeO₂NPs 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 CeO₂NPs 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 $\pm$ standard deviation (n=3).

Plant growth and seed harvest

116 The plant species used in this study was Rapid Cycling *B. rapa* (University of Wisconsin-
117 Madison, WI). The original *B. rapa* seeds (F_0) were purchased from Carolina Biological Supply
118 Company (Burlington, NC). The seeds were sterilized in 2.7% commercial Clorox bleach for 10
119 minutes, and washed three times with DI water. The F_0 seeds were placed in 100 mm x 15 mm
120 polystyrene petri dishes (10 seeds each) with a thin layer of DI water above the supporting filter
121 paper and three dishes were prepared altogether. The petri dishes were then sealed with parafilm,
122 and wrapped with aluminum foil. The stratification started with cold exposure at 4°C for three
123 days, after which petri dishes were unwrapped and incubated in a growth cart with 16-hour
124 photoperiod. The light intensity was $133 \mu\text{mol m}^{-2} \text{s}^{-1}$. The growth cart was exposed to ambient
125 temperature (~25°C) and humidity (10 - 30%). After cotyledons were fully open, seedlings were
126 gently transferred to pots (one plant per pot) containing 50 g of the mixture of topsoil/vermiculite
127 (1:1 v/v ratio). The topsoil/vermiculite mixture had been saturated with one quarter strength
128 Hoagland solution (Phyto Technology Lab, Shawnee Mission, KS) before transplant. The pots
129 were set in the same growth carts as used for seed germination. A 10 mL aliquot of CeO_2NP
130 suspension at concentrations of 0, 10, 100 or 1000 mg/L were supplied to the corresponding pots
131 daily. During irrigation, CeO_2NPs suspension or DI water was slowly and evenly poured on the
132 soil surface to uniformly distribute NPs in the media. As per the propagation instructions
133 provided with the seeds, in the early floral stage, dried bee sticks were used to facilitate
134 pollination between plants in the treatment scenario. Brassica plants in different treatment groups
135 were placed far enough to prevent outcrossing during pollination. All Brassica plants proceeded
136 through the 35 day lifecycle as well as additional five days for seed maturation. Irrigation was
137 stopped when the siliques were fully mature. Seeds were harvested after siliques were
138 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.

Seed germination

To assess initial seed quality, 30 seeds from each of the nine seed sources across three generations ($F_0 \times 1$, $F_1 \times 4$ and $F_2 \times 4$) 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.

Seedling growth

After the cotyledons fully opened, seedlings were transplanted to the soil mixture as described earlier for continued growth. CeO_2 NPs exposure started after the seedlings were transplanted. All seedlings were irrigated with either DI water or corresponding CeO_2 NPs 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 and their leaves collected for total chlorophyll analysis following the method developed by Reiss et al.¹⁴. The five plants randomly chosen at

each measurement date for biomass determination were not planted back and reused. At the end of the plant lifecycle (35 days), silique morphology, silique numbers and seed yield (number of seeds per silique) were recorded.

Hydrogen Peroxide Quantification and Visualization

To understand the effect of multigenerational exposure to CeO₂NPs on oxidative stress responses, 10-day (vegetative), 15-day (early flowering) and 20-day (late floral) old Brassica plants were harvested. On each date, fresh biomass of the whole plant was individually measured after the plant was removed from the soil and rinsed thoroughly with DI water. Then, the same plant was analyzed for H₂O₂ content with three replicates per treatment. The assay for H₂O₂ quantification followed the method reported by Velikova et al.¹⁵. The whole plant was homogenized in 0.1% (w/v) trichloroacetic acid (TCA) on ice. The homogenate was then poured into a 2 mL Eppendorf tube and centrifuged at 12,000 g for 20 min at 4 °C. The supernatant was then mixed with assay buffer containing 10 mM potassium phosphate (KH₂PO₄) at pH 7 and 1 M of potassium iodide (KI) in a total volume of 2 mL, containing 0.5 mL supernatant, 0.5 mL KH₂PO₄ and 1 mL KI. The absorbance of the mixture was immediately measured using a Varian CARY 50 Bio UV-Vis spectrophotometer at 390 nm. The H₂O₂ content was obtained based on a standard curve, which was generated from the absorbance of H₂O₂ at concentrations in a range of 0 - 200 µM H₂O₂ mixed in the same assay buffer. The H₂O₂ concentration in plant tissues was normalized to fresh weight of the tissue.

H₂O₂ was visualized in the tissue using 3,3'-diaminobenzidine (DAB) staining on exposure day 15 in order to minimize the interference from leaf senescence. Three leaves and three root sections (2 mm root tip) from three different plants were used for the H₂O₂ visualization. Visualization of H₂O₂ in root was performed following the method described

previously ¹¹. To visualize the H₂O₂ in leaf, a revised protocol in Thordal-Christensen's ¹⁶ study was adopted. All leaves on each plant were submerged in 1 mg/mL of DAB solution at pH 3.6 for 10 hours. Stained leaves were cleared of chlorophyll by boiling in absolute ethanol (200 proof) for no longer than 10 minutes. The cleared leaves were then examined using a Kruss MBL3000 light microscope equipped with Kruss digital camera.

Estimation of antioxidant enzymes activity

To investigate antioxidant enzymes, three plant replicates were used to analyze the activity of SOD, CAT and guaiacol peroxidase (GPX). After gently and thoroughly washing away soil residue from the root surfaces, the whole plant was weighed and snap frozen by liquid nitrogen, and then homogenized in 50 mM KH₂PO₄ buffer (pH 7.0) containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 1% (wt%) of polyvinylpolypyrrolidone (PVPP). The homogenate was then centrifuged at 15,000 g for 20 min at 4 °C. The supernatant was used to quantify the activities of SOD, CAT and GPX.

The SOD (EC1.15.1.1) activity was estimated by the inhibition of NADH (the reduced form of nicotinamide adenine dinucleotide)-phenazine methosulphate-nitroblue tetrazolium formazan production as per the method of Kakkar et al ¹⁷. A 200 µL aliquot of the supernatant was mixed with an assay buffer containing 1.2 mL of 25 mM sodium pyrophosphate (Na₂HPO₄, pH 8.3), 0.1 mL of 186 µM of phenazine methosulphate (PMS), 0.3 mL of 300 µM of nitroblue tetrazolium (NBT) and 0.2 mL of 780 µM of NADH. The absorbance of the 2 mL reagent mixture was continuously acquired at 10 s intervals for 90 s, using a Varian CARY 50 Bio UV-Vis spectrophotometer (Varian USA) at 560 nm. One unit of SOD is defined as the required amount of SOD that contributes 50% inhibition of NBT reduction (Blank control obtained via

NBT reduction in 90 s without SOD extract). The SOD activity is expressed as units/mg total protein.

The assay for CAT (EC1.11.1.6) activity was performed based on the decrease in absorbance at 240 nm, resulting from the H₂O₂ reduction catalyzed by CAT. The assay of GPX (EC1.11.1.7) was implemented based on the formation of tetraguaiacol through the oxidation of guaiacol. The activities of CAT and GPX were estimated following the protocol of Maehly et al¹⁸. A 15 µL aliquot of the extract was used for the estimations of CAT or GPX activity. The CAT activity was determined via continuously monitoring the decomposition of H₂O₂ for 60 s with time interval of 10 s in 1 mL assay buffer containing 982.5 µL 50 mM KH₂PO₄ buffer (pH 7.0), and 2.5 µL 10 mM H₂O₂, using a Varian CARY 50 Bio UV-Vis spectrophotometer at 240 nm. The pseudo-first order reaction constant (a') of the decrease of absorbance was obtained via linear regression analysis (slope of the 7 readings). The CAT activity was finally determined as pmol/mg total protein per min. For the GPX activity assay, the oxidation kinetics of guaiacol was measured in a buffer containing 200 µL of supernatant, 1.7 mL 50 mM KH₂PO₄ buffer (pH 7.0) containing 1 mM of EDTA, 2 µL of 10 mM guaiacol and 5 µL of 10 mM H₂O₂ at 470 nm wavelength for 1 min at the same time interval as CAT assay. The GPX activity was determined as µ mol tetraguaiacol formed / mg protein per min.

Estimation of Total Protein Content

Total protein content was determined using the NI (Non-Interfering) Protein Assay extraction kit (Geno Technology, St. Louis, MO, USA) according to the manufacturer's instructions. The fresh weight of the whole plant seedlings used for the protein analysis was determined beforehand and the total protein was normalized as g total protein/g fresh plant tissue. Three replicates were prepared for each treatment.

Lipid peroxidation

Plant lipid peroxidation was determined via thiobarbituric acid reactive substance (TBARS) method ¹⁹, in which malondialdehyde (MDA) was used as an indicator of the lipid peroxidation of the plasma membrane. To extract MDA, 200 mg fresh plant tissues (100 mg root and 100 mg shoot) were homogenized in 4 mL of 0.1% (w/v) trichloroacetic acid (TCA) after they were snap frozen in liquid nitrogen. The homogenate was then centrifuged at 8,000 g for 15 min at room temperature. A 1 mL aliquot of supernatant was transferred into a reaction buffer containing 2 mL of 10% (w/v) TCA and 2 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA). The mixture was heated at 95 °C for 20 min. The reaction was terminated by cooling the reaction tube on ice. The absorbance of the final mixture was obtained at 532 and 600 nm using a Varian CARY 50 Bio UV-Vis spectrophotometer. The value for non-specific absorption at 600 nm was subtracted from the value at 532 nm, and the MDA amount was finally calculated based on Beer-Lambert's Law with a MDA extinction coefficient of 155 mM⁻¹cm⁻¹.

Statistical analysis

One-way ANOVA test was performed on seed biomass analysis at each testing concentration (n=3-5). Two-way ANOVA was performed for all other parameters analyzed on day 10, 15 and 20, with the dosing concentration and the generation as two variables. If an interaction of generation and concentration was observed, one-way ANOVA followed by Tukey's post hoc analysis was performed to determine the statistical differences among three generations (F₀, F₁ and F₂) at each testing concentration (0, 10, 100 and 1000 mg/L). The results are mean ± standard error (SD). All statistical tests were processed with SAS 9.3 for Windows software package (SAS Institute Inc., Cary, NC).

Results

Seed quality

Exposure to CeO₂NPs significantly reduced seed biomass of the offspring generations compared with that of the parental generation (Figure 1). The generational reduction of seed biomass was more significant at higher concentrations. At 10 mg/L, only the third generation (F₂) seeds had significantly smaller biomass compared with those from the first generation (F₀), but both offspring generations had significantly smaller seed biomass at higher concentrations. The germination rate (lower panel, Figure 1) of the control seeds was comparable over three generations. The germination rate of the treated second generation seeds (F₁) in DI water was unaffected by the treatments to their parental plants. However, the germination rate of treated F₂ seeds in DI water decreased by approximately 20% compared with the first generation seeds (F₀) ($P < 0.001$) (Figure 1).

Plant biomass

Plant height was unaffected by multigenerational exposure to CeO₂NPs at the applied concentrations; all plants reached about 120-140 mm on day 20, the late floral stage (Supplementary Figure 1). However, CeO₂NPs significantly reduced the plant biomass of the treated F₁ and F₂ plants compared with the treated F₀ plants during the vegetative, early and late floral stage ($P < 0.001$ for all three stages) (Figure 2). The impact of exposure concentration varied with generations. For F₀ plants developed from the wild seeds, 1000 mg/L of CeO₂NPs increased the plant biomass by 47% and 43% compared with the F₀ controls at the early and late floral stages ($P < 0.05$), while the lower two concentrations did not show any effect throughout the plant growth stages. For F₁ plants developed from the F₁ seeds, exposure to CeO₂NPs at 1000 mg/L only significantly increased the F₁ plant biomass by 39% in the late floral stage when

compared with the F₁ controls ($P < 0.05$). The F₁ plant biomass was unaffected by other concentrations. The increase of plant biomass of the treated F₂ plants compared with the F₂ controls was not observed at any growth stage or at any concentration.

Chlorophyll content

Generation was a significant factor affecting the chlorophyll content, with the offspring generations generally containing a higher concentration of total chlorophyll (Supplementary Figure 2). F₁ plants treated with CeO₂NPs at 100 mg/L showed a higher chlorophyll a and b content than the F₀ plants under the same treatment during vegetative stage ($P < 0.05$). During the early floral stage, statistically significant differences were not observed between the three generations for all testing concentrations. During the late floral stage, 100 and 1000 mg/L CeO₂NPs significantly increased the total chlorophyll of F₂ plants compared with the F₀ plants.

Plant reproduction

Multigenerational exposure to CeO₂NPs significantly reduced seed yield in later generations. A comparison of the silique morphology across generations indicated that offspring generations produced smaller siliques than their parental plants after CeO₂NPs exposure at equivalent concentrations. Siliques were also visually thinner and smaller with increasing concentrations within a given generation (Supplementary Figure 3). Multigenerational exposure to CeO₂NPs not only reduced the size but also the numbers of siliques produced per plant by offspring generations (Figure 3). Seed formation was also significantly affected by CeO₂NPs. ($P < 0.001$ for both generation and concentration) (Figure 3). While the number of seeds formed per silique was unaffected by CeO₂NPs treatment at 10 mg/L, exposure to CeO₂NPs at 100 and 1000 mg/L significantly decreased the number of seeds per silique in the F₂ plants ($P < 0.05$ for both

concentrations). The F₁ plants exposed to CeO₂NPs at 100 mg/L also showed fewer seeds per silique as compared with F₀ plants under same treatment condition.

Hydrogen peroxide (H₂O₂) quantification and visualization

Exposure to CeO₂NPs significantly increased the H₂O₂ concentration of the F₁ and F₂ plants in vegetative stage ($P = 0.001$) (Figure 4). During the vegetative stage, F₁ plants treated by CeO₂NPs at 10 mg/L showed a higher H₂O₂ content than the F₀ plants. A significant increase in the H₂O₂ content was also observed in F₂ plants treated by 1000 mg/L of CeO₂NPs ($P < 0.05$). In the early floral stage, CeO₂NPs significantly increased the H₂O₂ content in F₁ plants at 10 and 100 mg/L of CeO₂NPs ($P < 0.05$ for both concentrations), but F₂ plants showed no differences compared with F₀ plants. An interaction between generation and concentration was not observed. During the late floral stage, H₂O₂ content in 100 and 1000 mg/L of CeO₂NPs treated F₁ and F₂ plants was significantly higher than that of the F₀ plants ($P < 0.001$); F₁ and F₂ plants exhibited similar H₂O₂ content level in this stage. Exposure to different concentrations of CeO₂NPs significantly increased the H₂O₂ content of the F₁ (76%, 92% and 43% respectively) and F₂ plants (95%, 63% and 45% respectively) at the late floral stage compared with their respective controls while H₂O₂ content in F₀ plants was unaffected by concentration. The DAB staining provided more information on the localization of H₂O₂ in leaves and root tips (Supplementary Figure 4-5). In leaves from the F₀ generation, H₂O₂ production was slightly elevated and found mostly in the primary leaf vasculature with some evidence of interveinal and marginal H₂O₂. The staining of peroxide was stronger for the 100 and 1000 mg/L treatments for F₁ leaves, with strong interveinal staining. Staining decreased for F₂ plants, with the additional H₂O₂ staining found on the leaf margins on the 1000 mg/L treatment. A much more discernable pattern of

H₂O₂ staining was evident in root tips. With increasing concentration and increasing number of generations, there was a greater staining evident, indicating significant H₂O₂ production.

Lipid peroxidation

Both concentration ($P < 0.001$) and generation ($P = 0.003$) significantly affected the malondialdehyde (MDA) production of the whole plant (Figure 5). The MDA concentration in the vegetative stage of the F₁ and F₂ plants was significantly higher than the F₀ plants and increased with the increase of CeO₂NPs concentration. The MDA concentration in F₂ plants treated by CeO₂NPs at 10 mg/L was increased by 42% compared with the F₀ plants, and both the F₁ and F₂ plants treated by 1000 mg/L of CeO₂NPs also showed significant increases in MDA concentrations. In the early floral stage, the MDA concentration of the F₂ plants was significantly higher than that of the F₀ plants while the F₁ plants showed an increased MDA concentration only at the 100 mg/L of CeO₂NPs exposure. At the late floral stage, the MDA concentration in the F₁ and F₂ plants was generally lower than that of the F₀ plants ($P = 0.001$).

Antioxidant enzyme activities

The assays showed that the enzymatic activity was altered in response to the CeO₂NPs exposures (Figure 6). In the vegetative stage, SOD activity of the F₁ plants treated by 100 mg/L of CeO₂NPs was significantly decreased compared with the F₀ plants. During the early floral stage, the F₁ plants showed increased SOD activity; the F₂ plants treated by CeO₂NPs at 100 mg/L also had notably higher SOD activity than the F₀ plants ($P < 0.05$). However, plants from all treatments exhibited similar SOD activity at the late floral stage. The CAT activity of F₂ plants treated by CeO₂NPs at 1000 mg/L was significantly higher than that of the F₀ and F₁ plants at the vegetative stage. CAT activity was comparable for plants across the treatment at the early floral stage, but F₁ generation activity was significantly higher from 100 and 1000 mg/L of

CeO₂NPs exposure than other two generations of plants. At the vegetative stage, F₁ and F₂ plants displayed significantly lower GPX activity compared with the F₀ plants treated with 10 and 100 mg/L of CeO₂NPs ($P < 0.001$). At the early floral stage, the 1000 mg/L of CeO₂NPs treated F₁ plants demonstrated significantly higher GPX activity than that of the F₀ plants. The GPX activity of the F₂ plants was significantly lower than that of the F₀ plants from the 10 and 100 mg/L of CeO₂NPs treatment groups at the late floral stage.

Discussion

Previous studies have reported that plant-ENPs (engineered nanoparticles) interactions influence plant growth and development, and can induce excessive production of H₂O₂ that can cause significant oxidative stress^{1, 20-24}. However, little information has been reported on the impact of multigenerational exposure to CeO₂NPs on plant progeny. This represents one of the first comprehensive studies showing that such multigenerational exposure to CeO₂NPs can inhibit plant growth, reduces reproduction, and leads to greater oxidative stress on subsequent plant generations.

Previous studies have reported that CeO₂NPs enhance plant growth of Arabidopsis²⁴, barley²⁵, cucumber, tomato, alfalfa, and corn⁵. Notably, all of these studies were performed over relatively short periods of time; certainly not longer than one generation. In agreement with these studies, our results also demonstrated that 1000 mg/L of CeO₂NPs enhanced plant biomass, as well as plant photosynthesis from increases in the total chlorophyll content in the F₀ generation. However, this enhancing effect was less significant in the F₁ generation and completely lost in the F₂ generation. A previous study also reported that after a lifecycle exposure to 10 mg/L of CeO₂NPs, the second generation tomato seedlings experienced stronger oxidative stress, and observed growth was stunted¹¹. The consistent pattern from these

multigenerational exposure is that even at relatively low concentrations, plant health and vigor was compromised. Notably, the multigenerational effects differed significantly from that observed during a single generation exposure, suggesting that caution must be used when extrapolating the environmental effect of CeO₂NPs from short term studies to predict the long term environmental health and safety.

Previous studies have reported that CeO₂NPs could be bioaccumulated by tomato, cucumber, rice and many other plant species^{11, 26-28}. Following uptake, the conversion between Ce³⁺ and Ce⁴⁺ oxidation states on the surface of CeO₂NPs due to the oxygen vacancy in the lattice structure was suggested to be the cause of reactive oxygen species (ROS) formation in plant tissues²⁹⁻³¹. Heckert et al.³² attributed the reduction of Ce⁴⁺ to Ce³⁺ as the primary mechanism for the ability of CeO₂NPs to convert superoxide radicals to H₂O₂, and presumed that Ce³⁺ sites on CeO₂NPs surface are the active sites for SOD mimetic activity. The authors also found that the SOD mimetic activity of CeO₂NPs increased with increasing Ce³⁺/Ce⁴⁺ ratio. It is known that plants can produce reducing substances such as sugars and phenolic compounds, which can trigger redox reactions^{7, 33}. Hence, we speculate that the higher H₂O₂ content in the early floral stage in the F₁ and the F₂ plants upon exposure to CeO₂NPs may have been partially caused by the increased SOD mimetic activity. The leaves of the F₁ plants irrigated by 100 and 1000 mg/L CeO₂NPs and the F₂ plants irrigated by 1000 mg/L CeO₂NPs displayed larger and darker DAB staining in the leaf blade and margin, suggesting the possibility of CeO₂NPs induced H₂O₂ production, perhaps via the photorespiration cycle³⁴. Additionally, the increased oxidative stress of the F₁ and the F₂ plants correlated with a concomitant increase in lipid peroxidation during the vegetative and the early floral stages. This finding agrees with previous observations, where increased oxidative stress caused greater lipid peroxidation²⁴. However, the

F₁ and F₂ plants during the late floral stage showed higher H₂O₂ content but lower lipid peroxidation, which may result from decreased unsaturated fatty acid content in the cell membrane. Rico et al.²⁶ reported that exposure to CeO₂NPs significantly increased the H₂O₂ content in the rice seedlings, but the cell membrane was undamaged. The authors found that the negligible lipid peroxidation was due to the low unsaturated fatty acid content in the cell membrane. Another possibility for the observed effects of CeO₂NPs over three generations could be the release and accumulation of Ce³⁺ in plant tissues which has been shown to affect plants. Clearly, further study is needed to understand the underlying mechanisms for the generational effects of CeO₂NPs.

Plants maintain the balance of ROS content via enzymatic and non-enzymatic processes to prevent oxidative stress/damage³⁵. A previous study with 10 day old rice seedlings grown in the presence of CeO₂NPs (62.5 to 500 mg/L) showed that exposure to 500 mg/L of CeO₂NPs increased the SOD and APX activities, but CAT activity was unaffected²⁶. Contrary to what was reported in that study, significantly increased CAT activity in the 1000 mg/L CeO₂NPs treated F₂ plants was observed, which showed that multigenerational exposure at this level induced stronger protective responses against oxidative stress. Lower GPX activity suggested that the H₂O₂ was probably generated in the peroxisome where CAT mainly resides. It is possible that the Rubisco oxygenase reaction and the photorespiratory pathway produced more H₂O₂ in the peroxisome as catalyzed by glycolate oxidase³⁴. Then, CAT rather than GPX was activated to remove the H₂O₂ in this cellular region. As plants continued to mature, H₂O₂ production in cytosol and other parts of the plant cell might have also increased, inducing higher GPX activity during early floral stage while CAT activity remained high. The results agree with Majumdar et al. where GPX diffused oxidative stress by balancing ROS production and scavenging processes

412 in kidney bean plants after 15 days of exposure to CeO₂NPs ³⁶. In our study, during the early
413 floral stage the F₁ plants showed higher GPX activity than the F₂ plants. It is possible that other
414 antioxidant proteins such as APX, dehydroascorbate reductase (DHAR), or glutathione reductase
415 (GR) were more active in the F₂ plants than in the F₁ individuals ²⁶. Antioxidant proteins were
416 not active during the late floral stage, where SOD and the GPX activity was lower in the later
417 generational plants; however, this may be caused by plant senescence. Antioxidant proteins are
418 known to start to degrade as plants approach the end of their lifecycle ³⁷. In summary,
419 multigenerational exposure to CeO₂NPs induced stronger enzymatic responses against oxidative
420 stress in later generation plants, which was in accordance with the higher H₂O₂ content observed
421 in the offspring generations.

422 Multigenerational exposure to CeO₂NPs caused a significant reduction of seed yield and
423 seed quality, which is critical for continued food security. It is particularly concerning to find
424 that CeO₂NPs at concentrations as low as 10 mg/L significantly reduced the silique numbers of
425 the third generation (F₂) Brassica. Underlying mechanisms for the reduced yield of later
426 generations by CeO₂NPs are still unknown. However, we suspect that this may be attributed to
427 the reduced uptake of some essential nutrients for seed formation. For example, Zhao et al. ⁷
428 reported that exposure to CeO₂NPs in soil at 400 and 800 mg/kg significantly reduced the Mo
429 content of cucumber plants, a critical element for seed formation. Another reason for the reduced
430 production may arise from the accumulation of NPs on the cell wall or within intercellular space,
431 which has been shown to block the water transport pathway ^{45, 46}. It has been noted that
432 significant amounts of CeO₂NPs were associated with the radish root surface, which
433 subsequently affected the water uptake by plant roots ⁴⁷. Accordingly, it is possible that
434 multigenerational exposure enhanced uptake of CeO₂NPs by offspring plants, with concomitant

inhibition of water absorption and transport. Our previous study also found that the second generation tomato seedlings typically accumulated higher concentrations of Ce element than the first generation seedlings even though the differences were not statistically significant¹¹. However, it remains unknown whether Due to limited biomass, Ce, Mo and other important elements in tissues were not quantified during the study; ongoing work is addressing this important point. The third possibility for the reduced seed formation may be associated with the signaling role of H₂O₂ on seed development. At the early seed production stage, H₂O₂ converts lipids to sugars. With the change of seed moisture content, H₂O₂ production varies dramatically until the end of seed germination⁵¹. Overproduced H₂O₂ can alter the redox balance in plants, switching the H₂O₂ molecule from a signaling agent to a toxicant, with subsequent negative impacts on the signaling pathway for seed production.

In conclusion, multigenerational exposure to CeO₂NPs altered the plant physiological and biochemical responses in subsequent generations of plants, and caused greater reductions in plant growth and development. In addition, the interaction between CeO₂NPs and plants also led to a reduction in seed production and seed quality over multigenerational exposures, which raises significant concerns over the impact of long term agricultural exposure to ENPs on food security. Ongoing studies are evaluating the mechanisms underlying these important observations, including the impact of multigenerational ENP exposure on water and nutrient uptake and distribution. These findings could have far reaching implications for the use or occurrence of ENPs in agricultural systems.

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461 **Reference**

- 462 1. Cassee, F. R.; van Balen, E. C.; Singh, C.; Green, D.; Muijsers, H.; Weinstein, J.; Dreher,
463 K. Exposure, health and ecological effects review of engineered nanoscale cerium and cerium
464 oxide associated with its use as a fuel additive. *Crit. Rev. Toxicol.* **2011**, *41* (3), 213-229.
- 465 2. Rico, C. M.; Morales, M. I.; McCreary, R.; Castillo-Michel, H.; Barrios, A. C.; Hong, J.;
466 Tafoya, A.; Lee, W. Y.; Varela-Ramirez, A.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L.
467 Cerium oxide nanoparticles modify the antioxidative stress enzyme activities and macromolecule
468 composition in rice seedlings. *Environ. Sci. Technol.* **2013**, *47* (24), 14110-14118.
- 469 3. Schwab, F.; Zhai, G.; Kern, M.; Turner, A.; Schnoor, J. L.; Wiesner, M. R. Barriers,
470 pathways and processes for uptake, translocation and accumulation of nanomaterials in plants -
471 Critical review. *Nanotoxicology* **2015**, 1-22.
- 472 4. Ma, Y.; Kuang, L.; He, X.; Bai, W.; Ding, Y.; Zhang, Z.; Zhao, Y.; Chai, Z. Effects of
473 rare earth oxide nanoparticles on root elongation of plants. *Chemosphere* **2010**, *78* (3), 273-279.
- 474 5. Lopez-Moreno, M. L.; de la Rosa, G.; Hernandez-Viezcas, J. A.; Peralta-Videa, J. R.;
475 Gardea-Torresdey, J. L. X-ray absorption spectroscopy (XAS) corroboration of the uptake and
476 storage of CeO₂ nanoparticles and assessment of their differential toxicity in four edible plant
477 species. *J. Agric. Food Chem.* **2010**, *58* (6), 3689-3693.
- 478 6. Zhao, L.; Sun, Y.; Hernandez-Viezcas, J. A.; Servin, A. D.; Hong, J.; Niu, G.; Peralta-
479 Videa, J. R.; Duarte-Gardea, M.; Gardea-Torresdey, J. L. Influence of CeO₂ and ZnO
480 nanoparticles on cucumber physiological markers and bioaccumulation of Ce and Zn: a life cycle
481 study. *J. Agric. Food Chem.* **2013**, *61* (49), 11945-11951.
- 482 7. Zhao, L.; Peralta-Videa, J. R.; Rico, C. M.; Hernandez-Viezcas, J. A.; Sun, Y.; Niu, G.;
483 Servin, A.; Nunez, J. E.; Duarte-Gardea, M.; Gardea-Torresdey, J. L. CeO₂ and ZnO
484 nanoparticles change the nutritional qualities of cucumber (*Cucumis sativus*). *J. Agric. Food*
485 *Chem.* **2014**, *62* (13), 2752-2759.
- 486 8. Zhao, L.; Sun, Y.; Hernandez-Viezcas, J. A.; Hong, J.; Majumdar, S.; Niu, G.; Duarte-
487 Gardea, M.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Monitoring the Environmental Effects
488 of CeO₂ and ZnO Nanoparticles Through the Life Cycle of Corn (*Zea mays*) Plants and in Situ μ-
489 XRF Mapping of Nutrients in Kernels. *Environ. Sci. Technol.* **2015**, *49* (5), 2921-2928.
- 490 9. Du, W.; Gardea-Torresdey, J. L.; Ji, R.; Yin, Y.; Zhu, J.; Peralta-Videa, J. R.; Guo, H.
491 Physiological and Biochemical Changes Imposed by CeO₂ Nanoparticles on Wheat: A Life
492 Cycle Field Study. *Environ. Sci. Technol.* **2015**, *49* (19), 11884-11893.
- 493 10. Wang, Q.; Ma, X.; Zhang, W.; Pei, H.; Chen, Y. The impact of cerium oxide
494 nanoparticles on tomato (*Solanum lycopersicum* L.) and its implications for food safety.
495 *Metallomics* **2012**, *4* (10), 1105-1112.
- 496 11. Wang, Q.; Ebbs, S. D.; Chen, Y.; Ma, X. Trans-generational impact of cerium oxide
497 nanoparticles on tomato plants. *Metallomics* **2013**, *5* (6), 753-759.
- 498 12. Geisler-Lee, J.; Brooks, M.; Gerfen, J. R.; Wang, Q.; Fotis, C.; Sparer, A.; Ma, X.; Berg,
499 R. H.; Geisler, M. Reproductive toxicity and life history study of silver nanoparticle effect,
500 uptake and transport in *Arabidopsis thaliana*. *Nanomaterials* **2014**, *4* (2), 301-318.
- 501 13. Williams, P. H.; Hill, C. B. Rapid-cycling populations of brassica. *Science* **1986**, *232*
502 (4756), 1385-1389.
- 503 14. Reiss, C. *Experiments in Plant Physiology*. 1st edition ed.; Benjamin Cummings: 1993.

15. Velikova, V.; Yordanov, I.; Edreva, A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. *Plant Science* **2000**, *151* (1), 59-66.
16. Thordal-Christensen, H.; Zhang, Z.; Wei, Y.; Collinge, D. B. Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. *The Plant Journal* **1997**, *11* (6), 1187-1194.
17. Kakkar, P.; Das, B.; Viswanathan, P. N. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* **1984**, *21* (2), 130-132.
18. Maehly, A. C.; Chance, B. The assay of catalases and peroxidases. *Methods Biochem Anal* **1954**, *1*, 357-424.
19. Heath, R. L.; Packer, L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of biochemistry and biophysics* **1968**, *125* (1), 189-198.
20. Burman, U.; Saini, M.; Kumar, P. Effect of zinc oxide nanoparticles on growth and antioxidant system of chickpea seedlings. *Toxicol. Environ. Chem.* **2013**, *95* (4), 605-612.
21. Castiglione, M. R.; Giorgetti, L.; Geri, C.; Cremonini, R. The effects of nano-TiO₂ on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. *Journal of Nanoparticle Research* **2011**, *13* (6), 2443-2449.
22. El-Temsah, Y. S.; Joner, E. J. Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil. *Environ. Toxicol.* **2012**, *27* (1), 42-49.
23. Frazier, T.; Burklew, C.; Zhang, B. Titanium dioxide nanoparticles affect the growth and microRNA expression of tobacco (*Nicotiana tabacum*). *Funct. Integr. Genomics* **2013**, 1-9.
24. Ma, C.; Chhikara, S.; Xing, B.; Musante, C.; White, J. C.; Dhankher, O. P. Physiological and Molecular Response of *Arabidopsis thaliana* (L.) to Nanoparticle Cerium and Indium Oxide Exposure. *ACS Sustainable Chem. Eng.* **2013**, 768-778.
25. Rico, C. M.; Barrios, A. C.; Tan, W.; Rubenecia, R.; Lee, S. C.; Varela-Ramirez, A.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Physiological and biochemical response of soil-grown barley (*Hordeum vulgare* L.) to cerium oxide nanoparticles. *Environ. Sci. Pollut. Res. Int.* **2015**, *22* (14), 10551-10558.
26. Rico, C. M.; Hong, J.; Morales, M. I.; Zhao, L.; Barrios, A. C.; Zhang, J. Y.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Effect of cerium oxide nanoparticles on rice: a study involving the antioxidant defense system and in vivo fluorescence imaging. *Environ. Sci. Technol.* **2013**, *47* (11), 5635-5642.
27. Zhang, Z.; He, X.; Zhang, H.; Ma, Y.; Zhang, P.; Ding, Y.; Zhao, Y. Uptake and distribution of ceria nanoparticles in cucumber plants. *Metallomics* **2011**, *3* (8), 816-822.
28. Zhao, L.; Peng, B.; Hernandez-Viezcas, J. A.; Rico, C.; Sun, Y.; Peralta-Videa, J. R.; Tang, X.; Niu, G.; Jin, L.; Varela-Ramirez, A.; Zhang, J. Y.; Gardea-Torresdey, J. L. Stress response and tolerance of *Zea mays* to CeO₂ nanoparticles: cross talk among H₂O₂, heat shock protein, and lipid peroxidation. *ACS Nano* **2012**, *6* (11), 9615-9622.
29. Dutta, P.; Pal, S.; Seehra, M. S.; Shi, Y.; Eyring, E. M.; Ernst, R. D. Concentration of Ce³⁺ and Oxygen Vacancies in Cerium Oxide Nanoparticles. *Chemistry of Materials* **2006**, *18* (21), 5144-5146.
30. Hernandez-Viezcas, J. A.; Castillo-Michel, H.; Andrews, J. C.; Cotte, M.; Rico, C.; Peralta-Videa, J. R.; Ge, Y.; Priester, J. H.; Holden, P. A.; Gardea-Torresdey, J. L. In situ

synchrotron X-ray fluorescence mapping and speciation of CeO₂ and ZnO nanoparticles in soil cultivated soybean (*Glycine max*). *ACS Nano* **2013**, 7 (2), 1415-1423.

31. Zhang, P.; Ma, Y.; Zhang, Z.; He, X.; Zhang, J.; Guo, Z.; Tai, R.; Zhao, Y.; Chai, Z. Biotransformation of ceria nanoparticles in cucumber plants. *ACS Nano* **2012**, 6 (11), 9943-9950.

32. Heckert, E. G.; Karakoti, A. S.; Seal, S.; Self, W. T. The role of cerium redox state in the SOD mimetic activity of nanoceria. *Biomaterials* **2008**, 29 (18), 2705-2709.

33. Sakihama, Y.; Cohen, M. F.; Grace, S. C.; Yamasaki, H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology* **2002**, 177 (1), 67-80.

34. Apel, K.; Hirt, H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **2004**, 55, 373-399.

35. Foyer, C. H.; Shigeoka, S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol.* **2011**, 155 (1), 93-100.

36. Majumdar, S.; Peralta-Videa, J. R.; Bandyopadhyay, S.; Castillo-Michel, H.; Hernandez-Viezcas, J.-A.; Sahi, S.; Gardea-Torresdey, J. L. Exposure of cerium oxide nanoparticles to kidney bean shows disturbance in the plant defense mechanisms. *J. Hazard. Mater.* **2014**, 278, 279-287.

37. Prochazkova, D.; Sairam, R. K.; Srivastava, G. C.; Singh, D. V. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Science* **2001**, 161 (4), 765-771.

38. Welch, R. M.; Shuman, L. Micronutrient nutrition of plants. *Critical Reviews in plant sciences* **1995**, 14 (1), 49-82.

39. Anderson, A. J.; Spencer, D. Molybdenum in nitrogen metabolism of legumes and non-legumes. *Australian journal of scientific research. Ser. B: Biological sciences* **1950**, 3 (4), 414-430.

40. Kaiser, B. N.; Gridley, K. L.; Ngair Brady, J.; Phillips, T.; Tyerman, S. D. The role of molybdenum in agricultural plant production. *Ann. Bot.* **2005**, 96 (5), 745-754.

41. Ma, F. W.; Hu, L. F.; Fu, P. R.; Hang, Q. F.; Zhang, C. X. The effect of manganese and molybdenum on the fruit setting and pollen germination of grape. *China Fruits* **1992**, 2, 19-20.

42. Anderson, A. J. Molybdenum deficiencies in legumes in Australia. *Soil Science* **1956**, 81 (3), 173-182.

43. Chatterjee, C.; Nautiyal, N. Molybdenum stress affects viability and vigor of wheat seeds. *Journal of plant nutrition* **2001**, 24 (9), 1377-1386.

44. Dietz, K. J.; Herth, S. Plant nanotoxicology. *Trends Plant Sci.* **2011**, 16 (11), 582-589.

45. Asli, S.; Neumann, P. M., Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport. *Plant Cell Environ* **2009**, 32, (5), 577-584.

46. Geisler-Lee, J.; Wang, Q.; Yao, Y.; Zhang, W.; Geisler, M.; Li, K.; Huang, Y.; Chen, Y.; Kolmakov, A.; Ma, X. Phytotoxicity, accumulation and transport of silver nanoparticles by *Arabidopsis thaliana*. *Nanotoxicology* **2012**, 7 (3), 323-337.

47. Zhang, W.; Ebbs, S. D.; Musante, C.; White, J. C.; Gao, C.; Ma, X. Uptake and accumulation of bulk and nanosized cerium oxide particles and ionic cerium by radish (*Raphanus sativus* L.). *J. Agric. Food Chem.* **2015**, 63 (2), 382-390.

48. Wang, C.; Zhang, S. H.; Wang, P. F.; Hou, J.; Zhang, W. J.; Li, W.; Lin, Z. P. The effect of excess Zn on mineral nutrition and antioxidative response in rapeseed seedlings. *Chemosphere* **2009**, 75 (11), 1468-1476.

- 595 49. Campbell, D. R.; Halama, K. J. Resource and pollen limitations to lifetime seed
596 production in a natural plant population. *Ecology* **1993**, 1043-1051.
- 597 50. Lee, T. D. Patterns of fruit and seed production. *Plant reproductive ecology: patterns and*
598 *strategies* **1988**, 179-202.
- 599 51. Bailly, C.; Bailly, C. Active oxygen species and antioxidants in seed biology. *Seed*
600 *Science Research* **2004**, *14*, (2), 93-108.
- 601

Figure 1: Total biomass of 30 Brassica seeds (top panel) and germination rate of Brassica seeds in DI water (bottom panel) collected from original standard fast-cycle seeds, first generation plants after lifecycle exposure to 0, 10, 100 and 1000 mg/L CeO₂NPs and the second generation plants after lifecycle exposure to CeO₂NPs at the same concentrations. Data are mean $\pm$ standard error (n=3 or 4). Letters represent statistical differences analyzed by one-way ANOVA with Tukey's post hoc test.

Figure 2. Biomass of F₀, F₁ and F₂ plants during vegetative (upper), early floral (middle) and late floral (bottom) stages. Data are mean $\pm$ standard error (n=5). Letters represent statistical differences analyzed by one-way ANOVA with Tukey's post hoc test.

Figure 3. Number of siliques formed in F₀, F₁ and F₂ Brassica plants (top panel) and number of seeds developed in individual silique harvested from F₀, F₁ and F₂ Brassica plants (bottom panel) after generational exposure to CeO₂NPs at concentrations of 0, 10, 100 and 1000 mg/L. Data are mean ± standard error (n=5). Letters represent statistical differences analyzed by one-way ANOVA with Tukey's post hoc test.

Figure 4. H₂O₂ concentration of F₀, F₁ and F₂ plants during vegetative (upper), early floral (middle) and late floral (bottom) stages. Data are mean $\pm$ standard error (n=3). Letters represent statistical differences analyzed by one-way ANOVA with Tukey's post hoc test.

Figure 5. Lipid peroxidation of F₀, F₁ and F₂ plants during vegetative (upper), early floral (middle) and late floral (bottom) stages. Data are mean $\pm$ standard error (n=3). Letters represent statistical differences analyzed by one-way ANOVA with Tukey's post hoc test.

Figure 6. Activities of SOD, CAT and GPX of F₀, F₁ and F₂ plants during vegetative (upper), early floral (middle) and late floral (bottom) stages. Data are mean $\pm$ standard error (n=3). Letters represent statistical differences analyzed by one-way ANOVA with Tukey's post hoc test.

Multigenerational Exposure to Cerium Oxide Nanoparticles: Physiological and Biochemical Analysis Reveals Transmissible Changes in Rapid Cycling *Brassica rapa*

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Graphical Abstract