**Damage Assessment for Soybean Cultivated in Soil with Either CeO₂ or ZnO
Manufactured Nanomaterials**

John H. Priester^{1,2,3}, Shelly Cole Moritz², Katherine Espinosa⁴, Yuan Ge^{1,2,3}, Ying Wang^{1,2,3},
Roger M. Nisbet^{2,3,5}, Joshua P. Schimel^{2,3,5}, A. Susana Goggi⁴, Jorge L. Gardea-Torresdey^{3,6}, and
Patricia A. Holden^{1,2,3*}

¹Bren School of Environmental Science and Management, University of California, Santa Barbara, CA 93106.

²Earth Research Institute, University of California, Santa Barbara, CA 93106.

³University of California Center for the Environmental Implications of Nanotechnology, University of California, Santa Barbara, CA 93106.

⁴Department of Agronomy, Iowa State University, Ames, IA 50011.

⁵Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106.

⁶Department of Chemistry, University of Texas at El Paso, El Paso, TX 79968.

*Corresponding Author: holden@bren.ucsb.edu, 805-893-3195 (phone), 805-893-7612 (fax).

ABSTRACT

With increasing use, manufactured nanomaterials (MNMs) may enter soils and impact agriculture. Herein, soybean (*Glycine max*) was grown in soil amended with either nano-CeO₂ (0.1, 0.5, or 1.0 g kg⁻¹ soil) or nano-ZnO (0.05, 0.1, or 0.5 g kg⁻¹ soil). Leaf chlorosis, necrosis, and photosystem II (PSII) quantum efficiency were monitored during plant growth. Seed protein and protein carbonyl, plus leaf chlorophyll, reactive oxygen species (ROS), lipid peroxidation, and genotoxicity were measured for plants at harvest. Neither PSII quantum efficiency, seed protein, nor protein carbonyl indicated negative MNM effects. However, increased ROS, lipid peroxidation, and visible damage, along with decreased total chlorophyll concentrations, were observed for soybean leaves in the nano-CeO₂ treatments. These effects correlated to aboveground leaf, pod, and stem production, and to root nodule N₂ fixation potential. Soybeans grown in soil amended with nano-ZnO maintained growth, yield, and N₂ fixation potential similarly to the controls, without increased leaf ROS or lipid peroxidation. Leaf damage was observed for the nano-ZnO treatments, and genotoxicity appeared for the highest nano-ZnO treatment, but only for one plant. Total chlorophyll concentrations decreased with increasing leaf Zn concentration, which was attributable to zinc complexes—not nano-ZnO—in the leaves. Overall, nano-ZnO and nano-CeO₂ amended to soils differentially triggered aboveground soybean leaf stress and damage. However, the consequences of leaf stress and damage to N₂ fixation, plant growth, and yield were only observed for nano-CeO₂.

INTRODUCTION

Manufactured nanomaterials (MNMs) are used in diverse applications (Roco, 2011) including electronics (Shulaker et al., 2013), medicine (Murday et al., 2009), cosmetics (Nazarenko et al., 2012) and textiles (Geranio et al., 2009). Such uses imply that MNMs are being released into the environment, where their accumulation is predicted (Liu and Cohen, 2014). MNMs are inherently chemically reactive, due to their small size (where at least one dimension is less than 100 nm) and associated high ratio of surface area to volume (Nel et al., 2006). Given such reactivity, MNMs could impact ecosystems (Holden et al., 2013) including terrestrial (Kwak and An, 2016) and specifically agricultural (Rico et al., 2011) environments.

As reviewed by others (Reddy et al., 2016), soils are projected to be a major destination for MNMs, and thus agricultural plants could be exposed to MNMs. Many MNM types have been shown to enter plants via roots, translocate as various forms through the plant (Aslani et al., 2014), and affect plant photosynthesis, growth, and reproduction (Ma et al., 2015b; Zuverza-Mena et al., 2016). If accumulated in plants, MNMs could transfer to higher trophic levels (Gardea-Torresdey et al., 2014). Metal oxide MNMs specifically are increasingly applied in agriculture, for example as fertilizer products, thus affording soil exposure and plant uptake (Du et al., 2016).

While measuring MNMs in soils remains challenging (Holden et al., 2016), the modeled concentrations of metal oxide MNMs in biosolids and soils span up to 1000 and 10 ppm, respectively, and nano-ZnO and nano-CeO₂ are in the top six of eleven highly produced MNMs (in decreasing order: carbon black; nano-SiO₂, -Al₂O₃, -TiO₂, -CeO₂, -ZnO, and -ZrO₂; carbon fiber; nano-CuO and -Ag) (Holden et al., 2014). Among the studied metal oxide MNMs, nano-ZnO and nano-CeO₂ may be particularly harmful to agricultural plants (Du et al., 2016). Some

metal oxide MNM studies regard soybean (*Glycine max*) (Du et al., 2016). However, more understanding of the potential impacts of nano-ZnO and nano-CeO₂ is needed, since soybean is a major global crop for both human and livestock consumption (Economic Research Service, 2012).

This study regards the damage of nano-ZnO and nano-CeO₂ to soil-grown soybean, focusing on foliar oxidative stress, impacts on chlorophyll production, and possible genotoxicity—all recognized potential effects of MNMs to plants (Reddy et al., 2016). This study builds on several prior reports for one greenhouse mesocosm study of soybean grown in nano-ZnO- or nano-CeO₂-amended soils (Ge et al., 2014; Hernandez-Viezcas et al., 2013; Peralta-Videa et al., 2014; Priester et al., 2012). Here we asked: Did either nano-ZnO or nano-CeO₂ cause oxidative stress and damage to leaves or beans? How were plant stress and damage indicators interrelated? Were plant stress and damage related to the MNMs translocated, either as nanoparticles or as metal complexes, into aboveground plant parts? If so, what were the consequences in terms of growth, yield and N₂ fixation potential for the entire plant? We hypothesized that translocated MNMs or associated metals caused oxidative stress and concomitant plant damage, which in turn contributed to impacts on plant growth, yield, and N₂ fixation potential, particularly for the nano-CeO₂ treatments. Herein, oxidative stress was measured as leaf reactive oxygen species (ROS, i.e. the combination of hydrogen peroxide or H₂O₂, superoxide or O₂⁻, and hydroxyl radical or •OH). Leaf oxidative stress was compared to other leaf stress and damage indicators: visible leaf damage (chlorosis or necrosis), changes in chlorophyll (*Chl*) content, or photosystem II (PSII) quantum efficiency, and DNA insertions or deletions. We assessed oxidative stress and leaf damage with respect to previously-reported plant growth (Priester et al., 2012), and plant uptake of MNM constituent metals (Priester et al., 2012)

or nanoparticles (Hernandez-Viezcas et al., 2013). The overall goal was to understand how MNMs impaired soil-grown soybean—as evidenced by decreased plant growth, yield, and N₂ fixation potential—via MNM uptake into plants and concomitant plant tissue damage.

MATERIALS AND METHODS

Nanoparticles, Plant Growth, and Visual Assessment of Leaf Damage

Soybean plants were cultivated in a greenhouse as described before (Priester et al., 2012). Briefly, seeds of dwarf soybean (variety early Hakucho, product no. 5555, Park Seed Company, Greenwood, SC) were inoculated with *Bradyrhizobium japonicum* strain USDA 110 (US Department of Agriculture). Inoculated seeds were germinated, and plants initially grown in peat pellets contained in PVC trays, until the emergence of true leaves (18 d, stage VC; http://extension.agron.iastate.edu/soybean/production_growthstages.html). The plantlets were transplanted into pots containing 2.4 kg of moist sandy loam soil obtained from an organic farm in Carpinteria, CA (N 34° 23' 40", W 119° 28' 40"). Prior to use, the soil was sieved (2 mm) and amended with either nano-ZnO or nano-CeO₂ (Meliorum Technologies, Rochester, NY). The soil characteristics were reported before (Priester et al., 2012), as were the total Zn (95 ppm) and total Ce (31 ppm) in the control soils (Hernandez-Viezcas et al., 2013).

Bare (neither capped nor coated) nano-ZnO and nano-CeO₂ were described by the manufacturer as having mean diameters of 10 nm and 8 nm, respectively; further characterization was described before (Priester et al., 2012). Unamended soils served as controls. In MNM treatments, the nanoparticle concentrations, on a mass per dry soil mass basis, were 0.05 (low), 0.1 (medium), and 0.5 (high) g kg⁻¹ for nano-ZnO, and 0.1 (low), 0.5 (medium), and 1.0 (high) g kg⁻¹ for nano-CeO₂. These MNM concentrations are within ranges commonly studied, and are in

the upper end of ranges predicted to occur in biosolids or soil (Holden et al., 2014), representing a potential future scenario of agricultural buildup (Holden et al., 2016) from the increasing use of metal oxide MNMs in agriculture (Du et al., 2016). Further, since similar concentrations were studied for hydroponic soybean (Lopez-Moreno et al., 2010), this study allows for understanding the bioavailability of nano-ZnO and nano-CeO₂ to plants growing in soil.

Four pots per treatment were planted, and four more pots per treatment were left as unplanted controls, allowing for a cross comparison (planted vs. unplanted) of MNM effects on soil microbial communities (Ge et al., 2014). After transplanting, the plants were grown in a greenhouse for another 48 d (to stage R5 to R6). The low nano-ZnO (0.1 g kg⁻¹) treatment had only three replicates, since one plant died shortly after transplanting. Following 48 d of growth (stage R5 to R6), plants were harvested as described previously (Priester et al., 2012). One day prior to harvest (stage R5 to R6), leaves were sampled for total ROS and chlorophyll measurements, as described below.

Trifoliate leaf emergence had first been observed in all treatments at day 8 (stage V1) post-transplanting (Priester et al., 2012). Thereafter, visible trifoliate leaf damage was recorded at weekly intervals during plant growth. Visible leaf damage was defined as observed chlorosis (Stevens et al., 2002) or necrosis (Chen et al., 2012; Stevens et al., 2002) as exemplified herein (Fig. S1). The percentage of visibly damaged leaves (number of damaged leaves divided by total leaf number, multiplied by 100) was calculated for each plant at each time point including at harvest (49 days, R5 to R6). Across all time points, the highest percentage within a replicate of a given treatment was deemed the maximum percentage of damaged leaves.

Leaf Chlorophyll and Fluorescence Measurements

Because leaf color, i.e. the degree of chlorosis, is related to chlorophyll content in soybean (Adams et al., 1999), chlorophyll content was quantified to further assess leaf damage and potential effects on photosynthesis. Photosynthetic function was separately assessed as quantum efficiency, using leaf chlorophyll fluorescence emission measurements.

One day before plant harvest (day 48, stage R5 to R6), 1.5 cm diameter leaf discs were acquired from random locations on each plant using a circular punch. For each plant, three leaf discs were pooled to serve as a single experimental replicate for chlorophyll measurement. The pooled discs were weighed and added to 100 mg of dried washed sea sand (Fisher Scientific, Pittsburgh, PA) with 100 μ L of 80% (v/v) acetone. The discs were ground with a mortar and pestle to a fine pulp (Bruinsma, 1963). The ground material was extracted (30 min) into 5 mL of 80% (v/v) acetone, vortexed (30 s), and centrifuged (10 °C, $2,600 \times g$, 5 min). The supernatant was decanted and added to 10 mL of 80% (v/v) acetone on ice. A subsample (5 mL) from each mixture (15 mL) was transferred to a glass spectroscopy tube wherein the absorbance was measured spectrophotometrically (646, 663, 710 and 750 nm). Chlorophyll *a* and *b* concentrations (mg L^{-1}) were calculated using the following equations (Lichtenthaler and Wellburn, 1983):

$$\text{Chlorophyll } a = (12.21 \times [(A_{663} - A_{750}) - (A_{710} - A_{750})]) - (2.81 \times [(A_{646} - A_{750}) - (A_{710} - A_{750})]) \quad (1)$$

$$\text{Chlorophyll } b = (20.13 \times [(A_{646} - A_{750}) - (A_{710} - A_{750})]) - (5.03 \times [(A_{663} - A_{750}) - (A_{710} - A_{750})])$$

(2)

where A_{646} , A_{663} , A_{710} and A_{750} are the absorbance values at 646 nm, 663 nm, 710 nm and 750 nm, respectively. The calculated chlorophyll amounts were normalized to dry leaf disk masses using the previously determined leaf water content (Priester et al., 2012). Total chlorophyll was calculated by summing chlorophyll *a* and chlorophyll *b*; the ratio of chlorophyll *a/b* was also calculated.

While measurements of chlorophyll from excised leaves can indicate plant damage, they do not provide information on the function of chloroplasts *in vivo* as can measurements of chlorophyll fluorescence (Maxwell and Johnson, 2000). We conducted leaf fluorescence measurements to calculate PSII quantum efficiency, the fluorescence parameter considered to be the most robust to problems (Adams III and Demmig-Adams, 2004; Murchie and Lawson, 2013). By using a portable pulse-modulated fluorometer with a long-pass sharp-cut red filter (maximum detection >680 nm) (Hansatech FMS 2, Norfolk, UK), leaf fluorescence emission was measured (Baker, 2008; Maxwell and Johnson, 2000; Schreiber et al., 1995) twice during the experiment: once after true leaves had emerged and expanded (day 18 post transplanting, stage V2), and just before plant harvest (day 45, stage R4 to R5). Maximum (F_m) and minimum (F_o) fluorescence were measured predawn on one dark-adapted leaflet to determine variable fluorescence (F_v) and maximum quantum efficiency of PSII ($F_v/F_m = (F_m - F_o)/F_m$) (Maxwell and Johnson, 2000; Schreiber et al., 1995). Diurnal measurements were made at 10:00, 12:00 and 16:00 hours (± 30 min) on two illuminated leaflets to capture light-adapted maximum fluorescence (F_m') and steady-state fluorescence (F_s), to determine PSII quantum or operating quantum efficiency (Φ_{PSII}) ($(F_m' - F_s)/F_m'$) under ambient conditions (Genty et al., 1989). Green

leaflets of the second or third trifoliate leaves were measured, providing the most fully developed, yet healthy appearing, leaves available across treatments.

Leaf ROS and Lipid Peroxidation Measurements

To investigate the combined effects of ROS production, detoxification, and damage, total ROS and lipid peroxidation (as a marker for ROS-mediated stress) were measured for leaf tissues. One day prior to harvest (day 48, stage R5 to R6), six leaf discs were acquired at random locations from separate individual leaves of each plant by the same approach used to sample leaves for chlorophyll. Total leaf ROS content was immediately determined according to a published method (Joo et al., 2005). The discs from each plant were pooled into groups of three, with one group used as an experimental replicate for the plant, and the second group used as a control for background fluorescence. Each group of three leaf discs was weighed, then ground on ice with a mortar and pestle after adding washed sea sand (100 mg; Fisher Scientific, Pittsburgh, PA) and sodium phosphate buffer (100 μ L, 25 mM). The ground tissue was rinsed from the mortar and pestle with sodium phosphate buffer (100 μ L, 25 mM), and transferred into centrifuge tubes containing either 2',7'-dichlorofluorescein diacetate solution (10 mL, DCFH-DA; prepared as described previously (Priester et al., 2009)), or sodium phosphate buffer (10 mL, 25 mM) to serve as a control for background fluorescence. The tubes were vortexed (30 s), incubated (1 h, room temperature), and centrifuged ($2,600 \times g$, 5 min). Supernatant (250 μ L) from each tube was transferred to a 96-well black (bottom and side) microplate. The fluorescence signal of the supernatant was measured using a microplate reader (model: Synergy 2; BioTek, Winooski, VT) at excitation and emission wavelengths of 480 and 530 nm, respectively. Fluorescence intensity units (FIU) for leaf ROS were recorded and normalized to

dry leaf masses to compare controls to MNM treatments. H_2O_2 standards were analyzed similarly, but FIU values were not converted to H_2O_2 equivalents. H_2O_2 equivalents have been reported for soybean plant leaves grown hydroponically (Muñoz et al., 2008; Prasad et al., 2005; Yan et al., 2007) or in soil (Weisany et al., 2012; Zhao et al., 2010). However, plant tissue ROS as H_2O_2 equivalents can vary by 1000-fold or more (Demidchik, 2015; Queval et al., 2008), depending on growth conditions, plant type (Cheeseman, 2006) and plant growth stage (Cheeseman, 2009). A contributing factor may be that total ROS originates from many ROS species with varying potencies relative to H_2O_2 (Setsukinai et al., 2003). Therefore, total ROS concentrations were reported as FIU normalized to dry leaf mass using the previously determined leaf water content (Priester et al., 2012), and compared across the treatments but not to other published studies.

At harvest (day 49, stage R5 to R6), randomly selected whole soybean leaves (separate from those used for total ROS and chlorophyll measurement) were weighed then frozen ($-80\text{ }^{\circ}\text{C}$) until processing for lipid peroxidation analysis. Just prior to analysis, the leaves were weighed and immediately transferred to separate disposable tissue grinders (Fisher Scientific, Pittsburgh, PA) containing extraction buffer (5 mL) on ice. The extraction buffer was composed of 0.1% (w/v) trichloroacetic acid (TCA) in phosphate buffered saline (PBS) (Loreto and Velikova, 2001). Each sample was homogenized (1 min) in the tissue grinders on ice, followed by vortexing (30 s). The homogenized samples were centrifuged ($10,000 \times g$, 15 min, $4\text{ }^{\circ}\text{C}$). Supernatant (4 mL) was transferred to clean centrifuge tubes and frozen ($-80\text{ }^{\circ}\text{C}$) until further analysis. Oxidative stress in soybean leaves was quantified according to the manufacturer's instructions for the OxiSelect TBARS Assay Kit (Cell Biolabs Inc., San Diego, CA), except that thiobarbituric acid (TBA) was dissolved in 20% (w/v) TCA instead of 20% acetic acid

(Loreto and Velikova, 2001). Briefly, the assay involves spectrophotometrically quantifying malondialdehyde (MDA, 72.06 g mol^{-1}). MDA, a byproduct of lipid peroxidation, binds to TBA in the assay and forms a compound that can be measured colorimetrically or fluorometrically (Rachmilevitch et al., 2006). The fluorescence signal of the MDA-TBA compound was measured using a microplate reader (model: Synergy 2; BioTek, Winooski, VT) at excitation and emission wavelengths of 480 and 530 nm, respectively. MDA concentrations were determined using a calibration curve based on standards. Measured MDA concentrations were normalized to dry leaf mass for each sample, using the previously determined leaf water content (Priester et al., 2012).

Leaf DNA Isolation and Analysis

Harvested leaf tissue (separate from that used for total ROS, chlorophyll, or lipid peroxidation measurements) was weighed then frozen in liquid nitrogen and stored (-80°C) until DNA extraction. Total DNA was isolated from freeze-dried leaf tissue (approximately 0.75 g) using a modified DNA extraction protocol (Edwards et al., 1991). DNA quantity and quality were assessed with an ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA). DNA was analyzed using 12 insertion-deletion (InDel) markers (Table S1) developed previously (Choi et al., 2007). The InDel markers were distributed across eight soybean chromosomes, and contain unique sequences when compared with the reference genome. These molecular markers consist of genomic DNA sequences between 12 to 48 nucleotides in length that are either present (insertion) or absent (deletion) in the genomic background. These InDel markers were selected because they are distributed across eight soybean chromosomes, and contain unique sequences when compared with the reference genome (Williams 82). Additional

criteria for selecting these markers were the small insertion and deletion fragment size—which increases the probability of detecting variation across the genome—and the polymerase chain reaction (PCR) reproducibility in the laboratory (Supplemental Information).

For InDel analyses, 10 ng of each DNA sample were used for each 25 µl reaction with 2X reaction *Taq* buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl₂, Triton x-100), 1 µl of each primer (0.4 µM), 2.5 mM of each dNTP, and 0.2 units of *Taq* DNA polymerase (GenScript USA Inc., Piscataway, NJ, USA) per reaction. Amplifications were performed using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Waltham, MA) programmed with the following conditions: temperature of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 7 min. The resulting PCR products were resolved on a 3% agarose/TBE gel stained with ethidium bromide (1 µg mL⁻¹) in 0.5X TBE at 100-120 V for 1 to 2 h. The band patterns were visualized and gels were photographed under UV light. Images were saved using a Fotodyne Archiver Imaging System (Fotodyne, Inc., Harland, WI), and analyzed with the open source ImageJ analysis software (Rasband WS. ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, imagej.nih.gov/ij/1997-2012). The fragment size of each PCR product was approximated by referencing the bands to a low molecular weight DNA ladder (New England BioLabs, Inc., Ipswich, MA).

Seed Protein Extraction, and Total Protein and Protein Carbonyl Quantification

Total protein content is an important measure of soybean seed food quality (Wang et al., 2003) that varies geographically (Karr-Lilienthal et al., 2004) but also varies with certain plant stressors (Medic et al., 2014). The carbonyl content of soybean proteins is an indicator of

oxidation (Moller et al., 2007; Shacter, 2000), and is a proxy for total protein quality (Cucu et al., 2013). Protein content was measured for soybean seeds that had been frozen (-80 °C) when plants were harvested (Priester et al., 2012). Without thawing, the seeds—several from each plant—were removed from the pods, and each was weighed separately to determine the moist mass. The moist masses were converted to dry masses using the moisture contents previously determined for harvested seeds (Priester et al., 2012). Seeds were ground (1 min) with dried washed sea sand (100 mg; Fisher Scientific, Pittsburgh, PA), using an ice-cold mortar and pestle. Following grinding, the seed tissues were homogenized (1 min) on ice in disposable tissue grinders (Fisher Scientific) containing extraction buffer (8.5 mL) composed of 83 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4.5 mM polyvinylpyrrolidone (PVPP), 0.9% (v/v) Triton X-100, 9% (v/v) glycerol, and 0.7% (v/v) protease inhibitor cocktail for plants (Sigma-Aldrich, St. Louis, MO) in Nanopure H₂O (Laing and Christeller, 2004). The homogenized samples were vortexed (30 s) then centrifuged (30,000 × *g*, 15 min, 4 °C). The supernatants were decanted, and 10 µL used immediately to quantify total protein amounts using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. The remaining supernatant was frozen (-80 °C) until further analysis. For comparison, soybeans from a frozen commercial product (Pictsweet Edamame Soybeans in the Pod, Bells, TN), and from a soybean field (Fairmont, MN, N 43° 35' 31.283", W 94° 28' 19.387"), were processed in the same manner.

Protein solutions extracted from seeds were thawed then concentrated (from 1.5 mL to 0.45 mL) using 3 kDa MWCO (molecular weight cutoff) Amicon Ultra-2 Centrifugal Filters (EMD Millipore, Billerica, MA) according to the manufacturer's instructions (7,500 × *g*, 90 min, 4 °C). The concentrated solutions were recovered from the filters by centrifugation (1,000 × *g*, 2

min, 4 °C). The solutions were concentrated again (from 0.45 to 0.2 mL) by centrifugation ($14,000 \times g$, 20 min) using 10 kDa MWCO Amicon Ultra-0.5 Centrifugal Filters (EMD Millipore). The final concentrates were recovered from the filters by centrifugation ($1,000 \times g$, 2 min), and frozen (-80 °C) until seed protein carbonyl measurement. Total protein concentrations in the extracted, concentrated, seed samples were $> 1 \text{ mg mL}^{-1}$ (as determined by the Quick Start Bradford Protein Assay Kit; see above), which was sufficient for further analysis. Oxidative damage in soybean seed protein was quantified according to the manufacturer's instructions for the OxiSelect Protein Carbonyl Fluorometric Assay (Cell Biolabs Inc., San Diego, CA). Briefly, the assay involves the binding of a fluorophore to the oxidized protein products, namely oxidized proline, arginine, lysine and threonine protein side chains (Dalle-Donne et al., 2003). The fluorescence signal of the fluorophore was measured using a microplate reader (model: Synergy 2; BioTek, Winooski, VT) at excitation and emission wavelengths of 480 and 530 nm, respectively, and converted to carbonyl concentration by using the fluorophore standards provided in the assay kit, according to the manufacturer's instructions. Measured carbonyl concentrations were normalized to total seed protein mass for each sample.

Statistical Analyses

Statistical data analyses were performed using Microsoft Excel software. Mean values across treatments were compared using the Student's *t*-test, assuming a two-tailed distribution and two-sample unequal variance. Correlations (Pearson linear of untransformed data, or of log transformed data in one case of a log-linear relationship) were evaluated using StatPlus for Mac within Excel across stress and damage measures, between stress and damage indicators for previously reported endpoint metal (Zn or Ce) contents, and for growth and endpoint plant

metrics reported previously (Priester et al., 2012) related to metal contents or to stress and damage indicators. Correlational analyses were performed with and without control data, to further assess the strength of the treatment effects with the applied MNM doses. Including or excluding controls in the correlational analyses was also in recognition that the forms of Ce or Zn (i.e. ionic, complexed, or nanoparticulate)—when quantifiable—likely differed between the control plants versus the treatments.

RESULTS

Visible Leaf Damage

Across all treatments, total numbers of trifoliolate leaves varied ($n = 1$ to 15 per plant), as did numbers of visibly damaged trifoliolate leaves per plant ($n = 0$ to 6 per plant) and, therefore, percentages of damaged leaves over time (Fig. 1). The leaf damage for plants grown in soil amended with nano-CeO₂ tended to manifest as necrosis (Fig. S1A), while leaves from plants grown in soil amended with nano-ZnO tended to show chlorosis (Fig. S1B). However, each damage type was present for each MNM, and therefore chlorosis and necrosis were not separately scored or quantified. Leaf damage was not visible until 29 days following transplanting (stage R1; Fig. 1C-D). In control plants, some leaves showed damage after 36 days (stage R1 to R2; Fig. 1C-D); this peaked at day 43 (stage R2). The magnitude of damage varied depending on whether plants were grown with nano-CeO₂ or nano-ZnO (Fig. 1C versus 1D).

The nano-CeO₂ treatments exhibited visible trifoliolate leaf damage, with increased soil nano-CeO₂ concentration corresponding to a shorter time to maximal damage (47, 43 and 36 days, equaling stages R5, R4, and R1 to R2, for low, medium and high concentrations, respectively) and a greater percentage of damaged leaves (12.5%, 29.1% and 47.6% for low,

medium and high concentrations, respectively, Fig. 1C). For the nano-CeO₂ treatments, there was a significant, albeit weak, positive correlation between maximum percent visible leaf damage and increasing soil dose ($r = 0.72$, $P=0.002$, including controls, $n = 16$; $r = 0.64$, $P = 0.03$, excluding controls, $n = 12$). The percent visible leaf damage at harvest was positively correlated to soil dose, but only when the controls were included in the analysis ($r = 0.59$, $P = 0.02$, $n = 16$). The maximum and at-harvest visible leaf damage percentages for the nano-CeO₂ treatments were significantly positively correlated to one another when either including ($r = 0.77$, $P < 0.001$, $n = 16$) or excluding ($r = 0.71$, $P = 0.01$, $n = 12$) the controls in the analyses.

The pattern of visible leaf damage for the high nano-ZnO treatment was similar to the control (Fig. 1D), i.e. first occurring after 36 days (stage R1 to R2) with a peak of $10.6 \pm 6.1\%$ at day 47 (stage R5). Visible damage was observed earlier (day 29, stage R1), and appeared greater in the medium (peak of $32.3 \pm 1.7\%$; day 43, stage R5) and low (peak of $22.2 \pm 12.8\%$, day 36, stage R3) nano-ZnO treatments (Fig. 1D), but the patterns in the two treatments did not differ, and were only significantly different from the controls at day 29 (stage R1; Fig. 1D). Within the nano-ZnO treatments, and whether or not controls were included in the analysis, neither the maximum nor at-harvest (49 days, stage R5 to R6) percentage visible leaf damage correlated with nano-ZnO soil dose. The maximum and at-harvest visible leaf damage percentages for the nano-ZnO treatments were significantly positively correlated to one another when either including ($r = 0.79$, $P < 0.001$, $n = 15$) or excluding ($r = 0.78$, $P = 0.005$, $n = 11$) the controls in the analyses.

Chlorophyll Content and Photosynthetic Function

The total chlorophyll content for control leaves (Table 1), when converted to a fresh leaf weight basis assuming 72% moisture content (Priester et al., 2012), was within range of previously reported soybean leaf chlorophyll concentrations for hydroponically-grown plants (Hu et al., 2014; Nair and Chung, 2014) or for plants exposed to iron MNMs (Ghafariyan et al., 2013). Except for the chlorophyll *a/b* ratio, the correlations between chlorophyll (*a*, *b* or total) measures were internally consistent across treatments and controls. Because of this internal consistency, we highlight patterns in total chlorophyll and chlorophyll *a/b* below, and investigate the relationship with total chlorophyll to the other plant variables.

Within the nano-CeO₂ treatment, the total chlorophyll concentration and chlorophyll *a/b* were reduced on average 2.6 mg/g and 0.3, respectively, in comparison to the control (Table 1). However, total chlorophyll did not decrease in a soil dose-dependent manner, and thus did not parallel the pattern of leaf damage (Fig. 1C). Rather, the total chlorophyll concentration was lowest for the low nano-CeO₂ treatment, i.e. 33% lower than the control. Within the nano-ZnO treatments, the total chlorophyll concentrations for the medium and high doses were significantly less than the control (Table 1; by 35 and 39% respectively). Chlorophyll *a/b* ratios in all doses were significantly less than the control (Table 1). There was no significant overall soil dose-dependent trend for either metric, whether or not the controls were included in the analysis.

We did not observe significant differences in predawn F_v/F_m between the control and treated plants, nor among nano-CeO₂ or nano-ZnO soil doses. Mean F_v/F_m values were remarkably similar and ranged from 0.84 to 0.87 (Table S2), thus above a value (0.83) that is typical for healthy, unstressed leaves not experiencing photoinhibition (Bjorkman and Demmig, 1987; Maxwell and Johnson, 2000). At 18 days (stage V2), plants in the high nano-CeO₂

treatment exhibited the lowest F_v/F_m value (0.84) while plants in the low nano-ZnO treatment exhibited the highest value (0.86). Except in the former treatment, all F_v/F_m values were higher at 45 days (stage R4 to R5; Table S2). Values for Φ_{PSII} in treated plants also did not significantly differ from the control or among concentrations within the nano-CeO₂ or nano-ZnO treatments at either measurement date or time of day (Student's t -test, $P > 0.10$; Fig. S2). Mean morning values were high (0.67 to 0.73) and diminished over the course of the day (0.38-0.44). However, the photosynthetic capacities fully recovered as indicated by the high predawn F_v/F_m values.

Leaf ROS and Lipid Peroxidation

Total leaf ROS concentrations (as FIU g⁻¹ dry leaf) in nano-CeO₂ treatments were higher for the medium and high nano-CeO₂ doses, relative to the control (Table 2). When the controls were included in the analysis (but not when they were excluded), leaf ROS increased significantly, albeit weakly, with nano-CeO₂ soil dose ($r = 0.58$, $P = 0.02$, $n = 16$). For the nano-ZnO treatments, ROS concentrations did not differ from the control (Table 2), and there was no relationship between ROS concentration and nano-ZnO soil dose whether controls were, or were not, included in the analyses.

Lipid peroxidation (as MDA concentration) was significantly elevated (by approximately 50%; Student's t -test, $P < 0.05$) for the low and medium nano-CeO₂ treatments when compared to the control (Table 2). There were no significant differences in MDA concentrations when comparing nano-ZnO treatments to the controls (Table 2). Further, there was no significant relationship between nano-CeO₂ or nano-ZnO soil doses and MDA concentrations, whether or not the controls were included in the analyses (data not shown).

In summary, the stress indicator of leaf ROS concentration increased with the concentration of soil-amended nano-CeO₂ (but not nano-ZnO). Leaf lipid peroxidation did not depend on nano-CeO₂ soil dose, but was elevated for the low and medium nano-CeO₂ soil doses.

Leaf Genotoxicity

PCR-based molecular analysis of soybean leaf tissue using 12 InDel markers showed that the plants were homozygous across all treatments. Amplicon sizes per InDel marker were determined for this genotypic background (Table S1). Nano-CeO₂ treatments did not cause detectable insertion/deletion events using these 12 InDel markers, since all band profiles were identical to the control (Fig. 2A). However, the analysis revealed that a high nano-ZnO exposed plant experienced a genetic change represented by an insertion event of 26 bp (InDel marker 19428; Fig. 2B).

Bean protein and protein carbonyl contents

Total seed protein ranged from 0.020 ± 0.007 to 0.031 ± 0.003 mg per mg dry mass (Table S3). These values are 10 to 20 times lower than typical values reported for soybean (Medic et al., 2014), likely owing to the extraction technique used herein. Total seed protein was similar to the control across all treatments including all soil doses, except in the medium nano-CeO₂ treatment, where protein was elevated (by ca. 29%) relative to the control and the other (low or high) nano-CeO₂ treatments (Table S3).

The low protein extraction efficiency was assumed to be similar across treatments, and thus was assumed to not affect the protein carbonyl measurement which, when normalized to total protein, was similar to that reported previously for soybean (Sun and Leopold, 1995; Wu et

al., 2009). Across the nano-CeO₂ and nano-ZnO treatments, seed protein carbonyl contents decreased as compared to the control, with values being significantly reduced (Student's *t*-test, $P < 0.05$) in the medium nano-CeO₂, high nano-CeO₂, low nano-ZnO, and high nano-ZnO treatments, by 51, 60, 66, and 72%, respectively (Table S3). For the nano-CeO₂ treatments, seed protein carbonyl contents decreased significantly with soil dose (ANOVA, $P = 0.01$). The patterns were similar when protein carbonyl masses were normalized to bean dry mass (Table S3). Given that oxidative damage to the seeds would result in increased protein carbonyl contents, these results suggest that, unlike for leaves, soybean seeds were not sites of nano-CeO₂ or nano-ZnO phytotoxicity.

Relationships between MNM dose, plant metal concentrations, and plant damage, growth, yield, and N₂ fixation potential

Differential leaf stress and damage were observed between the two MNM soil treatments. For example, leaf ROS and leaf damage (maximum, and harvest, percentages) increased with nano-CeO₂ soil dose. Leaf lipid peroxidation was elevated for two nano-CeO₂ soil doses (Table 2), although not in a dose-dependent fashion. Further, total chlorophyll was decreased for all of the nano-CeO₂ treatments and for the medium and high nano-ZnO treatments. To determine if the observed plant stress and damage was related to MNMs or associated metals that had translocated from soil into aboveground plant parts, we compared the data herein to the previously published plant growth and MNM concentration data. Interrelationships within the new data were also analyzed. As such, correlations were evaluated between: MNM soil dose and previously reported plant part metal concentrations (as total Ce or Zn; Tables S4 and S5); accumulated metal concentrations for the various plant parts; plant part accumulated metal

concentration and plant physiological metric (growth, yield, and N₂ fixation potential); plant stress or damage indicator and plant part metal concentration; plant stress or damage indicator and plant growth metric. The correlations were evaluated mostly separately for the two MNMs, since different plant growth effects had been observed for nano-CeO₂ versus nano-ZnO treatments (Priester et al., 2012).

MNM soil dose relationships to metal uptake in plant parts

To evaluate the relationships between leaf stress or damage indicators versus leaf metal or MNM contents, we first examined previously published leaf metal concentrations (Priester et al., 2012) for relationships to MNM soil doses. For the nano-ZnO treatments, large amounts of Zn translocated aboveground, including into leaves (Priester et al., 2012)(Table S5). Total Zn concentrations in leaves increased with nano-ZnO soil dose whether controls were ($r = 0.91$, $P < 0.001$, $n = 15$), or were not ($r = 0.89$, $P < 0.001$, $n = 11$), included in the analysis. Similarly positive and significant relationships between nano-ZnO soil dose and plant part Zn concentration held for root, nodule, stem and pod parts (Table S6, Fig. 3). However, it is unlikely that ZnO nanoparticles were present in the leaves, since the form of Zn in the stem and pods of the nano-ZnO treatments was mainly Zn-citrate (Hernandez-Viezcás et al., 2013).

Although small amounts of total Ce translocated to leaves (Priester et al., 2012) (Table S4), the amounts depended on nano-CeO₂ soil dose whether controls were ($r = 0.72$, $P = 0.002$, $n = 16$), or were not ($r = 0.78$, $P = 0.003$, $n = 12$), included in the analysis (Table S6, Fig. 4). Since nano-CeO₂ was identified as an important form of Ce in the beans for the nano-CeO₂ treatments (Hernandez-Viezcás et al., 2013), leaf Ce may have substantially been nano-CeO₂. Nodule (only when excluding the controls) and root Ce content were also significantly related to nano-CeO₂

soil dose. However, pod and stem Ce contents were not related to nano-CeO₂ soil dose (Table S6, Fig. 4).

Metal concentrations across plant parts were correlated, but differently for the two MNMs. For the nano-CeO₂ treatments, Ce concentrations were only correlated when comparing root to nodule, nodule to stem, and leaf to root (Table S7, Fig. 4). For the nano-ZnO treatments, the Zn concentrations in plants were significantly correlated across all plant parts (Table S8, Fig. 3).

Relationships of plant part metal concentrations to plant growth metrics

We tested for correlations of plant growth, yield, and N₂ fixation (Table S9)(Priester et al., 2012) to MNM soil dose, and to metal concentration in the plant parts (nodule, root, stem, leaf and pod). Fifteen plant metrics were included in the analyses. Six metrics (seeds per pod, pod length, pod width, pod length to width ratio, total aboveground dry biomass, total belowground dry biomass) were not included as they were mostly invariant across the treatments (Table S9). For the nano-CeO₂ treatments, the MNM soil concentration and the total Ce concentrations in leaves, nodules, and roots each negatively correlated with N₂ fixation potential (Table S10, Fig. 4). There were also significant positive correlations: between leaf Ce concentration and either the harvest leaf count or the stem dry biomass, and between nodule Ce concentration and stem elongation rate (Table S10, Fig. 4). The relationship between N₂ fixation potential and either nodule or root Ce concentration was logarithmic (shown graphically for nodule Ce concentration, Fig. S3). For the nano-ZnO treatments, growth metrics did not correlate with metal concentration in the plant except for one significant relationship: final leaf count was

negatively correlated to pod Zn concentration, but only when the controls were included in the analysis (Fig. 3; $r = -0.60$, $P = 0.02$, $n = 15$).

Interrelationships of plant damage and stress indicators, and relationships to bioaccumulated foliar metals

To determine if foliar stress and damage related to leaf uptake of MNMs, we evaluated leaf metal concentration versus the plant stress and damage indicators of ROS, lipid peroxidation, visible maximum and harvest percent leaf damage, and total chlorophyll content for each MNM type. PSII quantum efficiency, pod protein, and protein carbonyl contents were not evaluated for their relationships to plant metal content because there were no MNM treatment effects on these variables (Tables S2 and S3). Across the nano-CeO₂ treatments and controls, maximum and at-harvest (day 49, stage R5 to R6) percentages of visible leaf damage appeared to increase with leaf Ce concentration (Fig. S4). Although the correlations between the various at-harvest measures versus leaf Ce concentration were not significant (including controls, Fig. S4; excluding controls, not shown), the correlations for maximum visible percent leaf damage with leaf Ce concentration were significant whether the controls were ($r = 0.67$, $P = 0.005$, $n = 16$), or were not ($r = 0.67$, $P = 0.02$, $n = 12$), included in the analysis (Fig. 4). However, neither leaf ROS nor lipid peroxidation (as MDA) correlated directly with leaf Ce concentration, whether or not the controls were included in the analysis (Fig. 4). For the nano-ZnO treatments, the stress and damage metrics did not correlate with leaf Zn concentration (Fig. 3).

For the nano-CeO₂ treatments, only when controls were excluded from the analysis was leaf total chlorophyll positively correlated with total leaf Ce concentration (Fig. 4; $r = 0.59$, $P =$

0.04, $n = 12$). For the nano-ZnO treatments, total chlorophyll appeared to decrease in proportion to increasing leaf Zn concentration (Fig. S5), and total chlorophyll was significantly negatively correlated with increasing total leaf Zn concentration (Fig. 3; only when including controls in the analysis; $r = -0.53$, $P = 0.04$, $n = 15$).

To further assess interactions between leaf stresses (as indicated by ROS and lipid peroxidation) versus total chlorophyll concentration or visible leaf damage, correlations within and between the stress and damage measures were evaluated. Across all MNM treatments including the controls, leaf lipid peroxidation was positively, albeit weakly, correlated to leaf ROS ($r = 0.46$, $P = 0.02$; Fig. S6). The relationships were not significant when only evaluating the nano-CeO₂ treatments (with or without the controls), yet were significant when evaluating the nano-ZnO treatments alone whether the controls were ($r = 0.60$, $P = 0.02$, $n = 15$) or were not ($r = 0.61$, $P = 0.05$, $n = 11$) included in the analysis (Fig. 3). There was one other significant relationship for the nano-CeO₂ treatments: only when controls were included in the analysis, leaf ROS concentrations for the nano-CeO₂ treatments correlated with maximum percent visible leaf damage (Fig. 4; $r = 0.63$, $P = 0.01$, $n = 16$).

Relationships of plant damage and stress indicators to plant growth metrics

In response to soil-delivered MNMs we observed an increase in several of the stress or damage metrics. Did these measurements, however, relate to diminished plant growth or yield? To address, previously-collected (Priester et al., 2012) plant growth and yield data (Table S9) were tested for correlations to the leaf stress and damage indicators reported herein. For the nano-CeO₂ treatments, N₂ fixation potential was negatively correlated—while the stem elongation rate constant was positively correlated—to two stress and damage indicators: percent

visible leaf damage at harvest and leaf ROS (Table S11, Fig. 4). Harvest stem length and maximum leaf cover were also positively correlated to leaf ROS (Table S11, Fig. 4). Also for the nano-CeO₂ treatments, several plant metrics were negatively correlated to leaf lipid peroxidation: stem length, maximum and final leaf cover, harvest leaf and pod counts, and stem, leaf and total dry biomasses (Table S11, Fig. 4). There were also positive relationships within the nano-CeO₂ treatments: for total chlorophyll concentration versus stem growth rate constant, stem length (only when including the controls), stem dry biomass, and pod dry biomass (only when including the controls; Table S11, Fig. 4).

Although we did not observe significant nano-ZnO effects for most of the stress metrics, we found that the stem growth rate constant was negatively correlated with leaf ROS, and both the stem growth rate constant and leaf dry biomass were negatively correlated with lipid peroxidation (Table S12, Fig. 3). The leaf count at harvest was negatively correlated with maximum and at-harvest visible leaf damage percentages, and the pod count was negatively correlated with the maximum percent visible leaf damage (Table S12, Fig. 3). For the nano-ZnO treatments, the numbers of nodules per plant positively correlated with total chlorophyll (Table S12, Fig. 3).

DISCUSSION

With manufactured nanomaterial (MNM) use accelerating (Roco, 2011), more research is needed regarding MNM effects on plants (Ma et al., 2015a) including potential impacts to food crops (Rico et al., 2011) under realistic exposure conditions (Holden et al., 2014). Previously we described soybean plants grown in a greenhouse to full reproductive stage in soil amended with either nano-CeO₂ or nano-ZnO (Priester et al., 2012). We reported that root nodule N₂ fixation

potential was compromised at medium and high nano-CeO₂ doses; at the low nano-CeO₂ dose, N₂ fixation potential was not compromised although plant growth was decreased (Priester et al., 2012). Metal uptake into plants appeared to be dose-dependent, the bioconcentration factors varied with metal (Zn or Ce) and tissue type (root, nodule, stem, pod or leaf) (Hernandez-Viezcas et al., 2013), and total Zn and Ce concentrations were highest in the leaves and roots, respectively (Priester et al., 2012). Intact nano-CeO₂ particles were observed in the root nodules and beans of nano-CeO₂ treatments; various zinc complexes, but not ZnO nanoparticles, were identified within the leaves and beans of nano-ZnO treatments (Hernandez-Viezcas et al., 2013). Still, there were unresolved questions regarding potential stress and damage in aboveground plant parts from MNMs in the soil, the role of metals versus MNMs, and how plant stress and growth related to one another. While the answers vary by MNM, there is evidence that soil-administered MNMs translocated into aboveground plant parts where localized stress and damage occurred. Depending on the MNM, this was accompanied by systemic impacts to plant growth, N₂ fixation, and yield.

For both MNMs, metal bioaccumulation in various plant parts depended on MNM soil dose, but the patterns varied between the two MNMs. The nano-ZnO soil dose affected Zn concentrations in all below- or aboveground plant parts (Table S5, Fig. 3). Yet the nano-CeO₂ soil dose only affected Ce concentrations in the roots, nodules, and leaves (Table S6, Fig. 4). For the nano-ZnO treatments, Zn bioaccumulation appeared systemic (Table S5, Fig. 3), since Zn concentrations were correlated across all plant parts (Table S8, Fig. 3). For the nano-CeO₂ treatments, the distribution of Ce was patchy, as indicated by fewer significant correlations across plant part Ce concentrations (Table S7, Fig. 4). This may derive from the low overall Ce amounts translocated aboveground (Table S4). Regardless of the differences in MNM metal

bioaccumulation patterns, each MNM type caused aboveground effects, but with varying outcomes to plant growth and yield.

Overall, soybean growth and yield was resilient to the nano-ZnO treatments. This differs from a study using standard test soil, wherein soybean development and growth were impaired at similar soil doses of nano-ZnO (Yoon et al., 2014). Here, leaf chlorophyll and chlorophyll *a/b* decreased (Table 1) with increasing leaf Zn concentration (Fig. S5, Fig. 3). Yet, the plants did not show reduced quantum efficiencies, and biomass production was similar to the controls (Priester et al., 2012). Mukherjee et al. also reported decreased chlorophyll concentrations in leaves of soil-grown green peas (*Pisum sativum*) exposed to Zn (Mukherjee et al., 2014). The interpretation was that Zn substituted for essential metals in chlorophyll synthesis (Mukherjee et al., 2014). We observed that the predawn F_v/F_m ratios (Table S2) and the PSII quantum efficiencies (Fig. S2) were not impacted. In fact, the plants seemed to show typical responses to moderate Zn stress: chlorophyll impact and some visible leaf chlorosis, yet overall physiological resilience (Joshi and Mohandy, 2004). Many metals can diminish plant chlorophyll levels, either by degrading chlorophyll or impairing its synthesis, while not impacting the F_v/F_m ratio or PSII quantum efficiency (Joshi and Mohandy, 2004). Zn can substitute for Mg in RuBisCo and thereby impact photosynthesis (Joshi and Mohandy, 2004). Here, the leaves for nano-ZnO treated plants had similar Mg contents relative to control plant leaves (Peralta-Videa et al., 2014). As per a recent review, reductions in chlorophyll have been observed in other MNM plant studies without phytotoxicity or diminished growth (Rico et al., 2015).

We observed neither significant oxidative stress in leaves or soybeans, nor reductions in biomass, in response to nano-ZnO treatments. However, the plants with higher levels of foliar ROS also had reduced stem growth rates (Table S12, Fig. 3). For the nano-ZnO treatments, leaf

damage was related to lower final leaf counts, and fewer pods (Table S12, Fig. 3). Interestingly, leaf count decreased according to increasing pod Zn (Fig. 3), but this must derive from pod Zn being positively correlated to all plant part Zn concentrations, including the leaves (Table S8, Fig. 3). Mukherjee et al. reported high leaf lipid peroxidation in *P. sativum* at a Zn concentration equivalent to the highest concentration studied here (0.5 g kg^{-1} dry soil)(Mukherjee et al., 2014). However, they also reported significantly elevated leaf ROS relative to the control (Mukherjee et al., 2014) which was not observed here. The differences may reflect differential responses to Zn based on plant species or other conditions, since the accumulated Zn concentrations in leaves were similar across the two studies (Mukherjee et al., 2014; Priester et al., 2012). Still, the overall impression is that nano-ZnO treatments caused only moderate stress according to leaf damage and chlorophyll content.

There was evidence of genotoxicity from nano-ZnO, but only in one plant at the highest nano-ZnO dose (Fig. 2). Genotoxicity to beans that accumulate high Zn levels from nano-ZnO exposure could translate into effects in the next plant generation, as asserted previously for hydroponic soybean (Lopez-Moreno et al., 2010). Others (Atha et al., 2012) demonstrated DNA damage induced by nano-CuO in seedlings of radish (*Raphanus sativus*), perennial ryegrass (*Lolium perenne*), and annual ryegrass (*L. rigidum*). Similarly, the genotoxic effects of nano-Ag in root tip cells of onion (*Allium cepa*) were reported (Kumari et al., 2009). To our knowledge, however, few studies have detected genotoxicity through the use of molecular techniques, which benefit from the availability of the complete genome sequences for many plants, and the ability to detect single nucleotide changes in the plant genome. López-Moreno et al. detected a new DNA band in the random amplified polymorphic DNA (RAPD) profile of soybean roots treated with nano-ZnO at 4000 mg L^{-1} , wherein DNA changes may have arisen due to interactions with

nano-ZnO and/or dissolved Zn ions (Lopez-Moreno et al., 2010). However, while there are reports of nano-ZnO causing genotoxicity to aquatic organisms (Ali et al., 2012; Zhao et al., 2013), and to onion plant cells (Kumari et al., 2011), Zn was determined not to be nanoparticulate for the leaves in this study (Hernandez-Viezcás et al., 2013). Thus, the genotoxicity here was not specific to ZnO nanoparticles, but instead is likely attributable to Zn oxidative phytotoxicity, which can manifest in genotoxicity (Anjum et al., 2015).

Nano-CeO₂ affected soybean differently from nano-ZnO: the biochemical indicators of plant stress were different, the impacts to plants were different, and aboveground stresses were attributable to CeO₂ nanoparticles. Leaf chlorophyll increased with leaf Ce, i.e. the exact opposite of Zn effects on chlorophyll, and the plant stems in the nano-CeO₂ treatments elongated faster with greater leaf chlorophyll (Table S11, Fig. 4). With increased soil nano-CeO₂ (Priester et al., 2012), and concomitant increased nodule (Fig. S3) and root Ce, the N₂ fixation potential decreased (Table S10, Fig. 4). One interpretation is that nodule nano-CeO₂ impaired bacteroid function which shifted N sourcing from the atmosphere to soil, thus conserving photosynthate for aboveground biomass instead of fueling nodule symbioses (Priester et al., 2012). While bacteroids could have been directly impaired by visible nanoparticulate Ce in nodules (Priester et al., 2012), the data herein of increased leaf ROS with increasing soil nano-CeO₂ allows for considering an alternate explanation that begins with the plant leaves. Essentially, Ce that translocated as MNMs (Hernandez-Viezcás et al., 2013) may have done so early in plant growth, i.e. before nodule formation. Plants thus could have been responding to Ce-derived stress before plant infection by symbiotic bacteria. The prerequisites to nodulation, i.e. of plant root-bacterial signaling and of bacterial infection (Larrainzar et al., 2014), were apparently not impacted since neither nodule count nor nodule biomass were affected by the treatments (Tables S10 and S11,

Fig. 4). It is possible that there was local nodule control on N₂ fixation due to high nano-CeO₂ concentrations in nodules and concomitant inhibition of bacteroid function. It is also possible that there was a systemic response by plants to increasing nano-CeO₂ soil dose, whereby the plants deprived nodules of photosynthate in order to retain energy aboveground to support oxidatively stressed leaves. Clearly, there was evidence of that aboveground plant production was impaired by oxidative leaf damage (Table S11, Fig. 4). The stress manifested as peroxidized lipids (MDA) that could have accumulated throughout plant growth. ROS compounds are short-lived, although ROS mediated oxidative damage events, manifested as peroxidized lipids, would exert prolonged effects seen across the plant lifetime. Thus, the entire plant could have been involved in coping with oxidative stress initiated by nano-CeO₂ deposits in leaves, which could have led to controls on nodule function. The conservation of photosynthate aboveground would have allowed for positive interactions between stem growth and leaf oxidative stress (ROS) (Table S11, Fig. 4).

For the nano-CeO₂ treatments, the relationships between leaf area and mass, versus oxidative damage (by MDA concentrations; Table S11, Fig. 4), were reminiscent of the reductions in leaf area and mass that were observed in soil-grown soybean exposed to O₃ (Betzberger et al., 2012): simultaneous increase in antioxidant activity suggested that decreased leaf biomass was the result of oxidative stress processes within the leaves (Betzberger et al., 2012). For velvet mesquite (*Prosopis juliflora-velutina*) exposed to nano-ZnO (Hernandez-Viezcas et al., 2011) and corn (*Zea mays*) exposed to nano-CeO₂ (Zhao et al., 2012), catalase and ascorbate peroxidase both increased with MNM dose, presumably providing protection against ROS. This phenomenon has been described in rice plants (Rico et al., 2013), and suggests a protective response to nano-CeO₂ at certain concentrations. It is possible that this protection was

in place for the PSII machinery. However, although free radical scavenging enzymes were not measured here, based on systemic interactions that ultimately affected plant growth and yield, it appears that the soybeans in this study were not fully mitigating oxidative stresses from nano-CeO₂.

Also differently from the nano-ZnO treatments (Fig. 2), leaves from soybean plants grown in soil amended with nano-CeO₂ did not show evidence of genotoxicity, although ROS and oxidative damage were evident in the leaves of plants exposed to nano-CeO₂, and genetic damage from MNM exposure has been linked to oxidative damage (Singh et al., 2009). Previously, López-Moreno et al. reported new bands in the RAPD profiles of hydroponic soybean roots for seedlings treated with 2000 and 4000 mg L⁻¹ nano-CeO₂ (Lopez-Moreno et al., 2010). The lower nano-CeO₂ soil doses used in this study, by comparison (Lopez-Moreno et al., 2010), may explain the lack of genotoxicity herein. The present study also differs from Lopez-Moreno et al. in other ways: here, plants were grown in soil, and leaves were analyzed for genotoxicity (Lopez-Moreno et al., 2010). Growth in soil may have reduced the effective exposure concentration compared to hydroponic experiments (Lopez-Moreno et al., 2010), further avoiding a threshold concentration required for genetic damage. Genetic analysis of the leaves, which had at least 4,000 times less Ce as compared to the roots (Table S4), may have been insensitive to possibly infrequent genetic changes.

While the responses of soybeans to the two MNMs differed in many ways, they were similar in that seed quality and PSII quantum efficiency were unaffected. PSII is considered particularly sensitive to oxidative damage and can be one of the first indicators of stress (Maxwell and Johnson, 2000). Da Costa and Sharma found that rice plants grown in higher doses of nano-CuO (100 and 1000 mg/L) had significantly lower F_v/F_m values and photosynthetic

rates, corresponding with detrimental modifications to leaf and chloroplast anatomy (Da Costa and Sharma, 2016). Across the MNM treatments in our study, there was stress to the soybean leaves (i.e. reduction in leaf chlorophyll in both treatments, and increased ROS and leaf damage in nano-CeO₂ treated plants), but this did not appear to reduce the capacity or operating quantum efficiency of PSII either early or late in growth (Table S2, Fig. S2). Similar results were observed in barley seedlings treated with nano-CuO (Shaw et al., 2014): after 20 days of treatment, F_v/F_m was unaffected and Φ_{PSII} was either similar or higher in the nano-CuO treated seedlings. However, necrotic leaf spots were observed, chlorophyll content was significantly lower, and MDA and H₂O₂ were significantly higher in these seedlings (Shaw et al., 2014), just as we observed in soybeans grown in nano-CeO₂ treated soils. In response to drought (Ohashi et al., 2006) and ozone (Betzberger et al., 2012)—two other stresses to which soybeans are sensitive— PSII quantum efficiency (as either F_v/F_m or Φ_{PSII}) was also unaffected, but leaf area and photosynthetic rates were reduced. PSII is one of several photosynthetic components, and nanoparticles may interact selectively with the photosynthetic machinery. Recent studies have highlighted possible interactive mechanisms (Rico et al., 2015). For example, Conway et al. found that an annual wildflower grown in soils with nano-CeO₂ and nano-TiO₂ had reduced photosynthetic rates but maintained Φ_{PSII} values (Conway et al., 2015), possibly due to interference with electron transport from PSII to the Calvin cycle (Rico et al., 2015). Similarly, nano-Au is thought to accept excited electrons in PSII, thus increasing non-photochemical fluorescence quenching (Falco et al., 2011). In our study, PSII integrity appeared to be maintained and did not lead to photoinhibition and reduced F_v/F_m levels. Either a lack of toxicity at PSII or protective responses—such as greater antioxidant activity as observed in other studies (Du et al., 2016; Rico et al., 2015; Zhao et al., 2012)—could explain our findings.

Beyond how each MNM type affected soybean tissues and plants, it is important to understand the differential toxicity of MNMs as particles versus their metal constituents. Studies have not resolved the debate for bacterial populations. For example, while Ag MNMs appeared to intoxicate *Escherichia coli* and *Pseudomonas aeruginosa* due to Ag⁺ ions and associated complexes (Priester et al., 2014), Cu MNMs affected *E. coli* and *Lactobacillus brevis* distinctively from Cu²⁺ ions, their complexes, or larger Cu particles (Kaweeteerawat et al., 2015), and CdSe quantum dots inflicted nanoparticle-specific membrane damage and growth impairment to *P. aeruginosa* (Priester et al., 2009). As per a review of metal nanoparticle interactions with plants, there are many examples of nanoparticle-specific effects that are distinctive from constituent metals (Ma et al., 2015a). The use of non-nanoscale (negative) experimental controls, for example bulk ZnO (Mukherjee et al., 2014) or CeO₂ (Gomez-Garay et al., 2014), would allow for determining if the nano-scale imparts special toxicities to particulate metal oxides amended to planted soils.

In conclusion, we have shown that soybean, an important agricultural crop, can exhibit aboveground foliar stress and damage from MNMs amended to soil. However, PSII quantum efficiency and seed quality were maintained. Soil-grown soybeans bioaccumulated nano-CeO₂ and showed signs of oxidative damage in the leaves with consequences to the entire plant. Plants grown in soil amended with nano-ZnO contained Zn complexes throughout the plants. For nano-ZnO treatments, soybean leaf chlorophyll concentrations decreased but this did not correspond to diminished plant growth, yield, or N₂ fixation potential. Genotoxicity from nano-ZnO was evidenced in soil-grown soybean leaves for one plant at the highest nano-ZnO concentration. However, more studies are needed to confirm and explain this result including in the context of seed genetic variations. Plants grown with nano-CeO₂ appeared to protect their aboveground

biomass at the expense of the belowground symbioses, and the effects of nano-CeO₂ were systemic throughout the plant life cycle.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Reid G. Palmer (deceased) for his scientific contributions to this study. Laurie Van De Werfhorst assisted this research. This research was primarily funded by the National Science Foundation (NSF) and the U.S. Environmental Protection Agency (EPA) under Cooperative Agreements DBI-0830117 and 1266377 (to P.A.H, J.P.S., R.M.N., and J.L.G.-T.). Additional funding was provided by ASTA's Seed Research Foundation, the ISU Agronomy Endowment, and the Iowa Crop Improvement Association. Any opinions, findings, and conclusions expressed in this material are those of the author(s) and do not necessarily reflect those of either the NSF or the EPA. This work has not been subjected to EPA review and no official endorsement should be inferred.

FIGURE LEGENDS

Figure 1. Representative photographs of soybean leaves at harvest (A, B) and the percentage of leaves that were visibly damaged during plant growth with either nano-CeO₂ (C) or nano-ZnO (D). Photographs are of undamaged (A) and damaged (necrosis) (B) trifoliolate soybean leaves from control and high nano-CeO₂ treatments, respectively. Typical necrotic lesions are indicated by arrows (B). In the graphs (C, D), unfilled circles represent control treatments; filled squares, triangles and diamonds represent low, medium and high treatments, respectively. The error bars represent the standard error of the mean (n= 4 plants for each treatment, except n = 3 for low nano-ZnO).

Figure 2. Photographed gels from gene insertion/deletion (InDel) analysis of DNA extracted from single, randomly-selected, soybean leaves sampled from individual plants at harvest. The pictured gels show bands for InDel marker 19428 (Table S1) for nano-CeO₂ (A) and nano-ZnO (B) treatments. Control, low, medium and high treatments are underlined blue, yellow, orange and red, respectively, and replicate numbers (n = 4, except n = 3 for low nano-ZnO) appear below the lines. ND denotes the negative control (no DNA). The DNA ladder appears in the left lane. One plant from the High nano-ZnO treatment (3rd lane from the left, for this treatment category; white arrow) indicated a genetic change as per an insertion event of 26 bp.

Figure 3. Schematic of interactions for soybean plants grown in soil amended with nano-ZnO. Blue lines indicate significant ($P \leq 0.05$) pairwise (Pearson) correlations, whether the relationship was positive (solid line) or negative (dashed lines), and whether the controls were or were not included in the correlation analyses. Statistical values (regression coefficient and P values) are reported in the manuscript, or are tabulated (Tables S6, S8 and S12). Pearson correlation analyses were performed for arithmetic (not log-transformed) data that were paired within individual plants (n = 15, 4 replicates for 3 treatments including controls, and 3 replicates for the Low nano-ZnO treatment). Coloring indicates: pink = Zn reservoirs (including nano-ZnO in the soil, or total Zn in plant parts; Table S5); medium brown = nodule growth metric (count); green (dark for leaf, light for stem, olive for pod, bright for total biomass) = plant metrics (Table S9), bright yellow = stress and damage indicators, and light yellow = total chlorophyll. Plant metrics, and stress or damage indicators, that are not shown were not statistically significantly related to other variables. Chlorophyll *a* and *b*, and the ratio of *a/b* are not shown as these variables were not independent of total chlorophyll.

Figure 4. Schematic of interactions for soybean plants grown in soil amended with nano-CeO₂. Blue lines indicate significant ($P \leq 0.05$) pairwise (Pearson) correlations, whether the relationship was positive (solid line) or negative (dashed lines), and whether the controls were or were not included in the correlation analyses. Statistical values (regression coefficient and P values) are reported in the manuscript, or are tabulated (Tables S6, S7, S10 and S11). Pearson correlation analyses were performed for arithmetic (not log-transformed) data that were paired within individual plants (n = 16: 4 replicates for 4 treatments including controls). The relationship between N₂ fixation potential and nodule Ce concentration followed a power function (Fig. S3) and thus was linearized by log transforming the Y-axis data before Pearson correlation analysis. Box coloring indicates: pink = Ce reservoirs (including nano-CeO₂ in the soil, or total Ce in plant parts; Table S4); light brown = functional metric (N₂ fixation potential); green (dark for leaf, light for stem, olive for pod, bright for total biomass) = plant metrics (Table S9), bright yellow = stress and damage indicators, and light yellow = total chlorophyll. Plant

metrics, stress or damage indicators, or Ce reservoir (pod) that are not shown were not statistically significantly related to other variables. Chlorophyll *a* and *b*, and the ratio of *a/b* are not shown as these variables were not independent of total chlorophyll.

LITERATURE CITED

- Adams III WW, Demmig-Adams B. Chlorophyll fluorescence as a tool to monitor plant response to the environment. In: Papageorgiou GC, Govindjee, editors. Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. 19. Springer, Dordrecht, 2004, pp. 583-604.
- Adams ML, Philpot WD, Norvell WA. Yellowness index: an application of spectral second derivatives to estimate chlorosis of leaves in stressed vegetation. *Int J Remote Sens* 1999; 20: 3663-3675.
- Ali D, Alarifi S, Kumar S, Ahamed M, Siddiqui MA. Oxidative stress and genotoxic effect of zinc oxide nanoparticles in freshwater snail *Lymnaea luteola* L. *Aquatic Toxicology* 2012; 124, 83-90.
- Anjum NA, Singh HP, Khan MIR, Masood A, Per TS, Negi A, et al. Too much is bad-an appraisal of phytotoxicity of elevated plant-beneficial heavy metal ions. *Environmental Science and Pollution Research* 2015; 22: 3361-3382.
- Aslani F, Bagheri S, Julkapli NM, Juraimi AS, Hashemi FSG, Baghdadi A. Effects of engineered nanomaterials on plants growth: an overview. *Scientific World Journal* 2014.
- Atha DH, Wang HH, Petersen EJ, Cleveland D, Holbrook RD, Jaruga P, et al. Copper oxide nanoparticle mediated DNA damage in terrestrial plant models. *Environmental Science & Technology* 2012; 46: 1819-1827.
- Baker NR. Chlorophyll fluorescence: A probe of photosynthesis in vivo. *Annu Rev Plant Biol* 2008; 59: 89-113.
- Betzlberger AM, Yendrek CR, Sun JD, Leisner CP, Nelson RL, Ort DR, et al. Ozone exposure response for U.S. soybean cultivars: linear reductions in photosynthetic potential, biomass, and yield. *Plant Physiol* 2012; 160: 1827-1839.
- Bjorkman O, Demmig B. Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77-K among vascular plants of diverse origins. *Planta* 1987; 170: 489-504.
- Bruinsma J. The quantitative analysis of chlorophylls *a* and *b* in plant extracts. *Photochem Photobiol* 1963; 2: 241-249.
- Cheeseman JM. Hydrogen peroxide concentrations in leaves under natural conditions. *Journal of Experimental Botany* 2006; 57: 2435-2444.
- Cheeseman JM. Seasonal patterns of leaf H₂O₂ content: reflections of leaf phenology, or environmental stress? *Functional Plant Biology* 2009; 36: 721-731.
- Chen SG, Yin CY, Strasser RJ, Govindjee, Yang CL, Qiang S. Reactive oxygen species from chloroplasts contribute to 3-acetyl-5-isopropyltetramic acid-induced leaf necrosis of *Arabidopsis thaliana*. *Plant Physiol Bioch* 2012; 52: 38-51.
- Choi IY, Hyten DL, Matukumalli LK, Song QJ, Chaky JM, Quigley CV, et al. A soybean transcript map: gene distribution, haplotype and single-nucleotide polymorphism analysis. *Genetics* 2007; 176: 685-696.
- Conway JR, Beaulieu AL, Beaulieu NL, Mazer SJ, Keller AA. Environmental stresses increase photosynthetic disruption by metal oxide nanomaterials in a soil-grown plant. *ACS Nano* 2015; 9: 11737-11749.
- Cucu T, Devreese B, Kerkaert B, Mestdagh F, Sucic M, De Perre IV, et al. A comparative study of lipid and hypochlorous acid induced oxidation of soybean proteins. *Lwt-Food Sci Technol* 2013; 50: 451-458.

- Da Costa MVJ, Sharma PK. Effect of copper oxide nanoparticles on growth, morphology, photosynthesis, and antioxidant response in *Oryza sativa*. *Photosynthetica* 2016; 54: 110-119.
- Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta* 2003; 329: 23-38.
- Demidchik V. Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. *Environmental and Experimental Botany* 2015; 109: 212-228.
- Du W, Tan W, Peralta-Videa JR, Gardea-Torresdey JL, Ji R, Yin Y, et al. Interaction of metal oxide nanoparticles with higher terrestrial plants: Physiological and biochemical aspects. *Plant Physiology and Biochemistry* 2016.
- Economic Research Service USDoA. Soybeans and Oil Crops. 2015, 2012.
- Edwards K, Johnstone C, Thompson C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res* 1991; 19: 1349-1349.
- Falco WF, Botero ER, Falcao EA, Santiago EF, Bagnato VS, Caires ARL. *In vivo* observation of chlorophyll fluorescence quenching induced by gold nanoparticles. *Journal of Photochemistry and Photobiology a-Chemistry* 2011; 225: 65-71.
- Gardea-Torresdey JL, Rico CM, White JC. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environmental Science & Technology* 2014; 48: 2526-2540.
- Ge Y, Priester JH, Van De Werfhorst LC, Walker SL, Nisbet RM, An Y-J, et al. Soybean plants modify metal oxide nanoparticle effects on soil bacterial communities. *Environmental Science & Technology* 2014; 48: 13489-13496.
- Genty B, Briantais JM, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta* 1989; 990: 87-92.
- Geranio L, Heuberger M, Nowack B. The behavior of silver nanotextiles during washing. *Environ Sci Technol* 2009; 43: 8113-8118.
- Ghafariyan MH, Malakouti MJ, Dadpour MR, Stroeve P, Mahmoudi M. Effects of magnetite nanoparticles on soybean chlorophyll. *Environmental Science & Technology* 2013; 47: 10645-10652.
- Gomez-Garay A, Pintos B, Manzanera JA, Lobo C, Villalobos N, Martin L. Uptake of CeO₂ nanoparticles and Its effect on growth of *Medicago arborea in vitro* plantlets. *Biological Trace Element Research* 2014; 161: 143-150.
- Hernandez-Viezcás JA, Castillo-Michel H, Andrews JC, Cotte M, Rico C, Peralta-Videa JR, et al. In situ synchrotron X-ray fluorescence mapping and speciation of CeO₂ and ZnO nanoparticles in soil cultivated soybean (*Glycine max*). *ACS Nano* 2013; 7: 1415-1423.
- Hernandez-Viezcás JA, Castillo-Michel H, Servin AD, Peralta-Videa JR, Gardea-Torresdey JL. Spectroscopic verification of zinc absorption and distribution in the desert plant *Prosopis juliflora-velutina* (velvet mesquite) treated with ZnO nanoparticles. *Chemical Engineering Journal* 2011; 170: 346-352.
- Holden PA, Gardea-Torresdey JL, Klaessig F, Turco RF, Mortimer M, Hund-Rinke K, et al. Considerations of environmentally relevant test conditions for improved evaluation of ecological hazards of engineered nanomaterials. *Environmental Science & Technology* 2016; 50: 6124-6145.
- Holden PA, Klaessig F, Turco RF, Priester JH, Rico CM, Avila-Arias H, et al. Evaluation of exposure concentrations used in assessing manufactured nanomaterial environmental

- hazards: are they relevant? *Environmental Science & Technology* 2014; 48: 10541-10551.
- Holden PA, Nisbet RM, Lenihan HS, Miller RJ, Cherr GN, Schimel JP, et al. Ecological nanotoxicology: integrating nanomaterial hazard considerations across the subcellular, population, community, and ecosystems levels. *Accounts of Chemical Research* 2013; 46: 813–822.
- Hu H, Wang L, Wang Q, Jiao L, Hua W, Zhou Q, et al. Photosynthesis, chlorophyll, fluorescence characteristics, and chlorophyll content of soybean seedlings under combined stress of bisphenol A and cadmium. *Environmental Toxicology and Chemistry* 2014; 33: 2455-2462.
- Joo JH, Wang SY, Chen JG, Jones AM, Fedoroff NV. Different signaling and cell death roles of heterotrimeric G protein alpha and beta subunits in the *Arabidopsis* oxidative stress response to ozone. *The Plant Cell* 2005; 17: 957-970.
- Joshi MK, Mohanty P. Chlorophyll *a* fluorescence as a probe of heavy metal ion toxicity in plants. In: Papageorgiou GC, Govindjee, editors. *Chlorophyll a Fluorescence: A Signature of Photosynthesis*. Springer, Dordrecht, The Netherlands, 2004, pp. 637-661.
- Karr-Lilienthal LK, Grieshop CM, Merchen NR, Mahan DC, Fahey GC. Chemical composition and protein quality comparisons of soybeans and soybean meals from five leading soybean-producing countries. *J Agr Food Chem* 2004; 52: 6193-6199.
- Kaweeteerawat C, Chang CH, Roy KR, Liu R, Li R, Toso D, et al. Cu nanoparticles have different impacts in *Escherichia coli* and *Lactobacillus brevis* than their micro-sized and ionic analogues. *ACS Nano* 2015; 9: 7215-7225.
- Kumari M, Khan SS, Pakrashi S, Mukherjee A, Chandrasekaran N. Cytogenetic and genotoxic effects of zinc oxide nanoparticles on root cells of *Allium cepa*. *J. Hazard. Mater.* 2011; 190: 613-621.
- Kumari M, Mukherjee A, Chandrasekaran N. Genotoxicity of silver nanoparticles in *Allium cepa*. *Sci Total Environ* 2009; 407: 5243-5246.
- Kwak JI, An Y-J. The current state of the art in research on engineered nanomaterials and terrestrial environments: Different-scale approaches. *Environmental Research* 2016; 151: 368-382.
- Laing W, Christeller J. Extraction of proteins from plant tissues. In: Coligan JE, Dunn BM, Speicher DW, Wingfield PT, editors. *Current Protocols in Protein Science, Supplement 38*. John Wiley & Sons, Inc., Hoboken, NJ, 2004, pp. 4.7.1-4.7.7.
- Larrainzar E, Gil-Quintana E, Arrese-Igor C, Gonzalez EM, Marino D. Split-root systems applied to the study of the legume-rhizobial symbiosis: What have we learned? *Journal of Integrative Plant Biology* 2014; 56: 1118-1124.
- Lichtenthaler HK, Wellburn AR. Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochem Soc T* 1983; 11: 591-592.
- Liu HH, Cohen Y. Multimedia environmental distribution of engineered nanomaterials. *Environmental Science & Technology* 2014; 48: 3281-3292.
- Lopez-Moreno ML, de la Rosa G, Hernandez-Viezcas JA, Castillo-Michel H, Botez CE, Peralta-Videa JR, et al. Evidence of the differential biotransformation and genotoxicity of ZnO and CeO₂ nanoparticles on soybean (*Glycine max*) plants. *Environmental Science & Technology* 2010; 44: 7315-7320.
- Ma CX, White JC, Dhankher OP, Xing BS. Metal-based nanotoxicity and detoxification pathways in higher plants. *Environmental Science & Technology* 2015a; 49: 7109-7122.

- Ma YH, Zhang P, Zhang ZY, He X, Li YY, Zhang J, et al. Origin of the different phytotoxicity and biotransformation of cerium and lanthanum oxide nanoparticles in cucumber. *Nanotoxicology* 2015b; 9: 262-270.
- Maxwell K, Johnson GN. Chlorophyll fluorescence - a practical guide. *J Exp Bot* 2000; 51: 659-668.
- Medic J, Atkinson C, Hurburgh CR. Current knowledge in soybean composition. *Journal of the American Oil Chemists Society* 2014; 91: 363-384.
- Moller IM, Jensen PE, Hansson A. Oxidative modifications to cellular components in plants. *Annual Review of Plant Biology*. 58, 2007, pp. 459-481.
- Mukherjee A, Peralta-Videa JR, Bandyopadhyay S, Rico CM, Zhao LJ, Gardea-Torresdey JL. Physiological effects of nanoparticulate ZnO in green peas (*Pisum sativum* L.) cultivated in soil. *Metallomics* 2014; 6: 132-138.
- Muñoz N, González C, Molina A, Zirulnik F, Luna CM. Cadmium-induced early changes in $O_2^{\bullet-}$, H_2O_2 and antioxidative enzymes in soybean (*Glycine max* L.) leaves. *Plant Growth Regulation* 2008; 56: 159-166.
- Murchie EH, Lawson T. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of Experimental Botany* 2013; 64: 3983-3998.
- Murday JS, Siegel RW, Stein J, Wright JF. Translational nanomedicine: status assessment and opportunities. *Nanomed-Nanotechnol* 2009; 5: 251-273.
- Nair PMG, Chung IM. A mechanistic study on the toxic effect of copper oxide nanoparticles in soybean (*Glycine max* L.) root development and lignification of root cells. *Biological Trace Element Research* 2014; 162: 342-352.
- Nazarenko Y, Zhen HJ, Han T, Liou PJ, Mainelis G. Potential for inhalation exposure to engineered nanoparticles from nanotechnology-based cosmetic powders. *Environ Health Persp* 2012; 120: 885-892.
- Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science* 2006; 311: 622-627.
- Ohashi Y, Nakayama N, Saneoka H, Fujita K. Effects of drought stress on photosynthetic gas exchange, chlorophyll fluorescence and stem diameter of soybean plants. *Biologia Plantarum* 2006; 50: 138-141.
- Peralta-Videa JR, Hernandez-Viezcas JA, Zhao L, Diaz BC, Ge Y, Priester JH, et al. Cerium dioxide and zinc oxide nanoparticles alter the nutritional value of soil cultivated soybean plants. *Plant Physiology and Biochemistry* 2014; 80: 128-135.
- Prasad SM, Dwivedi R, Zeeshan M. Growth, photosynthetic electron transport, and antioxidant responses of young soybean seedlings to simultaneous exposure of nickel and UV-B stress. *Photosynthetica* 2005; 43: 177-185.
- Priester JH, Ge Y, Mielke RE, Horst AM, Moritz SC, Espinosa K, et al. Soybean susceptibility to manufactured nanomaterials with evidence for food quality and soil fertility interruption. *Proceedings of the National Academy of Sciences* 2012; 109: E2451-E2456.
- Priester JH, Singhal A, Wu B, Stucky GD, Holden PA. Integrated approach to evaluating the toxicity of novel cysteine-capped silver nanoparticles to *Escherichia coli* and *Pseudomonas aeruginosa*. *Analyst* 2014; 139: 954-963.
- Priester JH, Stoimenov PK, Mielke RE, Webb SM, Ehrhardt C, Zhang JP, et al. Effects of soluble cadmium salts versus CdSe quantum dots on the growth of planktonic *Pseudomonas aeruginosa*. *Environmental Science & Technology* 2009; 43: 2589-2594.

- Queval G, Hager J, Gakiere B, Noctor G. Why are literature data for H₂O₂ contents so variable? a discussion of potential difficulties in the quantitative assay of leaf extracts. *Journal of Experimental Botany* 2008; 59: 135-146.
- Rachmilevitch S, DaCosta M, Huang B. Physiological and biochemical indicators for stress tolerance. In: Huang B, editor. *Plant-Environment Interactions*, Third Edition. CRC Press, Boca Raton, FL, 2006, pp. 321-355.
- Reddy PVL, Hernandez-Viezcas JA, Peralta-Videa JR, Gardea-Torresdey JL. Lessons learned: Are engineered nanomaterials toxic to terrestrial plants? *Science of the Total Environment* 2016; 568: 470-479.
- Rico CM, Majumdar S, Duarte-Gardea M, Peralta-Videa JR, Gardea-Torresdey JL. Interaction of nanoparticles with edible plants and their possible implications in the food chain. *Journal of Agricultural and Food Chemistry* 2011; 59: 3485-3498.
- Rico CM, Morales MI, McCreary R, Castillo-Michel H, Barrios AC, Hong J, et al. Cerium oxide nanoparticles modify the antioxidative stress enzyme activities and macromolecule composition in rice seedlings. *Environmental Science & Technology* 2013; 47: 14110-14118.
- Rico CM, Peralta-Videa JR, Gardea-Torresdey JL. Chemistry, biochemistry of nanoparticles, and their role in antioxidant defense system in plants. In: Siddiqui MH, Al-Whaibi MH, Mohammad F, editors. *Nanotechnology and Plant Sciences*. Springer International Publishing, 2015, pp. 1-17.
- Roco MC. The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *J. Nanopart. Res.* 2011; 13: 427-445.
- Schreiber U, Bilger W, Neubauer C. Chlorophyll fluorescence as a noninvasive indicator for rapid assessment of in vivo photosynthesis. In: Schulze E-D, Caldwell MM, editors. *Ecophysiology of Photosynthesis*. Springer-Verlag, Berlin, 1995, pp. 49-70.
- Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *Journal of Biological Chemistry* 2003; 278: 3170-3175.
- Shacter E. Quantification and significance of protein oxidation in biological samples. *Drug Metab Rev* 2000; 32: 307-326.
- Shaw AK, Ghosh S, Kalaji HM, Bosa K, Brestic M, Zivcak M, et al. Nano-CuO stress induced modulation of antioxidative defense and photosynthetic performance of Syrian barley (*Hordeum vulgare* L.). *Environmental and Experimental Botany* 2014; 102: 37-47.
- Shulaker MM, Hills G, Patil N, Wei H, Chen HY, PhilipWong HS, et al. Carbon nanotube computer. *Nature* 2013; 501: 526-+.
- Singh N, Manshian B, Jenkins GJS, Griffiths SM, Williams PM, Maffei TGG, et al. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. *Biomaterials* 2009; 30: 3891-3914.
- Stevens G, Motavalli P, Scharf P, Nathan M, Dunn D. Crop nutrient deficiencies and toxicities. University of Missouri, 2002.
- Sun WQ, Leopold AC. The Maillard reaction and oxidative stress during aging of soybean seeds. *Physiologia Plantarum* 1995; 94: 94-104.
- Wang TL, Domoney C, Hedley CL, Casey R, Grusak MA. Can we improve the nutritional quality of legume seeds? *Plant Physiol* 2003; 131: 886-891.

- Weisany W, Sohrabi Y, Heidari G, Siosemardeh A, Ghassemi-Golezani K. Changes in antioxidant enzymes activity and plant performance by salinity stress and zinc application in soybean (*Glycine max* L.). *Plant Omics J* 2012; 5: 60-67.
- Wu W, Zhang CM, Kong XZ, Hua YF. Oxidative modification of soy protein by peroxy radicals. *Food Chem* 2009; 116: 295-301.
- Yan SR, Huang XH, Zhou Q. Effect of lanthanum (III) on reactive oxygen metabolism of soybean seedlings under supplemental UV-B irradiation. *Journal of Rare Earths* 2007; 25: 352-358.
- Yoon SJ, Kwak JI, Lee WM, Holden PA, An YJ. Zinc oxide nanoparticles delay soybean development: a standard soil microcosm study. *Ecotoxicology and Environmental Safety* 2014; 100: 131-137.
- Zhao L, Peng B, Hernandez-Viezcas JA, Rico C, Sun Y, Peralta-Videa JR, et al. Stress response and tolerance of *Zea mays* to CeO₂ nanoparticles: cross talk among H₂O₂, heat shock protein, and lipid peroxidase. *ACS Nano* 2012; 6: 9615–9622.
- Zhao TH, Wang JL, Wang Y, Sun JW, Cao Y. Effects of reactive oxygen species metabolic system on soybean (*Glycine max*) under exogenous chitosan to ozone stress. *Bulletin of Environmental Contamination and Toxicology* 2010; 85: 59-63.
- Zhao X, Wang S, Wu Y, You H, Lv L. Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. *Aquatic Toxicology* 2013; 136: 49-59.
- Zuverza-Mena N, Martínez-Fernández D, Du W, Hernandez-Viezcas JA, Bonilla-Bird N, López-Moreno ML, et al. Exposure of engineered nanomaterials to plants: Insights into the physiological and biochemical responses-A review. *Plant Physiology and Biochemistry* 2016; in press.

TABLES

Table 1. Leaf chlorophyll (*a*, *b*, *a/b* ratio, and total) content, normalized to dry leaf mass, for soybean plants at harvest. Measurements were made for a sample from three randomly-selected leaf discs per replicate plant (*n* = 4 per treatment, except *n*=3 for 0.05 g per kg soil nano-ZnO). Means $\pm$ one standard error of the mean are tabulated. ^{a-d} Like letters in each column denote insignificant differences between mean values (Student's *t*-test, *P* > 0.05).

Treatment (g per kg soil)	Chlorophyll <i>a</i> (mg per g dry mass)	Chlorophyll <i>b</i> (mg per g dry mass)	Chlorophyll <i>a/b</i>	Total chlorophyll (mg per g dry mass)
Control	8.0 $\pm$ 0.4 ^a	2.9 $\pm$ 0.1 ^a	2.7 $\pm$ 0.0 ^a	10.9 $\pm$ 0.5 ^a
0.1 nano-CeO ₂	5.2 $\pm$ 0.2 ^b	2.1 $\pm$ 0.0 ^b	2.5 $\pm$ 0.1 ^{b,c}	7.3 $\pm$ 0.2 ^b
0.5 nano-CeO ₂	6.6 $\pm$ 0.2 ^c	2.7 $\pm$ 0.2 ^{a,c}	2.4 $\pm$ 0.1 ^{b,c}	9.3 $\pm$ 0.4 ^c
1.0 nano-CeO ₂	5.9 $\pm$ 0.3 ^d	2.5 $\pm$ 0.1 ^c	2.3 $\pm$ 0.0 ^b	8.4 $\pm$ 0.4 ^{c,d}
0.05 nano-ZnO	5.3 $\pm$ 0.9 ^{b,c,d}	2.3 $\pm$ 0.4 ^{a,b,c,d}	2.3 $\pm$ 0.1 ^{b,c}	7.6 $\pm$ 1.4 ^{a,b,c}
0.1 nano-ZnO	4.8 $\pm$ 0.4 ^b	2.3 $\pm$ 0.4 ^{a,b,c,d}	2.1 $\pm$ 0.1 ^{b,c}	7.1 $\pm$ 0.7 ^{b,d}
0.5 nano-ZnO	4.8 $\pm$ 0.2 ^b	1.9 $\pm$ 0.1 ^d	2.6 $\pm$ 0.0 ^c	6.7 $\pm$ 0.3 ^b

Table 2. Leaf total reactive oxygen species (ROS) reported as fluorescence intensity units (FIU), and leaf lipid peroxidation reported as malondialdehyde (MDA) concentrations, each normalized to dry leaf mass, for soybean plants at harvest. Measurements were made for a sample from three randomly-selected leaf discs per replicate plant (n = 4 per treatment, except n=3 for 0.05 g per kg soil nano-ZnO). Means $\pm$ one standard error of the mean are tabulated. ^{a-d} Like letters in each column denote insignificant differences between mean values (Student's *t*-test, *P* > 0.05). Individual values for each treatment are regressed (ROS vs. MDA) in Figure S6.

Treatment (g per kg soil)	Total ROS (FIU per g dry mass)	Lipid Peroxidation (mg MDA per g dry mass)
Control	2.32E6 $\pm$ 1.54E5 ^a	0.20 $\pm$ 0.03 ^{a,b}
0.1 nano-CeO ₂	2.48E6 $\pm$ 1.59E5 ^{a,b}	0.29 $\pm$ 0.03 ^{c,d}
0.5 nano-CeO ₂	2.79E6 $\pm$ 1.32E5 ^{b,c}	0.30 $\pm$ 0.04 ^{c,d}
1.0 nano-CeO ₂	2.83E6 $\pm$ 1.53E5 ^{b,c}	0.21 $\pm$ 0.02 ^{a,b}
0.05 nano-ZnO	2.17E6 $\pm$ 2.53E4 ^a	0.20 $\pm$ 0.01 ^a
0.1 nano-ZnO	2.36E6 $\pm$ 4.42E5 ^{a,c}	0.20 $\pm$ 0.05 ^{a,d}
0.5 nano-ZnO	2.25E6 $\pm$ 9.59E4 ^a	0.23 $\pm$ 0.01 ^{b,c}

Figure 1. Representative photographs of soybean leaves at harvest (A, B) and the percentage of leaves that were visibly damaged during plant growth with either nano-CeO₂ (C) or nano-ZnO (D). Photographs are of undamaged (A) and damaged (necrosis) (B) trifoliate soybean leaves from control and high nano-CeO₂ treatments, respectively. Typical necrotic lesions are indicated by arrows (B). In the graphs (C, D), unfilled circles represent control treatments; filled squares, triangles and diamonds represent low, medium and high treatments, respectively. The error bars represent the standard error of the mean (n= 4 plants for each treatment, except n = 3 for low nano-ZnO).

Figure 2. Photographed gels from gene insertion/deletion (InDel) analysis of DNA extracted from single, randomly-selected, soybean leaves sampled from individual plants at harvest. The pictured gels show bands for InDel marker 19428 (Table S1) for nano-CeO₂ (A) and nano-ZnO (B) treatments. Control, low, medium and high treatments are underlined blue, yellow, orange and red, respectively, and replicate numbers ($n = 4$, except $n = 3$ for low nano-ZnO) appear below the lines. ND denotes the negative control (no DNA). The DNA ladder appears in the left lane. One plant from the High nano-ZnO treatment (3rd lane from the left, for this treatment category; white arrow) indicated a genetic change as per an insertion event of 26 bp.

Figure 3. Schematic of interactions for soybean plants grown in soil amended with nano-ZnO. Blue lines indicate significant ($P \leq 0.05$) pairwise (Pearson) correlations, whether the relationship was positive (solid line) or negative (dashed lines), and whether the controls were or were not included in the correlation analyses. Statistical values (regression coefficient and P values) are reported in the manuscript, or are tabulated (Tables S6, S8 and S12). Pearson correlation analyses were performed for arithmetic (not log-transformed) data that were paired within individual plants ($n = 15$, 4 replicates for 3 treatments including controls, and 3 replicates for the Low nano-ZnO treatment). Coloring indicates: pink = Zn reservoirs (including nano-ZnO in the soil, or total Zn in plant parts; Table S5); medium brown = nodule growth metric (count); green (dark for leaf, light for stem, olive for pod, bright for total biomass) = plant metrics (Table S9), bright yellow = stress and damage indicators, and light yellow = total chlorophyll. Plant metrics, and stress or damage indicators, that are not shown were not statistically significantly related to other variables. Chlorophyll a and b , and the ratio of a/b are not shown as these variables were not independent of total chlorophyll.

Figure 4. Schematic of interactions for soybean plants grown in soil amended with nano-CeO₂. Blue lines indicate significant ($P \leq 0.05$) pairwise (Pearson) correlations, whether the relationship was positive (solid line) or negative (dashed lines), and whether the controls were or were not included in the correlation analyses. Statistical values (regression coefficient and P values) are reported in the manuscript, or are tabulated (Tables S6, S7, S10 and S11). Pearson correlation analyses were performed for arithmetic (not log-transformed) data that were paired within individual plants ($n = 16$: 4 replicates for 4 treatments including controls). The relationship between N₂ fixation potential and nodule Ce concentration followed a power function (Fig. S3) and thus was linearized by log transforming the Y-axis data before Pearson correlation analysis. Box coloring indicates: pink = Ce reservoirs (including nano-CeO₂ in the soil, or total Ce in plant parts; Table S4); light brown = functional metric (N₂ fixation potential); green (dark for leaf, light for stem, olive for pod, bright for total biomass) = plant metrics (Table S9), bright yellow = stress and damage indicators, and light yellow = total chlorophyll. Plant metrics, stress or damage indicators, or Ce reservoir (pod) that are not shown were not statistically significantly related to other variables. Chlorophyll a and b , and the ratio of a/b are not shown as these variables were not independent of total chlorophyll.

TOC Art