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DEVELOPMENTAL PHYTOTOXICITY OF METAL OXIDE NANOPARTICLES TO *ARABIDOPSIS THALIANA*

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Abstract—Phytotoxicity is an important consideration to understand the potential environmental impacts of manufactured nanomaterials. Here, we report on the effects of four metal oxide nanoparticles, aluminum oxide (nAl_2O_3), silicon dioxide ($nSiO_2$), magnetite (nFe_3O_4), and zinc oxide (nZnO), on the development of *Arabidopsis thaliana* (Mouse-ear cress). Three toxicity indicators (seed germination, root elongation, and number of leaves) were quantified following exposure to each nanoparticle at three concentrations: 400, 2,000, and 4,000 mg/L. Among these particles, nZnO was most phytotoxic, followed by nFe_3O_4, $nSiO_2$, and nAl_2O_3, which was not toxic. Consequently, nZnO was further studied to discern the importance of particle size and zinc dissolution as toxicity determinants. Soluble zinc concentrations in nanoparticle suspensions were 33-fold lower than the minimum inhibitory concentration of dissolved zinc salt ($ZnCl_2$), indicating that zinc dissolution could not solely account for the observed toxicity. Inhibition of seed germination by ZnO depended on particle size, with nanoparticles exerting higher toxicity than larger (micron-sized) particles at equivalent concentrations. Overall, this study shows that direct exposure to nanoparticles significantly contributed to phytotoxicity and underscores the need for eco-responsible disposal of wastes and sludge containing metal oxide nanoparticles. Environ. Toxicol. Chem. 2010;29:669–675.

Keywords—Nanomaterials Nanotoxicology Phytotoxicity Metal oxide nanoparticles *Arabidopsis*

INTRODUCTION

Nanotechnology is a rapidly growing industry that is expected to reach a market size of approximately 2.6 trillion dollars by 2015 ([1]; http://cohesion.rice.edu/centersandinst/ICON/emplibrary/Nanomaterial%20Volumes%20and%20Applications%20%20Holman,%20Lux%20Research.pdf). Increasing numbers of commercial products, from cosmetics to medicine, incorporate manufactured nanomaterials (MNMs) that can be accidentally or incidentally released to the environment [2,3]. Concern over the potentially harmful effects of such nanoparticles has stimulated the advent of nanotoxicology as a unique and significant research discipline [4–9]. However, the majority of the published nanotoxicology articles have focused on mammalian cytotoxicity or impacts to animals and bacteria, and only a few studies have considered the toxicity of MNMs to plants [4,10]. Developmental phytotoxicity of MNMs is a critical knowledge gap because nanoparticles entering wastewater streams may predominantly be incorporated into sewage sludge and applied to agricultural fields [11].

The impact of MNMs on different plant species can vary greatly, and there are reports of both positive and negative effects. Among positive effects, expedited soybean germination and growth was promoted by a mixture of nano-sized silicon dioxide ($nSiO_2$) and nano-titanium dioxide ($nTiO_2$) at low concentrations, which increased nitrate reductase activity, enhanced the ability to absorb water and fertilizer, and stimulated the antioxidant system [12]. The addition of $nTiO_2$ at 2.5 to 40 g/kg of soil promoted the growth of spinach, likely by protecting the chloroplasts from aging during long-term illumination [13,14]. Similarly, $nSiO_2$ enhanced the growth of Changbai larch (*Larix olgensis*), and the enhancement increased with the $nSiO_2$ concentration up to 500 mg/L [15]. In contrast, root growth inhibition by 2,000 mg/L nano-aluminum oxide (nAl_2O_3) was reported for five plant species: corn, cucumber, soybean, cabbage, and carrot [16]. Another study investigated the effects of five types of nanoparticles—multiwalled carbon nanotubes (MWCNT), nAl, nAl_2O_3, nano-zinc (nZn), and nano-zinc oxide (nZnO)—suspended in deionized (DI) water on seed germination and root growth of six higher plant species: radish, rape, ryegrass, lettuce, corn, and cucumber [4]. That study reported significant inhibition of ryegrass germination by 2,000 mg/L nZn. Similarly, 2,000 mg/L nZnO or nAl_2O_3 inhibited corn germination, whereas no inhibition was observed for 2,000 mg/L of MWCNT. Interestingly, nAl caused both positive and negative effects on root elongation, depending on the plant species [4]. Overall, the current phytotoxicity profile of nanomaterials is highly empirical and preliminary, and the effects of nanoparticle elemental composition, size, and stability are poorly understood.

In the present study, we investigated the developmental phytotoxicity exerted by four different metal oxide nanoparticles—nAl_2O_3, iron oxide (magnetite, nFe_3O_4), $nSiO_2$, and nZnO—to address the effect of elemental composition. *Arabidopsis thaliana*, which is new to the nanotoxicology literature, was selected as test plant species for various reasons. Its quick

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germination and short lifespan facilitate life-cycle toxicity screening [17]. Its small seed size results in a relatively large surface area to volume ratio, which is conducive to higher sensitivity to toxicants [18]. *Arabidopsis thaliana* is also the first plant to have its genome sequenced [19], which facilitates future work on its molecular response to nanomaterials. To discern phytotoxicity caused by exposure to metal oxide nanoparticles versus larger particles or released (soluble) metals, both micron-sized ZnO particles and soluble Zn salts (added as $ZnCl_2$) were tested separately. In doing so, we addressed an outstanding etiological issue by differentiating toxicity due to dissolved metals from that due to metal oxide nanoparticles themselves.

MATERIALS AND METHODS

Nanoparticles and micron-scale zinc oxide

Nano-scale silicon dioxide ($nSiO_2$) and nFe_3O_4 particles were purchased from Sigma-Aldrich, nano-scale nAl_2O_3 from Inframat Advanced Materials, nZnO from BASF, and micron-scale ZnO from Sigma-Aldrich. The particles' properties are summarized in Table 1. Size distribution and zeta potentials were determined in the plant growth medium (pH 5.8) using both dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments) and small-angle X-ray scattering (SAXS). All particle size ranges were corroborated by transmission electron microscopy (TEM) using a JEOL 2010 microscope operating at 120 kV. Samples for TEM samples were prepared by placing drops of nanoparticle suspensions on 300-mesh copper grids (Ted Pella) and allowing them to dry overnight before imaging.

Total dissolved zinc concentrations were measured to assess the role of soluble metal in phytotoxicity. Nano-scale ZnO suspensions at 400 mg/L and 4,000 mg/L were autoclaved for 15 min at 120°C, then centrifuged at 150 *g* for 10 min. The supernatants were subsequently filtered through 0.2-μm glass filters, acidified by 0.5% trace metal grade nitric acid (HNO_3), and stored at 4°C until elemental analyses of Zn by inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 4300DV). All measurements were carried out in the axial mode using yttrium as an internal standard for calibration. The detection limits of the ICP for this element were 0.01 mg/L, and the relative standard deviation of three replicate analyses was less than 5%.

Table 1. Characteristics of metal oxide nanoparticles and larger zinc oxide particles used in this study (the particles were characterized in plant growth medium, pH 5.8)

Particle	Purity (%)	Particle size (nm)	Hydrodynamic diameter (nm)[a]
nAl_2O_3	99.8	~150[b]	2,025 ± 8
$nSiO_2$	99.6	42.8 ± 3.9[c]	1,060 ± 89
nFe_3O_4	98	<50[b]	2,853 ± 411
nZnO	99.5	44.4 ± 6.7[c]	927 ± 34
Larger ZnO particles	99.99	820 ± 8[b]	2,311 ± 304

[a] Measured by dynamic light scattering. The values are listed as means ± standard deviation where provided by manufacturer or measured.
[b] Size provided by the manufacturer.
[c] Measured by small-angle X-ray scattering.

Preparation of seeds

Wild-type *Arabidopsis thaliana*, Col-0 seeds were purchased from Arabidopsis Biological Resource Center, Ohio State University and stored in a dry opaque envelope at room temperature. The seeds were transferred into 2-ml collection tubes, soaked in 1 ml of autoclaved DI water for 30 min, and centrifuged (9,000 rpm) for 30 s to soften the seed coat. Seeds were sterilized by washing once with 1 ml of 70% ethanol for 1 min, centrifuging for 30 s, once with 1% sodium hypochlorite for 1 min, centrifuging for 30 s, then four times with 1 ml of autoclaved DI water, and centrifuging for 30 s. Prior to transferring to plates for toxicity experiments, the seeds were suspended in autoclaved 0.1% agar solution in collection tubes in a dark container for 5 d at 4°C. All procedures were conducted under a Steriguard® laminar hood to prevent microbial contamination.

Plant growth conditions

The nanoparticles were dispersed in one-half strength Murashige and Skoog (MS) agar rather than hydroponic solution used in several previous studies with *A. thaliana*. This medium was selected to avoid aggregation and precipitation commonly reported for the latter medium [10]. The agar medium contained 2.2 g (half-strength) MS Basal Medium with Gamborg's vitamins (Sigma-Aldrich), 7 g Bacto agar, and 20 g sucrose per liter. Three nanoparticle concentrations (400 mg/L, 2,000 mg/L, 4,000 mg/L) were selected to encompass the previously reported toxic doses of nAl_2O_3, 2,000 mg/L [16], and to cover one order of magnitude for other metal oxide nanoparticles of unknown inhibitory concentrations. The nanoparticles were added to the growth media and stirred for 10 min. Appropriate amounts of hydrochloric acid (HCl) or sodium hydroxide (NaOH) were added to achieve pH 5.8, optimum for *A. thaliana*'s development (Supplemental Data, Table S1) [20]. Six soluble zinc concentrations (50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L, and 500 mg/L) were prepared in half-strength MS medium (pH 5.8) by adding appropriate amounts of $ZnCl_2$ (EMD Chemicals) to identify the minimum inhibitory concentration of released soluble zinc from nZnO. Unlike previous studies that did not adjust pH and allowed exposure at varying pH values caused by the addition of metal oxides [4,16], pH was eliminated as a confounding factor for toxicity studies [21]. Growth media were sterilized by autoclaving for 15 min at 120°C prior to nanoparticle characterization (Table 1). Sterile media were then poured into 100 mm × 15 mm Petri dishes. The plants were incubated at 22 to 23°C with 100 $\mu E/m^2/s$ lighting to provide optimal growth conditions [20]. Although it is important to recognize the role of the available surface area of nanoparticles in toxicity evaluations, bioavailable surface area of the nanoparticles was expected to change during the course of experiments as a result of agglomeration, precipitation, and interactions with organic matter [22]. Therefore, the present study focused on dose response based on nanoparticle concentration rather than surface area.

The particles were observed in dried agar samples by scanning electron microscopy (SEM). Agar nanoparticle suspensions were autoclaved for 15 min at 120°C then allowed to solidify at room temperature. Solid agar media were dried onto filter paper with the Welch GEM vacuum system Model 8890.

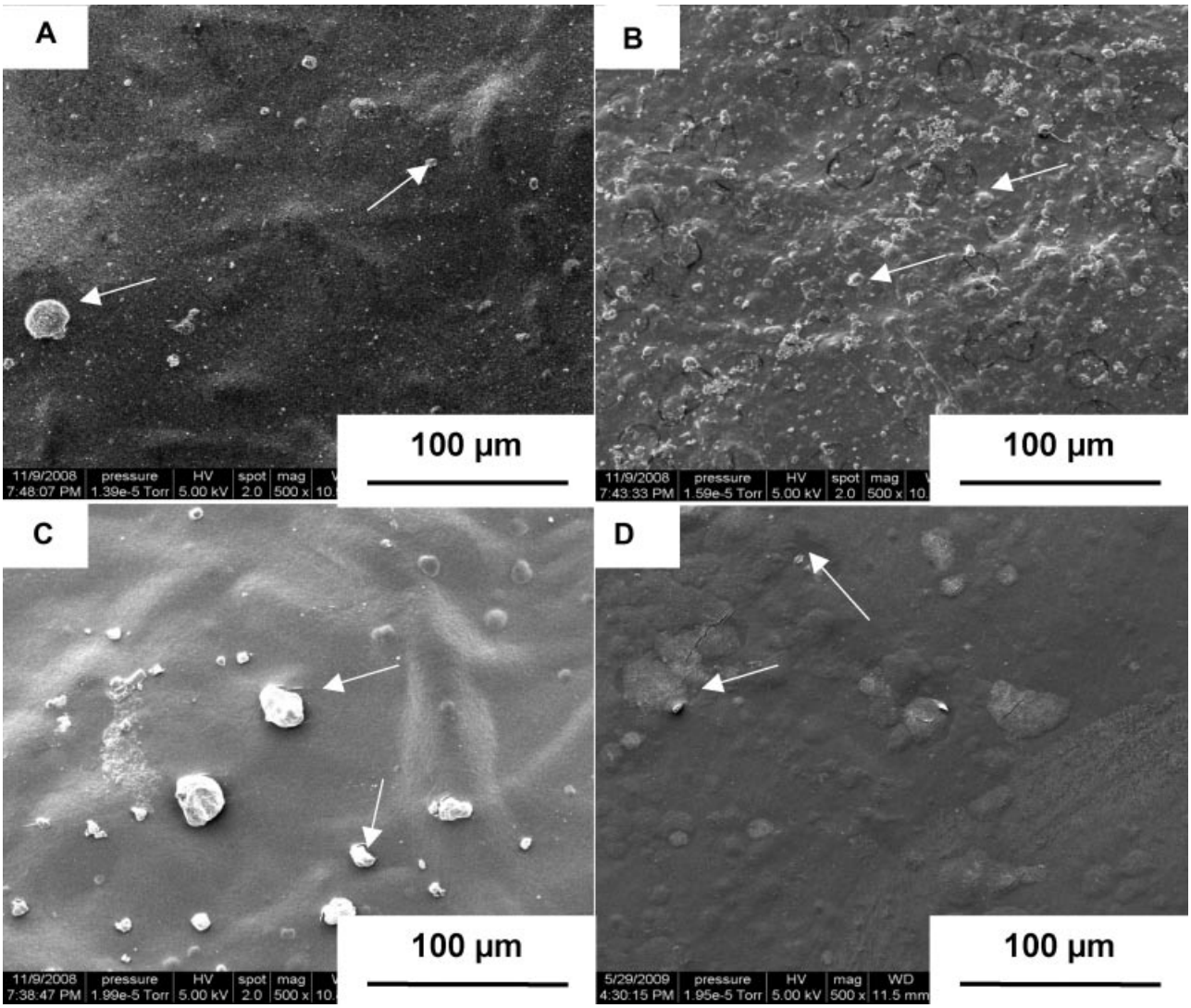

Fig. 1. Scanning electron micrographs showing uniform dispersion of metal oxide nanoparticles suspended in agar media. (**A**) nAl_2O_3, (**B**) $nSiO_2$, (**C**) nFe_3O_4, (**D**) nZnO. Arrows show nanoparticle aggregation in the media.

The samples were coated with 20 nm of Au (CrC-150 Sputtering System; TORR International), and viewed with a SEM (FEI Quanta 400 ESEM FEG, 5 kV). All nanoparticles were well dispersed in the agar suspension. Although aggregation occurred in all samples, the dispersion of all nanoparticles throughout the agar medium was relatively uniform (Fig. 1).

Measurements of toxic effects

Germination percentage, number of leaves, and primary root elongation, were measured 18 d after planting, toward the full mature vegetative growth stage of the *A. thaliana* life cycle. Germination percentages were determined by comparing the numbers of seeds that developed a primary root of at least 1 mm to the total number of seeds planted in each dish. Primary root elongation was determined by extracting the plants from MS medium, and measuring the straightened primary root from the bottom of the stem (crown) to the end of the primary root. Relative root growth inhibition was calculated as the difference between average primary root length of the unexposed control plants and test plants' average root length divided by the primary root length of the control. The number of leaves were counted and recorded.

Statistical analysis

Each treatment was conducted in at least triplicate, and the results were presented as mean values with respective standard deviations. Phytotoxicity endpoints for all treatments were compared to those of unexposed controls using the Student's *t* test paired two sample for means. This test was selected because all seeds came from the same population. Statistical significance of differences between treatments was determined at the 95% confidence level.

RESULTS AND DISCUSSION

Effect of nanoparticles on seed germination

Germination percentage is widely used to test phytotoxicity of chemicals ([23]; http://www.epa.gov/opptsfrs/publications/

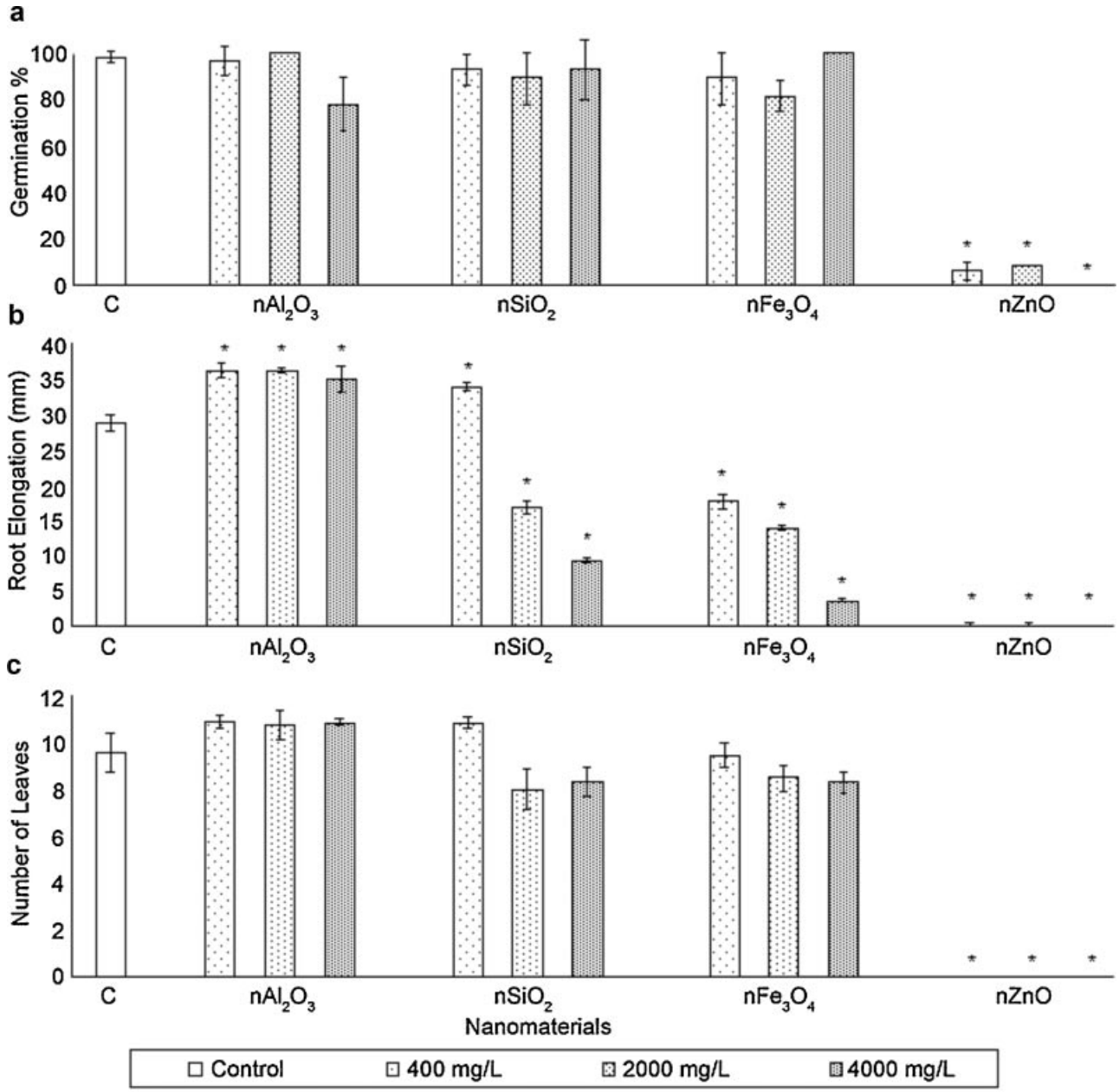

Fig. 2. *Arabidopsis thaliana* seeds exposed to various concentrations of metal oxide nanoparticles. (**a**) germination percentage, (**b**) root elongation, (**c**) number of leaves. The values are given as mean ± one standard deviation of triplicate samples with nine seeds each (with the exception of nZnO; those values are determined from quadruplicate samples with 13 seeds each). *Denotes significant differences ($p < 0.05$) from the unexposed control (C).

OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-4200.pdf). All tested concentrations of nZnO significantly inhibited seed germination, whereas the other nanoparticles had no significant effect (Fig. 2a). Because seed coats have pores that exhibit selective permeability, the interaction between solid or particulate constituents and the plant may be limited until the radicles emerge and come into direct contact with the growth medium [24]. However, intracellular spaces (<10 μm) in seed coat parenchyma may be filled with aqueous media facilitating the transport of soluble nutrients as well as small particles to the embryo [25]. This may explain the significant inhibition exerted by the smaller, monodisperse, nZnO particles (Table 1). Interestingly, despite the measured average hydrodynamic diameter of $nSiO_2$ (1,060 nm) being close to that of nZnO (927 nm), the germination percentage was not impacted by $nSiO_2$, indicating that the elemental composition as well as particle size may play a significant role in developmental phytotoxicity.

Effect of nanoparticles on plant development

A significant positive influence on root elongation was observed for all tested concentrations of nAl_2O_3 and for 400 mg/L $nSiO_2$. On the other hand, nFe_3O_4 and nZnO exerted inhibitory effects at all concentrations, as did $nSiO_2$ at 2,000 mg/L and 4,000 mg/L (Fig. 2b). Thus, $nSiO_2$ exhibited

a dual behavior that had not been reported in the literature: promoting root elongation at lower concentrations in accordance with previous studies [12,15], and exerting toxicity at high concentrations as predicated by Shelford's law of tolerance [26]. The positive influence of nAl_2O_3 on root elongation was unexpected because previous phytotoxicology studies show that these particles have either neutral or inhibitory effects on plant growth [4,16]. Lin and Xing [27] showed that *A. thaliana* and several other plant species display higher tolerance (showing no inhibition of root growth) toward nAl_2O_3. The mechanism responsible for enhanced root elongation is unclear; although it might reflect that the agar medium is nonporous and limits oxygen diffusion and root elongation, subjecting the root to similar stresses as those observed in water-clogged soils. Root stress might be relieved by inert nAl_2O_3, which could serve similar functions as nano-sized perlite, which enhances gas transfer, prevents water loss, and hinders soil compaction [28].

The number of leaves has not been previously used as a phytotoxicology endpoint for nanomaterials. However, it is an accurate and commonly used nondestructive method for determining the physiological state of *A. thaliana*, and a decrease in the number of leaves accurately corresponds to inhibition of *A. thaliana* growth [29]. Significantly fewer leaves were present on plants exposed to all tested concentrations of nZnO (Fig. 2c), corroborating the potential phytotoxicity of nZnO.

Role of dissolved species versus particles in phytotoxicity

Previous studies have suggested that the toxicity of metal and metal oxide nanoparticles may be caused by their dissolution and subsequent release of toxic metal ions [4,30,31]. Heavy metals are widely acknowledged to inhibit seed germination, growth, and development of plants, and disturb their biochemical and physiological processes [32]. To determine the role of the dissolved metal species in causing nanoparticle toxicity, we measured the total soluble Zn concentrations released from the nZnO particles (displaying highest toxicity among the tested materials) as well as larger (micron-scale) ZnO particles (Table 2).

Exposure to 400 mg/L nZnO, which released 14.6 mg/L of soluble Zn, prevented 94% of the seeds from germinating (Fig. 2a) and completely halted root elongation (Fig. 2b). In contrast, exposure to equivalent concentrations of soluble Zn (added as $ZnCl_2$ salt, without nanoparticles) resulted in significantly lower toxicity (Fig. 3). For instance, soluble Zn concentrations up to 250 mg/L did not hinder seed germination (Fig. 3a), although it took 50 mg/L total soluble Zn to hinder root elongation by 75% relative to unexposed controls (Fig. 3b). Only the highest concentration (500 mg/L) of soluble Zn, which is one order of magnitude higher than the amount released by toxic levels of nZnO, inhibited germination (100% of seeds failed to germinate) (Fig. 3a). These data suggest that the phytotoxicity of nano-scale metal oxides cannot be explained solely by the dissolved metal species, and that the particles themselves also contribute to phytotoxicity.

Table 2. Total dissolved zinc released from nZnO and larger ZnO particles suspended in Murashige and Skoog Basal medium at pH 5.8

	Total dissolved zinc (mg/L)	
ZnO added (mg/L)	nZnO	Larger ZnO particles
0	0.9 ± 0.01 mg/L[a]	0.9 ± 0.01 mg/L[a]
400	14.6 ± 0.14 mg/L	12.89 ± 0.11 mg/L
4,000	96.9 ± 1.22 mg/L	32.74 ± 0.24 mg/L

[a] The plant growth medium contained zinc and other trace elements (Supplemental Data, Table S2).

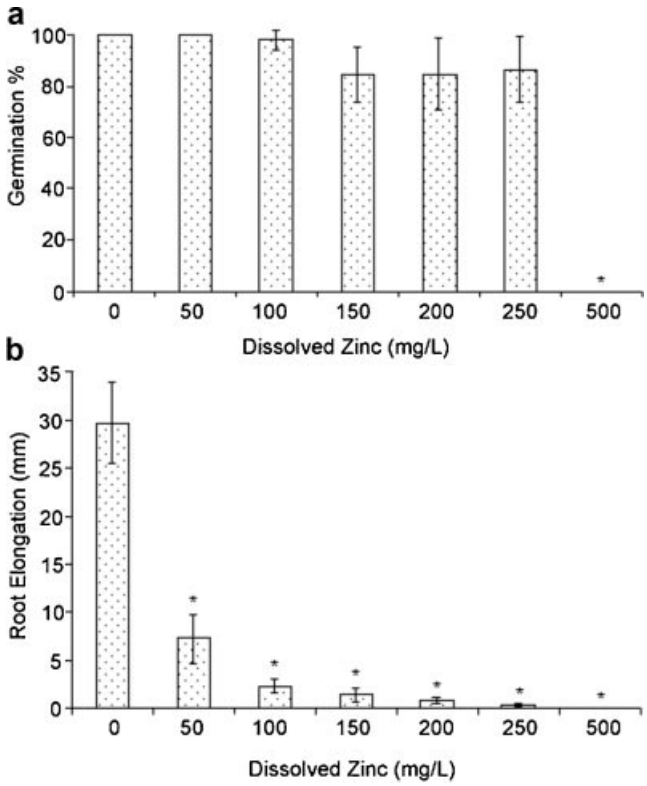

Fig. 3. (**a**) Germination percentage and (**b**) root elongation of *Arabidopsis thaliana* seeds exposed to various concentrations of soluble zinc (added as $ZnCl_2$) for 18 d. The inhibitory effect of soluble zinc was more pronounced for root elongation. The values are given as mean ± one standard deviation of quadruplicate samples of 13 seeds each. *Denotes significant differences ($p < 0.05$) from the unexposed control.

To confirm that nanoparticles played an important role in the observed phytotoxicity, we also exposed *A. thaliana* to micron-sized ZnO as controls. Whereas the average nominal particle size of nZnO was 44.4 ± 6.7 nm, the particles formed 927 ± 34 nm-sized aggregates when suspended in half-strength MS agar medium. Despite significant aggregation, these nanoparticles were much more toxic that the larger ZnO particles ($2{,}311 \pm 304$ nm), and caused complete germination failure at 4,000 mg/L compared to 4,000 mg/L for micron-sized ZnO (Fig. 4). Whether the plants' immunological response (triggered by exposure to the nanoparticles) contributed to toxicity was not investigated. Immune responses may depend on the composition, size, shape, zeta potential, density, thickness, and stability of the nanomaterials [33].

It is unknown whether intracellular uptake is a requirement for causing phytotoxicity. A recent study showed significant uptake of nano-sized copper (nCu) by *Phaseolus radiatus* (Mung bean) and *Triticum aestivum* (wheat), with reported bioaccumulation factors of 8 and 32 L/kg, respectively [10]. Transmission electron microscopy analysis showed that nCu was absorbed and agglomerated into the cytoplasm of the root cells and the extent of absorption depended on the concentration

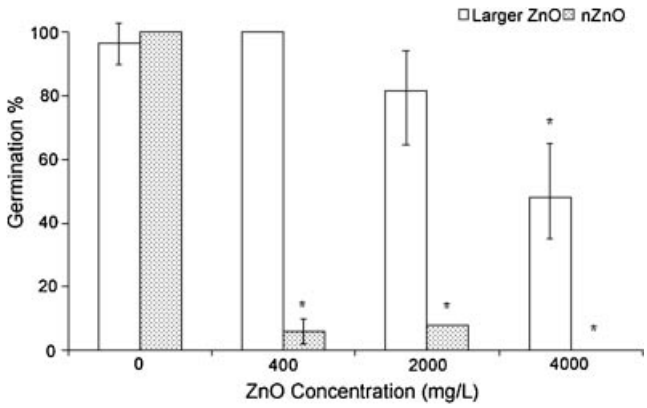

Fig. 4. Inhibition of *Arabidopsis thaliana* seed germination by nZnO and larger ZnO particles. The values are given as mean ± one standard deviation of quadruplicate samples of 13 seeds each for nZnO and triplicate samples of nine seeds for the larger ZnO particles. *Denotes significant differences ($p < 0.05$) from the unexposed control.

of the nCu deposits on the roots' surface. Another study found individual nZnO particles in endodermal and vascular cells of ryegrass exposed to 1,000 mg/L of nZnO [34]; the translocation factor (defined as Zn content ratio of shoot to root) was 0.01 to 0.02. Significant uptake, translocation, and accumulation of nFe_3O_4 in the roots and leaves of *Cucurbita maxima* (pumpkin) has also been reported without any effect on growth and development of the test species [35]. Therefore, some uptake of nanoparticles by plants is very possible. However, little is known about the maximum nanoparticle size amenable for plant uptake, and how uptake kinetics and toxicity are affected by plant type and rhizospheric chemistry. Recent research highlights the importance of transition metals that adsorb to nanoparticles and promote oxidative stress [36], whereas natural organic matter in soil or pore water can sorb, coat, or stabilize nanoparticle suspensions and affect their mobility, bioavailability, reactivity, and toxicity [37–39]. This illustrates the daunting challenge of quantifying and predicting the nanoparticle properties and bioavailable concentration to which plant roots may be exposed in nature.

Overall, the present study demonstrates possible adverse effects of metal oxide nanomaterials on plants, which underscores the need for ecologically responsible disposal of wastes and sludge containing metal oxide nanoparticles and calls for further research on the potential impacts of manufactured nanoparticles on agricultural and environmental systems.

SUPPLEMENTAL DATA

Table S1. Final pH of agar suspensions after the addition of nanoparticles.

Table S2. Murashige and Skoog Medium with Gamborg's Vitamins ingredients.

Table S3. BET surface area of four of five particles used in this study.

Figure S1. Transmission electron micrographs of metal oxide nanoparticles. (415 KB PDF)

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