Functional Analysis of TiO₂ Nanoparticle Toxicity in Three Plant Species

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Abstract Titanium dioxide nanoparticles (nano-TiO₂) are manufactured and used worldwide in large quantities. However, phytotoxicity research on nano-TiO₂ has yielded confusing results, ranging from strong toxicity to positive effects. Therefore, in this research, the effects of nano-TiO₂ on the germination and root elongation of seed and seedlings were studied. Additionally, the uptake and physiological responses of mature plants were investigated. Physical chemistry data were analyzed to assess the availability of nano-TiO₂. Finally, a hydroponic system designed to overcome nano-TiO₂ precipitation was used to reproduce the environmental conditions of actual fields. Nano-TiO₂ did not have any effect on seed germination or on most of the plant species tested. Nano-TiO₂ had positive effects on root elongation in some species. No physiological differences in enzyme activities or chlorophyll content were detected, even though the plants absorbed nano-TiO₂. Physical chemistry data showed that nano-TiO₂ agglomerated rapidly and formed particles with much bigger hydrodynamic diameters, even in distilled water and especially in a hydroponic system. Furthermore, agglomerated nano-TiO₂ formed precipitates; this would be more severe in an actual field. Consequently, nano-TiO₂ would not be also readily available to plants and would not cause any significant effects on plants. Our results and other reports suggest that titanium itself is not phytotoxic, even though plants absorb titanium. In conclusion, nano-TiO₂ is not toxic to the three plant species, in vitro or in situ.

Keywords Nanoparticle · Titanium dioxide · Phytotoxicity · Physical chemistry · Plant species

Introduction

Manufactured nanoparticles (NPs) have attracted tremendous attention because of their positive effects in consumer products, pharmaceutics, cosmetics, transportation, energy, and agriculture [1]. However, the unique properties of NPs could lead to unpredicted biological effects, such as toxicity. Some former studies have shown that NPs have toxic effects on plants [1–3], animals [4, 5], and microorganisms [6, 7]. Some NPs, such as silver nanoparticles, are even manufactured for their toxicity (i.e., antibacterial activity). However, despite recent progress in understanding the health and environmental consequences of NPs, the toxicity of some NPs is still controversial; conflicting results are often reported. Therefore, challenges remain for future research [8]. Additionally, although there are many studies on the toxicity of NPs in animals and bacteria, studies assessing on the toxicity of nanomaterials in higher plants are limited [1, 9].

Titanium dioxide nanoparticles (nano-TiO₂) are manufactured worldwide in large quantities for cosmetics, especially sunblock, in which nano-TiO₂ helps protect the skin from UV light [10]. Nano-TiO₂ is also widely used in antibacterial products and for decomposing organic matter in wastewater [1]. Several articles reported on the toxic effects of nano-TiO₂ in animals, such as DNA damage [10], neurotoxicity [11], and cytotoxicity [12]. However, some studies, investigating cytotoxicity, genotoxicity, acute toxicity, and sensitization, have reported opposite results, finding no toxicity [13] and no evidence of significant penetration of nano-TiO₂ [14]. Therefore, even for animals, the toxicity of nano-TiO₂ remains

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controversial. Hence, further studies must be done to analyze the biological effects of nano-TiO$_2$ [14]. Only a small number of articles researching on the effects of nano-TiO$_2$ on plants were found in a recent search of the literature [1, 15–17]. Furthermore, two important studies testing on the effects of nano-TiO$_2$ on seed germination and root growth, the most important basic toxicity research tools for plants, showed opposite results: one study showed positive effects of nano-TiO$_2$ [17], and the other study showed negative effects [1]. Therefore, the effects of nano-TiO$_2$ on plants are not clear. Further research is needed. Furthermore, in addition to basic toxicity testing tools, the material properties of nano-TiO$_2$, its uptake by plants, and the physiological responses of plants should be studied.

In this study, the uptake of nano-TiO$_2$ and its effects on germination, root elongation, and physiological responses, such as antioxidant enzyme activities and chlorophyll content, were investigated. The physical properties (agglomeration/aggregation and particle diameter) of nano-TiO$_2$ were considered when designing research methods to test the toxicity of nano-TiO$_2$ on plants. Finally, a new approach to overcome methodological limits caused by the precipitation of testing materials and to reproduce the environmental conditions in actual fields was tested.

Material and Methods

Nanoparticles

Manufactured nano-TiO$_2$ from Evonik Industries, Germany (product name: AEROXIDE TiO2 P 25) was used for the studies. The properties of the nano-TiO$_2$ were as follows: aerosol; purity≥99.5 %; anatase/rutile=80:20; and particle size=27 nm. For additional studies, nano-TiO$_2$ from Daejoo Electronic Materials, Korea (powder; purity≥99.0 %; 55 m^2/g specific surface area; and particle size=10–20 nm) was also tested.

Plant Species and Preparation

Seeds of *Brassica campestris* ssp. *napus* var. *nippo-oleifera* Makina (oilseed rape), *Lactuca sativa* L. (lettuce), and *Phaseolus vulgaris* var. *humilis* (kidney bean) were selected to test the toxicity of nano-TiO$_2$. The species were selected because they are common, easy to obtain, and, above all, included among the species recommended for the testing of chemicals in the Organisation for Economic Co-operation and Development guidelines [18] and the Ecological Effects Test Guidelines provided by the United States Environmental Protection Agency [19]. Seeds were purchased from a local Syngenta agent (Syngenta AG, Switzerland). The seeds were

vernalized for 2 weeks and were sterilized for 10 min in 10 % sodium hypochlorite solution [19] before application.

Seed Germination and Root Elongation

For germination and Ti uptake studies, the seeds were soaked in nano-TiO$_2$ solutions for 48 h [17] in the dark at room temperature with gentle shaking on an orbital shaker at 150 rpm to enhance mixing. Subsequently, the seeds were washed with distilled water (DW). Most of the seed were transferred to 10-mm Petri dishes containing a piece of filter paper (90 mm) and 5 mL of DW [9], and some seeds were used for uptake analysis. The seeds were tested for germination in a growth chamber under a range of conditions established by the OECD guidelines [18]: temperature, 24 °C; humidity, 70±25 %; photoperiod, 18-h light; and light intensity, 300 μE m^{-2} s^{-1} with protection from drying. Each Petri dish (n=10) contained five seeds, and germination rates were investigated every 3 days (five times altogether). NP-treated seeds were dried for uptake analysis, and the surface (seed coat and endosperm) of additional *P. vulgaris* seeds was removed by scalpel to a depth of 1 mm to investigate the internal absorption of Ti. The weight of *L. sativa* was too low to provide enough mass for inductively coupled plasma (ICP) analysis.

The seeds were soaked in nano-TiO$_2$ solutions with concentrations of 0, 100, 500, 1,000, 2,500, and 5,000 mg/L. Because a previous study on nano-TiO$_2$ solutions used concentrations up to 4,000 mg/kg [1] and found significant effects with 2,000 mg/L [1], 5,000 mg/mL was considered sufficient to test toxicity. Another previous study on nano-TiO$_2$ solutions, however, used concentrations up to 6,000 mg/kg [17] and did not report on any negative effects. Concentrations greater than 6,000 mg/kg are considered unrealistic in natural systems because other studies on nano-TiO$_2$ used concentrations of 500 mg/kg [10] or lower [11, 13, 14]. We placed five seeds into each Petri dish, with ten replicates, and measured germination rates five times, once every 3 days.

For root elongation studies, seeds were germinated in Petri dishes. After 2 days (3 days for *P. vulgaris*), germinated seeds were moved to new Petri dishes. Each Petri dish contained five seedlings (ten Petri dishes, a total of 50 replicates of seedlings) and 5 mL of the test medium. The root lengths of the seedlings were measured every 3 days (six times altogether). However, *P. vulgaris* was measured only four times because it began to extend outside the Petri dishes and to dry. Other conditions, including concentrations and the chamber conditions, were the same as for the germination study described above. After the first root elongation study, we conducted the root elongation study again with *B. campestris*, using additional concentrations of 50 and 10,000 mg/L (50 replicates).

Pot Experiment

Plants were grown in a 50-hole pot tray with each hole filled with 10 g of commercial soil (Sunshine Mix #5, Sun Gro, Canada). After a 5-week growing period from the seedling stage, 10 mL of nano-TiO$_2$ solution at 1,000, 2,500, and 5,000 mg/L was added to each pot. Solutions were administered with 1-mL pipettes several times to avoid leaching. Therefore, the growing media contained exactly 1,000, 2,500, and 5,000 mg/kg nano-TiO$_2$. The antioxidant enzyme activities and chlorophyll content of the plants were measured after a week. Two weeks after treatment, the plants were harvested to assess TiO$_2$ accumulation.

Circular Hydroponic System

Global commercial soil is widely used for testing the toxicity of NPs. However, NPs such as nano-TiO$_2$, which are principally used in cosmetics, are mainly released to the environment via the sewage system [20, 21], and plants would be expected to encounter NPs in water systems. Therefore, even if complicated, another hydro-method to apply NPs to plants would be useful. A short-term hydroponic system was used to predict the actual reactions of toxic materials in the field [22]. A hydroponic system enhances the mixing of the solutions and can reduce other variables, except diffusion through a boundary layer of roots [23]. Thus, hydroponics could be useful for testing the toxicity of NPs. However, during experiments, nano-TiO$_2$ always formed precipitates, which altered the concentration of the solution. Therefore, a new approach to prevent precipitation was required to enable the use of hydroponics for NP research.

A water pump was installed in the bottom of a 10-L (25 cm×20 cm×20 cm) plastic container filled with 9 L of Hoagland solution [24] containing different concentrations of nano-TiO$_2$ to create a circular flow inside the container and prevent precipitation. The solution was then pumped (by another water pump installed in the sidewall of the plastic container) to a pot container (long rectangular parallelepiped, 50 cm×8 cm×8 cm) with six pot holes (each of 6 cm in diameter). When the pot container was filled with nano-TiO$_2$ solutions, the solutions were discharged to a 10-L plastic container by gravity, making the system circular. The water level of the pot container remained at approximately 4 cm when the system was activated.

Among the species used in this study, *L. sativa* was selected as the testing plant because it is frequently used for hydroponics cultivation [25]. Seedlings of *L. sativa* were carefully placed in pots filled with smooth pearlite. Plants were grown in the hydroponics for 5 weeks before treatment. Then, the Hoagland solution was replaced by the Hoagland solution [24] with different concentrations (100, 1,000, and 5,000 mg/L) of nano-TiO$_2$. One week after nano-TiO$_2$ application, one leaf of each plant was harvested for analysis of antioxidant enzyme activity and chlorophyll content. After 2 weeks, plants were harvested to assess TiO$_2$ accumulation.

Chlorophyll, Antioxidant Enzyme Activity, and Ti Analysis

The antioxidant enzyme activities (total antioxidant capacity and superoxide dismutase activity) of the plants were measured by the protocols of Song and Lee [26]. The chlorophyll content of the plants was measured by a DMSO extraction method [27]. The Ti content of nano-TiO$_2$-treated plant seeds and leaves was analyzed using an ICP emission spectrometer (ICPS-7510, Shimadzu, Japan) [28]. Plant samples were washed thoroughly with distilled water to remove nano-TiO$_2$ from surface. Then, samples were oven dried at 70 °C for 5 days. Dried plant samples were ground and digested by open-vessel digestion using nitric acid for ICP analysis [29]. One gram of samples (0.1 g for seedling samples) was moved into Pyrex tubes, and 10 mL of nitric acid (analytical grade) were added and left for a cold soak for approximately 2 h. Then the tubes are placed into a heating block. Temperature was slowly raised up to 600 °C and heated for 4 h with minimum supply of distilled water to prevent total vaporization during heating. Then the tubes were slowly cooled to room temperature. Cooled digests were diluted to 20 mL with distilled water and filtered with a filter paper (Whatman no. 44) and finally diluted to 40 mL for ICP–emission spectrometry (ES) analysis. The wavelength used for Ti analysis in ICP-ES was 334.941 nm. Obtained results were recalculated considering diluted volume (40 mL) and original plant weight.

Physical Chemistry Analysis

The physical chemistry (Pchem) properties (hydrodynamic diameter and zeta potential) of the nano-TiO$_2$ in solution were measured by electrophoretic light scattering (ELS-Z2, Otsuka Electronics, Japan) under dark conditions at 24 °C.

Additional Experiments Using Different Nano-TiO$_2$

After first testing nano-TiO$_2$ from Evonik Industries, the toxicity of nano-TiO$_2$ from a different source was also tested. In the second round of experiments, we used a powdered form of nano-TiO$_2$ from Daejoo Electronic Materials with a smaller particle size (10–20 nm particle size). Seed germination, root elongation, antioxidant enzyme activity, and Pchem properties using *B. campestris* were assessed.

Statistical Analyses

A one-way ANOVA was performed to identify significant differences between treatments. Upon detection of a significant difference, Tukey's studentized range (HSD) test was

applied post hoc and assessed using an SAS 9.3 program (SAS Institute Inc., USA). Differences were considered significant when $p<0.05$.

Results

Germination, Root Elongation, and Ti Uptake by Seeds

The various treatments did not affect the germination rates of the three species tested (*B. campestris*, *L. sativa*, and *P. vulgaris*) (Fig. 1). No significant differences were found in any measurement. No toxic effects of nano-TiO$_2$ on seed germination were observed.

After treatment with nano-TiO$_2$, significant differences in root elongation were observed only in *L. sativa* (Fig. 2). However, compared to control, the 5,000-mg/L treatment significantly decreased root elongation, whereas the other treatments (100, 500, 1,000, and 2,500 mg/L) significantly increased root growth (Fig. 2 b). Nano-TiO$_2$ did not have any effects on *B. campestris* and *P. vulgaris* (Fig. 2a, c).

The seeds of *B. campestris* had Ti contents ranging from 0.54 to 1.64 mg/kg (Table 1). The Ti content was three times higher in seeds treated with 1,000 mg/L nano-TiO$_2$ than in seeds treated with a higher concentration of 5,000 mg/L (1.64 vs. 0.54 mg/kg). Seeds of *P. vulgaris* had similar Ti contents without and with their surfaces removed (Table 1), with values of approximately 0.75 mg/kg Ti for the 5,000-mg/kg treatment.

Physiological Responses and Ti Uptake by Plants

The chlorophyll content of plants was affected by neither nano-TiO$_2$ treatments (Table 2) nor the hydroponic system. The average total chlorophyll content was 10.7 mg/L for *B. campestris* and 6.7 mg/L for *L. sativa* (including hydroponics). The plants did not show any significant differences in total antioxidant capacity (TAC) and superoxide dismutase activity (SOD) in response to the different nano-TiO$_2$ treatments, including those administered with hydroponics (Fig. 3). Plants did not show any physiological differences in response to nano-TiO$_2$ treatments.

Table 3 shows the Ti uptake of plants administered with nano-TiO$_2$ by pot (*B. campestris* and *L. sativa*) or hydroponics (*L. sativa*). In the hydroponic system, the Ti content was significantly higher in *L. sativa* treated with 1,000 mg/L nano-TiO$_2$. Ti absorption by plants ranged from approximately 4 to 10 mg/kg in the pot experiments and up to 12.8 mg/kg in the hydroponic system.

Pchem Analysis

The hydrodynamic diameter of nano-TiO$_2$ in DW ranged from more than 200 nm to over 650 nm (Fig. 4a), much bigger than
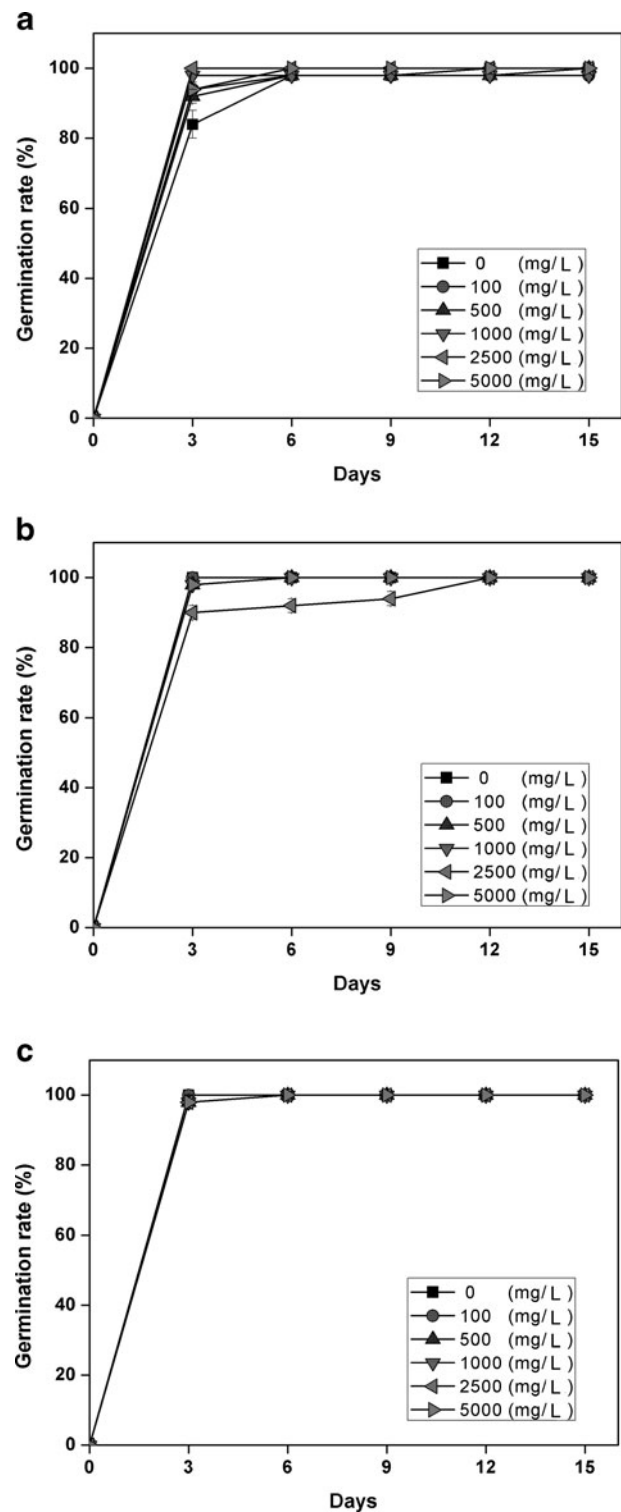

Fig. 1 Germination rates of **a** *B. campestris*, **b** *L. sativa*, and **c** *P. vulgaris* seeds after nano-TiO$_2$ treatment. *Symbols* and *bars* represent the mean ± SE of ten replicates. No significant differences were observed

the original size (27 nm). Nano-TiO$_2$ at a concentration of 5,000 mg/L had the smallest diameter among the treatment concentrations and the highest zeta potential (Fig. 4b),
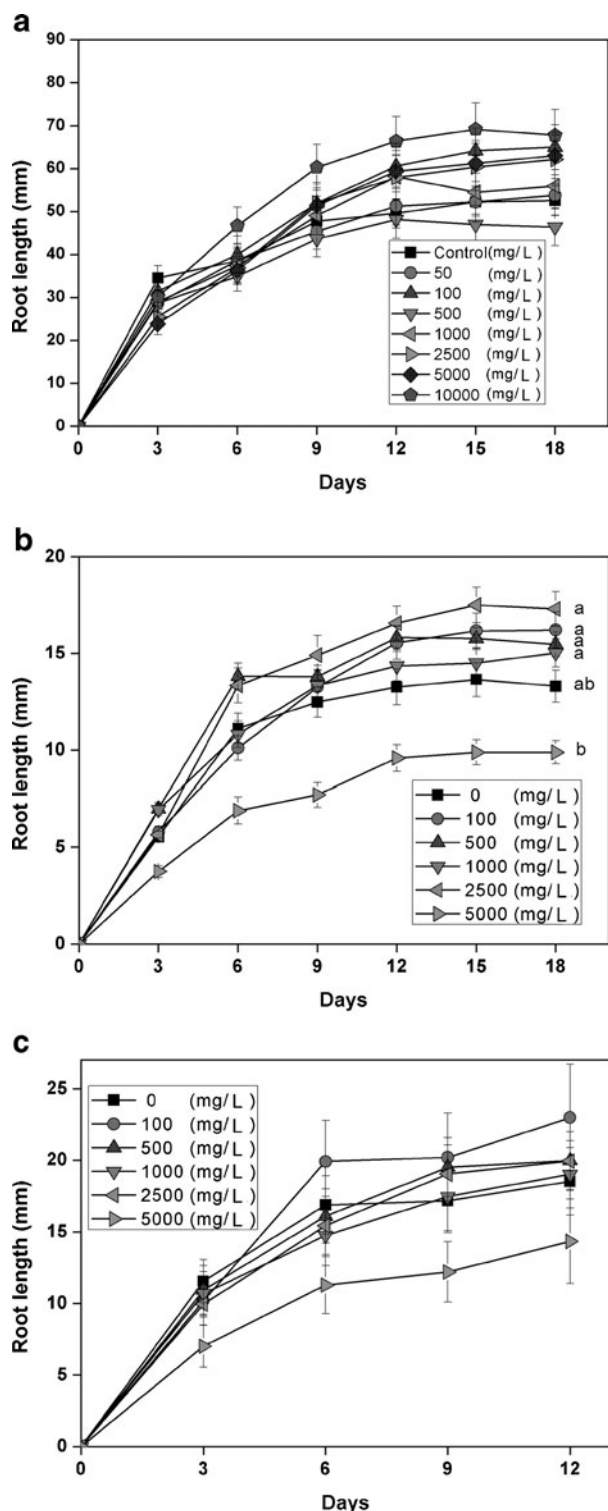
Fig. 2 Effect of nano-TiO₂ on the root elongation of **a** *B. campestris*, **b** *L. sativa*, and **c** *P. vulgaris*. *Symbols* and *bars* represent the mean ± SE of 50 replicates. *Symbols with the same letter* are not significantly different at the 0.05 level

Table 1 Titanium uptake of *B. campestris* and *P. vulgaris* seeds 48 h after exposure (in milligram per kilogram)

TiO₂ treatment (mg/L)	B. campestris	P. vulgaris	P. vulgaris (SR)
100	0.76±0.20	1.08±0.21	1.00±0.12
1,000	1.64±0.56	0.92±0.23	0.59±0.15
5,000	0.54±0.05	0.75±0.15	0.74±0.05

Values represent mean ± SE of three replicates. No significant differences were observed

SR surface removed

over 9,000 nm (Fig. 4c), more than 300 times the original diameter. The zeta potential of 100 mg/L nano-TiO₂ in the Hoagland solution was over 10 mV, whereas the zeta potential of 1,000 mg/L nano-TiO₂ in the Hoagland solution dropped below zero (Fig. 4d).

Experiments Using Nano-TiO₂ from a Different Source (Daejoo)

The germination rates of *B. campestris* did not differ significantly between treatments (Fig. 5a). Root elongation differed significantly, with the longest root length in the 500-mg/L treatment (Fig. 5b). The TAC values (in millimolar per milligram protein) of *B. campestris* were 0.16±0.02 for control, 0.19±0.02 for the 1,000-mg/L treatment, and 0.20±0.01 for the 5,000-mg/L treatment (mean ± SE, $n=5$). The differences were not significant. The SOD values (in units per milligram protein) did not differ significantly, with 0.94±0.00 for control, 0.97±0.01 for the 1,000-mg/L treatment, and 0.95±0.01 for the 5,000-mg/L treatment (mean ± SE, $n=5$).

Discussion

The germination rates of three species (*B. campestris*, *L. sativa*, and *P. vulgaris*) did not differ between treatments (Fig. 1). Every treatment group showed almost 100 % germination. In this study, seeds were treated longer (48 h) than in a previous study [1], which showed negative effects of nano-TiO₂ on germination with a 24-h treatment. Table 1 shows that nano-TiO₂ was absorbed into the seed, not only attached to the seed coat, because nano-TiO₂ was found even in seeds with their surface removed. Before analyzing the metal uptake of seeds, the authors considered the possibility that nano-TiO₂ was not absorbed into the plant system (inside seed tissues) because treatment with nano-TiO₂ did not affect the germination rate. However, because even seeds with their surface removed contained nano-TiO₂, this possibility was ruled out. Though many studies reported on heavy metal accumulation in seeds after heavy metal treatment of the plant, it was difficult to locate studies in international journals reporting

indicating that particles repelled each other more than in the other treatments. Nano-TiO₂ in the Hoagland solution had a much larger hydrodynamic diameter, ranging from 2,000 to

Table 2 Chlorophyll content of plants 7 days after exposure to TiO_2

TiO_2 treatment (mg/L)	Brassica campestris			Lactuca sativa			Lactuca sativa (HS)		
	Chl-a	Chl-b	Total Chl	Chl-a	Chl-b	Total Chl	Chl-a	Chl-b	Total Chl
0	9.23±0.47	3.06±0.07	12.29±0.53	4.39±0.39	2.00±0.20	6.40±0.50	4.17±0.24	2.32±0.12	6.49±0.14
100	7.18±0.57	2.57±0.15	9.75±0.72	4.88±0.26	1.95±0.09	6.83±0.35	4.45±0.23	2.54±0.16	6.98±0.19
2,500	7.26±0.46	2.66±0.13	9.92±0.58	4.94±0.41	2.05±0.10	6.99±0.50	4.62±0.39	2.38±0.21	7.00±0.38
5,000	7.99±0.33	2.86±0.10	10.85±0.43	4.03±0.45	2.24±0.09	6.26±0.49	4.17±0.08	2.32±0.18	6.49±0.20

Values represent mean ± SE of five replicates. No significant differences were observed

HS hydroponics system

on the heavy metal content of seeds after heavy metal treatment of the seed itself. Thus, it is difficult to determine by comparison if the values in Table 1 are high or low. Nevertheless, nano-TiO_2 was absorbed into seed tissues, and it did not have any negative effects on germination. To our knowledge, this is the first report on NPs absorbed into seed tissues, but nano-TiO_2 did not show any toxicity. Additionally, although there were no statistically significant differences

Fig. 3 Total antioxidant capacity and superoxide dismutase activity of plants after nano-TiO_2 treatment. **a** TAC of B. campestris, **b** TAC of L. sativa, **c** TAC of L. sativa (hydroponics system (HS)), **d** SOD of B. campestris, **e** SOD of L. sativa, and **f** SOD of L. sativa (HS). The bars and error bars represent the mean ± SE of five replicates. No significant differences were observed

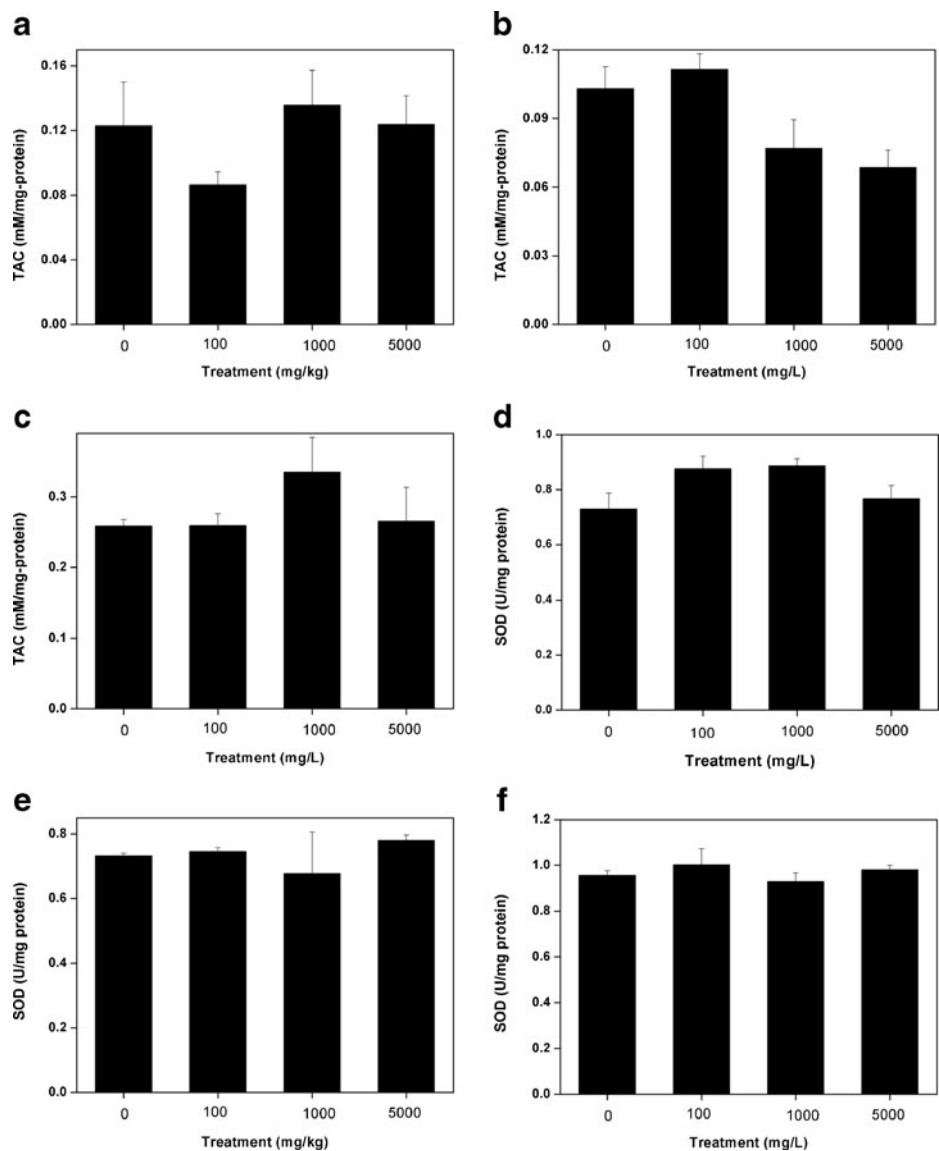**Table 3** Titanium uptake by plant leaves 2 weeks after exposure (in milligram per kilogram)

TiO$_2$ treatment (mg/L)	*Brassica campestris*	*Lactuca sativa*	*Lactuca sativa* (HS)
100	4.10±0.45	3.78±0.21	6.46±0.06 b
1,000	8.08±2.02	3.89±0.42	12.82±1.46 a
5,000	9.94±2.92	4.02±0.34	5.42±0.83 b

Values represent mean ± SE of three replicates. Values with the same letter are not significantly different at the 0.05 level

HS hydroponics system

between the Ti levels in *B. campestris* seeds after the various treatments, the Ti concentration after the 1,000-mg/L treatment was three times higher than after the 5,000-mg/L treatment (they showed significant differences when compared only with each other). This result indicates that a higher concentration does not always lead to higher absorption.

Among the three species tested, only *L. sativa* showed significant differences in root elongation with the different treatments (Fig. 2). However, because our first experiments using up to 5,000 mg/L nano-TiO$_2$ did not show any effects of nano-TiO$_2$ on the other species, we conducted experiments with *B. campestris* again using much higher (10,000 mg/L) and lower (50 mg/L) concentrations (as well as other concentrations, Fig. 2a). We tested 50 mg/L nano-TiO$_2$ because even at 100 mg/L, nano-TiO$_2$ had a high hydrodynamic diameter (Fig. 4a), indicating agglomeration. However, neither treatment

showed any effect. Therefore, even at high concentrations, nano-TiO$_2$ does not seem toxic.

For *L. sativa*, only treatment with 5,000 mg/L nano-TiO$_2$ significantly decreased root elongation; the other treatments (100, 500, 1,000, and 2,500 mg/L nano-TiO$_2$) significantly increased root growth compared to control (Fig. 2b). This might indicate that low levels of nano-TiO$_2$ are not harmful—they may even sometimes have positive effects on plant growth—whereas too high concentrations could have negative effects. Treatment with 2,500 mg/L nano-TiO$_2$ induced the longest root elongation (Fig. 2b), supporting this hypothesis. However, given that other species did not show signs of toxicity even when exposed to higher concentrations, we conclude that the toxicity of nano-TiO$_2$ at high concentrations is species-dependent. Moreover, root elongation in plants exposed to 5,000 mg/L nano-TiO$_2$ was only 25 % less than that in control; thus, toxicity would not be fatal.

Nano-TiO$_2$ at 5,000 mg/L had the highest zeta potential (Fig. 4b), which indicated that particles repelled each other more than in the other treatments. Consequently, particles in the 5,000 mg/L nano-TiO$_2$ solution had the smallest diameter among the treatment concentrations (Fig. 4 a). However, even nano-TiO$_2$ particles in DW with the smallest hydrodynamic diameter ranged from more than 200 to over 650 nm (Fig. 4a), much bigger than original particle size (27 nm). The zeta potential of nano-TiO$_2$ at higher concentrations was close to zero. Particles with surface charges near zero have higher aggregation potential [30]. Therefore, although the 100 and 1,000 mg/L nano-TiO$_2$ solutions had fewer particles, these

Fig. 4 Physical chemistry (Pchem) analysis (hydrodynamic diameter and zeta potential) of nano-TiO$_2$ in solution. **a** Hydrodynamic diameter of nano-TiO$_2$ in distilled water. **b** Surface charge (zeta potential) of nano-TiO$_2$ in distilled water. **c** Hydrodynamic diameter of nano-TiO$_2$ in Hoagland's solution. **d** Surface charge (zeta potential) of nano-TiO$_2$ in Hoagland's solution. Values represent the mean ± SE of 150 replicates for hydrodynamic diameter and nine replicates for zeta potential
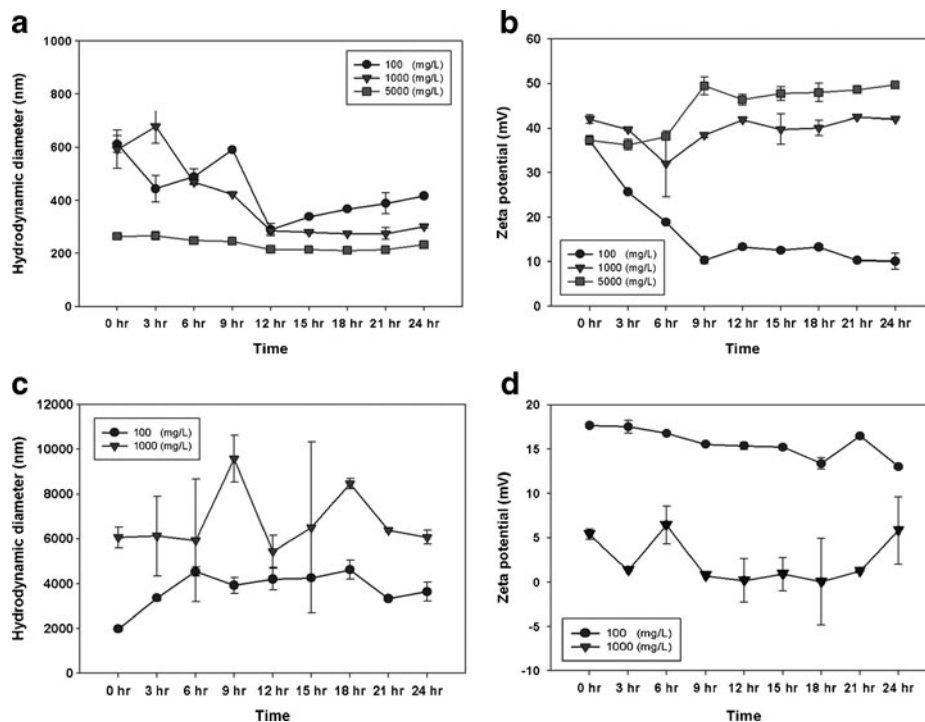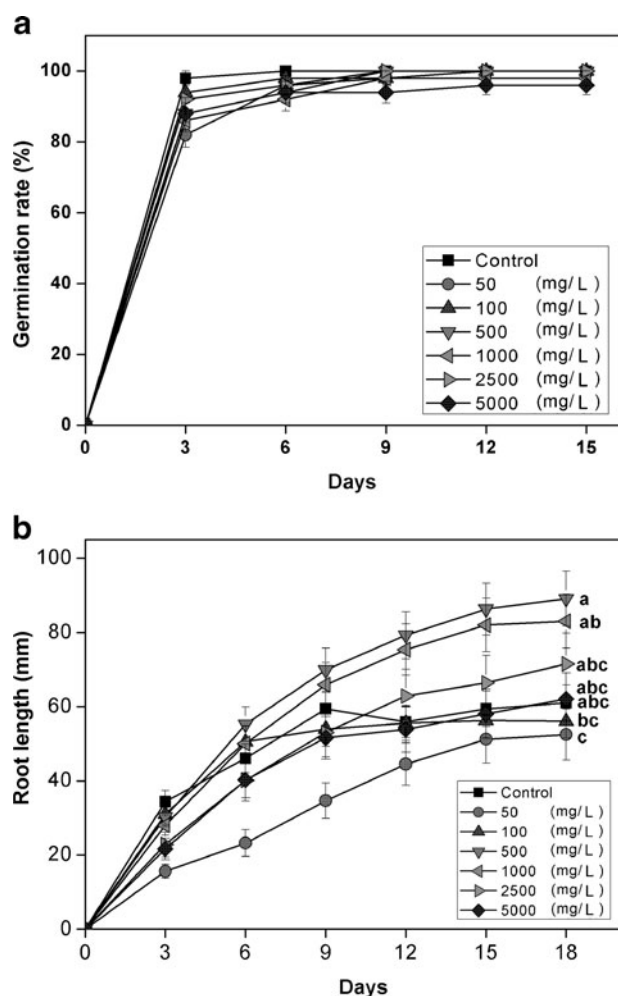
Fig. 5 **a** Germination rates and **b** root elongation of *B. campestris* after nano-TiO$_2$ (Daejoo) treatment. *Symbols* and *bars* represent the mean ± SE of ten replicates for germination rate and 50 replicates for root length. *Symbols with the same letter* are not significantly different at the 0.05 level

particles eventually agglomerated and formed particles with larger hydrodynamic diameters, similar to the particles observed at high concentrations (5,000 mg/L). Therefore, at every concentration, particles agglomerated and created particles of greater diameter; their absorption may be lower than expected for NPs. This characteristic of NPs is an important factor for nanotoxicity studies [31], since increased particle size (by agglomeration) would reduce uptake by plants. Furthermore, although we analyzed the characteristics of nano-TiO$_2$ for 24 h by Pchem analysis, we examined root elongation for more than 2 weeks, during which time the particles would have the chance to agglomerate further. Therefore, though they start as nanosized particles, NPs would become larger and not affect plants as expected.

Usually, NP phytotoxicity research investigates the seed or seedling stage of plants. It was difficult to locate studies in international journals reporting on the phytotoxicity of NPs in mature plants. One study investigated plants grown from seeds over 2 months, but the plants were exposed to NPs from the beginning [32]. However, in natural conditions, plants more likely encounter NPs when mature (after the seedling stage). Additionally, it is important to determine if NPs are toxic to grown plants. Therefore, we used plants aged over 5 weeks to test phytotoxicity in pot experiments and a hydroponic system. Although the hydroponic system more closely resembles natural conditions than the soil medium experiment, with regard to how nano-TiO$_2$ contacts plants [20, 21], and thus can be used to predict the actual reactions of toxic materials in the field [22], the circular flow inside the container to prevent precipitation affects the physical characteristics of nano-TiO$_2$, causing nano-TiO$_2$ to agglomerate more. However, because sewage systems and hydrological transport of NPs would also have physical effects on nano-TiO$_2$, our circular hydroponic system even more closely resembles natural conditions. In this research, nano-TiO$_2$ showed some precipitation even at 5,000 mg/L. Despite the circular flow created, in the 5,000-mg/L treatment, particles of nano-TiO$_2$ precipitated and agglomerated during the experiment. However, this would approximate results when nano-TiO$_2$ at such a high concentration was released into a natural system.

The chlorophyll content of plants was not affected by nano-TiO$_2$ treatment or the hydroponic system (Table 2). Plants did not show any significant differences in TAC and SOD activity when exposed to the various nano-TiO$_2$ treatments, including those administered with hydroponics (Fig. 3). Plants did not show any physiological differences after nano-TiO$_2$ treatments. Because SOD activity, which is sensitive to heavy metals, did not change [26], nano-TiO$_2$ seemed to have no effect on mature plants. Table 3 shows Ti uptake by plants administered with nano-TiO$_2$ by pot (*B. campestris* and *L. sativa*) or hydroponics (*L. sativa*). In the hydroponic system only, the Ti content of *L. sativa* was significantly higher after the 1,000 mg/L treatment. The amount of Ti absorbed by the plants ranged from 4 to 10 mg/kg in pot experiments and up to 12.8 mg/kg in the hydroponic system. In a previous study, *L. sativa* cultured in soil concentrated with 1,000 mg/kg of lead, cadmium, or copper absorbed 6.7 mg/kg (lead) to 21.7 mg/kg (copper) of heavy metal [33]. Considering the exposure time (2 weeks), it seems that Ti was absorbed more readily than the other heavy metals, but not extremely so. However, a previous research showed an increase in heavy metal values with 3,000 mg/kg treatment (200 % for lead and cadmium, 30 % for copper), whereas our experiments showed no increase or significantly lower concentrations in some 5,000 mg/L treatments (Table 3). Nano-TiO$_2$ in the Hoagland solution had a much larger hydrodynamic diameter, ranging from 2,000 to over 9,000 nm (Fig. 4c), more than 300 times the original diameter. Therefore, in natural conditions, where water has solutes, nano-TiO$_2$ would have a larger particle size and not

have NP characteristics (small size) anymore. This explains why plants in hydroponics do not have notably high concentrations of nano-TiO$_2$ (Table 3) and do not exhibit any NP-induced stress (Table 2 and Fig. 3). The zeta potential of 100 mg/L nano-TiO$_2$ in the Hoagland solution was over 10 mV, whereas the zeta potential of 1,000 mg/L nano-TiO$_2$ in the Hoagland solution dropped below zero (Fig. 4d). These values indicated that NPs in the Hoagland solution have a larger cohesive force, thereby explaining the larger particle diameter in the Hoagland solution. We were not able to measure the Pchem properties of 5,000-mg/L nano-TiO$_2$ in the Hoagland solution because too many precipitates formed. However, plants treated with 5,000 mg/L of nano-TiO$_2$ had a lower Ti concentration (Table 3) than plants treated with 1,000 mg/L, suggesting that nano-TiO$_2$ in the 5,000-mg/L treatment agglomerated, thereby limiting its absorption by plants.

Plants grown in growing soil showed relatively lower concentrations of Ti than those grown in the hydroponic system (Table 3), but they showed certain concentration, considering the exposure time (2 weeks). However, the values were also not surprisingly high like the hydroponics system and even lower. Because the agglomeration of NPs correlates with transportability in soil, and electrostatic attraction exists between the NPs and soil [34], nano-TiO$_2$ is not well absorbed by plants in soil. Additionally, unlike heavy metals that easily becomes ionized [35], aerosolized nano-TiO$_2$ cannot travel through soil particles, and soil acts as a filter to block contact between nano-TiO$_2$ and plant roots. Overall, plants grown in nano-TiO$_2$-treated soil did not accumulate notable amounts of Ti or demonstrate stress.

Because the aerosolized form of nano-TiO$_2$ did not demonstrate phytotoxicity, a powdered form of nano-TiO$_2$ (Daejoo) was also tested to confirm our results. The powdered form of nano-TiO$_2$ did not significantly alter the germination rate (Fig. 5a), and some treatments significantly increased root elongation compared with control (Fig. 5b). However, treatment with low concentrations (50 and 100 mg/L) significantly reduced root elongation, compared with the control (Fig. 5b). This result might indicate that plants more readily absorb nano-TiO$_2$ at low concentrations (less agglomeration), but the Pchem results (Fig. 6) showed that particles in the 100-mg/L solution were not distinctively small. Compared with control, low concentrations (50 and 100 mg/L) of nano-TiO$_2$ decreased root elongation by 15 %, whereas the 500-mg/L treatment increased the root length more than 40 %. Some studies reported on the positive effects of nano-TiO$_2$ on plant growth [17] and nitrogen photoreduction [16]. Therefore, our results suggest that nano-TiO$_2$ has positive effects on plants, rather than negative. Furthermore, TAC and SOD values in *B. campestris* were not significantly different after treatment with the powdered form of nano-TiO$_2$. Therefore, the powdered form of nano-TiO$_2$ also does not appear to be phytotoxic.

The result of seed germination and root elongation shows that among two previous opposite results on nano-TiO$_2$ research,
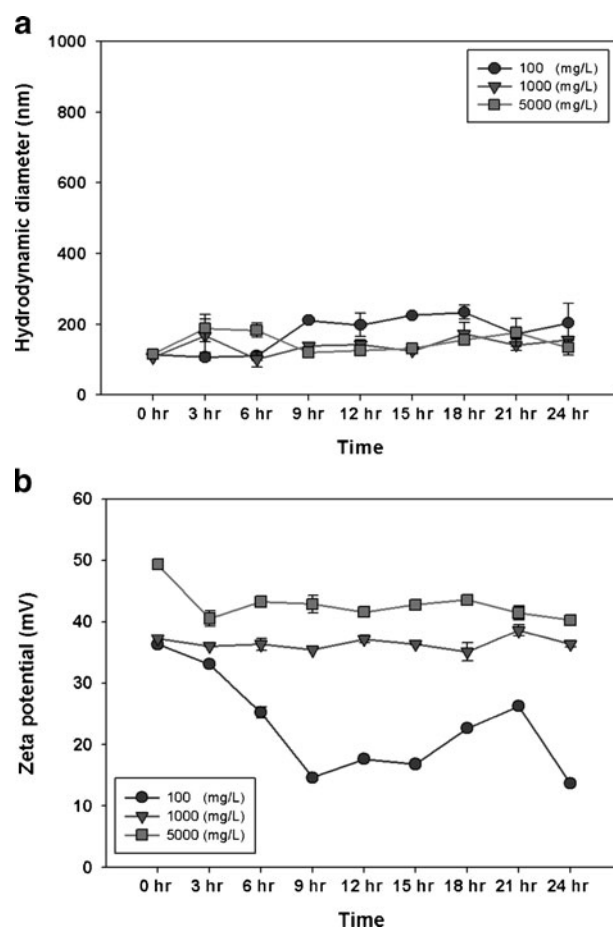

Fig. 6 Pchem analysis (hydrodynamic diameter and zeta potential) of Daejoo-TiO$_2$ in DW. **a** Hydrodynamic diameter of nano-TiO$_2$ in distilled water. **b** Surface charge (zeta potential) of nano-TiO$_2$ in distilled water. Values represent the mean ± SE of 150 replicates for hydrodynamic diameter and nine replicates for zeta potential

both researches showing positive effects of nano-TiO$_2$ [17] and negative effects of nano-TiO$_2$ [1] are possible. The results of NP phytotoxicity studies are highly dependent on the application method because apparent differences in the phytotoxicity of nanoparticles may arise from the properties of nanoparticles, plant species and ages, exposure time, and concentrations [36]. However, our results support the view that nano-TiO2 is not toxic (100 % germination; most species showed positive effects of nano-TiO$_2$; no significant changes in antioxidant enzyme activities and chlorophyll content). There could be long-term negative effects of nano-TiO$_2$, such as genotoxicity [37]. However, in actual fields, precipitation and agglomeration would reduce the possibility of nano-TiO$_2$ uptake, judging by our Pchem and hydroponic system studies. Additionally, after the seedling stage, plants showed high tolerance to nano-TiO$_2$, indicating less possibility of nano-TiO$_2$ phytotoxicity. Furthermore, given that very few studies have reported on phytotoxicity of Ti itself [38] and the United States Environmental Protection Agency has not announced ecological soil screening levels of Ti, Ti itself appears to be not toxic. Moreover, many papers have

reported on the positive effects of Ti on plant metabolism and physiology [39]. Ti is present in the soil at relatively high concentrations, but Ti is poorly available to plants because it is present mostly in the form of minerals that are insoluble in water, like TiO_2 or $FeTiO_3$ [39]. Therefore, nano-TiO_2 is not readily available to plants. Also, not only considering that nano-TiO_2 is difficult to accumulate to plants, our results show that even nano-TiO_2 penetrated the seed coat and accumulated inside seed tissues, it showed no toxic effects; and plants grown in soil and hydroponics system also showed certain uptake of TiO_2 but showed no phytotoxicity. These results indicate that even after uptake of TiO_2 into plant metabolic systems, Ti has no toxic effects to plants, which corresponds to results of previous Ti research [38] and USEPA. Overall, based on our results, nano-TiO_2 is not toxic to plants.

References

1. Ruffini Castiglione M, Giorgetti L, Geri C, Cremonini R (2011) The effects of nano-TiO_2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. J Nanopart Res 1(6):2443–2449. doi:10.1007/s11051-010-0135-8

2. Geisler-Lee J, Wang Q, Yao Y, Zhang W, Geisler M, Li K, Huang Y, Chen Y, Kolmakov A, Ma X (2013) Phytotoxicity, accumulation and transport of silver nanoparticles by *Arabidopsis thaliana*. Nanotoxicol 7(3):323–337. doi:10.3109/17435390.2012.658094

3. Lee W-M, An Y-J, Yoon H, Kweon H-S (2008) Toxicity and bio-availability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): Plant agar test for water-insoluble nanoparticles. Environ Toxic Chem 27(9):1915–1921. doi:10.1897/07-481.1

4. Griffitt RJ, Weil R, Hyndman KA, Denslow ND, Powers K, Taylor D, Barber DS (2007) Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Environ Sci Tech 41(23):8178–8186. doi:10.1021/es071235e

5. Bar-Ilan O, Albrecht RM, Fako VE, Furgeson DY (2009) Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. Small 5(16):1897–1910. doi:10.1002/smll.200801716

6. Rai M, Yadav A, Gade A (2009) Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 27(1):76–83. doi:10.1016/j.biotechadv.2008.09.002

7. Jones N, Ray B, Ranjit KT, Manna AC (2008) Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS Microbiol Lett 279(1):71–76. doi:10.1111/j.1574-6968.2007.01012.x

8. Wiesner MR, Lowry GV, Alvarez P, Dionysiou D, Biswas P (2006) Assessing the risks of manufactured nanomaterials. Environ Sci Tech 40(14):4336–4345. doi:10.1021/es062726m

9. Lin D, Xing B (2007) Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. Environ Pollut 150(2):243–250. doi:10.1016/j.envpol.2007.01.016

10. Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH (2009) Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res 69(22):8784–8789. doi:10.1158/0008-5472.can-09-2496

11. Long TC, Saleh N, Tilton RD, Lowry GV, Veronesi B (2006) Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. Environ Sci Tech 40(14):4346–4352. doi:10.1021/es060589n

12. Kang SJ, Kim BM, Lee YJ, Chung HW (2008) Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. Environ Mol Mutagen 49(5):399–405. doi:10.1002/em.20399

13. Nohynek GJ, Dufour EK, Roberts MS (2008) Nanotechnology, cosmetics and the skin: is there a health risk? Skin Pharmacol Phys 21(3):136–149

14. Newman MD, Stotland M, Ellis JI (2009) The safety of nanosized particles in titanium dioxide- and zinc oxide-based sunscreens. J Am Acad Dermatol 61(4):685–692. doi:10.1016/j.jaad.2009.02.051

15. Hong F, Yang F, Liu C, Gao Q, Wan Z, Gu F, Wu C, Ma Z, Zhou J, Yang P (2005) Influences of nano-TiO_2 on the chloroplast aging of spinach under light. Biol Trace Elem Res 104(3):249–260. doi:10.1385/bter:104:3:249

16. Yang F, Hong F, You W, Liu C, Gao F, Wu C, Yang P (2006) Influence of nano-anatase TiO_2 on the nitrogen metabolism of growing spinach. Biol Trace Elem Res 110(2):179–190. doi:10.1385/bter:110:2:179

17. Zheng L, Hong F, Lu S, Liu C (2005) Effect of nano-TiO_2 on strength of naturally aged seeds and growth of spinach. Biol Trace Elem Res 104(1):83–91. doi:10.1385/bter:104:1:083

18. (OECD) OECD (2003) OECD guidelines for the testing of chemicals: proposals for updating guideline 208-terrestrial plant test: seedling emergence and seedling growth test. http://wwwoecdorg/dataoecd/11/31/33653757pdf. Accessed 20 Apr 2011. doi: 10.1016/j.buildenv.2013.04.024

19. United States Environmental Protection Agency (USEPA) (1996) Ecological effects test guidelines (OPPTS 850.4200): seed germination / root elongation toxicity test. http://wwwepagov/opptsfrs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-4200pdf. Accessed 25 Mar 2011. doi: 10.1016/j.buildenv.2013.04.024

20. Gottschalk F, Sonderer T, Scholz RW, Nowack B (2009) Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, Fullerenes) for different regions. Environ Sci Tech 43(24):9216–9222. doi:10.1021/es9015553

21. Kim B, Murayama M, Colman BP, Hochella MF (2012) Characterization and environmental implications of nano- and larger TiO_2 particles in sewage sludge, and soils amended with sewage sludge. J Environ Monitor 14(4):1128–1136

22. Watson C, Pulford ID, Riddell-Black D (2003) Screening of willow species for resistance to heavy metals: comparison of performance in a hydroponics system and field trials. Int J Phytoremediat 5(4):351–365. doi:10.1080/15226510309359042

23. Gent MPN, White JC, Eitzer BD, Mattina MI (2007) Modeling the difference among *Cucurbita* in uptake and translocation of *p,p′*-dichlorophenyl-1,1-dichloroethylene. Environ Toxicol Chem 26(12):2476–2485. doi:10.1897/06-258.1

24. Hoagland D, Arnon D (1950) The water culture method for growing plants without soil. California Agri Exp Sta Circ 347(4):1–39. doi:10.1007/s12374-010-9112-0

25. Lee JG, Lee BY, Lee HJ (2006) Accumulation of phytotoxic organic acids in reused nutrient solution during hydroponic cultivation of lettuce (*Lactuca sativa* L.). Sci Hortic 110(2):119–128. doi:10.1016/j.scienta.2006.06.013

26. Song U, Lee E (2010) Ecophysiological responses of plants after sewage sludge compost applications. J Plant Biol 53(4):259–267. doi:10.1007/s12374-010-9112-0

27. Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57(12):1332–1334. doi:10.1139/b79-163

28. Jeffryes C, Gutu T, Jiao J, Rorrer GL (2008) Metabolic insertion of nanostructured TiO$_2$ into the patterned biosilica of the diatom *Pinnularia* sp. by a two-stage bioreactor cultivation process. ACS Nano 2(10):2103–2112. doi:10.1021/nn800470x

29. Rodushkin I, Ruth T, Huhtasaari Å (1999) Comparison of two digestion methods for elemental determinations in plant material by ICP techniques. Anal Chim Acta 378(1–3):191–200. doi:10.1016/S0003-2670(98)00635-7

30. Badawy AME, Luxton TP, Silva RG, Scheckel KG, Suidan MT, Tolaymat TM (2010) Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions. Environ Sci Tech 44(4):1260–1266. doi:10.1021/es902240k

31. Warheit DB (2008) How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol Sci 101(2):183–185. doi:10.1093/toxsci/kfm279

32. Doshi R, Braida W, Christodoulatos C, Wazne M, O'Connor G (2008) Nano-aluminum: transport through sand columns and environmental effects on plants and soil communities. Environ Res 106(3):296–303. doi:10.1016/j.envres.2007.04.006

33. Smilde KW (1981) Heavy-metal accumulation in crops grown on sewage sludge amended with metal salts. Plant Soil 62(1):3–14. doi:10.1007/BF02205020

34. Darlington TK, Neigh AM, Spencer MT, Guyen OTN, Oldenburg SJ (2009) Nanoparticle characteristics affecting environmental fate and transport through soil. Environ Toxicol Chem 28(6):1191–1199. doi:10.1897/08-341.1

35. Lake DL, Kirk PWW, Lester JN (1984) Fractionation, characterization, and speciation of heavy metals in sewage sludge and sludge-amended soils: a review. J Environ Qual 13(2):175–183. doi:10.2134/jeq1984.00472425001300020001x

36. Ma X, Geiser-Lee J, Deng Y, Kolmakov A (2010) Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation. Sci Total Environ 408(16):3053–3061. doi:10.1016/j.scitotenv.2010.03.031

37. Ghosh M, Bandyopadhyay M, Mukherjee A (2010) Genotoxicity of titanium dioxide (TiO$_2$) nanoparticles at two trophic levels: plant and human lymphocytes. Chemosphere 81(10):1253–1262. doi:10.1016/j.chemosphere.2010.09.022

38. Wallace A, Alexander GV, Chaudhry FM (1977) Phytotoxicity of cobalt, vanadium, titanium, silver, and chromium. Commun Soil Sci Plant Anal 8(9):751–756. doi:10.1080/00103627709366769

39. Tlustoš P, Cígler P, Hrubý M, Kužel S, Száková J, Pavlíková D, Balík J (2005) The role of titanium in biomass production and its influence on essential elements contents in field growing crops. Plant Soil Environ 51:19–25