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SHORT COMMUNICATION

Titanium oxide nanoparticle effects on composition of soil microbial communities and plant performance

David J. Burke · Shun Zhu · Michele P. Pablico-Lansigan · Charlotte R. Hewins · Anna Cristina S. Samia

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Abstract We examined the effect of TiO_2 nanoparticles (NPs) on the growth of maize and soybean plants and associated soil microbial communities. Plants were grown in a greenhouse, and low levels of undoped or nitrogen-doped TiO_2 NPs were applied. Plant growth and nutrient content were determined, and effects of NPs on composition of soil microbial communities were examined using terminal restriction fragment length polymorphism analysis (TRFLP) of rDNA. We found no significant effects of TiO_2 NPs on plant growth, nutrient content, or the composition of bacterial communities within the rhizosphere. However, arbuscular mycorrhizal fungal communities were affected by application of undoped and nitrogen-doped TiO_2 NPs. This observation may be partially attributed to the small but significant TiO_2 NP uptake levels in the root tissues of both plants. Our results suggest that even low concentrations of TiO_2 NPs may influence some important groups of soil microbes, such as mycorrhizal fungi, but changes in the composition of microbial communities may not affect plant growth under conditions of adequate moisture and nutrients.

Keywords *Glycine max* · Microbial communities · Nanoparticles · Soil · Titanium oxide · *Zea mays*

Introduction

There is increasing interest in the potential beneficial and detrimental effects of engineered nanoparticles (NPs) on agricultural plants. Although one of the most widely used and studied NP is titanium oxide (TiO_2), the effects of TiO_2 NPs on plant and microbial communities are not thoroughly understood. Studies on the effects of TiO_2 NPs on plants have reported mixed results, with positive, negative, and neutral effects for plant growth and physiology (Asli and Neumann 2009; Ghosh et al. 2010; Hong et al. 2005; Lu et al. 2002; Seeger et al. 2009) with some reporting reduced toxic effects of TiO_2 NPs as compared to other NPs such as AgO_2 and ZnO (Boonyanitipong et al. 2011; Kim et al. 2011).

Nanoparticles could also alter the composition of soil microbial communities in the rhizosphere since some NPs, such as TiO_2 and AgO_2, have antimicrobial properties (e.g., they are used in antimicrobial soaps). There is also evidence that toxic effects on microbes could be selective and depend on the type of NP and specific bacterial community or specific microbial functional groups (Emami-Karvani and Chehrazi 2011; Priester et al. 2012). Since rhizosphere microbial communities perform key functions in terms of plant growth promotion, plant protection, and enhanced nutrient uptake (Hodge et al. 2001; Smith and Read 2008), the selective inhibition of certain groups within rhizosphere microbial communities may impact plant nutrient uptake. However, few studies have simultaneously examined the effects of NPs on both plant growth and rhizosphere microbial communities (Feng et al. 2013). Additional comprehensive studies are needed to better understand how NPs affect complex

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D. J. Burke (✉) · C. R. Hewins
The Holden Arboretum, 9500 Sperry Road, Kirtland, OH 44094, USA
e-mail: dburke@holdenarb.org

D. J. Burke
Department of Biology, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106, USA

S. Zhu · M. P. Pablico-Lansigan · A. C. S. Samia (✉)
Department of Chemistry, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106, USA
e-mail: anna.samia@case.edu

interactions within the plant–soil system that can affect plant growth through alteration in soil fertility (Feng et al. 2013).

In this study, we report the results of a greenhouse experiment testing the effects of TiO_2 NPs on the growth of maize and soybean and the composition of their rhizosphere microbial communities. We hypothesized that treatment of soil with TiO_2 NPs would (1) have negative effects on plant root growth through direct toxic effects as observed in previous studies; (2) increase plant absorption of TiO_2 NPs but NPs would be restricted to root tissue; and (3) alter the composition of rhizosphere microbial communities. Previous studies have examined effects of high concentration of NPs on plants and microbes, which could result from accumulation of NPs in soil after many years of exposure (Priester et al. 2012). However, low to moderate concentrations of NPs could also have detrimental effects on both plants and important soil microbial groups, but low exposure studies are underreported in the literature. Consequently, our greenhouse study was meant to assess toxic effects of low to moderate NP application on plants and the composition of soil microbial communities and examine uptake and translocation of NPs by plants which have been understudied. We also wanted to examine how the form of NP could affect toxic properties of the compound and the uptake by plants so undoped and N-doped TiO_2 (N–TiO_2) NPs were used.

Materials and methods

We examined the response of plants and microbial communities using two widely planted agricultural crop species: *Zea mays* L. (maize—peaches and cream hybrid) and *Glycine max* (L.) Merr (soybean—variety Envy). Seeds were planted into 15-cm plastic pots filled with potting soil mix (Fafard, Agawam, MA, USA; 3B middle weight mix) that was heat sterilized (40 min at 121 °C) and then 100-g dry weight of field-collected soil was incorporated into each pot to serve as a microbial inoculant. Seeds of either maize or soybean were planted into each pot (24 pots maize, 24 pots soybean) on October 17, 2011 and were grown in a greenhouse. Supplemental lighting was used to maintain a 12-h day/night cycle that mimics spring growing conditions, and temperature was maintained between 18 and 27 °C.

TiO_2 and N-doped TiO_2 NPs were prepared using a modified sol–gel synthetic approach according to previously published procedures (Burda et al. 2003; Chen et al. 2005; Clouser et al. 2008). The size and morphology, crystallinity, and presence and percentage of N doping of the TiO_2 NPs were investigated using transmission electron microscopy (TEM), dynamic light scattering (DLS), powder X-ray diffractometry (XRD), and X-ray photoelectron spectra (XPS) analysis following standard procedures (Burda et al. 2003; Chen et al. 2005). The obtained X-ray diffraction patterns demonstrated that TiO_2 NPs indexed well with the anatase crystalline form, with some peaks due to the presence of both the brookite and rutile phases. XPS analysis of the N-doped TiO_2 sample revealed an average of 11 % N doping, which is reflected in the change in the optical response of the TiO_2 NPs (Supplemental Fig. 2A). From the UV–Vis reflectance measurements, the N-doped sample exhibits a strong absorption in the visible spectral region (Supplemental Fig. 2B) indicating the presence of N species occupying the oxygen positions in the lattice. The NPs used in our study formed aggregates in the 40- to 60-nm size range as observed from TEM and DLS analysis (see Supplemental Fig. 1).

After 30 days, plants were arranged on the greenhouse bench and treatments applied to selected pots. Pots of either maize or soybean designated as nontreated controls received 100 mL of distilled water, while treated pots received 100 mg of synthesized undoped or N-doped TiO_2 (N–TiO_2) NPs dissolved in 100 mL of distilled water. This resulted in a final concentration of less than 200 mg NP kg^{-1} soil fresh weight. Shallow (2 cm) plastic trays were placed under each planted pot to catch any liquid that passed through the pot and prevent loss of NP during watering. Our experimental set-up allowed for a 2×3 factorial design that tested for the effect of NP (nontreated, TiO_2-treated, N–TiO_2-treated) and plant species (maize or soybean) on microbial community composition. Eight replicated pots were established for each nanoparticle/plant treatment combination (2 plant species × 8 replicates × 3 nanoparticle treatments = 48 pots). One week after treatment initiation, plants were fertilized with 100 mL of half strength Peters water soluble general purpose fertilizer (20–20–20) (Scotts Company LLC, Marysville, OH, USA). This was the only supplemental fertilization performed over the study.

After 6 weeks of plant growth under treated conditions (10 weeks from sowing), all above- and belowground biomass was collected and separated from the pots, dried at 60ºC for 1 week and weighed. Total plant C and N was determined using dry combustion on an ECS 4010 CHNSO elemental analyzer (Costech Analytical, Valencia, CA, USA) and total leaf and root P was determined through acid digestion by sulfuric acid (Moore 1992) and colorimetric analysis (Kuo 1996). Above- and belowground plant biomass was separately acid-digested at 150 °C for 4 h in pressurized Teflon vessels using 70 % nitric acid, diluted to a final acid concentration of 2 % nitric acid, and subsequently analyzed for Ti content using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Soil adhering tightly to fine roots was collected (i.e., rhizosphere soil) at the time of harvest and retained for microbial analysis. Total soil DNA was extracted using a bead beating protocol followed by phenol–chloroform extraction (Burke et al. 2012) except additional gel purification was not used. DNA was suspended in 100 μL 1 M TE (Tris-EDTA) buffer and amplified by PCR targeting the 16S rDNA using labeled

primers 338f and 926r and conditions described by Burke et al. (2006). For analysis of arbuscular mycorrhizal fungal communities, we targeted the 18S rRNA gene using universal eukaryotic primer NS31 (Simon et al. 1992) and AM specific primer AM1 following general procedures reported by Helgason et al. (1998). Primers were labeled either with the fluorochrome 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX) or 6-carboxyfluorescein (6FAM). PCR products were digested with restriction enzymes *Msp*I and *Hinf*I (Promega, Madison, WI, USA) for bacteria and fungi, respectively, and terminal restriction fragment length polymorphisms (TRFLP) was completed through the Cornell Bioresource Center using an Applied BioSystems 3730xl DNA Analyzer. The TRFLP profiles were analyzed using the GS600 LIZ size standard and Peak Scanner™ Software (version 1.0, Applied Biosystems 2006). Only peaks that accounted for greater than 1 % of the relative peak area were included in this analysis (i.e., major TRFs; Burke et al. 2008).

Differences in plant biomass and nutrient content were analyzed by two-way ANOVA using SigmaStat 3.5 (Systat Software Inc., CA, USA). Effects of plant species and treatment on bacterial and mycorrhizal community structure were analyzed using nonmetric multidimensional scaling (NMS) and multiresponse permutation procedures (MRPP) as described by Burke et al. (2012).

Results and discussion

Plant growth responses and plant chemical content

Plant above- and belowground biomass followed the same patterns with significant differences between species (F= 316.2, P<0.001; F=111.5, P<0.001 above- and belowground, respectively) but NP treatments had no significant effects on biomass (Table 1). Both above- and belowground plant C, N, and P content were unaffected by nanoparticle treatment but significant differences existed between plant species (Table 1). These results are in agreement with previous studies that have found little negative effect of TiO_2 NPs on plant growth (Boonyanitipong et al. 2011; Kim et al. 2011; Seeger et al. 2009). Although the growth and elongation of rice roots were negatively affected by ZnO, rice was unaffected by TiO_2 NPs at similar concentrations (Boonyanitipong et al. 2011). In another study using duckweed (*Lemna paucicostata*), Kim et al. (2011) found that toxic effects of TiO_2 NPs were only evident at very high concentrations (>250 mg kg^{-1}) whereas toxic effects of AgO were evident at lower concentrations (>1 mg kg^{-1}). Both of these studies indicate that although TiO_2 NPs may be toxic to plant cells, the toxicity may be substantially lower as compared to other widely used NPs.

However, we observed increases in Ti content in roots with the TiO_2 NP treatment. Control soybean and maize roots had low levels of Ti, with 14 and 19 μg Ti per gram of root tissue. However, levels rose for N-doped TiO_2 treatments where soybean and maize roots had 41 and 35 μg Ti per gram of root, respectively, and for TiO_2 treatments, soybean and maize Ti levels rose to 103 and 69 μg Ti per gram of root. However, only very low levels of Ti were detected in the above ground tissue. Levels of Ti in aboveground tissue ranged from not detected to 2.4 μg Ti per gram of plant tissue, and no differences were observed between control and TiO_2 treatments. The NPs used in our study form aggregates in the 40- to 60-nm size range (Supplemental Fig. 1), which should prevent the transport of the NPs into the plant system and limit their chemical reactivity. Although roots were washed and cleaned of soil and extraneous matter before digestion, it is possible that metal concentration reflect both root uptake and adhesion of NPs to external root surfaces (Seeger et al. 2009).

Table 1 Plant chemistry and biomass data for *Z. mays* and *G. max* grown under greenhouse conditions with either titanium oxide or titanium oxide + N nanoparticle applied to soil

	Z. mays			*G. max*		
	Control	TiO_2	TiO_2 + N	Control	TiO_2	TiO_2 + N
	Aboveground tissue					
Biomass (g mass/pot)	8.05 (0.49)	8.13 (0.41)	8.11 (0.52)	2.77 (0.20)	2.94 (0.21)	2.80 (0.16)
C (g C/100 g biomass)	43.721 (0.303)	43.652 (0.286)	43.424 (0.303)	43.229 (0.303)	43.108 (0.303)	43.197 (0.324)
N (g N/100 g biomass)	0.851 (0.156)	0.886 (0.147)	1.093 (0.156)	2.704 (0.156)	2.775 (0.156)	2.586 (0.167)
Leaf P (μg/g dry weight)	2,135 (344)	2,561 (324)	1,864 (344)	4,444 (344)	4,622 (344)	3,966 (367)
	Belowground tissue					
Biomass (g mass/pot)	0.70 (0.05)	0.69 (0.05)	0.70 (0.07)	0.22 (0.03)	0.27 (0.05)	0.25 (0.04)
C (g C/100 g biomass)	43.868 (0.162)	43.985 (0.127)	43.853 (0.166)	43.439 (0.599)	43.098 (0.548)	42.632 (0.686)
N (g N/100 g biomass)	0.899 (0.051)	0.775 (0.029)	0.844 (0.040)	2.190 (0.235)	2.232 (0.293)	2.355 (0.204)
Leaf P (μg/g dry weight)	725 (51)	674 (41)	649 (24)	3,203 (1,449)	2,066 (387)	2,171 (473)

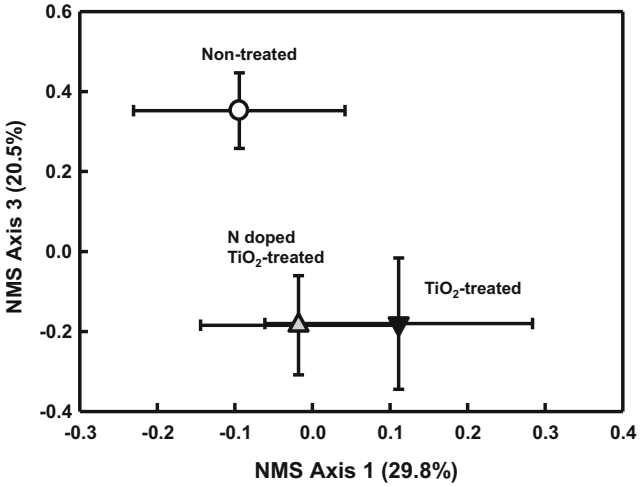

Fig. 1 Nonmetric multidimensional scaling ordination of arbuscular mycorrhizal community composition in maize and soybean rhizosphere using mean values for each treatment. Maize and soybean data were pooled to examine differences in NP treatment on AMF communities. *Symbols* represent mean and standard error of axis scores for a given treatment. When we excluded outliers from our analysis (4 samples excluded, 44 analyzed), we found clear separation in AM communities between maize and soybean with the ordination producing a 3-dimensional solution with a final stress of 14.8 % and a cumulative coefficient of determination of 0.813

Nonetheless, we did observe differences in NP association with roots by both plant species, with roots in the undoped TiO_2 NP treatment associated with higher NP levels.

The levels of Ti we observed are comparable to previously reported levels of Ce found in roots of soybean after exposure to nano-CeO_2 NPs where very low levels of Ce were found in leaves (Priester et al. 2012). Our results and those by Priester et al. (2012) suggest that metal NPs can become available to plant roots, and that the behavior of the NP aggregates may change in the rhizosphere initiating their uptake by the plant. Our finding is surprising, given that the initial size of the NP should have prevented plant uptake. How NP aggregates behavior changes, and the extent of potential uptake and translocation will require additional study.

Microbial community response

Although there were significant differences between the composition of bacterial communities within rhizosphere soil of maize and soybean, no differences were observed between NP treatments (Supplemental Fig. 3). We did find evidence of a weakly significant effect of NP treatment on the composition of AM communities ($P<0.01$, $A=0.024$; MRPP). When both plants were analyzed together, control samples had AM community composition that was weakly significantly different from TiO_2 treatment ($P=0.02$, $A=0.023$; MRPP) and from TiO_2+ N treatment ($P=0.01$, $A=0.031$) (Fig. 1). This suggests that treatment with TiO_2 altered the community composition of AM fungi in the rhizosphere as compared to nontreated controls and is in agreement with previous studies that found effects of NP on the composition of AM fungi (Feng et al. 2013). Although previous studies have reported antimicrobial properties of NPs (Coleman et al. 2005; Emami-Karvani and Chehrazi 2011; Li et al. 2008), we only found impacts of TiO_2 NPs on the composition of AM fungal communities at the concentrations used in our study. Ge et al. (2011, 2012) found impacts of TiO_2 NPs on microbial community structure but only at high concentrations (>500 mg kg^{-1}) and in one study only after 60 days of exposure (Ge et al. 2012). In addition, NP effects can vary with soil type (Frenk et al. 2013) and our soil conditions may have mitigated the toxic effect of TiO_2 NPs on microbial community structure.

It is possible that binding of TiO_2 NPs to plant roots, as observed by Seeger et al. (2009), or increases in actual concentration of Ti in root tissue as we observed, could have impacted the composition of AM fungi to a greater degree than bacteria community composition since the AM fungi colonize the interior of the root system and may have been exposed to higher levels of Ti. But actual quantity of AM fungi in the rhizosphere and roots cannot be determined using the techniques employed here. AM fungi are important plant mutualists increasing plant nutrient gain and growth (Smith and Read 2008). In addition, AM taxa can have differential effects on plant growth and affect the functional properties of the root system (Avio et al. 2006), so changes in the composition of AM communities brought about by TiO_2 NPs could have large effects on plant growth and nutrient uptake, but this will require further study. It is also important to note that our greenhouse study grew plants under relatively controlled and optimal conditions, with plants supplied adequate amounts of water and nutrients and protected from pathogens and insects, which were not observed in the greenhouse during our study. Future work should address effects of TiO_2 NPs on microbial community composition and plant growth performance under conditions where nutrient and water availability may limit plant growth and where alterations of microbial community composition, and especially AM fungal communities may become more important to plant performance.

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