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RESEARCH ARTICLE

Evaluation of zinc oxide nanoparticles on lettuce (*Lactuca sativa* L.) growth and soil bacterial community

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Abstract

The wide spread of nanoparticles (NPs) has caused tremendous concerns on agricultural ecosystem. Some metallic NPs, such as zinc oxide (ZnO), can be utilized as a nano-fertilizer when used at optimal doses. However, little is known about the responses of plant development and concomitant soil bacteria community to ZnO NPs. The present pot experiment studied the impacts of different doses of ZnO NPs and bulk ZnO (0, 1, 10, 100 mg ZnO/kg), on the growth of lettuce (*Lactuca sativa* L.) and the associated rhizospheric soil bacterial community. Results showed that at a dose of 10 mg/kg, ZnO NPs and bulk ZnO, enhanced the lettuce biomass and the net photosynthetic rate; whereas, the Zn content in plant tissue was higher in NPs treatment than in their bulk counterpart at 10 mg/kg dose or higher. For the underground observations, 10 mg/kg treatment doses (NPs or bulk) significantly changed the soil bacterial community structure, despite the non-significant variations in alpha diversity. Taxonomic distribution revealed that some lineages within *Cyanobacteria* and other phyla individually demonstrated similar or different responses to ZnO NPs and bulk ZnO. Moreover, some lineages associated with plant growth promotion were also influenced to different extents by ZnO NPs and bulk ZnO, suggesting the distinct microbial processes occurring in soil. Collectively, this study expanded our understanding of the influence of ZnO NPs on plant performance and the associated soil microorganisms.

Keywords Nanoparticle · Rhizosphere · Bacterial community · 16S rDNA · Net photosynthetic rate

Introduction

Nanoparticles (NPs) are defined as particles that have at least one dimension less than 100 nm. During the last decade, NPs have been widely applied in the fields of commerce, industry, and agriculture due to their unique characteristics, e.g., the small size and the high surface-to-volume ratio (Dimkpa et al. 2013). Due to the anti-microbial and anti-tumor properties, zinc oxide (ZnO) is among the most frequently used nanoparticles, and therefore its release into the environment is inevitable (Raguvaran et al. 2015). In plants, existing data suggest that ZnO NPs can inhibit different developmental stages, such as seed germination and root elongation (Ma et al. 2010), subsequently reducing plant biomass/yield (Lin and Xing 2007). Additionally, in soil, ZnO NPs are reported to hamper soil respiration, ammonification, and soil dehydrogenase activity (Shen et al. 2015). Further, Chai et al. (2015) note that the proliferation of some key bacteria for soil fertility (such as Azotobacter, P-solubilizing, and K-solubilizing bacteria) is also inhibited by ZnO NPs. However, it should be pointed out that the unrealistically high doses (400–2000 mg/kg) at which these previous studies were carried out may have been the cause of toxicity in their study systems (Liu et al. 2015).

On the other hand, ZnO NPs may serve as a micronutrient for plants or a carrier of conventional chemical fertilizers (i.e., nanocarriers) for efficient nutrient utilization (Ditta and Arshad 2016; Monreal et al. 2016). For example, Dimkpa et al. (2015) recently found that ZnO NPs at 100 mg/kg dose stimulate shoot growth of common beans (*Phaseolus*

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vulgaris). In addition, the nutrient use efficiency in peanuts (*Arachis hypogaea*) can be improved by ZnO NPs in the presence of NPK fertilizers (Prasad et al. 2012). Those studies demonstrate the potential of using ZnO NPs as an agricultural nano-fertilizer. Thus far, most research in this area focuses on the impact of ZnO NPs on plants, with fewer concerns for the concomitant response of soil microorganisms and their potential feedback on plant communities. Soil microorganisms are considered as both relevant and sensitive indicators of soil perturbations because of their key roles in biogeochemical cycling, crop production, and biodegradation of pollutants (Simonin and Richaume 2015). Therefore, understanding the soil microbial response would help us to better evaluate the influence of ZnO NPs on environment.

In this study, we hypothesized that when used in moderation, ZnO NPs would improve plant growth, and some key bacterial guilds would adapt to such perturbation accordingly. In order to clarify the roles of particle size, effects of bulk ZnO were analyzed in parallel. To this end, we conducted a greenhouse pot-based experiment, whereby an agriculturally relevant plant was exposed to gradient doses (0, 1, 10, and 100 mg ZnO /kg soil) of both ZnO NPs and bulk ZnO. The lettuce plant (*Lactuca sativa* L.) was selected as it is cultivated worldwide, easily stored, and eaten in a raw form by people because of its nutrient content and health properties (Trujillo-Reyes et al. 2014). The lettuce traits (including fresh biomass, photosynthetic rate of plant leaves, and Zn content) were evaluated. High-throughput sequencing was used to determine the response of soil bacterial components to ZnO NPs. These results would provide a relative full view of the impact of ZnO NPs on the plant-soil ecosystem.

Materials and methods

ZnO particles

Uncoated ZnO NPs (advertised mean particle size of 90 ± 10 nm, purity 99.8%) and the bulk ZnO particles were both purchased from Sigma-Aldrich (Shanghai, China). Before use, the morphologies of ZnO particles (nano-sized and bulk) were characterized by scanning electron microscopy (SEM, JEOL, JSM-5610LV). The generated SEM images showed that the diameters of ZnO NPs and bulk ZnO were about 90 and 300 nm, respectively (Fig. S1). The zeta potential (ζ) of ZnO NPs was approximately − 21.1 ± 1.0 mV at pH 6.53, as measured with a zeta potential analyzer (Delsa 440SX, Beckman). As determined by a dynamic light scattering (DLS) instrument (Malvern Instruments Ltd., Britain), the average hydrodynamic diameters of ZnO NPs suspended in deionized water at 0.001, 0.01, and 0.1% (*w*/*w*) were 95, 134, and 272 nm, respectively, reflecting the presence of aggregation of ZnO NPs.

Experimental design and procedure

The pot experiment was carried out in the greenhouse at the Station of Agricultural Meteorology (32° 14′ N, 118° 42′E), Nanjing University of Information Science and Technology, Nanjing, China. Soil was collected from the surface horizon (0–20 cm depth) of an arable field near the university (refer to Table S1 for main physicochemical properties of the tested soil), where no history of industrial or urban contamination has been documented. After being air-dried and sieved through a 2-mm mesh, the soil was used for the following pot-based experiment.

For each pot, 1.5 kg of sieved soil were mixed well with 20.0 g organic fertilizer (straw-amended cow manure compost: total organic carbon 36.2%, pH 7.2) and 1.0 g chemical compound fertilizer ($N:P_2O_5:K_2O = 15:15:15$). Three doses of ZnO NPs were prepared as reported by Antisari et al. (2013): 1 mg ZnO/kg soil (nZnO1), 10 mg ZnO/kg soil (nZnO10), and 100 mg ZnO/kg soil (nZnO100). In order to interpret the respective effect of particle size, three corresponding doses of bulk ZnO were set in parallel (mZnO1, mZnO10, mZnO100). The mixing procedures were as follows: first, 0.00, 0.01, and 0.1% (*w*/*w*) of ZnO NPs or bulk ZnO suspensions were prepared in deionized water, followed by ultrasonic dispersion (600 W, 20 min). Then 150 ml of the respective suspension were well-mixed with the soil from each pot, leading to n/mZnO1, n/mZnO10, and n/mZnO100, respectively. A negative control that received 150 ml water only (no added ZnO) was also established (Control). Each treatment was replicated in three independent pots.

Ten seeds of lettuce (*Lactuca sativa* L.) were sown directly in the soil in each pot. After the emergence of the second cotyledons of the plant, four plants were allowed to grow until being harvested during the 7th week. Weekly, each pot was watered with deionized water to maintain 70% water holding capacity.

Plant growth parameters

At the 7th week before harvest, the net photosynthetic rates (NPR) of the fully expanded leaves were measured in the morning (9:00am–12:00am) by using a portable open-flow, gas-exchange system (LI-6400; LICOR Biosciences, Lincoln, NE, USA). Ambient conditions: CO_2 concentration was 380 ± 10 μmol/mol (supplied from a CO_2 steel gas cylinder) and light intensity of approximately 1000 μmol m^{-2} s^{-2} (provided by an LED red/blue light source). A total of four leaves per pot were measured, and the measurements were repeated four times for each leaf.

Total Zn content in plant tissue

The oven-dried lettuce plants were ground to pass through a 0.422-mm mesh sieve. Powdered lettuce samples were digested with concentrated HNO_3 and 30% H_2O_2 (in a 1:4 ratio) using a microwave digestion system (CEM Corp., Mathews, NC, USA). The digestion solution was analyzed for Zn content by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin-Elmer, USA). The Zn content in plant tissue was expressed as mg of Zn/kg fresh plant weight.

Soil pH measurement

Soil pH (with a soil-to-water ratio of 1:5) was determined with a pH meter based on an oven-dried (105 °C, 24 h) soil weight.

Soil DNA extraction

In each pot, the soil loosely adhering to the root systems of the plants was discarded by vigorous shaking (Wen et al. 2017). Each root system still held some rhizospheric soil (tightly adhering soil), which was collected. Total soil DNA was extracted from 0.5 g of soil, using the FastDNA ® SPIN Kit for Soil (MP, Santa Ana, CA, USA) and purified using the PowerClean DNA Cleanup Kit (MoBio, Carlsbad, CA, USA), following the manufacturer's instructions. The extracted soil DNA was stored at − 20 °C until further use.

High-throughput sequencing of bacterial 16S rRNA gene

Illumina MiSeq sequencing was performed by amplifying the V4–V5 hypervariable region of the bacterial 16S rRNA gene using individually bar-coded forward primer 519F (5'-CAGCMGCCGCGGTAATWC-3′) and reverse primer 907R (5'-CCGTCAATTCMTTTRAGTTT-3′) (Biddle et al. 2008) for each DNA sample (amplicon size ~500 bp). PCR reactions were carried out in 50 μL reaction volumes containing each of the four deoxynucleotide triphosphates (dNTPs) at a concentration of 1.25 μM per dNTP, 2 μL of 15 μM forward and reverse primers, and 2 U of Taq DNA polymerase (TaKaRa, Japan), and each reaction mix received 1 μL (i.e., 50 ng) of genomic community DNA as template. Thermal cycling was as follows: 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, extension at72°C for 1 min, and a final extension at 72 °C for 7 min. Negative controls using sterilized water instead of soil DNA were included to avoid primer or sample DNA contamination. Each DNA sample was amplified in three technical replicates. Reaction products were then pooled, purified using the QIAquick PCR Purification Kit (QIAGEN), and quantified using NanoDrop ND-1000 (Thermo Scientific, USA). The bar-coded PCR products (library) from all samples were normalized and pooled together in equimolar ratio, then prepared for sequencing using TruSeq™ DNA Sample Prep LT Kit. The library pool was diluted and denatured according to the standard MiSeq protocol, and 11 pM was loaded for sequencing on the Illumina MiSeq using the 500 cycle MiSeq Reagent Kit v3.

Processing of the high-throughput sequencing data

The raw sequencing data were processed through the Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0) pipeline using default parameters unless otherwise noted (Caporaso et al. 2010). Primers, barcodes, sequences with a quality score less than 25, and sequences shorter than 200 bp or containing any unresolved nucleotides were excluded from analysis. Then, the sequencing data were first denoised followed by chimera identification and removal. In total, 307,417 high-quality sequences were obtained from all 21 samples with 7846 and 27,603 sequences per sample. Each sample was rarefied to the same number of reads (7800) for the downstream analyses using QIIME pipeline. Operational taxonomic units (OTUs) were selected using the UPARSE pipeline with a sequence similarity cutoff of 97% (USEARCH software V8) (Edgar 2017). A representative sequence was picked up from each OTU and the Silva 119 database (http://www.arb-silva.de/download/archive/qiime/) was used to assign taxonomic information. The sequencing reads were deposited in the DNA Data Bank of Japan (DDBJ) under accession number DRA006133.

Statistical analysis

All statistical analyses were carried out in R (Version 3.3.3) (R Core Team 2017) and SPSS 19.0 for Windows (IBM Corporation, NY, USA). The differences among treatments were analyzed with one-way analysis of variances (ANOVA) followed by Tukey's post-hoc test. Differences were considered statistically significant at $p < 0.05$. The complexity of species diversity was determined within each sample (alpha diversity), using the Chao1 and Faith's phylogenetic diversity (PD) indices, both of which were calculated with QIIME (Caporaso et al. 2010). To visualize the similarity in terms of community composition between the different samples, non-metric multidimensional scaling (NMDS) on OTUs table was performed using the "metaMDS" function in R's vegan package (Oksanen et al. 2013), based on the Bray-Curtis distance metric. Additionally, we tested for significant differences in OTU/bacterial composition via the adonis function within vegan, using permutations of the Bray-Curtis distance matrices. The number of permutation tests was set at 999, while all other arguments used the default values set in the adonis function. The OTUs explaining most differences

between mZnO10 and Control, as well as between nZnO10 and Control, were analyzed using similarity percentage (SIMPER) analysis in R's vegan package. The OTUs that most contributed to the bacterial community differences, revealed by the SIMPER analysis, were selected to carry out response ratio analyses following the statistical method of Luo et al. (2006).

Results

Net photosynthetic rate of plant leaves

In the NPs treatments, only nZnO10 enhanced net photosynthetic rate (NPR) significantly (ANOVA, $F_3 = 3.7$, $p = 0.027$), with 6.2% higher NPR than Control (Fig. 1). A similar trend was found for the bulk ZnO treatments. NPR in mZnO10 was 6.0% higher than that in Control, and the difference was statistically significant (ANOVA, $F_3 = 3.7$, $p = 0.031$). The difference between the NPs and the bulk counterpart NPR was not statistically significant at any dose of ZnO.

Fresh biomass of plant

In the ZnO NPs treatments, a significant increase of 6.2% was detected for nZnO10 compared to Control (ANOVA, $F_3 = 5.1$, $p = 0.03$); whereas, no significant increases were found for the other NPs treatments (Fig. 1). A similar trend was observed for the bulk ZnO treatments. No significant differences were found between the ZnO NPs and bulk treatments at any dose.

Zn accumulation in plant tissues

In the ZnO NPs treatments, no significant difference was observed between nZnO1 and Control (Fig. 1, $p > 0.05$), but at the other doses, nZnO10 and nZnO100 significantly increased the plant Zn content by 83.3 and 208.3% as compared to Control (ANOVA, $F_3 = 592.1$, $p = 0.00$). By contrast, in the bulk ZnO treatments, only mZnO100 demonstrated significantly higher Zn content than Control. It was noteworthy that nZnO10 and nZnO100 both significantly increased the Zn content in comparison with their respective bulk counterpart's, with increments of 67.5 and 6.0%.

Soil pH

In the NP treatments, soil pH increased gradually with increasing ZnO doses. A significantly higher pH value was observed in nZnO100 compared to that in Control (ANOVA, $F_3 = 3.9$, $p = 0.016$, Fig. 1). A similar trend was shown for the bulk ZnO treatments. No significant pH differences were found between the ZnO NPs and bulk treatments at any dose.

General taxonomic distribution of rhizospheric soil bacterial community

The MiSeq platform was used to evaluate the taxonomic distribution of the rhizospheric soil bacterial community. After annotation, there were totally 25 phyla classified. The relative abundances of the dominant phyla and the *Proteobacterial* orders were shown in Fig. 2. *Proteobacteria* (39.26–48.79%) were the most prevalent phylum across all samples, followed by *Bacteroidetes* (10.48–15.77%), *Cyanobacteria* (5.79–18.62%), *Actinobacteria* (5.96–8.99%), *Firmicutes* (5.94–11.24%), *Acidobacteria* (5.22–6.68%), *Chloroflexi* (1.90–2.45%), *Gemmatimonadetes* (1.7–2.15%), *Planctomycetes* (1.4–2.17%), and *Nitrospirae* (0.79–1.43%). Other phyla, such as *Armatimonadetes*, *Elusimicrobia*, *Chlorobi*, and *Fibrobacteres*, collectively represented only 0.01–0.39% of the total sequences per sample. In the phylum *Proteobacteria*, *Alpha-*, *Beta-*, *Gamma-*, and *Delta-* were the dominant classes, accounting for 12.05–13.84%, 10.85–14.60%, 2.15–2.82%, and 13.58–18.50% of the total sequences per sample, respectively.

According to the one-way ANOVA test of the relative abundances of the dominant phyla, the most remarkable variation was reflected in the phylum *Cyanobacteria*, whose proportion was significantly higher in nZnO10 and mZnO10 compared to Control (ANOVA, $F_3 = 4.7$, $p < 0.05$). By contrast, no significant differences were found for all the dominant phyla between Control and the other doses (1 and 100 mg/kg), regardless of whether ZnO NPs or bulk ZnO.

The Chao1 and phylogenetic diversity (PD) indices were calculated to represent the total OTU diversity and community richness, respectively. However, there were no significant differences among any treatments using these indices (Fig. S2).

Shifts in bacterial community structure

Variations in the bacterial community across different samples were evaluated using a matrix of an NMDS ordination plot, with the Bray-Curtis distance metric (Fig. 3, stress = 0.12). In general, the nZnO10 and mZnO10 treatments separated from all others. Moreover, a division of the nZnO10 and mZnO10 bacterial communities was also observed, illustrating the dissimilarity of their bacterial community structure. In contrast, at the other doses (1 and 100 mg/kg), ZnO NPs and the bulk treatments clustered together along with Control. Such variations were consistent with the results from adonis analyses (Table S2). These pairwise comparisons showed that nZnO10 and mZnO10 had the greatest effects on the rhizospheric bacterial community, as they had significantly lower p values in comparison to other treatments ($p < 0.05$). In this regard, the bacterial compositions of these two treatments were further characterized in comparison with Control.

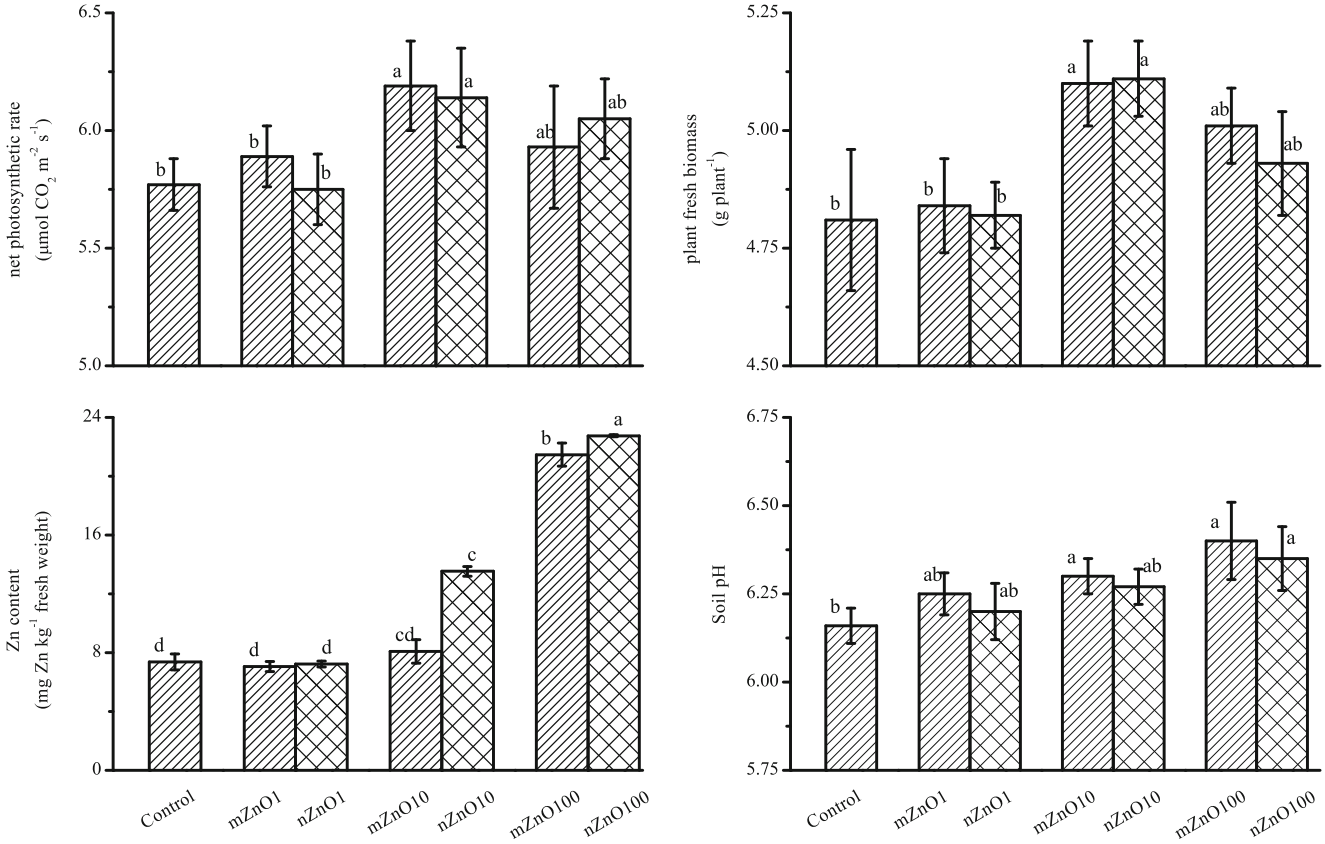

Fig. 1 Influence of ZnO NPs and bulk ZnO at gradient concentrations on the net photosynthetic rate (NPR) of plant leaves, plant biomass, Zn content in lettuce tissues, and soil pH. Error bars represent the standard deviation of the mean ($n = 3$). Different letters indicate significant differences among treatments at the 0.05 level

Bacterial OTU variations between nZnO10 and mZnO10

SIMPER analysis was used for pairwise comparison of the variations in rhizospheric bacterial community (at OTU level) between mZnO10 and Control, as well as between nZnO10 and Control. Top 50 OTUs contributed to approximate 37.7 and 41.5% of the total variation for mZnO10 and nZnO10, respectively (Tables S3 and S4). Among those, 39 OTUs were shared by mZnO10 and nZnO10, and the subsequent analyses of response ratio (Luo et al. 2006) were carried out among them (Fig. 4). The 95% confidence intervals (CI) were calculated to determine the effects of nZnO10 or mZnO10 on the specific groups. If the OTU's 95% CI range is higher than zero (without overlapping zero), the treatment can be concluded to have a positive and significant effect on the specific bacterial group. Alternatively, negative 95% CI equates to negatively significant effect. As compared to Control, nZnO10 positively influenced some genera within *Cyanobacteria* [such as *Nostoc* (OTU8), *Scenedesmus* (OTU28), and *Scenedesmus*

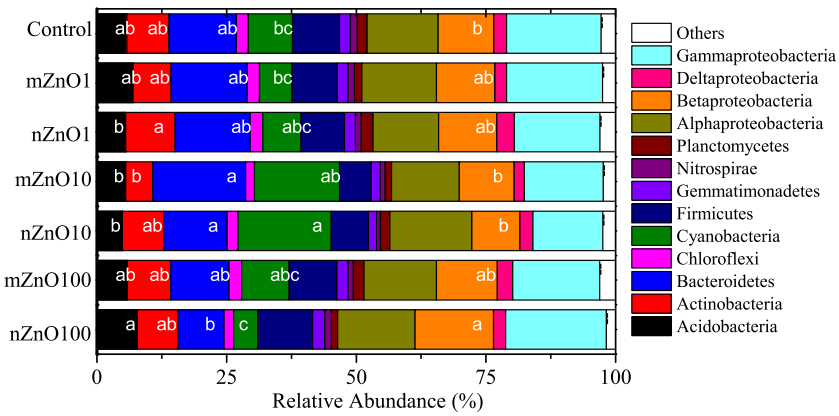

Fig. 2 The 100% stacked column chart of relative abundances of dominant bacterial phyla (or Proteobacterial class) derived from 16S rRNA genes in soil. Different letters for the same phyla (or Proteobacterial class) indicate significant differences at the 0.05 level

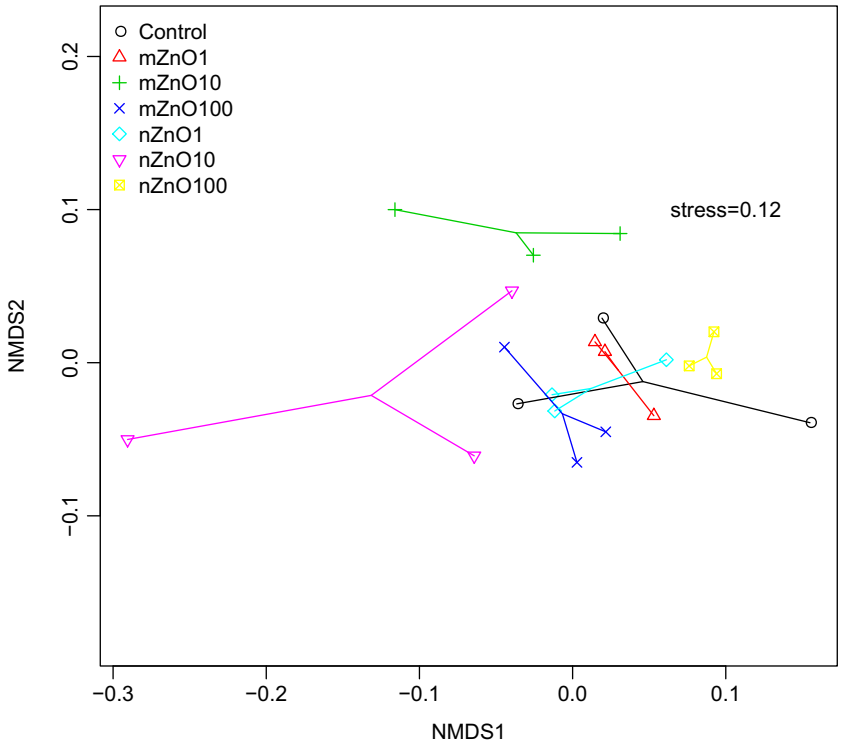

Fig. 3 Non-metric multidimensional scaling (NMDS) plots obtained from pairwise Bray-Curtis to compare the bacterial communities in different treatments

(OTU28)], but inhibited others [*Geitlerinema* (OTU13) and *Leptolyngbya* (OTU154)]. In other phyla, *Xanthomonadales* (OTU3), *Cytophagaceae* (OTU4) were positively influenced by both nZnO10 and mZnO10.

Some lineages displayed different responses to nZnO10 than they did to mZnO10. For example, *Rhodanobacter* (OTU1), *Chitnophagaceae* (OTU10), *Rhizomicrobium* (OTU45), and *Nitrospira* (OTU14) responded negatively to

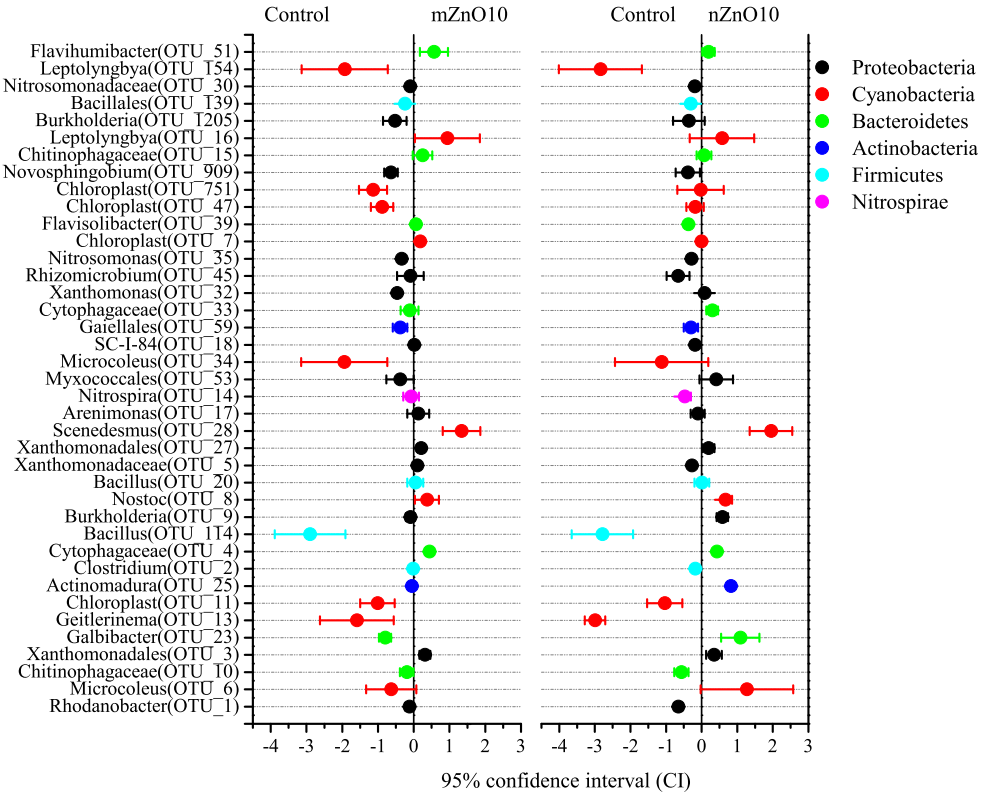

Fig. 4 Significantly changed OTUs in the responsive lineages in the presence of bulk ZnO (left) and ZnO NPs (right) by using the response ratio method at 95% confidence interval (CI). If the 95% CI range of a specific group is higher than zero (without overlapping zero), the treatment can be concluded to have a positively significant effect on the specific group. Alternatively, if the 95% CI range of a specific group is lower than zero (without overlapping zero), the treatment can be concluded to have a negatively significant effect on the specific group

nZnO10 but were not significantly influenced by mZnO10. Alternatively, *Galbibacter* (OTU23) and *Burkholderia* (OTU9) responded positively to nZnO10 but negatively to mZnO10.

Discussion

ZnO NPs influence plant development in a dose-dependent manner

Zn is an important micronutrient for plant growth due to its roles in photosynthesis, protein synthesis, fertility, and seed production, as well as in defense against disease (Frassinetti et al. 2006). ZnO NPs may dissolve to Zn ions, becoming available for organisms to utilize. A previous study shows that amendment with ZnO NPs increases cucumber leaf count and enhances the dry weight of cucumbers plant by 10.5%, even at a dose of 400 mg ZnO/kg soil (Zhao et al. 2013). Alternatively, a negative influence of ZnO NPs is found for corn plant under the same dose (Zhao et al. 2015). In this study, the greatest increase of lettuce plant biomass was only ~6% (for mZnO10 and nZnO10). This suggests that the impact of ZnO NPs on plant growth may strongly depend on the plant species (Garcia-Gomez et al. 2017). Moreover, Zn compounds influence the plant development in a dose-dependent manner (Reddy et al. 2016). This is because most plants require only 0.05 mg/L of Zn in soil solution for normal growth (Liu and Lal 2015). At optimal dose, Zn shows a potential for propitious impact on plants. Overdosing of Zn could delay the plant development by increasing the abnormal plant cells (Shaymurat et al. 2012), damaging the plant's photosynthetic apparatus (Mousavi Kouhi et al. 2015), and decreasing the chlorophyll content of plants (Zhao et al. 2015). As to the dose gradients tested herein, only 10 mg ZnO/kg soil (regardless of whether NPs or bulk) significantly benefited the plant biomass and NPR (Fig. 1), suggesting10 mg/kg as the optimal dose for lettuce growth. Although plant physiological parameters were not determined in this study, the decreasing trends in plant growth and NPR from 10 to 100 mg/kg dose implied a potentially negative effect of ZnO particles on plants (Fig. 1). Similarly, adverse effects of ZnO NPs were also found on soybean (Yoon et al. 2014) and alfalfa plants (Bandyopadhyay et al. 2015).

We further found that at doses of 10 and 100 mg/kg (Fig. 1), ZnO NPs significantly enhanced the amount of Zn in plant tissues as compared to their bulk counterpart. Rico et al. (2011) show that aside from zinc ions, ZnO NPs are bioavailable to plants. Thus, it is deserved to discuss whether the higher Zn content in plant tissue is caused by the ZnO NPs themselves, increased dissolution to Zn ions, or some combination thereof (Bandyopadhyay et al. 2015; Liu et al. 2015). NPs are found to be prone to interact with the soil components (such as organic matter and clay) through agglomeration and sorption (Frenk et al. 2013; Ge et al. 2011; Heggelund et al. 2014; Mohd Omar et al. 2014; Servin et al. 2015), which reduce their possibility of direct absorption by plants. Moreover, considering the size of the ZnO nanoparticle used in this study (90 nm; Fig. S1), and that the plants are more likely to preferentially take up Zn ions instead of ZnO NPs (Liu et al. 2015), we infer that dissolution of ZnO NPs to Zn ions is what led to the higher Zn content in plant tissues, and not the ZnO NPs themselves. Previous studies also show that ZnO NPs often released more dissolved Zn ions than the bulk ZnO at the same dose (Savoly et al. 2016; Zhai et al. 2017), i.e., approximately 90 mg/L Zn ions in ZnO NPs suspension (pH 6.3) versus 60 mg/L in bulk counterpart under the same concentration of this study. This trait potentially explained our observation that the plant Zn content was higher in the ZnO NPs treatment.

Another concern is whether the higher Zn content in plants could pose a risk to human health upon consumption. In fact, ZnO is generally recognized as a safe (GRAS) chemical when at reasonable doses (Dimkpa 2014), and the safety level for Zn in most frequently consumed food products is 26 mg/kg according to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (WHO/FAO 2011). The Zn concentrations in plants (21.5–22.7 mg/kg fresh weight) observed here were under the WHO/FAO safety level, which suggests the safety of ZnO application herein.

ZnO NPs impact the rhizospheric bacterial community composition

Metal oxides, including nano and bulk ZnO, influence specific microorganisms or the soil bacterial community in a dose-dependent manner (Kasemets et al. 2009; Simonin et al. 2017). As to the soil bacterial community, such dose-dependent pattern, is not a classical curve (i.e., linear or sigmoidal) and is instead more a hump-shaped curve. Research thus suggests that studies should include very low doses of NPs when evaluating their impacts (Simonin et al. 2017). In this study, although no significant variations were detected for the alpha-diversity indices of soil bacterial community (Fig. S2), the community compositions were indeed influenced by the low dose of ZnO NPs, especially at 10 mg/kg dose, as reflected by NMDS (Fig. 2) and adonis analyses (Table S2). Since plant exerts selection on the microbial community in the rhizosphere based on particular functional traits (Yan et al. 2017), and plant developments here responded profoundly to the 10 mg/kg dose, we supposed that the most likely reason for the broad effects of 10 mg/kg doses were associated with plant response. For this reason, the following taxonomic comparisons were conducted between the10 mg/kg NPs/bulk treatment and Control.

When soil was exposed to ZnO particles, some bacterial lineages responded similarly to both the NPs and the bulk counterparts. The most remarkable variations were reflected in the phylum *Cyanobacteria* and some of its subgroups [i.e., *Nostoc* (OTU8) and *Scenedesmus* (OTU28)]. This phenomenon might be ascribed to the important role of Zn in promoting the proliferation of *Cyanobacteria* (Xu and Juneau 2016). Considering that both ZnO NPs and the bulk ZnO primarily influence the microorganism through the release of Zn ions (Kasemets et al. 2009), such release processes are likely to mask the size-related effects of ZnO, resulting in the insignificant differences of *Cyanobacterial* groups between nZnO10 and mZnO10. Besides, ZnO particles, regardless of whether NPs or bulk, elevated the soil pH (Fig. 1). This change could result in the negative response of some lineage preferring low pH value, such as *Rhodanobacter* (Gammaproteobacteria) (Wen et al. 2017).

Despite those, ZnO NPs influenced some lineages to a greater extent than the bulk counterpart. For example, lineages such as *Rhizomicrobium* (*Alphaproteobacteria*) and *Nitrospira* (*Nitrospirae*) were inhibited by nZnO10, but not mZnO10. One possible reason was that ZnO NPs have higher rates in dissolving to Zn ions compared to bulk ZnO (Savoly et al. 2016; Zhai et al. 2017). Also, ZnO NPs may produce reactive oxygen species (ROS) which can damage cellular lipids, carbohydrates, proteins, and DNA, leading to cellular oxidative stress response (Kasemets et al. 2009; Xia et al. 2008). Thus, the inhibitory effects on those lineages were more obvious in the NPs treatment.

Given the increased plant biomass and NPR in nZnO10 and mZnO10, it was possible that some bacterial groups positively associated with plant growth could be stimulated. Indeed, some lineages (such as *Cyanobacteria*, *Burkholderia*, and *Xanthomonadales*) responded positively to nZnO10 and/or mZnO10, which might potentially influence the C pool as well as the nutrient cycling in soil. For instance, *Cyanobacteria* and *Burkholderia* (Reddy et al. 1993; Zhang et al. 2016) are capable of fixing atmosphere N. *Xanthomonadales* possess solubilization and oxidative ability to increase the phosphate and/or sulfur contents within soil (Campbell et al. 2010; Jangid et al. 2008). Some plant promoting traits are also reported for *Nostoc* (Kennedy et al. 2005; Sciuto and Moro 2015). The varying responses of those lineages to ZnO NPs and the bulk ZnO suggested the different microbial mechanisms involved in improving plant growth. Given the complexity of soil matrix and the functional redundancy of soil microorganisms, we surmise that the improved plant performances might be the outcome of the trade-offs of rhizospheric soil microbiota.

Conclusions

Collectively, our study demonstrates that at an optimal dose (10 mg/kg), ZnO NPs could improve plant growth (i.e., increase plant biomass and net photosynthetic rate). A higher Zn content was observed for the NPs treatment as compared to the bulk ZnO at doses of 10 mg/kg or higher. As for underground observations, the 10 mg/kg dose impacted the soil bacterial community structure more profoundly (regardless of NPs or bulk ZnO). Some lineages within *Cyanobacteria* and other phyla individually demonstrated similar or different responses to ZnO NPs and bulk ZnO. In short, the improved plant growth, together with the shifted bacterial community components, suggested the potential benefit of ZnO NPs at the optimal dose (10 mg/kg) in agriculture. Further research associated with the bacterial functions would be greatly helpful for elucidating the influence of ZnO NPs on the plant-soil ecosystem.

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