



Long-term effects of ZnO nanoparticles on exoenzyme activities in planted soils

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) have been used as additives in a variety of consumer products. While these particles may enter the environment, only a limited number of studies have investigated the effects of ZnO NPs on soil exoenzymes. Here, we investigate the long-term effects of ZnO NPs at concentrations of 50 and 500 mg/kg on the activities of six soil exoenzymes in planted soils: Dehydrogenase, fluorescein diacetate (FDA) hydrolase, urease, acid phosphatase, arylsulfatase, and β -glucosidase. Significant effects were observed at one or more time points for all enzymes except for FDA hydrolase. These effects included both decreases and increases in enzyme activity. Our results suggest that ZnO NP treatments of 50 and 500 mg/kg can adversely affect soil enzymes, particularly acid phosphatase and urease, and thus, these data may have implications for phosphorous and nitrogen cycles in the soil.

Keywords: Exoenzyme, OECD standard soil, Soil, Zinc oxide nanoparticles

1. Introduction

Zinc oxide nanoparticles (ZnO NPs) have been widely used in sunscreens and cosmetic products and as catalysts, gas sensors, and food additives [1, 2]. The widespread use of ZnO NPs increases the probability of their entry into the environment through production, transportation, and disposal activities [3].

Soil exoenzyme activities are related to the dynamic properties of soil ecosystems, including the soil microbial communities and nutrient cycles [4], and such enzymes have been used as indicators of soil quality [5]. Recently, several studies have been conducted to evaluate the effects of NPs on various enzyme activities of soil microbes. He et al. [6] investigated the short-term effects of single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) on soil enzymes activity and soil respiration, and then observed the inhibition of alkaline phosphatase and invertase. Tong et al. [7] reported that fullerene (C₆₀) had no adverse effects on the activities of dehydrogenase, phosphatase, β -glucosidase, and urease. Hänsch and Emmerling [8] did not observe any effects of silver NPs on the activities of leucine-aminopeptidase, β -cellobiohydrolase, acid phosphatase, β -glucosidase, chitinase, and xilosidase; however, Shin et al. [9] observe negative effects of silver NPs on the activities of de-

hydrogenase, fluorescein diacetate (FDA) hydrolase, urease, acid phosphatase, arylsulfatase, and β -glucosidase. Chung et al. [10] showed that 5,000 μ g of MWCNTs per gram of soil could inhibit the activities of phosphatase, β -N-acetylglucosaminidase, β -glucosidase, cellobiohydrolase, and xylosidase. Cullen et al. [11] and Fang et al. [12] found positive or negligible effects of zero-valent iron NPs and α -Fe₂O₃ and Fe₃O₄ NPs on dehydrogenase, FDA hydrolase, urease, acid phosphatase, amylase, and catalase. Du et al. [13] found negative effects of TiO₂ and ZnO NPs on the activities of protease, catalase, and peroxidase, but no effects were observed on urease activity in the soil after harvest of the planted wheat. Kim et al. [14] reported that ZnO NPs can affect the activities of dehydrogenase and acid phosphatase, but they found no significant effects on β -glucosidase in the soil after the harvest of *Cucumis sativus*. While soils sustaining plant growth affect the soil microbial communities, we could only find three studies that have evaluated soil enzyme activities using NPs in planted soils [13-15].

In this study, the toxic effects of ZnO NPs were evaluated in planted soils by evaluating the extent of inhibition of the activities of six exoenzymes: Dehydrogenase, FDA hydrolase, urease, acid phosphatase, arylsulfatase, and β -glucosidase. General microbial activities were studied by measuring the activities of de-



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hydrogenase and FDA hydrolase, whereas the dynamics of nitrogen, phosphorus, sulfur, and carbon cycles were assessed by the activities of urease, acid phosphatase, arylsulfatase, and β -glucosidase, respectively. To the best of our knowledge, this is the first study showing the effects of ZnO NPs during the evaluations of exoenzyme activities in the OECD (Organization for Economic Co-operation and Development) standard soil.

2. Materials and Methods

2.1. Nanoparticle Preparation in Soil

The ZnO NPs were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), and the material had a purity of > 97% and a particle size < 50 nm. The ZnO NPs were mixed at specified doses into the test soil, which was composed of OECD standard soil [16]. Per OECD recommendations, peat moss was used as the organic matter component. However, because peat moss can contain enzyme activity, it was air-dried at room temperature for 3 days prior to use. Soil was prepared with 0, 50, and 500 mg ZnO NPs per kg soil (on a dry soil basis) from stock soils containing 100 times of each test concentration. Stock soils of ZnO NPs were made by alternating layers of OECD standard soil with ZnO NP powder, and then, the test soil was mixed evenly using a hand mixer for 10 min and a roller mixer for 4 h. Although tested concentration of 500 mg ZnO NPs per kg soil may be not environmentally relevant, accidental exposure scenario was considered in this study.

2.2. Plantings

Soybean seeds (*Glycine max* (L.) Merrill) were purchased in 2010 from Paju Agricultural Products Corporation (Kyunggi-do, Korea). The seeds were surface-sterilized in a 5% sodium hypochlorite solution for 3 min and then rinsed five times in sterile distilled water. Each pot was filled with 2 kg (dry weight) of OECD standard soil enriched with ZnO NPs (as mentioned above). In all treatments, 1,111 mL of N-free Hoagland's solution [17] was sprayed onto the soil, and then, the soil was aged under dark conditions in a greenhouse for 9 days. After aging, holes for planting the seeds were made at a depth of 1 cm from the surface soil, and a seed was planted in each hole. Five pots having one seed were prepared for each treatment. Every 3 ± 1 d, water with N-free Hoagland's solution (pH 6.0) or tap water was added to maintain overall 35% (v/w) water content. The pots were rotated every day and maintained in a greenhouse at 25-30°C. Details of plantings and effects of ZnO NPs on soybean plants were described in the previous study from same research group [18].

2.3. Soil Sampling for the Exoenzyme Assay

Measurements of soil exoenzyme activities were performed on samples from all the pots on days 0 (after aging), 28, and 56. Composite test soil samples were collected from each pot in the cardinal directions at depths of 3-4 cm from the surface of the soil. A total of five replicates (one replicate represents a single pot) were prepared for each treatment. The soil samples were stored in a refrigerator at 4°C prior to analyses.

2.4. Assay for Soil Exoenzymes

This study evaluated the activities of six different soil exoenzymes including dehydrogenase (EC 1.1), FDA hydrolase, urease (EC 3.5.1.5), acid phosphatase (EC 3.1.3.2), arylsulfatase (EC 3.1.6.1), and β -glucosidase (EC 3.2.1.21). Acid phosphatase, arylsulfatase, and β -glucosidase activities were determined according to the procedures described in Tabatabai et al. [19]. Dehydrogenase activity was measured following the procedure described in Gong [20] and Brohon et al. [21]. FDA hydrolase activity was determined according to the procedures described in Adam and Duncan [22]. Urease was measured following the procedure described in Kandeler and Gerber [23]. TRIS buffer (1M, pH 8.0), potassium phosphate buffer (pH 7.6), borate buffer (0.05M, pH 10.0) and acetate buffer (pH 5.8) solution were used to measure the activities of dehydrogenase, FDA hydrolase, urease and arylsulfatase, respectively. MUB (pH 6.5) was used for acid phosphatase and β -glucosidase assays. All tests for soil exoenzyme activities included blanks for each treatment to exclude the absorbance of the soil, and these sterile blanks were prepared by autoclaving samples at 121°C for 15 min or by injecting formaldehyde into the sample. The results for soil exoenzyme activities were corrected with the results obtained from the blank samples (i.e., blank data were subtracted from sample data). In addition, the results were quantified in manner that allowed for consideration of the water content; this was done by use of a calibration curve.

2.5. Statistical Analyses

Dunnett's test was used to determine which treatment results were different from the control values; a 95% significance level ($p < 0.05$) was used for all comparisons [24].

3. Results and Discussion

3.1. Effects of ZnO NPs on the Activities of Acid Phosphatase and Urease

Fig. 1(a) and Table 1 show activities of acid phosphatase throughout the exposure periods. Nannipieri et al. [25] reported that the quantity of acid phosphatase in natural soils was 0.05-86.33 $\mu\text{mol } p\text{-nitrophenol per gram per hour}$. Compared to that study [25], the concentrations of acid phosphatase measured in this study for OECD standard soil were of a reasonable level (average, approximately 34.9-44.4 $\mu\text{g } p\text{-nitrophenol per gram}$, which can be converted to be 0.89-1.14 $\mu\text{mol } p\text{-nitrophenol per gram}$). Activities of acid phosphatase were mostly inhibited during exposures to 500 mg ZnO NPs per kg dry soil for 28 and 56 d. Specifically, the activities were inhibited by 35% after 28 d and by 46% after 56 d. Kim et al. [14] reported that the acid phosphatase activities decreased during exposures to 2,000 mg ZnO NPs per kg dry soil in post-harvest soil after planting for 8 weeks. Overall, these results indicate that ZnO NPs can affect the hydrolysis capacity for the conversion of organic phosphate ester to orthophosphate in the phosphorous cycle, which may influence the amount of phosphorus available to living organisms in the soil over long-periods of time.

Table 1. Activities of Six Soil Exoenzymes (Acid Phosphatase, Urease, β -glucosidase, Arylsulfatase, Dehydrogenase, Fluorescein Diacetate Hydrolase) in the Presence of ZnO Nanoparticles (NPs) in Planted Soil on 0, 28, and 56 d ($n = 5$). Soil Enzyme Activities Were Expressed as Products (μg) Released by Enzyme-substrate Reactions in Dry Soil

Enzyme	NPs (mg/kg)	Product (μg) released by enzyme-substrate reactions in dry soil ¹		
		Day 0	Day 28	Day 56
Acid phosphatase	0	44.1 (34.1-54.1)	44.4 (39.2-49.6)	34.9 (27.7-41.9)
	50	58.9 (51.6-66.0)	39.3 (34.9-43.7)	33.9 (25.3-42.5)
	500	33.6 (22.1-45.0)	28.9 (26.5-31.3)	19.6 (13.6-25.6)
Urease	0	2.1 (1.8-2.4)	2.6 (2.4-2.8)	4.7 (3.4-6.0)
	50	2.4 (1.8-3.0)	2.5 (1.5-3.5)	3.7 (3.2-4.2)
	500	1.6 (1.5-1.7)	1.9 (1.7-2.1)	5.2 (4.1-6.3)
β -Glucosidase	0	3.6 (3.4-3.8)	2.7 (2.1-3.3)	4.6 (3.5-5.7)
	50	3.8 (3.6-4.0)	2.4 (2.1-2.7)	4.4 (3.2-5.6)
	500	2.5 (2.4-2.6)	2.2 (1.8-2.6)	4.1 (3.2-5.0)
Arylsulfatase	0	1.2 (1.0-1.4)	2.1 (1.8-2.4)	2.6 (1.8-3.4)
	50	1.3 (1.0-1.6)	1.5 (1.0-2.0)	1.9 (1.4-2.4)
	500	1.3 (0.7-1.9)	2.1 (1.8-2.4)	2.4 (1.9-2.9)
Dehydrogenase	0	9.1 (8.8-9.4)	14.7 (11.7-17.7)	7.0 (5.4-8.6)
	50	9.6 (9.3-9.9)	14.6 (13.7-15.5)	8.8 (6.2-11.4)
	500	9.0 (8.5-9.5)	16.7 (16.3-17.1)	12.5 (11.7-13.3)
Fluorescein diacetate (FDA) hydrolase	0	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.3 (0.1-0.5)
	50	0.2 (0.1-0.3)	0.3 (0.1-0.5)	0.4 (0.2-0.6)
	500	0.1 (0.0-0.2)	0.3 (0.1-0.5)	0.4 (0.2-0.6)

¹ Units of measured values are μg iodinitrotetrazolium formazan g^{-1} dry soil for dehydrogenase, μg fluorescein g^{-1} dry soil for FDA hydrolase, μg NH_4 g^{-1} dry soil for urease, and μg p -nitrophenol g^{-1} dry soil for acid phosphatase, arylsulfatase, and β -glucosidase.

Activities of urease throughout the exposure periods are shown in Fig. 1(b) and Table 1. Activities of urease decreased during exposures to 500 mg ZnO NPs per kg dry soil for 0 and 28 d. Specifically, the activities were inhibited by 23% on day 0 and by 25% after 28 d. Increased urease activities on day 56 were likely due to adaptation responses to chemical stress that are known occur in microbial communities [26-28].

3.2. Effects of ZnO NPs on the Activities of β -glucosidase, Arylsulfatase, Dehydrogenase, and FDA Hydrolase

Activities of β -glucosidase, arylsulfatase, dehydrogenase, and FDA

hydrolase throughout the exposure periods are shown in Fig. 1 and Table 1. Activities of β -glucosidase decreased by 29% in the presence of 500 mg ZnO NPs per kg dry soil on day 0. Except for these results, the effects of ZnO NPs on β -glucosidase were not significant throughout the experimental period. Additionally, the activities of arylsulfatase decreased by 31% in planted soils treated with 50 mg ZnO NPs per kg dry soil for 28 d, but except for this case, no other effects were found for this enzyme. No inhibitory effects of ZnO NPs on dehydrogenase activities were observed in this study. We observed that the activities of dehydrogenase in planted soils

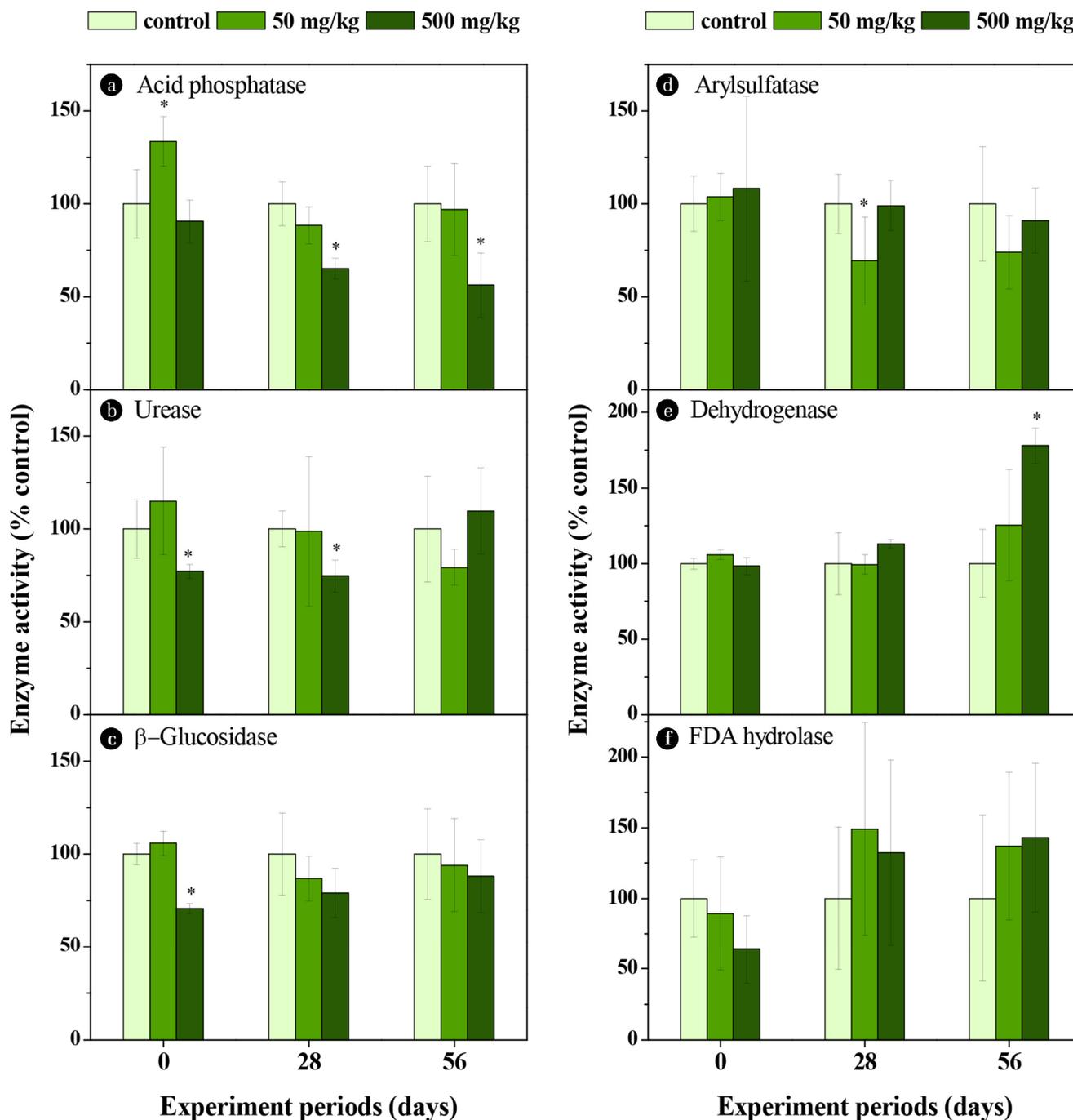


Fig. 1. Relative activities of (a) acid phosphatase, (b) urease, (c) β -glucosidase, (d) arylsulfatase, (e) dehydrogenase, and (f) fluorescein diacetate (FDA) hydrolase as a percentage of the mean of the control treatments exposed to 0, 50, and 500 mg ZnO nanoparticles (NPs) per kg soil for 0, 28, and 56 d. Values are the means of five replicates \pm standard deviation. (*Asterisks represent significantly lower or higher activity than the control at different ZnO NP concentrations, where $p < 0.05$).

treated with 500 mg ZnO NPs per kg dry soil for 56 d increased. The reason is unclear, however, it may be related to the soil pH. The optimum pH for dehydrogenase activities is 8.0, and the soil pHs of exposed and control soils were 6.2 ± 0.2 , and

5.6 ± 0.2 , respectively. Furthermore, only very small amounts of FDA hydrolases were found in the soil used in this study, and no differences were observed between the blank and test group enzyme activities.

4. Conclusions

This study evaluated the effects of ZnO NPs on six soil exoenzymes and found that the activities of acid phosphatase and urease were particularly sensitive to the inhibitory effects of the NP treatments used in this study. Given the past biochemical studies on zinc [29-31], it is likely that zinc interacted with the sulfhydryl groups at the active site of urease, which then led to decreases in the catalytic activity, but there are few mechanistic studies detailing how zinc reacts with acid phosphatase. Generally, metals can affect enzyme activities by interfering with the enzyme-substrate complex through a variety of changes such as alterations in the proteins conformational structure and displacements of metals at the active sites [32-36]. Future mechanistic studies aimed at elucidating how ZnO NPs interact with acid phosphatase would be valuable. Lastly, the adverse effects of ZnO NPs on soil enzymes observed in this study suggest that these contaminants may influence the phosphorous and nitrogen cycles in the soil. This warrants future research attention as well.

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