



Bioavailability of coated and uncoated ZnO nanoparticles to cucumber in soil with or without organic matter



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ABSTRACT

There is a gap of knowledge for the fate, effects and bioavailability of coated and uncoated ZnO nanoparticles (NPs) in soil. Moreover, little is known about the effects of soil properties on effects of NPs on plants. In this study, the availability ZnO NPs in two soils with different organic matter content (one treated with cow manure (CM) and the other as untreated) was compared with their bulk particles. Results showed that coated and uncoated ZnO NPs can be more bioaccessible than their bulk counterpart and despite their more positive effects at low concentration ($< 100 \text{ mg kg}^{-1}$), they were more phytotoxic for plants compared to the bulk ZnO particles at high concentration (1000 mg kg^{-1}) in the soil untreated with CM. The concentration of 1000 mg kg^{-1} of ZnO NPs, decreased shoot dry biomass (52%) in the soil untreated with CM but increased shoot dry biomass (35%) in CM-treated soil compared to their bulk counterpart. In general, plants in the CM-treated soil showed higher Zn concentration in their tissues compared with those in untreated soil. The difference in shoot Zn concentration between CM-treated and untreated soil for NPs treatments was more than bulk particles treatment. This different percentage at 100 mg kg^{-1} of bulk particles was 20.6% and for coated and uncoated NPs were 37% and 32%, respectively. Generally, the distribution of ZnO among Zn fractions in soil (exchangeable, the metal bound to carbonates, Fe-Mn oxides, organic matter and silicate minerals and the residual fraction) changed based on applied Zn concentration, Zn source and soil organic matter content. The root tip deformation under high concentration of NPs (1000 mg kg^{-1} treatment) was observed by light microscopy in plants at the soil untreated with CM. It seems that root tip deformation is one of the specific effects of NPs which in turn inhibits plant growth and nutrients uptake by root. The transmission electron microscopy image showed the aggregation of NPs inside the plant cytoplasm and their accumulation adjacent to the cell membrane.

1. Introduction

Nanoparticles (NPs) are suggested to be used as a source of nutrients required for plant growth (Monreal et al., 2015). Available data suggest that NPs are more bioreactive than bulk counterpart due to their smaller size and greater surface area (Dietz and Herth, 2011). These properties can also increase the NPs risks for environment health. Previous studies have shown both positive and negative effects of NPs on plants (Moghaddasi et al., 2013; Zhang et al., 2007). Adverse effects of copper oxide NPs i.e., growth retardation, reduced biomass production, lower root length, shrinking of root tip, and high collapse of root epidermal and cortical cells of *Arabidopsis thaliana*, have been reported (Nair and Chung, 2014). The effect of NPs on plants depends

on their properties, dose and method of application and plant species (Lin and Xing, 2007; Zhang et al., 2007; Torney et al., 2007). Dissolution, aggregation and change in the surface properties of ZnO NPs in solid matrices might modify bioactivity of these particles (Moghaddasi et al., 2015). The main reactions of NP in soil-plant systems seems to be dissolution into ions, aggregation of individual NPs into the larger size units, the dissolution of aggregation in soils, interaction with root exudates or labile soil organic matter and adsorption on root surfaces (Monreal et al., 2015).

To date, a great number of studies have been conducted on artificial plant growth media while the final destination of all engineered NPs is in soil. In addition, several factors in soil influence the fate and effects of NP. For example soil contains a wide variety of solutes and colloidal

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materials including dissolved organic carbon, which may coat engineered nanomaterials (NMs) and thus, alter their interactions with soil and plant (Lowry et al., 2010). The translocation factor of cerium in the nano-CeO₂ exposed plants grown in organic matter enriched soil was twice as the plants grown in low organic matter soil (Majumdar et al., 2016). The absence of ZnO NPs in shoot of wheat may be explained by their attachment to the soil particle (Dimkpa et al., 2013). In addition, plant uptake of metals from soil depends on both the soluble fraction of total metal and the capability of soil solid pools to release the metals into solution (Backes et al., 1995). Metal availability to plant is, therefore, considerably affected by soil properties particularly the distribution of metal among different fractions. To better understand the metal phytoavailability, knowledge on the metal fractionation in soil solid phase in relation with plant uptake is useful. The method of Tessier et al. (1979) to estimate the bioavailability of heavy metals in soil to plants, provides a potential useful approach to evaluate the bioavailability of engineered NPs. This 5-steps sequential examination method separates the metal of interest into five fractions i.e., the exchangeable, water/acid soluble metal (F₁), the metal bound to carbonates (F₂), the metal bound to Fe-Mn oxides (F₃), the metal bound to organic matter (F₄), and finally the metal bound to silicate minerals and the residual fraction (F₅).

ZnO NPs are one of the most widely used NPs. These NPs are used in personal care products e.g., sunscreens as well as in coatings and paints due to their ultra violet (UV) absorption efficiency and transparency (Lin and King, 2007). On the other hand, the potential adverse effects of NPs on ecosystems as well as human health have increased. Moghaddasi et al. (2013) showed the positive effects of NPs having ZnO at low concentration (1–25 mg kg⁻¹) on cucumber was grown in hydroponic and negative effects of them at higher concentration (250 mg kg⁻¹). According to previous research and more effects of elements on hydroponic systems the concentration of Zn in this study were chosen. Cucumber plant was selected for this experiment due to high growth speed and good response to Zn concentration.

The aim of the present study was to evaluate (1) uptake and toxicity of coated and uncoated ZnO NPs in comparison with bulk ZnO on plant growth, (2) the fractionation and bioavailability of ZnO NPs in comparison with bulk counterpart (3) the impact of organic matter on translocation of Zn supplied in the form of ZnO NPs and their counterpart.

2. Materials and methods

2.1. ZnO and its Nanoparticles

Bulk ZnO powder (99.9%, nominal diameter < 1 μm) was purchased from Sigma–Aldrich. ZnO NPs were synthesized according to the method presented by Hosseini et al. (2011). According to the atmospheric pressure solution evaporation method, 5 g of zinc acetate [Zn (CH₃COO)₂·2H₂O] with 99.5% purity, was heated to 80 °C temperature. After temperature stabilization of the precursor solution, 450 ml stoichiometric ethylene glycol solution (90% purity), was added to the suspension on a drop-by-drop basis. The stirring and heating of the reaction mixture was continued for 3–4 h. The suspension was dried in an oven at 100 °C for 12 h, and then crystallized at 600 °C for 1 h.

For coating NPs, humic acid (HA) solution (500 mg L⁻¹) was obtained by dissolving solid HA (HA technical, purchased SIGMA-ALORICH) in 0.1 M NaOH. The solution pH was adjusted to 5.0 with 0.1 M HCl. Nano-ZnO particles (5 g) were mixed with 1L HA solution in a bottle and shaken for 2 days, after which the suspension was centrifuged at 3500 rpm for 30 min. Wet material due to precipitation was rinsed with deionized water, freeze-dried (Model FD-4, Pishtaz engineering co., Iran), and ground gently (Yang et al., 2009). Characterization of the NPs has been presented in a study by Moghaddasi et al. (2017). Transmission electron microscopy (TEM) (Model EM10C-80KV, Germany) was used to examine the primary particle size distributions of

NPs (coated and non-coated) obtained by randomly sampling 50 particles in their corresponding TEM images. The sample was prepared by placing a droplet of the colloidal suspension (200 mg L⁻¹) on a Cu mesh TEM grid and left to air drying. The zeta potential and hydrodynamic (agglomerate) particle sizes of NP aqueous suspensions were measured by dynamic light scattering (DLS) method using Zetasizer Nano ZS system (Malvern Instruments Ltd.).

2.2. Soil preparation and cucumber cultivation

A bulk soil (100 kg) with silty clay loam texture was collected from surface layer (0–20 cm) of the field of Research Station of Isfahan University of Technology, Isfahan, Iran. Soil samples were air-dried and sieved through a 1 mm mesh prior to experimental use. Half of the oven dried soil was thoroughly mixed with 1% fully matured cow manure. The rest of the soil was considered as un-amended soil. Cow manure was obtained from a local farm with pH 7.3, C:N 3.1, % OC and total N 6.6 and 2.2 respectively. Before adding to the soil, collected manure was air dried for 3 days and sieved through a 2 mm sieve. The soil texture was determined using the wet sieve analysis and hydrometer test (Bouyoucos, 1962). Soil pH was measured potentiometrically in 1 M KCl after 24 h in the liquid/soil ratio of 2.5. The total nitrogen (Nt) was determined by the Kjeldahl's method. The concentration of K, Mg, Ca, and Na were determined according to procedures by Van Reeuwijk, (2002). Total organic carbon (TOC) was determined by TOC-VCSH (SHIMADZU) with Solid Sample Module SSM-5000. Selected characteristics of both untreated and CM treated soils are described in Table 1.

Triplicate subsamples (20, 200 and 2000 g) of coated and uncoated NPs and bulk ZnO were sonicated for 30 min (25 °C, 100 W, 40 kHz) and applied through spraying (100 ml) to the soils to achieve final concentrations of 10, 100, 1000 mg ZnO per kg soil. The control treatment was pots without ZnO application.

Seeds of *Cucumis sativus* L. cv. Dominus were placed in rows on wet blotting paper to germinate for three days. Uniform seedlings were transplanted into plastic pots filled with 2 kg soil and continued their growth for 30 days under greenhouse conditions. The daily photoperiod in the greenhouse was 12 h, with average daily maximum and minimum temperature of 30 °C and 18 °C, respectively. During cultivation, soil moisture was kept near field capacity by weighting the pots. At harvest (30 days after seeding), shoot and roots were separated, washed with deionized water, dried at 70 °C for 48 h and weighed. Plant materials digested by 2 M HCl and Zn concentration in the extracts, were measured by atomic absorption spectrophotometer (AAS) (Perkin Elmer, Model 3030).

Table 1

Physiochemical properties of the study soils. n/a: not available. All values are mean ± SD (n = 3). All measurement had tree replicate.

Parameters	CM-treated soil	Untreated soil	Unit
Coordinates	32°29'N, 52°10'E	32°29'N, 52°10'E	–
pH(CaCl ₂)	5.6 ± 0.3	6.8 ± 0.2	–
Carbonate concentration	0.5 ± 0.06	0.4 ± 0.04	g/100 g dw
Total N	0.5 ± 0.02	n/a	g/100 g dw
Chloride concentration	15 ± 0.6	5.5 ± 0.04	mg/kg dw
Sulfate concentration	8 ± 0.7	n/a	mg/kg g dw
Sulfur concentration	530 ± 28	75 ± 9	mg/kg dw
Total organic carbon	6.5 ± 0.9	0.9 ± 0.01	g/100 g dw
Water holding capacity	35 ± 3	31 ± 2	%
Calcium concentration	21.6 ± 19	3.2 ± 0.7	Cmol(+)/kg
Magnesium concentration	4.2 ± 0.05	1.3 ± 0.01	Cmol(+)/kg
Potassium concentration	0.9 ± 0.02	0.1 ± 0.02	Cmol(+)/kg
Sodium concentration	0.1 ± 0.01	< 0.1 ± 0.01	Cmol(+)/kg

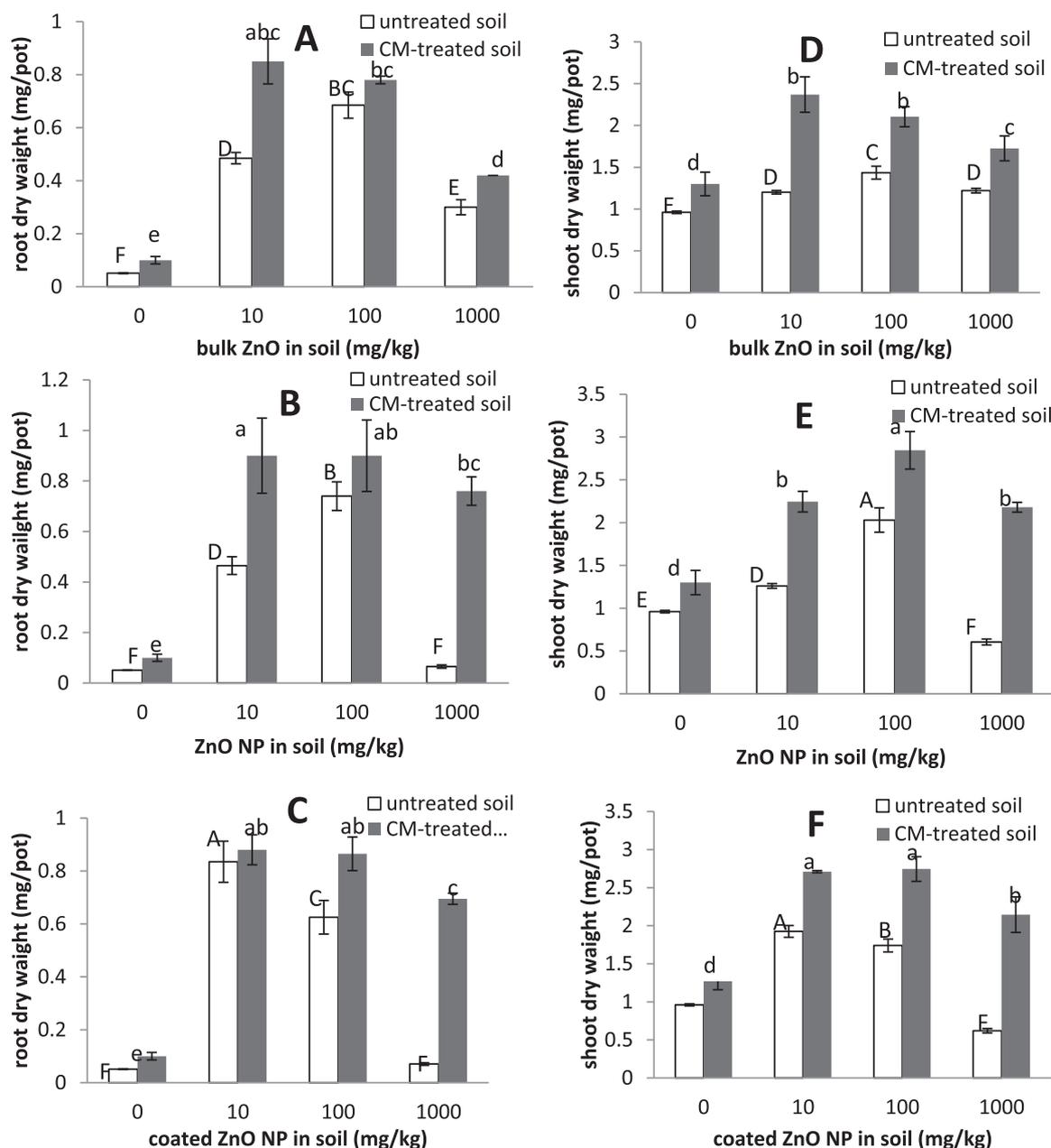


Fig. 1. Root (A, B, C) and shoot (D, E, F) dry biomass percentage of cucumber plants grown in CM-treated or untreated soil treated with 10, 100, 1000 mg of Zn per kilogram soil. The error bars present standard error ($n = 3$). Different letters in root or shoot are separated, in lower case and upper case represent significant differences between the treatments in reached and unreached soil respectively (< 0.05).

2.3. Microscopic observations

The effect of ZnO NPs on root tips of cucumber was observed by light microscopy (LM) (Model E100, German). At harvest, the first 4 cm plant rootlets were longitudinally sliced (500 nm thick), washed with distilled water and prefixed with alcohol (70%) and glycerin. Sliced root samples were washed in sodium hypochlorite (20%) and acetic acid and embedded in carmine for 15 min. The samples were then washed with deionized water and embedded in green methyl for 30 s.

Root and stem samples of cucumber plants from all treatments were washed by running tap water and rinsed with deionized water before scanning Electron Microscopy (SEM) observations. Small cross section roots and stems were dehydrated in an acetone series. SEM photographs were carried for the samples using Philips XL30 Robeahi attached with energy dispersive X-ray unit, with an accelerating voltage of 20 kV.

Root samples were also prepared for TEM according to a standard

procedure (Ni et al., 2005). The cucumber roots were prefixed in 2–4% glutaraldehyde, washed in 0.1 mol/L pH 7.0 phosphate buffer, prefixed in 1% osmium tetroxide, and dehydrated in acetone. Cross sections (65 nm thick) of the root tips were cut for TEM (Model EM10C-80KV, Germany) using a microtome with a diamond knife.

2.4. Zn fractionation in soil

After plant harvesting, rhizosphere soil samples were collected from each pot, air dried and submitted to sequential extraction. The distribution of Zn in different soil solid fractions was determined according to the method described by Tessier et al. (1979) with minor modifications. For this purpose, 1 g of soil was used for initial extraction and after that, in each step the residue sample of last step was used for next extraction. The extracted solutions were stored in 50 ml centrifuge tubes and supernatants were separated from the solid fraction by

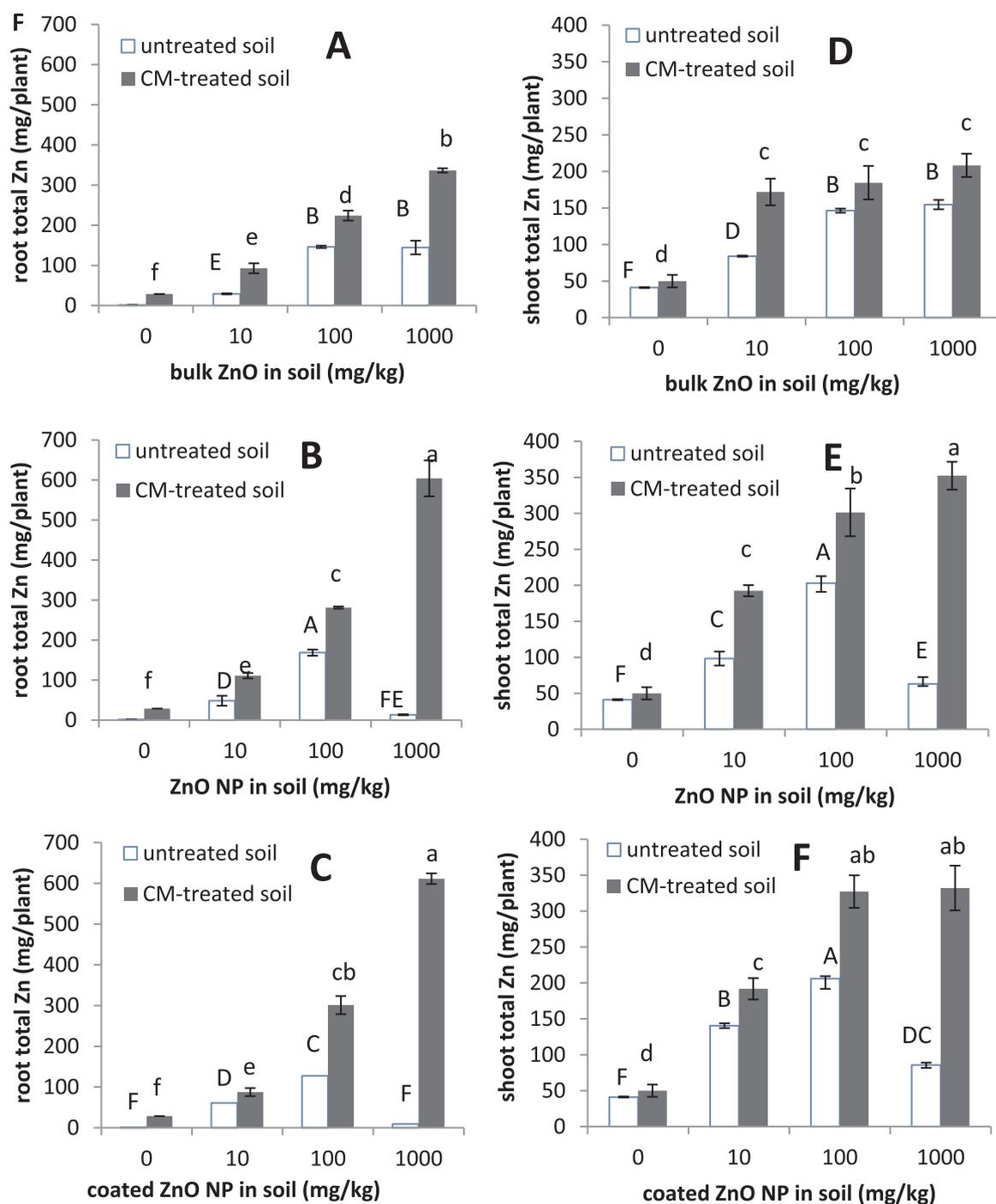


Fig. 2. Root (A, B, C) and shoot (D, E, F) Zn uptake of cucumber plants grown in CM-treated or untreated soil treated with 10, 100, 1000 mg of Zn per kilogram soil. The error bars present standard error ($n = 3$). Different letters in root or shoot are separated in lower case and upper case represent significant differences between the treatments in reached and unreached soil respectively (< 0.05).

centrifugation (15 min at 10,000 g). Water and exchangeable soluble were extracted at room temperature for 1 h with 10 ml of distilled water and magnesium chloride solution (1.0 M) respectively. The residue solution were extracted at room temperature for 1 h with 10 ml of sodium acetat (1.0 M) at pH 5 and named carbonate bound fraction. For extracting Fe/Mn bound, the residual solution were extracted by 10 ml 0.4 M $\text{NH}_2\text{OH}\cdot\text{HCL}$ in 25% (v/v) acetic acid (pH 2.0) with agitation at 96 °C in water bath for 5 h. For extracting organic bound, the residual solution were extracted by 7.5 ml of 30% (v/v) hydrogen peroxide with pH 2 that was heated 85 °C for 5 h. Then 2.5 ml of 3.2 M ammonium acetate in 20% (v/v) nitric acid was added, shaken for 0.5 h and centrifuged. For extracting residual bound fraction, residue from last

fraction were digested with a mixture of 5 ml concentrated HNO_3 (HNO_3 , 70% w/w), 10 ml of hydrofluoric acid (HF, 40% (w/w)) and 10 ml of perchloric acid (HClO_4 , 60% w/w). The overall recovery of zinc at the end of the multi-step procedure was about 90%, and losses were probably due to adsorption to centrifuge tubes, pipettes and funnels. Concentration of Zn in the extracted samples was measured by AAS (Perkin Elmer, Model 3030).

2.5. Statistical analysis

The pot experiment was set up in a completely randomized design with three treatments in triplicates. Treatments effects were analyzed

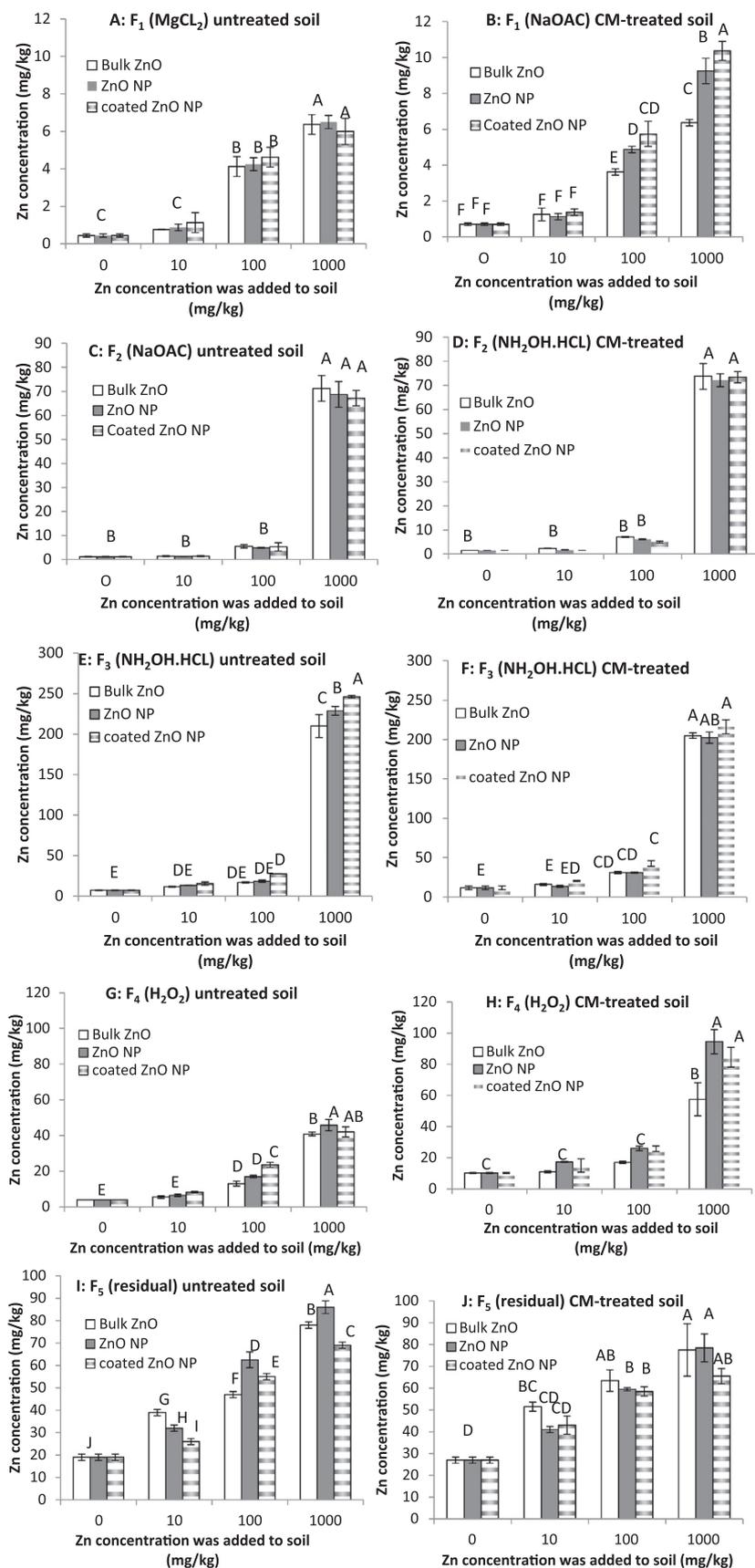


Fig. 3. Relative distribution of ZnO in sequential extraction fractions CM-treated and untreated soils were spiked with either coated, uncoated NPs and their bulk counterparts. The error bars present standard error (n = 3). Identical letters indicate treatments that do not differ significantly (P > 0.05).

by analysis of variance using the GLM procedure. Means were compared using least significant differences (LSD) at $P < 0.05$ (25).

3. Results

3.1. Size and ζ -potential of ZnO NPs

The size of ZnO NPs estimated by TEM was 75 nm. The size of ZnO NPs increased from 75 nm to 100 nm after coating with HA while hydrodynamic diameter of NPs was decreased by coating from 441 to 328 nm. The ζ -potential of non-coated ZnO NPs (20 mg/L ZnO NPs) was +3.1 while it was -15.4 for ZnO NPs coated with HA.

3.2. Root and shoot dry mass

The results revealed an inverse U-shape response of root and shoot to Zn concentrations (Fig. 1). Both 0 mg kg⁻¹ and high (1000 mg kg⁻¹) Zn concentrations resulted in inhibition of plant growth due to Zn deficiency and toxicity, respectively. Increasing Zn concentration in soil, up to 10 or 100 mg kg⁻¹, increased root and shoot dry mass of cucumber, however, application of higher Zn levels had no significant effect or even decreased plant growth.

At high concentration of Zn (1000 mg kg⁻¹) in the soil untreated with CM, phytotoxicity of Zn from NPs (coated and uncoated) was higher as compared with bulk counterpart. Application of 1000 mg kg⁻¹ coated and uncoated ZnO NPs in the soil, untreated with CM, resulted in more than % reduction of shoot growth compared with control. The shoot growth reduction in comparison with the maximum shoot biomass which was obtained at the 10 or 100 mg kg⁻¹ ZnO, was 110% and 230%, respectively (Fig. 1). In contrast to NPs, bulk ZnO at the Zn concentration of 1000 mg kg⁻¹, resulted in 21% higher dry biomass compared with control.

In the soil untreated with CM, the maximum root growth at the coated NPs treatments was more than the uncoated NPs and bulk particle treatments. However in CM-treated soil, no significant difference was found between the coated and uncoated NPs treatment for root dry biomass. In both CM-treated and untreated soils, also no significant difference in root dry biomass was found between the uncoated NPs and their bulk counterparts except at 1000 mg kg⁻¹ treatment. At high Zn rate (1000 mg kg⁻¹), the values of root growth in CM-treated soil were significantly more from the source of ZnO NPs compared to bulk counterparts. In contrast, in untreated soil, root growth reduction from NPs was more than their bulk counterparts.

Regardless of soil organic matter content, no significant difference was found between the coated and uncoated NP treatments for the maximum and minimum shoot biomass, although shoot growth increase at low Zn concentration was greater from NPs (coated and uncoated) than from the bulk ZnO. The stimulating effect of NPs on shoot growth at < 100 mg kg⁻¹ was more than bulk ZnO (32% in untreated soil with CM and 25% in treated soil with CM). The higher concentration of ZnO NPs (coated and uncoated) (1000 mg kg⁻¹) decreased shoot dry biomass (about 52%) when the soil was untreated with CM, however increased it (about 35%) for the CM-treated soil, compared to their bulk counterparts.

3.3. Zn uptake by root and shoot

The effect of ZnO on Zn uptake by cucumber varied upon the soil organic matter content and the source of Zn (Fig. 2). In the soil untreated with CM, irrespective of Zn source, by increasing Zn levels to 100 mg kg⁻¹, its concentration in root and shoot increased. By increasing Zn level to 1000 mg kg⁻¹, based on Zn source, no change (by the bulk ZnO) or significant decrease (by the coated and uncoated NPs) in tissue Zn concentration occurred. In the CM-treated soil, addition of ≥ 10 mg kg⁻¹ Zn in the form of ZnO NPs resulted in higher root and shoot Zn concentration (Fig. 2).

In general, plants at the CM-treated soil had higher Zn concentration in their tissues compared with those at the untreated soil (except at 10 mg kg⁻¹ Zn level in the form of coated ZnO NPs). The difference in shoot Zn concentration between CM-treated and untreated soil for NPs treatments was more than bulk particles treatment. This different percentage at 100 mg kg⁻¹ of bulk particles was 20.6% and for coated and uncoated NPs were 37% and 32%, respectively. In CM-treated soil, at most of Zn levels, the increase in root Zn was greater from the NPs than bulk counterpart. The total shoot Zn uptake treated with NPs was higher than bulk particles at 10 and 100 mg kg⁻¹ unreach soil and 100 and 1000 mg kg⁻¹ reached soil. In soil untreated with CM at 1000 mg kg⁻¹, plants grown at the bulk particles treatment had higher Zn concentration in root and shoot compared with those at the NPs treatments. As shown in Fig. 2, no significant difference in reached soil was found between the coated and uncoated NPs treatments in root and shoot Zn concentration.

3.4. Zn fractionation in soil

The actual concentrations of Zn in each individual fraction of soil are illustrated in Fig. 3. Generally, the distribution of ZnO among these five fractions changed based on applied Zn concentration, Zn source and soil organic matter content. Regardless of applied Zn source, up to concentration of 100 mg Zn kg⁻¹, the residual fraction was the dominant fraction of Zn in the solid phase. While at 1000 mg kg⁻¹, Zn bound to Fe and Mn oxides was the dominant fraction. Approximately 5% of added Zn remained bound with soil carbonate but this value increased up to 70% by application of 1000 mg kg⁻¹ Zn compared with the 100 mg kg⁻¹ Zn treatment. Unlike what was seen for F1 and F4, the value of Zn bound with Fe and Mn, increased extremely by increasing ZnO concentration (1000 mg kg⁻¹) in soil.

In the soil untreated with CM, no significant difference was found between the coated and uncoated NPs and bulk particle treatments in the exchangeable Zn fraction. At the CM-treated soil, distribution of Zn in the exchangeable fraction was greater at 10 and 100 mg kg⁻¹ Zn concentrations in the form of NPs (coated and uncoated) compared with that in the form of bulk ZnO. At 1000 mg kg⁻¹ Zn, exchangeable Zn fraction was greater at the coated NPs treatment than that by uncoated NPs treatment.

In both CM-treated and untreated soils, the source of ZnO (coated and uncoated NPs and bulk particles) had no significant effect on distribution of Zn in the carbonate-bound fraction.

In CM-treated soil, no significant difference was found between NPs and their bulk counterparts (except at 1000 mg kg⁻¹ Zn level) in the organically bound fraction of Zn. At the soil untreated with CM and Zn rate of 100 mg kg⁻¹, the concentration of organic matter bound Zn in coated NPs was more than uncoated NPs and bulk particles. At the untreated soil with CM, the fraction extracted with hydroxylamine (easily reducible material, e.g. Mn/Fe oxides) was larger when 100 mg kg⁻¹ Zn was applied in the form of NPs (coated and uncoated) than when the same amount of Zn was applied in the form of bulk ZnO particles. In general, the CM-treated soil contained higher Zn in the oxidizable fraction (F3) than untreated soil.

At CM-treated soil, no significant difference in the residual fraction of Zn was found between NPs and their bulk counterparts while at the soil untreated with CM, at all Zn levels (except 100 mg kg⁻¹), the residual fraction of Zn for the coated NPs treatment was greater than that of uncoated and bulk particles treatments. The highest distribution of Zn in the residual fraction was observed for uncoated NPs treatment at the 100 and 1000 mg kg⁻¹ Zn concentrations.

3.5. Microscopy observations

The TEM image (Fig. 4) showed the aggregation of NPs inside the plant cytoplasm and their accumulation adjacent to the cell membrane. The presence of nano-particles in cucumber stem indicated the possible

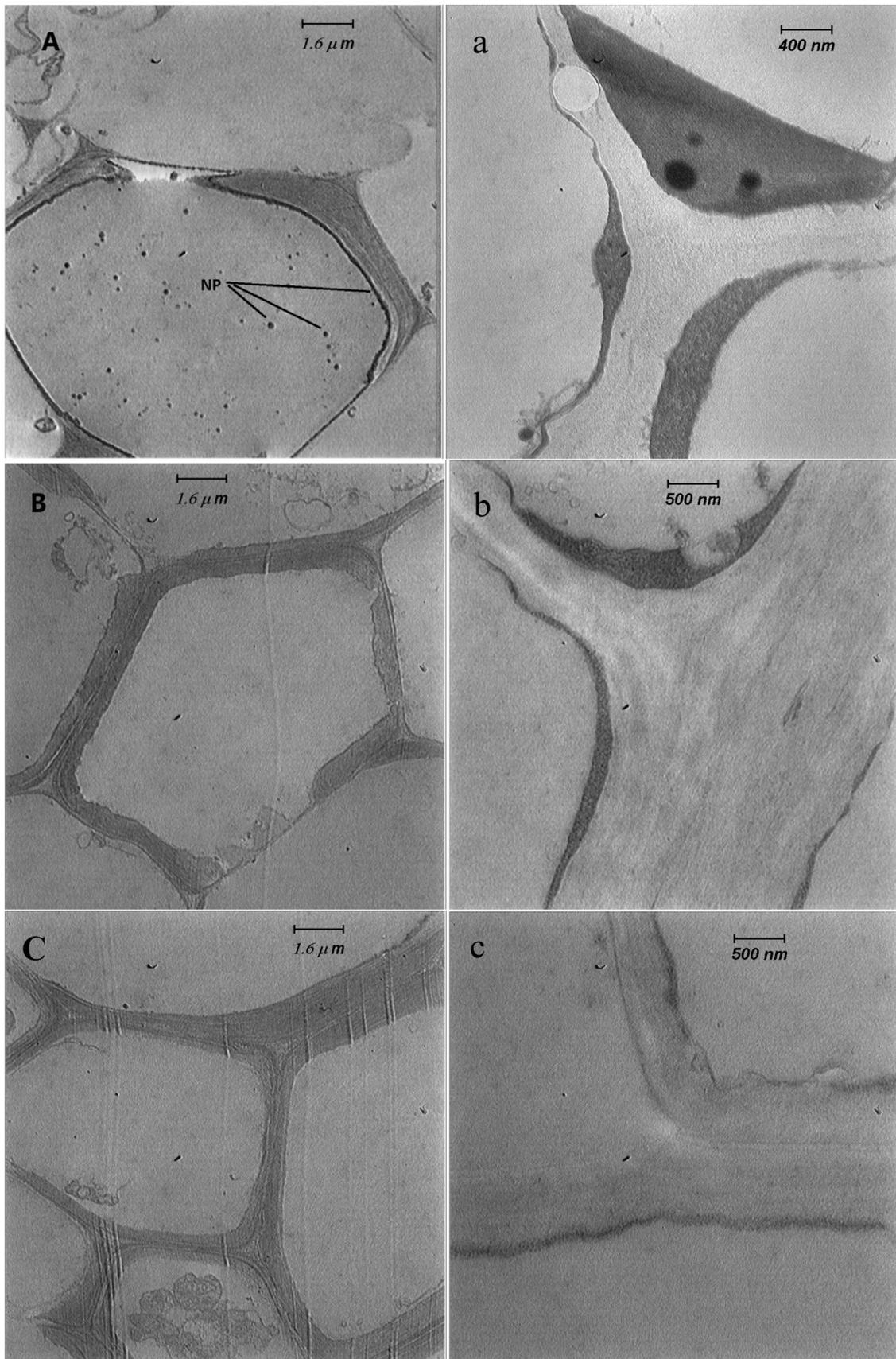


Fig. 4. TEM image of root (NPs of ZnO treatment (A) bulk ZnO (B) and, control (C)) and shoot cells (NPs of ZnO treatment (a), bulk (b) and control (c)) indicates the penetration of ZnO NPs into the root and shoot cells and precipitation on cell wall.

transport of NPs from the root to the shoot (Fig. 4); although a small part of ZnO NPs seems to be transported from root-to-shoot. These dark NPs were observed in all tree replicates of nano treatments but not seen under either the control and bulk ZnO treatments.

4. Discussion

Decreasing hydrodynamic size of nanoparticle after coating with humic acid (HA) can be explained by more negative charge of coated NPs. Adsorption of humic acid on the surface of ZnO nanoparticles leads to increased electrostatic repulsion. The decrease in the ζ -potential of NPs after coating with HA could be attributed to the negative charges of adsorbed HA on ZnO surfaces. Ligand exchange of hydroxyl groups on surface of ZnO NPs with HA, which may provide less hydroxyl groups for protonation, could also be partly responsible for decrease in ζ -potential (Tombacz et al., 1999). In addition, organic anions could increase the negative charge density adjacent to mineral particle surface and cause a shift in position of the shear plane further away from the surface, which could lead to decrease in ζ -potential of nano-oxide (Yang et al., 2009). However, our data showed that the zeta potential still fell within the range of flocculation (-30 to $+30$), therefore the higher negative charge alone might not be able to explain less aggregation of coated ZnO NPs; other factors such as influence of steric forces due to the coating might be involved. For example, Leong (1997) proposed a quantitative particle-pair interactions model incorporating steric and hydrophobic interactions for the behavior of ZrO_2 suspensions containing organic acids.

Although previous studies provided significant valuable information on the fate and effects of NPs (Lin and Xing, 2007) in hydroponic systems, there is still little information on the fate and bioavailability of these particles to terrestrial plants in soil. In addition to the type and concentration of NPs, various soil characteristics (e.g. soil pH, organic matter content and EC) affect availability of the NPs in soil. In this study, the availability ZnO NPs in two soils with different organic matter content (one treated with CM and the other as untreated) was compared with their bulk particles. Results showed that coated and uncoated ZnO NPs can be more bio-accessible than their bulk counterpart and despite their more positive effects in low concentration ($< 100 \text{ mg kg}^{-1}$), they were more phytotoxic for plants compared to the bulk ZnO particles at high concentration (1000 mg kg^{-1}) in the soil untreated with CM. Similar to this result, Stampoulis et al. (2009) found that ZnO NPs were more toxic than their bulk particles and greater ion dissolution from the NPs only partly explained the toxicity. The root tip deformation under high concentration of NPs (1000 mg kg^{-1} treatment) was observed by LM in plants at the soil untreated with CM. It seems that root tip deformation is one of the specific effects of NPs that inhibits plant growth and nutrients uptake. At the highest Zn concentration (1000 mg kg^{-1}), ZnO NPs, despite the higher phytotoxicity, resulted the same or even lower Zn accumulation in plant tissues compared with the ZnO bulk particles. This indicates that plants growth inhibition by NPs at high Zn concentration (1000 mg kg^{-1}) was not only associated with Zn^{2+} toxicity but also due to specific effects of NPs on plant. Pokhrel and Dubey (2013) showed that exposure to ZnO NPs at concentration of 1000 mg kg^{-1} , caused 'tunneling-like effect' in the primary root tip of maize. They suggested that the tender cells of epical meristem upon contact with ZnO NPs suspension led to cell dissolution from cellular structural disintegration.

Higher organic matter content in the CM-treated soil resulted in smaller plant growth retardation in the presence of ZnO NPs at the 1000 mg kg^{-1} Zn level. This is possibly at least in part due to decrease of NPs specific effects on plant. It has been reported that the phytotoxicity of NPs even at high applied doses in soil could be negated, dependent on soil properties (Watson et al., 2015). Lee et al. (2011) showed that covering the surface of NPs by organic matter prevents the homo-aggregation (repulsive forces) of NPs, but it also reduces their availability to living organisms. Organic compounds are rich in various

functional groups and they can form complexes with NPs, which may also contribute to reduction of their bioavailability (Lee et al., 2011). Jos'ko and Oleszczuk (2013) showed that ZnO did not cause any inhibition of root growth of *Lolium sativum* in soil with higher concentration of total organic carbon. In the present study, higher Zn uptake by plants at the CM-treated soil in comparison with those at the untreated soil indicated the role of organic matter in increasing Zn availability for plants. Therefore, other mechanisms resulted in lower phytotoxicity of HM-coated ZnO NPs than uncoated particles. Trapping ZnO NPs by HA and enhancing accumulation of Zn in root apoplastic spaces or soil particles surfaces can explain lower phytotoxicity of coated NPs than uncoated NPs. Navarro et al. (2012) reported that humic acid enhanced accumulation of synthesized NPs onto the root surface of *Arabidopsis thaliana* however the mechanism is not clear yet. On the other hand, coating with HA increased the zeta potential (increasing negative charge), dispersion, ionization and uptake bioavailability of ZnO. Zeta potential is a factor determining the fate of NPs because it significantly affects their tendency to agglomeration. Therefore, HA and other organic matters as dispersion agents have more effects on NPs compared to their bulk counterparts. This suggested that dispersion of NPs not only increases ionization of these particles but also affects their penetration into the plant root cells. It can also explain higher root and shoot growth and Zn uptake by cucumber under the coated NPs treatments than the uncoated ones.

In the CM-treated soil, the presence of relatively high organic matter content decreased specific effects of nanoparticles on plant and higher dry biomass was produced by plants supplied with NPs ZnO than those supplied with bulk ZnO. In contrast, in the soil untreated with CM, specific effects of coated and uncoated NPs at the 1000 mg kg^{-1} Zn level resulted in lower dry biomass production at the ZnO NPs compared to the bulk ZnO. In both CM-treated and untreated soils at low concentration of Zn ($\leq 100 \text{ mg kg}^{-1}$), ZnO NPs produced higher shoot dry biomass of cucumber than bulk particles. Greater plant shoot dry biomass and Zn uptake at the CM-treated soil compared to the untreated soil can be due to nutrient balance, easier Zn transport, uptake and improved soil properties in presence of organic matter. Lower Zn uptake from the ZnO NPs compared to the ZnO bulk particles can be due to root shrank induced by NPs. It seems that root destruction at 1000 mg kg^{-1} NPs was more than bulk counterparts and it is in line with obtained dry biomass. At this Zn concentration, root and shoot dry biomass were greatly lower at the NPs treatments compared to the bulk treatment. Moghaddasi et al. (2013) showed that in hydroponic culture, shoot Zn concentration of cucumber plant treated with synthesized NPs at 125 mg kg^{-1} was lower than 25 mg kg^{-1} of Zn concentration due to root deformation at high Zn level.

Greater shoot Zn uptake from NPs than their bulk counterparts can be due to higher solubility of Zn in soil or more penetration of NPs into root cells. The TEM images of cucumber roots indicated the presence of ZnO NPs inside the root endodermal cells (Fig. 4). Current studies have shown that nanoparticles can be taken up by plant roots as intact particles, with potential for release in planta (Monreal et al., 2015; Lin and zing, 2008; Ghafariyan et al., 2013; Moghaddasi et al., 2013). However the NPs penetration and transport mechanisms are not quite clear and further evidences are required to clarify this aspect. Apparently, both NPs and their aggregates were taken up into plant root and shoot (Fig. 4). Nanoparticles can be potentially taken up by plant root and transport to shoots through vascular systems depending upon the composition, dose, shape, and size of nanoparticles and plant species (Ma et al., 2010).

According to this study results, coating with HA, size, and concentration of NPs and soil characteristics were significant factors affecting the fractionation of ZnO NPs in soil. Low solubility of bulk ZnO and strong adsorption of ZnO NPs on the surface of soil particles are possible reasons for small distribution of Zn in exchangeable fraction (F_1). Different hydrodynamic size of ZnO particles at different applied concentrations may also affect distribution of Zn among various

fractions in the solid phase (Zhang et al., 2015). According to our results, differences in soil Zn fractionation between NPs and bulk ZnO sources at the applied level of 1000 mg kg⁻¹ was more than other contractions. In most soils, the surface charges of quartz and feldspars, which are the main components of sand and silt, are negative (Yin and Drelich, 2008). Fe-Mn oxides, considered as secondary minerals and exist primarily in clay (Fields and Swindale, 1954). So it suggests that different zeta potential of particles caused different electrostatic adsorption on soil different components. Higher percentage of coated NPs in Fe-Mn oxides compared to uncoated NPs and their bulk counterparts at high concentration of Zn in untreated soil can be due to higher negative charges of these particles. Lin et al. (2010) showed that natural organic matter can enhance the mobility of NPs in soil by increasing charge and steric stabilization. Zhang et al. (2015) showed that the consistently higher cerium concentration in roots of radish grown in loamy sand was consistent with organic matter. Soil organic matter is important factor affecting the soil fractionation and subsequently bioavailability of ZnO NPs to plants. Increasing organic fraction especially for NPs enhances plant growth and Zn uptake in CM-treated soil and there is also high correlation between soil organic matter amount and Zn uptake in plants. In summary, coated and uncoated ZnO NPs can be more bio-accessible than their bulk counterpart and despite their more positive effects in low concentration (< 100 mg kg⁻¹), they can be more phytotoxic for plants compared to the bulk ZnO particles at high concentration (1000 mg kg⁻¹) in the soil, depending on soil organic matter. In general soil coating with HA, size, and concentration of NPs and soil characteristics were significant factors affecting the fractionation, fate and transport of ZnO NPs in soil.

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