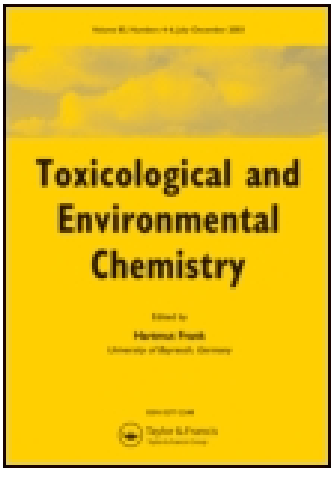

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Comparative phytotoxicity of ZnO nanoparticles, ZnO microparticles, and Zn^{2+} on rapeseed (Brassica napus L.): investigating a wide range of concentrations

Seyed Mousa Mousavi Kouhi[a], Mehrdad Lahouti[a], Ali Ganjeali[a] & Mohammad H. Entezari[b]

[a] Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

[b] Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

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Comparative phytotoxicity of ZnO nanoparticles, ZnO microparticles, and Zn^{2+} on rapeseed (*Brassica napus* L.): investigating a wide range of concentrations

Seyed Mousa Mousavi Kouhi[a]*, Mehrdad Lahouti[a], Ali Ganjeali[a] and Mohammad H. Entezari[b]

[a]*Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran;*
[b]*Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran*

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The effects of ZnO nanoparticles (NPs), ZnO microparticles (MPs), and zinc ions (Zn^{2+}) on some growth parameters of rapeseed (*Brassica napus*) seedlings have been studied. The growth inhibition by ZnO NPs was not stronger than that by ZnO MPs while treatment with Zn^{2+} inhibition was clearly stronger.

Keywords: rapeseed (*Brassica napus*); ZnO nanoparticles; phytotoxicity; root elongation

Introduction

Recent advances in nanotechnology have raised concerns about nanoparticles (NPs) release into the environment and their adverse biological effects on organisms (Hernandez-Viezcas et al. 2011). An increased application of ZnO NPs in different fields can lead to their release into the different environmental matrices (Kumari et al. 2011). Interaction of NPs with plants, an essential base component of all ecosystems, is one of the most important aspects of risk assessment of NPs (Ma et al. 2010). Plants play a critical role in the fate and transport of NPs in the environment due to the uptake and bioaccumulation of NPs (Kumari et al. 2011).

Although a number of studies have found that ZnO NPs have negative effects on plant systems, most of the studies have not simultaneously investigated the effects of corresponding MPs and ionic form of corresponding metal. However, comparative effects of ZnO NPs and MPs (Stampoulis, Sinha, and White 2009), and ZnO NPs and Zn^{2+} (Lin and Xing 2008) have been studied. Seed germination and root elongation (RE) are one of the most useful phytotoxicity tests of chemical substances in plants (Munzuroglu and Geckil 2002).

Rapeseed was selected as test species because the seeds are relatively small and thus uniformly treated in Petri dishes (Lin and Xing 2007). The aim of this investigation is to evaluate the phytotoxicity of ZnO NPs in comparison with ZnO MPs and Zn^{2+} on seed germination and some growth parameters to answer the questions whether the inhibitory effects of ZnO NPs are stronger than those of ZnO MPs or the ionic form of Zn^{2+}, whether the impacts of ZnO are related to Zn^{2+} or are NP-specific.

*Corresponding author. Email: mousavi.m@stu.um.ac.ir

Materials and methods

Characterization of ZnO particles

ZnO NPs and MPs were purchased from Sigma-Aldrich (St Louis, USA); purity of ZnO NPs was >97% and particle size was <50 nm. ZnO NPs and MPs were suspended in deionized water (250 mg L^{-1}) and sonicated for 30 min and their hydrodynamic diameters were analyzed by dynamic light scattering (DLS), using a particle size analyzer (VASCO 3, Cordouan, Pessac, France) at 25 °C. The size and morphology of ZnO NPs and ZnO MPs were also characterized using transmission electron microscopy (TEM) (Model 912 AB, LEO, Cambridge, UK) and scanning electron microscopy (SEM) (Model 1450VP, LEO, Cambridge, UK).

Preparation of particle suspensions and zinc ion solution

Stock suspensions of 500 mg L^{-1} ZnO NPs or MPs were prepared in deionized water and to avoid aggregation the suspensions were sonicated (GEX 750-5B Ultrasonic Processor VCX 750 Watt, 20 kHz, Cole Parmer, Vernon Hills, IL, USA) for 30 min, and suspensions with concentrations of 5, 10, 25, 50, 75, 100, 125, 250, and 500 mg L^{-1} were prepared. The pH after dispersion was 6.5. Solutions of Zn^{2+} at equivalent concentrations, i.e., 4, 8, 20.1, 40.2, 60.2, 80.3, 100, 201, and 402 mg L^{-1}, were prepared by dissolving calculated amounts of $ZnSO_4.7H_2O$ in deionized water.

Determination of Zn^{2+} ions released from ZnO suspensions

Stock suspensions of ZnO NPs or MPs were centrifuged at 12,000 rpm for 10 min. The supernatants were collected and filtered using a 0.22 μm syringe filter with a nylon membrane (Jet Biofil, Guangzhou, China) (Kumari et al. 2011). The Zn^{2+} concentrations in the filtrates were measured by atomic absorption spectrophotometry (AA-670, Shimadzu Company, Kyoto, Japan).

Germination assay

Seeds of rapeseed cultivar Hayola 401 were procured from Agricultural and Natural Resources Research Center of Khorasan – Razavi, Mashhad, Iran. Seeds were soaked in 10% sodium hypochlorite solution for 10 min and then rinsed thoroughly five times with deionized water. The seeds were soaked in deionized water as control, ZnO NPs suspensions, ZnO MPs suspensions, and Zn^{2+} solutions for about 2 h. Glass Petri dishes (100 × 15 mm) were washed with deionized water and sterilized in an oven at 70 °C for 1 h. Filter papers sterilized in an oven at 70 °C for 1 h were placed in each dish and 5 mL test medium or deionized water was added. Seeds were then transferred onto the paper filters in the Petri dishes containing the test media with 20 seeds per dish and 1 cm or larger distance between each seed. The dishes were sealed with tape and placed in an incubator in the dark at 25 °C. Seeds with radicles emerging from the seed coat were recorded as being germinated (Lin and Xing 2007). After six days, the germination was halted and the germination percentage (GP) was calculated. Root and shoot lengths of seedling, and dry weight of root (DWR) and shoot (DWS) were measured after being dried in an oven at 70 °C for 24 h.

Statistical analyses

There were three replicate for each treatment arranged in a complete randomized design. All data were reported as mean ± standard deviation (SD). A one-way ANOVA test followed by Duncan's multiple range test was performed with MSTAT-C software. In all cases, the significant difference was defined when the probability of p was less than 0.05.

Results and discussion

TEM images of ZnO NPs and SEM images of ZnO MPs showed that both were cube and rod shaped, and the size of ZnO NPs was verified to be <50 nm (Figure 1(b)) while that of most MPs was more than 100 nm, some of them more than 1 μm (Figure 1(d)).

Since in aqueous medium the particles form agglomerations, their hydrodynamic size may increase. DLS is one of the best tools to determine the hydrodynamic diameter of colloidal particles (Ma et al. 2010). The hydrodynamic diameters of ZnO NPs and MPs determined by DLS were about 155 ±10 nm and 590 ± 27 nm, respectively (Figure 1(a) and 1(c)). Larger sizes of the particles compared with the microscopic results are due to the formation of aggregates in suspensions, with the agglomeration of NPs being more extensive than that of MPs.

The effects of ZnO NPs, ZnO MPs, and Zn^{2+} on GP are shown in Figure 2(a). Although GP was not affected significantly by any treatment except for 402 mg L^{-1}

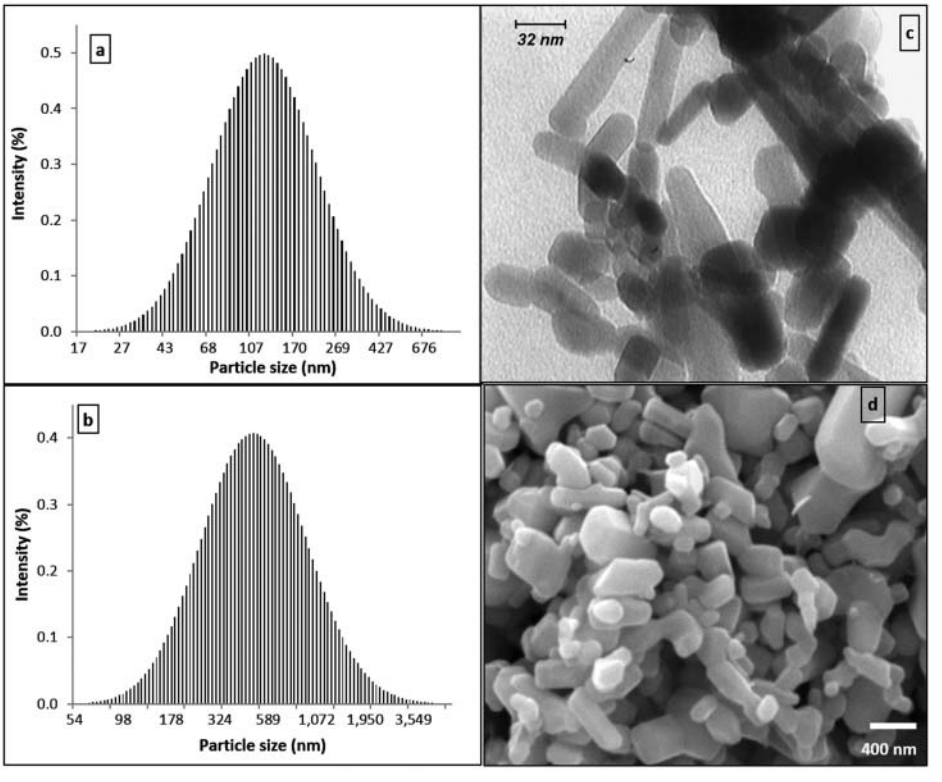

Figure 1. Hydrodynamic diameter of ZnO NPs (a) and ZnO MPs (b) determined by DLS and expressed as intensity percentage, (c) TEM image of ZnO NPs, and (d) SEM image of ZnO MPs.

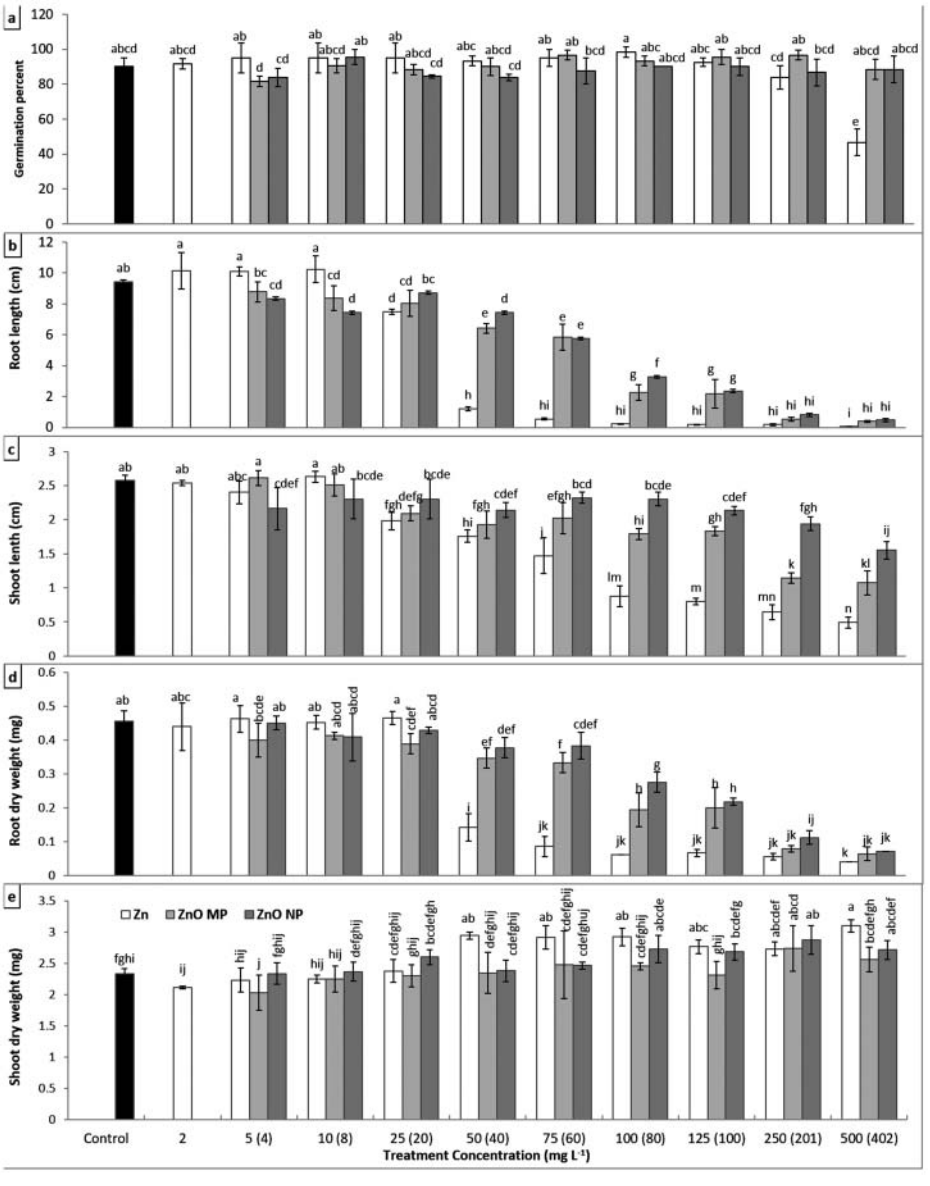

Figure 2. Effects of ZnO NPs, ZnO MPs, and Zn^{2+} on (a) germination percentage, (b) root elongation, (c) shoot elongation, (d) dry weight of root, and (e) dry weight of shoot of the rapeseed seedling. Different letters indicate significant differences ($p < 0.05$). Concentration of 2 mg L^{-1} Zn^{2+} is equivalent to the Zn^{2+} released by 500 mg L^{-1} ZnO NPs and MPs. Numbers in parentheses are equivalent concentrations of Zn^{2+}.

Zn^{2+}, RE was inhibited by all three types of treatment (ZnO NPs, ZnO MPs, and Zn^{2+}) (Figure 2(b)). While the effects of ZnO NPs and ZnO MPs on RE were similar (Figures 2(b) and 3), the inhibitory effects of Zn^{2+} were clearly stronger than those of ZnO NPs or MPs. While the fifty percent inhibitory concentration (IC_{50}) of Zn^{2+} was 27 mg L^{-1}, it was about 83 and 75 mg L^{-1} for ZnO NPs and MPs, respectively. Stronger inhibitory effects of Zn^{2+} compared with those of ZnO NPs have also been reported by

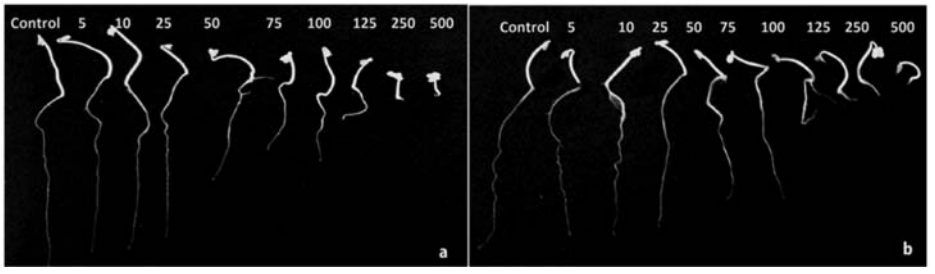

Figure 3. Effects of (a) ZnO MPs and (b) ZnO NPs on root and shoot elongation of the rapeseed seedlings.

Lin and Xing (2008) for *Lolium perenne*. At the highest concentrations of 250 [201] and 500 [402] mg L^{-1}, all three types of treatments inhibited RE (Figure 2(b)) by more than 90%. The fact that GP was not significantly affected by any treatment while RE was retarded by all three treatment types in dose-dependent manner suggests that the seed coat may prevent passage of chemical substances to affect germination, while the radicles extending through the seed coat are in direct contact with the media (Lin and Xing 2007). The effects on DWR were similar (Figure 2(d)); at Zn^{2+} concentrations greater than 40 mg L^{-1}, DWR was reduced by more than 75%, being more strongly inhibitory than ZnO NPs or ZnO MPs for which the DWR was reduced by more than 75% at concentrations of 250 and 500 mg L^{-1}. ZnO NPs and ZnO MPs were of the same inhibitory strength.

Inhibitory effects of all three types of treatments on shoot elongation (SE) were less severe (Figure 2(b) and 2(c)). The IC_{50} on SE was 66, 220, and 650 mg L^{-1} for Zn^{2+}, ZnO MPs, and ZnO NPs, respectively. Clearly, SE is less sensitive to the treatments than RE. Since the radicles extending from the seed coat are first to be exposed to the test media, their retardation is stronger than that of shoots (Sresty and Rao 1999). Overall, the inhibitory effect on SE by Zn^{2+} was also stronger than that of ZnO NPs and MPs. At concentrations higher than 25 mg L^{-1}, the negative effect of ZnO MPs suspensions was more than that of ZnO NPs suspensions being significant ($p < 0.05$) except for 25 and 50 mg L^{-1}.

It was interesting that DWS was increased at high concentrations of all three types of treatment (Figure 2(e)), being more increased by Zn^{2+} than by ZnO NPs or ZnO MPs. Since seed storage constitutes a major part of the seed dry weight of rapeseed (Hu et al. 2013) and since during the heterotrophic (nonphotosynthetic) growth in the seed storage is utilized by seedling (Taiz and Zeiger 2010), the dry weight of seedling is gradually decreased during the heterotrophic growth in the germination stage. However, correlated with reducing growth of root and shoot by later treatments, more storage of seeds has remained unused in cotyledonary leaves and has resulted in appearing increased DWS relative to control. Since shoots contain cotyledonary leaves, it is expected that the DWS (rather than root) is decreased with the increasing heterotrophic growth.

Although zinc is an essential element for plants, when the zinc supply is excessive, zinc toxicity can be induced in plants resulting in functional and structural disorders. Since phytotoxicity of Zn^{2+} has been studied extensively, it could be a good index for evaluating the intensity of ZnO NPs and MPs phytotoxicity. Inhibition of RE is one of the disorders under zinc toxicity, being very sensitive parameter (Marschner 1995). The IC_{50} of Zn^{2+} on biomass has reported to vary from 43 to 996 mg L^{-1} to various plant species (Paschke, Perry, and Redente 2006). Many previous studies have reported phytotoxicity

of zinc on seed germination and growth of roots, stems, and leaves in a variety of plants (Munzuroglu and Geckil 2002; El-Ghamery, El-Kholy, and El-Yousser 2003), and that Zn^{2+} toxicity can induce decreased or stunted growth (El-Ghamery, El-Kholy, and El-Yousser 2003). Stampoulis, Sinha, and White (2009) reported such symptoms in *Cucurbita pepo* exposed to both ZnO MPs and NPs. Similar symptoms were observed in *Lolium perenne* under both ZnO NPs and Zn^{2+} treatments (Lin and Xing 2008).

Zn^{2+} dissolution by stock suspensions of ZnO NPs or MPs was 2 ± 0.14 mg L^{-1}. Zn^{2+} dissolution from 5, 10, 25, 50, 75, 100, 125, and 250 mgL^{-1} suspensions of ZnO NPs or ZnO MPs was calculated to be about 0.02, 0.04, 0.10, 0.2, 0.3, 0.4, 0.5, and 1.0 mg L^{-1}, respectively. These values are too low to be responsible for NPs or MPs phytotoxicity. Consistent with this logic, the results showed that 2 mg L^{-1} Zn^{2+} (a concentration equivalent to the Zn^{2+} released by stock suspension of ZnO NPs or ZnO MPs) did not significantly affect the growth parameters of *B. napus* seedlings. Zn^{2+} dissolution from ZnO NPs or MPs suspensions in other studies has been measured to be very low (De la Rosa et al. 2011; Dimkpa et al. 2012). For instance, in a study by De la Rosa et al. (2011) the Zn^{2+} released by 4000 mg ZnO NPs L^{-1} suspension were 8 mg L^{-1}.

While on the basis of size, the absorption of ZnO MPs by roots must be naturally less than that of ZnO NPs, their inhibitory effects were more. This indicates that the inhibitory effects of ZnO particles could not be completely attributed to their absorption and subsequent chemical effects on the plant. The inhibitory effects of ZnO MPs or ZnO NPs may be, at least in part, due to the increased Zn dissolution induced by root exudates, or may also result from the physical interactions between ZnO particles and plant roots (Ma et al. 2010).

In this study, no positive effect of ZnO NPs, ZnO MPs, or Zn^{2+} on investigating parameters was observed. Overall, the inhibitory effect of ZnO NPs was not more than ZnO MPs, but it was lower in some parameters. In some studies that have reported negative impact of NPs on plants species, high concentrations of NPs up to 1000 mg L^{-1} and even more were investigated, such as the effects of 2000 mg L^{-1} ZnO and Zn NPs on *B. napus, Raphanus sativus, Lolium perenne, Lactuca sativa, Zea mays,* and *Cucumis sativus* (Lin and Xing 2007), effects of 1000 mg L^{-1} of five nanomaterials (MWCNTs, Ag, Cu, ZnO, Si) on *Cucurbita pepo* (Stampoulis, Sinha, and White 2009), effect of up to 4000 mg L^{-1} ZnO and SiO_2 NPs on *Arabidopsis thaliana* (Lee et al. 2010), effect of up to 4000 mg L^{-1} ZnO NPs on *Prosopis juliflora-velutina* (Hernandez-Viezcas et al. 2011), effect of 1000 mg L^{-1} ZnO NPs on *B. oleracea* (Pokhrel and Dubey 2013), effects of 4000 mg L^{-1} ZnO NPs on *Fagopyrum esculentum* (Lee et al. 2013), and effect of 200−1500 mg L^{-1} ZnO NPs on *B. juncea* (Rao and Shekhawat 2014). However, like in this work, most of the studies have been limited to early stages of the plant life cycle. More work and detailed experiments need to be done on the long-term effects of NPs on plants and other organisms to investigate the chronic effects of NPs.

Conclusion

Comparative study on the effects of ZnO NPs, ZnO MPs, or Zn^{2+} on *B. napus* in this study has helped to appropriately evaluate the phytotoxicity of NPs. Moreover, investigating a wide range of concentrations has provided the possibility of investigating both positive and negative effects of NPs on the plant. The inhibitory effects of the treatments on *B. napus* were in the order Zn^{2+} > ZnO MPs > ZnO NPs, indicating that the inhibitory effects of ZnO particles could not be completely attributed to their absorption by roots, and that they may be, at least in part, due to the increased Zn dissolution induced by root

or due to the physical interactions of ZnO particles with roots. More detailed experiments need to determine the mechanisms by which NPs could affect plant growth and development.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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