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Research article

Toxicity of CuO and ZnO nanoparticles and their bulk counterparts on selected soil-borne fungi

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Abstract

The antifungal effects of copper oxide (CuO) and zinc oxide (ZnO) in bulk and nano forms were tested *in vitro* against important soil-borne fungi and one fungal-like oomycete. Potato dextrose agar amended with 500 parts per million (ppm), 1,000 ppm and 1,500 ppm and broth with 250 ppm of either metal oxide were inoculated with fungi aged 7 d. The percentage growth inhibition and biomass were measured after 7 d. Mycelial alterations and cell membrane damage were recorded using scanning electron microscopy and fluorescence microscopy. The results showed that most of the test fungi were susceptible to CuO in both forms rather than to ZnO. Exposure to CuO and ZnO resulted in shriveling of mycelia and possible damage to the cell membrane in *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). Compound properties, not size, were the main cause of toxicity to the test fungi. Exposure and concentration were vital in the metal-oxide toxicity mechanism. It was recommended that a range of sizes be used and that the use of nanoparticles in relation to their bulk counterparts should be thoroughly reviewed.

Introduction

Metal oxide nanoparticles (NPs) are one group of nanomaterials utilized in commercial products including pigments, sunscreens, coatings and semiconductors (Zhang et al., 2008). The growing preference for nano-scale metal oxides within the size range of 1–100 nm is due to the properties conferred by the small size which suits various applications. Low stability observed in bulk materials may change to high stability in their nano-form (Zhang et al., 2008). Consequently, metal oxides, although previously used only in their normal size and form, are now being engineered to form nanomaterials to confer them with enhanced essential properties for processing, manufacturing and eventual commercial consumption. Copper oxide (CO) and zinc oxide (ZnO) NPs are among the two most widely used metal oxide NPs. For example, CuO NPs are used extensively as a sterilization agent, anti-bacterial agent and arsenic removal agent in water (Ananth et al., 2015). CuO NPs are cheaper and have relatively stable physical and chemical properties. On the other hand, ZnO NPs have applications in the biomedical field such as in bioimaging, drug delivery, gene delivery and biosensing, as well as in formulations of sunscreens (Zhang et al., 2013).

However, these same features have raised concerns on their potential environmental and health consequences and the risks associated with the engineering and handling of metal oxide nanomaterials have been the topic of reviews (Hallock et al., 2009;

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Oberdörster et al., 2009). Moreover, the rate and quantity of the production of these NPs and their ensuing disposal may exceed the buffering capacity of sinks such as the water and the soil ecosystems.

An increasing amount of the literature has provided useful and consistent insights on the possible interactions of these NPs with the soil microbiota. Metal oxide NPs have shown to alter the soil bacterial community (Ge et al., 2011; He et al., 2011), and to have a wide range of impacts on algae and fungi (Navarro et al., 2008). While CuO and ZnO NPs are utilized for the control of diseases caused by fungal plant pathogens, beneficial species may still be adversely affected by the toxicity spectrum when NPs enter the soil ecosystem.

Soil amendments do not often discriminate between pathogenic or beneficial soil microorganisms. As the amendment is foreign to the soil environment, it may provide a blanket effect on all soil microbiota. Most engineered NPs lack a "toxic-by-design" concept; thus, they may operate on a blanket effect strategy. Individual morphological and physiological characteristics of organisms, such as the different fruiting bodies and conidioma of fungi, may provide useful inferences on how fungal taxa may respond differentially to metal oxide NPs (Anahid et al., 2011; Sirelkhatim et al., 2015).

Thus, the current study tested the ability of fungal representatives to tolerate metal oxide NPs *in vitro*. First, beneficial soil-borne fungi (both pathogenic and saprophytic) were tested for their CuO and ZnO growth inhibitory potential. Second, the plant pathogens *Rhizoctonia solani*, *Sclerotium rolfsii,* and *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) were used as test organisms in antifungal assays and for elucidating morpho-cultural alterations after exposure to the metal oxides. Finally, a comparison was made between the bulk (non-nano) and nano forms of CuO and ZnO in terms of their effects on test fungi.

Materials and Methods

Fungal specimen and metal oxides

Rhizoctonia solani J.G. Kuhn (isolated from rice), *Sclerotium rolfsii* Sacc. (from pepper), *Fusarium oxysporum* f.sp. *lycopersici* W.C. Snyder & H.N. Hansen (from tomato), P*hytophthora palmivora* Butler (from durian), *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg and *Paecilomyces lilacinus* (Thom) Samson were obtained from the culture collection of the Plant Pathology Department of the Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. *Chaetomium globosum* Kunze, *Emericella nidulans* (Eidam) Vuill. and *Schizophyllum commune* Fries were provided by the mycology laboratory of the Department of Plant Pathology, University of the Philippines Los Baños, Philippines. All isolates were maintained in potato dextrose agar (PDA) or in broth (PDB) at room temperature.

The copper oxide and zinc oxide used in this research were obtained from Sigma-Aldrich® (Singapore). To verify the shape and size, samples were observed using transmission electron microscopy (TEM) at the Center for Advanced Studies of Kasetsart University, Bangkok, Thailand.

Determination of fungal growth in solid media and biomass in liquid media

Preliminary antifungal assay used concentrations based on He et al. (2011), Pradhan et al. (2011) and Karimiyan et al. (2015). Final concentration was determined thereafter. Autoclaved PDA (Himedia®, Ghatkopar, Mumbai, India) prepared following the manufacturer's instruction was amended with 500 parts per million (ppm), 1,000 ppm and 1,500 ppm of metal oxides. An agar plug (6 mm) of fungal mycelia taken from the growing margins of culture aged 7 d was inoculated at the center of the plate and incubated at room temperature. Observation and measurement of the diameter of fungal colonies was done on day 7 following the method of He et al. (2011) and expressed as percentage inhibition ± SD computed as shown in Equation 1:

$$([\text{Radial growth of control} - \text{radial growth of treated}] / \text{radial growth of control}) \times 100 \quad (1)$$

PDB containing 250 ppm of CuO or ZnO were likewise inoculated with fungal mycelia (6 mm agar disc) and incubated for seven days. Biomass was determined by weighing air-dried fungal mycelia and weight was expressed in grams ± SD.

Scanning electron microscopy

Fungal plant pathogens were grown in PDB for 7 d, both in amended and unamended conditions. Fungal mycelia were collected, washed with 1× phosphate buffer solution (PBS) to remove debris. Mycelia were then cut into small pieces and fixed with 1% glutaraldehyde for 2 hr and transferred to 3% glutaraldehyde for the next 24–48 hr as modified from Gardea-Torresday et al. (1997). Mycelia were then washed with PBS and serially dehydrated in 10%, 20%, 30%, 50%, 70%, 80%, 95% and absolute ethanol, each for 15 min. Mycelia were then critically dried, coated with gold in a sputter coater (Quorum SC7620, East Sussex, United Kingdom) for 3 min and observed using SEM.

Plasma membrane integrity

Plasma membrane integrity was assessed using the membrane-impermeable dye propidium iodide (PI; Molecular Probes; Eugene, OR) according to Azevedo et al. (2007). Mycelia exposed to metal oxides were dissociated into small pieces in phosphate buffer (1× PBS, pH 7.4) and incubated with PI (final concentration, 0.005 µg/uL) for 15 min at room temperature and scanned under an epifluorescence microscope (Axioskop connected to an AxioCam HRc camera; Zeiss, Singapore).

Statistical analysis

All data were statistically analyzed using analysis of variance and Tukey's honest significant difference post-hoc test and p-values were determined.

Results

Characterization of copper oxide and zinc oxide bulk and nano forms

Transmission electron microscopy showed a similarity in the shape of bulk forms with their nano forms. The CuO bulk and nano forms had a mix of short and elongated particles with pointed tips. The CuO-bulk particle size was in the range 100–430 nm while the CuO-nano particles were in the range 50–80 nm. The ZnO-bulk and nanoforms were in the shape of short and long rods or cylinders. The particle size of ZnO-bulk was in the range 300–500 nm while the ZnO-nano particles were in the range 20–50 nm. The sizes of CuO-nano and ZnO-nano particles used in this study were within the defined range for a nanomaterial of 100 nm in diameter or smaller and were consistent with the manufacturer's material information sheet.

Percentage inhibition to radial growth in solid media

At 500 ppm concentration, the fungi tested on CuO and ZnO showed differential responses based on the percentage inhibition to growth (Table 1). These results suggested that all, except *Fol*, were susceptible to CuO in both forms. *Fol* showed susceptibility only to CuO-nano. ZnO produced lower growth inhibition than CuO, even partially improving the growth of *Fol* and *P. lilacinus*. Except for *R. solani*, both bulk and nano forms of ZnO had statistically similar inhibitory potentials. *T. asperellum, P. palmivora* and *E. nidulans* were unaffected by ZnO.

At 500 ppm concentration, the saprophytes were highly affected by CuO, along with the pathogenic *P. palmivora* and the biocontrol *T. asperellum*, dismissing the role-based effects of metal oxides to fungus. In addition, the phylum did not show an overarching trend among its members, was the case for the basidiomycetes *R. solani, S. rolfsii* and *S. commune*. Among the ascomycetes, the coelom-forming *C. globosum* and *E. nidulans* responded interchangeably. For *C. globosum*, further growth was prevented by ZnO while for *E. nidulans* further growth was prevented by CuO. Among the hyphomycetes (*T. asprellum, P. lilacinus, Fol*), it appeared that ZnO had from no effect to slight growth-inducing effects.

Because of the agricultural importance of CuO and ZnO in controlling plant disease, higher concentrations were tested on the plant pathogens R. *solani, S. rolfsii,* and *Fol* (Table 2). While CuO-bulk prevented further growth of *R.solani*, increasing the concentration did not improve the levels of inhibitory potential. On the other hand, CuO-nano was ineffective. Both forms were likewise ineffective against *Fol*. Only *R. solani* was partially susceptible to higher concentrations of ZnO. Higher concentrations of ZnO in both forms resulted in growth of *S. rolfsii* comparable with the control.

Rhizoctonia solani on CuO-nano was comparable to the control. However, the mycelial masses were different when viewed on plates (Fig. 1), with the control plates being fuller (Fig. 1.A1, B1, C1 and D1) while the mycelia on CuO-nano were less dense. Interestingly, while mycelial growth of *R.solani* in 500 ppm ZnO-bulk was statistically comparable to the control plates, this concentration was able to induce the formation of sclerotial bodies (Fig. 1.C2) as early as 4 days after inoculation. These were not observed in any of the control or other treated plates.

Concentrations of 1,000 and 1,500 ppm of CuO and ZnO were statistically similar in *S. roflsii*, suggesting that any further increase would not result in improved inhibitory potential (Fig. 2). There was also evidence that *S. rolfsii* was able to solubilize ZnO (Fig. 2 C2, D2) to render it less toxic or beneficial; however, the exact mechanism for this was not furthered investigated in the current study.

A clearing zone similar to *S. rolfsii* was also observed around mycelia of *Fol* grown on ZnO, most notably in ZnO-bulk 1,000 ppm (Fig. 3.C3) and ZnO-nano 1,500 ppm (Fig. 3.D4). For all three fungi, CuO and ZnO only delayed and did not suppress growth; it appeared that CuO and ZnO were fungistatic rather than fungicidal.

However, there was evidence that some morphological and physiological changes occurred among the fungi. Culturally, the mycelia of *Fol* treated with CuO, in both bulk and nano-forms, had lobate-to-filiform margins, flat colonies and appeared greenish to orange. Colonies treated with higher concentrations had greenish colonies compared to the purple colonies of the control. Those growing on ZnO-nano and bulk were similar to the control in being cottony to velvety (Fig. 3).

Table 1 Percentage inhibition of CuO and ZnO at 500 parts per million concentration among tested fungi

Treatment	Percentage growth inhibition								
	Plant pathogenic fungi / oomycete				Biocontrol fungus			Saprophyte	
	R. solani	*S. rolfsii*	*Fol*	*P. palmivora*	*T. asperellum*	*P. lilacinus*	*C.globosum*	*E. nidulans*	*S. commune*
Control	0.00±0.00^{d}	0.00±3.23^{c}	0.00±4.91^{b}	0.00±5.54^{b}	0.00±12.73^{b}	0.00±0.00^{bc}	0.00±8.89^{c}	0.00±0.92^{b}	0.00±0.00^{c}
CuO-Bulk	69.41±4.19^{a}	15.16±4.38^{b}	-21.11±0.79^{d}	93.48±0.00^{a}	93.48±0.00^{a}	28.74±9.89^{a}	10.80±1.76^{b}	97.40±1.84^{a}	100.00±0.00^{a}
CuO-Nano	7.06±0.96^{c}	9.42±5.80^{b}	12.78±2.08^{a}	100.00±0.00^{a}	100.00±0.00^{a}	14.94±3.25^{ab}	7.51±5.43^{bc}	74.80±24.82^{a}	100.00±0.00^{a}
ZnO-Bulk	5.88±0.00^{c}	27.87±2.53^{a}	-2.22±5.50^{bc}	0.00±1.54^{b}	0.01±14.26^{b}	-14.94±1.63^{c}	60.09±1.33^{a}	6.37±7.44^{b}	70.98±3.88^{b}
ZnO-Nano	17.65±1.66^{b}	28.69±3.62^{a}	-8.89±3.14^{c}	0.00±4.07^{b}	0.01±14.75^{b}	-10.35±12.28^{c}	58.22±0.66^{a}	4.23±6.74^{b}	68.63±2.77^{b}
p-value	0.0000	0.0001	0.0000	0.0000	0.0000	0.0167	0.0000	0.0000	0.0000

Values (mean±SD) followed by different lowercase superscripts in the same column differ significantly at the indicated p-value using Tukey's honest significant difference test.

Table 2 Percentage inhibition of radial growth of test fungi on potato dextrose agar using different concentrations of CuO and ZnO in bulk and nano forms

Treatment	Concentration (ppm)	Percentage growth inhibition		
		Rhizoctonia solani[2]	*Sclerotium rolfsii*[2]	*Fusarium oxysporum* f.sp. *lycopersici*[3]
Control	0	0.00±0.00 ^{f}	0.00±3.23 ^{de}	0.00±0.00 ^{ab}
CuO-Bulk	500	69.41±4.19 ^{a}	15.16±4.38 ^{bc}	-21.11±0.79 ^{b}
	1,000	66.67±2.77 ^{a}	34.42±4.06 ^{a}	-3.33±2.36 ^{ab}
	1,500	72.16±1.47 ^{a}	37.29±2.66 ^{a}	5.00±7.07 ^{ab}
CuO-Nano	500	7.06±0.96 ^{f}	9.42±5.80 ^{cde}	12.78±2.08 ^{ab}
	1,000	8.24±0.96 ^{def}	-2.46±2.90 ^{e}	4.44±12.64 ^{ab}
	1,500	13.73±3.37 ^{cdef}	4.91±3.23 ^{cde}	8.33±4.91 ^{ab}
ZnO-Bulk	500	5.88±0.00 ^{ef}	27.87±2.53 ^{ab}	-2.22±5.50 ^{ab}
	1,000	26.27±4.74 ^{bc}	-3.69±0.58 ^{e}	17.78±18.07 ^{ab}
	1,500	27.06±1.92 ^{bc}	-0.82±3.01 ^{e}	15.56±25.76 ^{ab}
ZnO-Nano	500	17.65±1.66 ^{cde}	28.69±3.62 ^{a}	-8.89±3.14 ^{b}
	1,000	34.90±11.13 ^{b}	3.68±4.06 ^{cde}	17.78±20.43 ^{ab}
	1,500	21.57±4.00 ^{bcd}	12.70±4.38 ^{cd}	42.78±24.51 ^{a}
p-value		0.0000	0.0000	0.0157

Values (mean±SD; n=3) followed by different lowercase superscripts in the same column differ significantly at the indicated p-value using Tukey's honest significant difference test.

[2] observation on day 3 after inoculation on amended potato dextrose agar (PDA).

[3] observation on day 7 after inoculation on amended PDA.

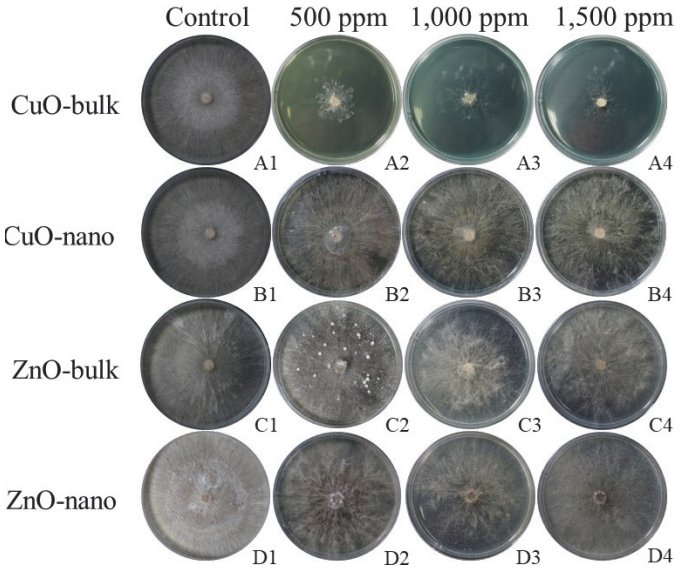

Fig. 1 *Rhizoctonia solani* at 4 d after inoculation on unamended (control) potato dextrose agar (PDA) and amended PDA [CuO-bulk (A), CuO-nano (B), ZnO-bulk (C), ZnO-nano (D)] in increasing concentrations in parts per million, with sclerotial body formation in C2

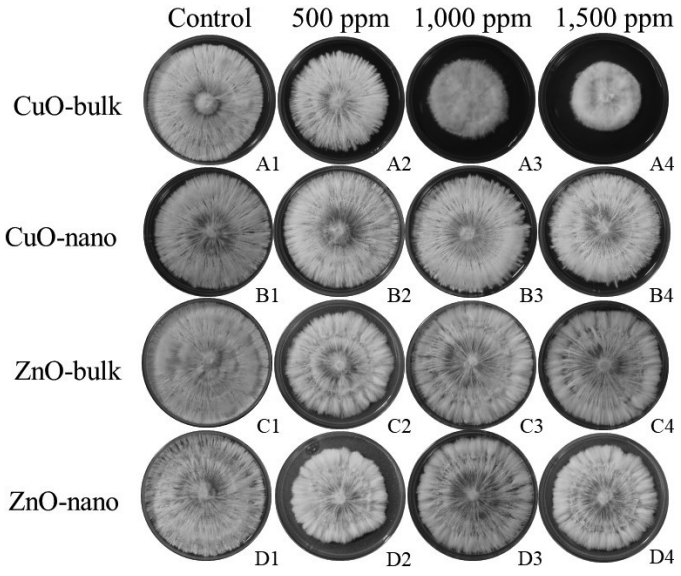

Fig. 2 *Sclerotium rolfsii* at 4 d after inoculation on unamended potato dextrose agar (PDA) (control) and amended PDA [CuO-bulk (A), CuO-nano (B), ZnO-bulk (C), and ZnO-nano (D)] in increasing concentrations in parts per million

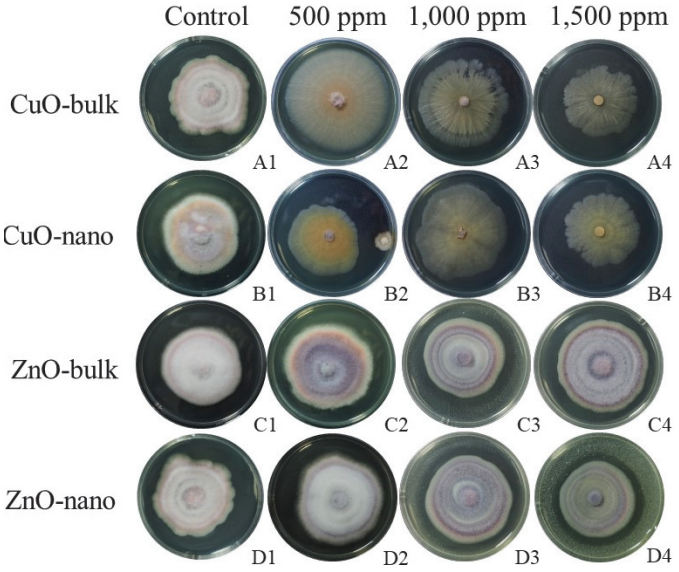

Fig. 3 *Fusarium oxysporum* f.sp. *lycopersici* at 7 d after inoculation on unamended potato dextrose agar (PDA) (control) and amended PDA [CuO-bulk (A), CuO-nano (B), ZnO-bulk (C), and ZnO-nano (D)] in increasing concentrations in parts per million

Hence, sporulation of *Fol* was checked 10 and 15 d after inoculation (Fig. 4). The fungi grown in CuO were not sporulating but were forming chlamydospores. Both forms of ZnO delayed sporulation until day 15. It was inferred that the mycelial and spore development of *Fol* was affected by CuO, possibly to compensate for the metal stress. Chlamydospores are thick-walled resting spores, formed by species of *Fusarium* and other fungi in conditions unfavorable for growth (Gadd et al., 1987). These structural modifications have been observed among *Fusarium* species subjected to metals such as Cr (Guria et al., 2014) and Cu (Pan et al., 2010). Repression of sporulation and the formation of chlamydospore are typically found under environmental stress, including metal exposure.

Biomass in liquid media

The results from the biomass in liquid media (Table 3) corroborated the results in solid media, especially for *S. rolfsii* and *Fol*. ZnO in both forms was ineffective in reducing the growth of *S. rolfsii* while ZnO-bulk improved the growth of *Fol*. This strengthened the hypothesis that *S. rolfsii* was able to solubilize Zn and transform it to less-toxic forms (Fig. 4). Although *S. rolfsii* is already known to solubilize phosphate-containing compounds (Das et al., 2012), there have been no known reports on Zn solubilization of this fungus.

Cell membrane damage

The effects of the metal oxides on mycelia were further investigated by examination of the fluorescent micrographs of *R. solani* (Fig. 5), *S. rolfsii* (Fig. 6) and *Fol* (Fig. 7). High fluorescence, suggesting membrane disruption, was seen in all treated *R. solani.* No observations were possible for CuO-bulk because the fungus was unable to grow in the amended media. With *S. rolfsii*, the nano form did not create as much membrane damage as its bulk counterpart. Very little damage was seen in *Fol* treated with ZnO-bulk.

Morphological alterations to fungal mycelia

The scanning electron micrographs showed prominent morphological alterations in the mycelia of the test fungi. The mycelia of *R. solani* were observed to shrink when in contact with CuO, in both the bulk and nano forms, and a more roughened texture was observed for those in contact with ZnO (Fig. 8). The surfaces of the *S. rolfsii* mycelia (Fig. 9) were roughened compared to the control, but the bulk forms of the metal oxides had more prominent alterations than the nano-forms. In *Fol*, chlamydospores (Fig. 10) were very clear following exposure to CuO in both forms. Shrinking of the mycelia compared to the control was likewise observed in those treated with ZnO.

Discussion

It appeared that the antifungal effects of CuO and ZnO nanoparticles in the current study were mainly due to the properties of the compound but did not have much to do with the sizes, which for CuO and ZnO NPs were 50–80 nm and 20–50 nm, respectively. These were much larger than those used effectively against *R. solani* and *S. rolfsii* in the studies by Rubina et al. (2017) using CuO NPs with diameters of 2–3 nm. Likewise, Jamdagni et al. (2018) used ZnO NPs in the range 12–32 nm, which were effective against *F. oxysporum*. Similar observations suggested that a smaller size induce sensitivity of fungi to CuO NPs such as 28–32 nm (Vanathi et al., 2016) and ZnO NPs with size range of 12–15 nm (Narendhran and Sivaraj, 2016) against *F. oxysporum*.

However, Espitia et al. (2013), the ZnO NPs described were much larger than 100 nm, with the smallest being 259 nm. Dispersion of the NPs in the media became the ultimate factor. The ZnO NPs, as seen in the current study, have a tendency to precipitate and thus become unavailable to the fungi. Non-dispersion of the nanoparticles in the media may have caused the lack of antifungal activity. Evidence derived from this current research also suggest that the fungi, especially S. *rolfsii*, had the ability to solubilize ZnO and thus make it less toxic to the growing mycelia. Baldrian (2003) also noted that members of the phylum Basidiomycota require minimal amounts of Zn for their growth and development. This may explain the zone of solubilization surrounding *S. rolfsii* and the inability of ZnO to fully suppress growth of *R. solani and S. commune* at 500 ppm.

Ivask et al. (2013) suggested that particle dissolution had an effect on the toxicity of metal-containing NPs such as CuO and ZnO, with toxicity in most cases being due to dissolved Cu and Zn ions, as these become more reactive than the oxide form. This was in agreement with the findings of Bondarenko et al. (2013) that the ionic form of CuO and ZnO after dissolution caused toxicity to the test organisms. These reports strengthen the argument of the current study implicating the chemical nature of CuO and ZnO and not the size as the source of toxicity among the fungi tested.

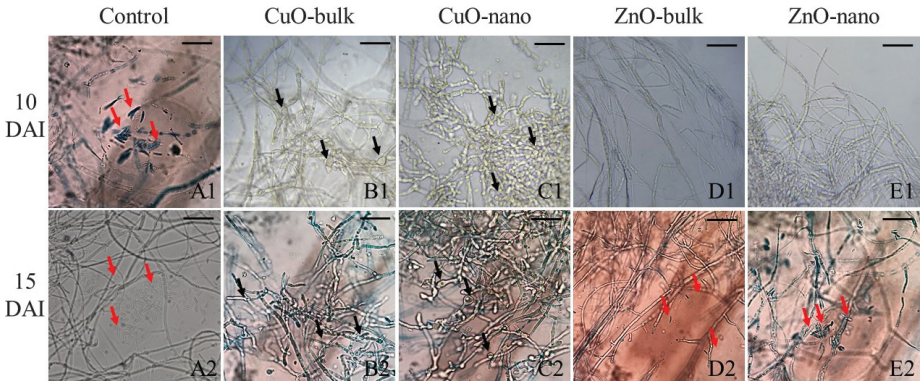

Fig. 4 *Fusarium oxsporum* f.sp. *lycopersici* 10 and 15 d after inoculation growing on unamended potato dextrose agar: (A) sporulation (red arrow); chlamydospores (black arrow) of *Fol* grown on CuO-bulk (B) and CuO-nano (C); normal mycelia without sporulation of *Fol* grown on ZnO-bulk (D) and ZnO-nano (E). Scale bar = 40 µm

Table 3 Biomass of fungal plant pathogens in liquid media amended with metal oxides

Treatment	Biomass (g) at 250 ppm concentration		
	Rhizoctonia solani	*Sclerotium rolfsii*^{ns}	*Fusarium oxysporum* f.sp. *lycopersici*
Control	0.23±0.001^{a}	2.06±0.47	0.35±0.07^{b}
CuO-bulk	0.00±0.00^{b}	0.63±0.87	0.06±0.01^{b}
CuO-nano	0.05±0.004^{b}	1.70±0.96	0.51±0.56^{b}
ZnO-bulk	0.03±0.002^{b}	2.35±0.55	1.41±0.44^{a}
ZnO-nano	0.01±0.001^{b}	1.37±0.25	0.41±0.00^{b}
p-value	0.0015	0.1812	0.0168

ppm = parts per million; ns = not significant..

Values (mean±SD) followed by different lowercase superscripts in the same column differ significantly at the indicated p-value using Tukey's honest significant difference test.

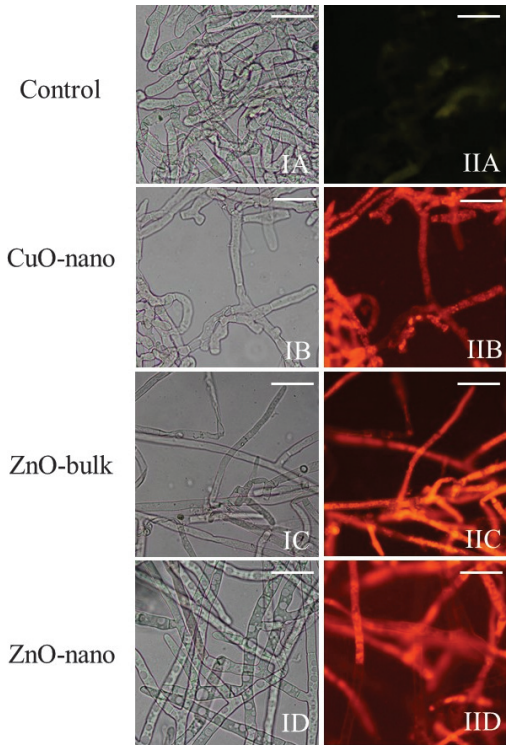

Fig. 5 *Rhizoctonia solani* viewed under normal light (I) and fluorescent (II) microscopy, where membrane damage is evident in all treatments, with vacuolation in mycelia treated with ZnO-nano. Scale bar = 50 µm

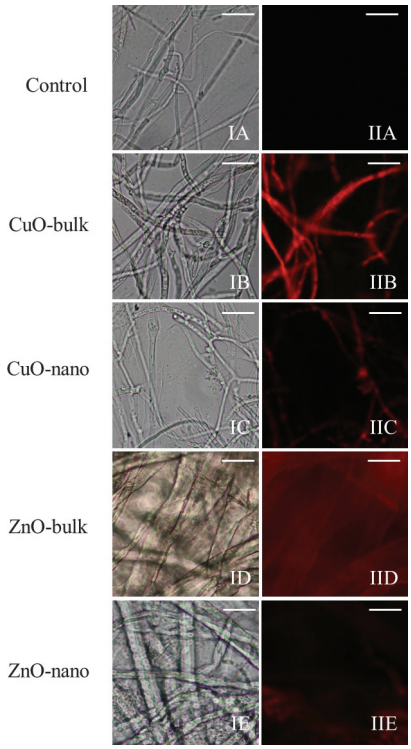

Fig. 6 *Sclerotium rolfsii* viewed under normal light (I) and fluorescent (II) microscopy, where high membrane damage is visible in all treatments with bulk forms exceeding their nanoform counterparts. Scale bar = 50 µm

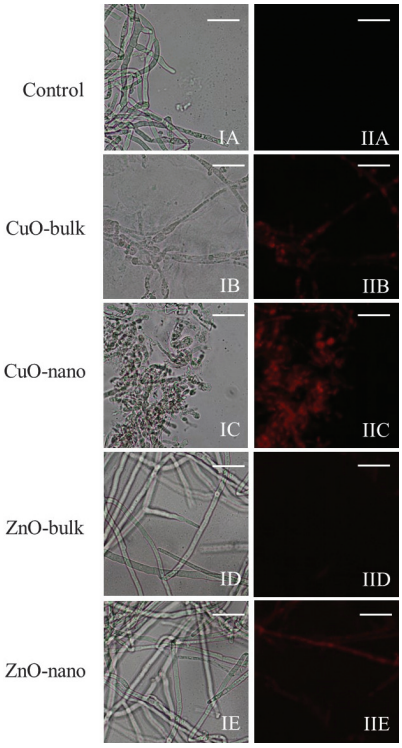

Fig. 7 *Fusarium oxysporum* f.sp. *lycopersici* viewed under normal light (I) and fluorescent (II) microscopy with minimal membrane damage in all samples. Scale bar = 40 µm

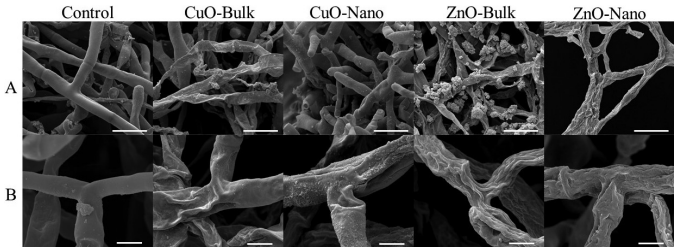

Fig. 8 Scanning electron micrographs of *Rhizoctonia solani* exposed to 500 parts per million of bulk and nano forms of CuO and ZnO, with mycelia showing general shriveling across treatments relative to untreated (control) mycelia and scale bars: A = 20 µm, B = 5 µm

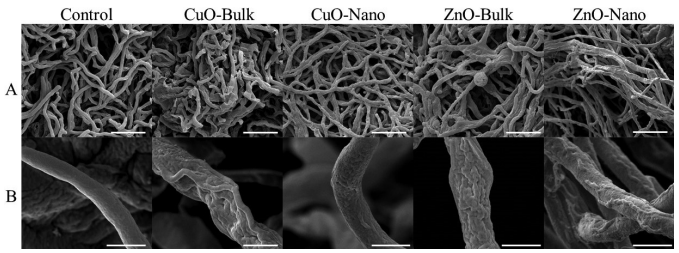

Fig. 9 Scanning electron micrographs of *Sclerotium rolfsii* mycelia exposed to 500 parts per million of bulk and nano forms of CuO and ZnO showing rough mycelial surfaces across treatments compared to control, with scale bars: A = 20 µm, B = 5 µm

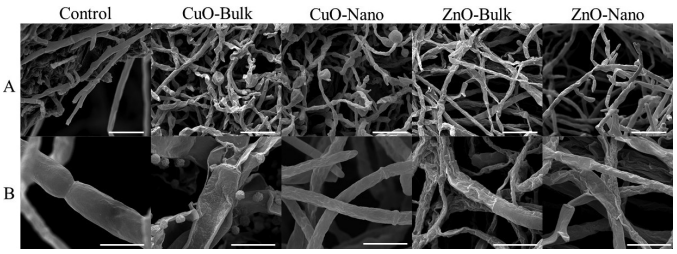

Fig. 10 Scanning electron micrographs of *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) exposed to 500 parts per million of bulk and nano forms of CuO and ZnO with chlamydospores evident in CuO-exposed fungus and absent in fungi exposed to ZnO and scale bars: A = 20 µm, B = 10 µm

Most often, the concentration and size together increase the sensitivity of fungi to metal oxide nanoparticles. However, far too often, the concentration is overemphasized as the main factor of toxicity. While this may be true, contact may be as vital in the toxicity mechanisms of the metal oxides. As seen in this research, mere contact with metal oxides altered the mycelia of the test fungi and was sufficient to elicit morphological differences from the control when observed under electron scanning microscopy.

Non-inhibition of growth in fungi due to metal oxides such as CuO and ZnO should only be the minimum standard for toxicity studies. For example, CuO and ZnO did not show promise as control mechanisms against *Fol* in terms of growth, but they did significantly alter sporulation. This may be utilized in a strategy for disease control. Therefore, it is recommended that other toxicity mechanisms be tested.

The differential responses of test organisms make studying the effect of metal oxides on fungi complex. However, the current research conformed with the literature that CuO is more toxic than ZnO. *In vitro* studies such as this current research can only foreshadow limited possibilities when all factors are controlled. However, when these metal oxides find their way to the soil, physico-chemical properties of the receiving soil medium and its intricate biota complicate species-specific studies. The soil ecosystem is not a fungal monopoly but rather a diverse interaction among bacteria, nematodes, soil animals and other organisms. Physico-chemical factors such as the pH and moisture can intervene in the translocation and eventual fate of ZnO and CuO in the soil (Dimkpa et al., 2013; Rajput et al., 2018). The environment can modify the nanoparticles through factors such as organic matter coating, agglomeration and disaggregation (Simonet and Valcárcel, 2009) which may render them inert, toxic or anything in between (Nowack and Bucheli, 2007). The soil, being the receiving medium of the metal oxides, can profoundly affect the fate of the CuO and ZnO as well as their bioavailability and behavior (Navarro et al., 2008).

The results of the current study point to the importance of CuO and ZnO NPs in agriculture. The trend in nanoparticle manufacturing as a promising agricultural innovation must be thoroughly reviewed. When NPs find their way into the soil, they may have a gradual but lasting effect. Soil amendments do not often discriminate between pathogenic or beneficial soil microorganisms. “Toxic by design”, as multiple

reviews have explained, is crucially anchored by an understanding of toxicity mechanisms, the range of affected organisms and the eventual fate and behavior of NPs in the environment.

Conflict of Interest

The authors declare no conflict of interest.

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