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# Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens



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# HIGHLIGHTS

# GRAPHICAL ABSTRACT

- NPs are effective against both mycelial growth and spore germination.
- Mode of fungitoxic action of Cu-NPs is apparently different than that of their bulk counterparts.
- Ag-NPs and Cu-NPs significantly suppressed *B. cinerea* symptoms *in vivo*.
- NPs tested exhibit promising potentials as nano-fungicides.



# A R T I C L E I N F O

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# ABSTRACT

Nano-fungicides are expected to play an important role in future plant disease management as eco-friendly alternatives of conventional synthetic fungicides. In the present study, the sensitivity of seven fungal species, known to cause foliar and soil-borne diseases, to nanoparticles (NPs) containing copper (Cu-NPs, CuO-NPs), silver (Ag-NPs) and zinc (ZnO-NPs) was assessed in vitro. Mycelial growth assays revealed that Cu-NPs with mean inhibition rates, EC<sub>50</sub>, ranging between 162 and 310 µg/mL were most effective among the NPs tested in inhibiting fungal growth, followed by ZnO-NPs with EC<sub>50</sub> ranging between 235 and 848 µg/mL. All fungal species were practically insensitive to CuO-NPs and Ag-NPs except for B. cinerea, which was equally sensitive to Ag-NPs and Cu-NPs (EC<sub>50</sub> = 307 µg/mL). Cu-NPs were more fungitoxic in terms of mycelial growth, to almost all species tested, than a protective fungicide containing Cu(OH)<sub>2</sub>, which was used as a reference. Fungitoxicity experiments with the NPs tested and bulk size reagents containing the respective metals revealed that ZnO-NPs were more toxic to all fungal species tested than ZnSO<sub>4</sub>, whereas Cu-NPs were more fungitoxic than CuSO<sub>4</sub> in all cases, except for B. cinerea, A. alternata and M. fructicola. The existence of a positive correlation between Cu-NPs and CuO-NPs toxicity and, at the same time, the absence of any correlation between NPs tested and their respective bulk metal counterparts indicated potential differences in the mode of action between bulk and nanosized antifungal ingredients. Although there was considerable variation between fungal species, all NPs were generally 10 to 100 fold more fungitoxic to spores than hyphae and in the majority of cases more effective than Cu (OH)<sub>2</sub>, as revealed by colony formation bioassays. NPs significantly suppressed grey mold symptoms on plum fruit, especially Ag-NPs, which completely inhibited disease development. Consequently, tested NPs have the potential to be used as protective antifungal agents.

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# 1. Introduction

Crop losses due to plant parasites are a considerable challenge that the current agricultural production system faces worldwide with diseases accounting for at least 25% of the losses (Pandey et al., 2018). Conventional synthetic fungicides are largely considered as the most effective and cost efficient means for disease management. However, the intensity of usage and the site-specific mode of action of most synthetic fungicides eventually lead to resistance problems and an increased environmental cost due to elevated drug residues to water reservoirs. Nanoparticles (NPs) are expected to play an important role in resolving this challenge in the future (Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). NPs provide a novel eco-friendly alternative to synthetic chemical fungicides, due to their promising properties that improve drug delivery, slow active ingredient release, and increase effectiveness in lower doses. NPs containing silver (Ag-NPs) have proven to exert a wide range of antimicrobial activity against bacteria, fungi and viruses (Huang et al., 2018). Bulk copper compounds have been exploited to protect agricultural crops from many pests, including those causing a wide range of bacterial and fungal infections, due to their low cost, protective activity, and reduced risk for resistance development controlled by their multi-site mode of action against pathogens (Keller et al., 2017). The observed lack of systemic movement and ease of residue removal from plant tissues has enabled copper fungicides to be a part of disease control management in organic farming (Keller et al., 2017). Taking advantage of the antifungal and antimicrobial properties of [Cu<sup>+2</sup>], applications of NPs containing copper (Cu-NPs) in agriculture and food preservation are readily emerging in an attempt to exploit their unique nano-scale properties (Keller et al., 2017; Park et al., 2016).

Metal oxide NPs, compared to their bulk counterparts, are more stable in extreme conditions, exhibit antimicrobial activity at low concentrations and low or no toxicity to humans (Król et al., 2017). NPs containing zinc (ZnO-NPs) are very effective antibacterial agents against a broad spectrum of bacterial species, due to their high surface to volume ratio and unique physicochemical properties (Sun et al., 2018). Several studies have demonstrated the fungistatic potential of ZnO-NPs against fungal pathogens including *Fusarium* sp., *Botrytis cinerea*, *P. expansum, Aspergillus niger* and *Rhizopus stolonifer* (He et al., 2011; Król et al., 2017; Ashajyothi et al., 2016).

Air-borne fungal strains tested in the present study include pathogens that cause important pre- and post-harvest plant diseases such as Alternaria tomato brown leaf spot, Grey mold caused by Botrytis cinerea, Monilia stone fruit Brown rot, and strawberry Anthracnose caused by Colletotrichum spp. Most of the above pathogens require a large number of fungicide applications for their control, while fungicide resistance development is seriously impairing effective control (FRAC, 2013; Malandrakis et al., 2013, 2018; Ziogas et al., 2003). Soil-borne pathogens also included in this study are causal agents of wilting (V. dahliae) or root rots (FORL and F. solani) in a wide range of hosts and are difficult to control with conventional fungicides except for high-cost fumigants (Paplomatas et al., 2005). Thus, it is essential to consider alternative compounds suitable for disease suppression with suitable properties to alleviate control disadvantages against the above pathogens, and at the same time minimize the environmental impact of conventional pesticides. Although numerous studies, especially in the case of silver, have examined metal nanoparticle fungitoxic activity, there are only very few data available concerning the efficacy of NPs in inhibiting both mycelial growth and spore germination of plant pathogens in vitro and even fewer in vivo (Nemati et al., 2015; Pandey et al., 2018; Kah et al., 2018; Sun et al., 2018). Under this light, in vitro and in vivo evaluation bioassays were carried out with the main objective of: (a) determining the mean sensitivity (in terms of EC<sub>50</sub> values) of Alternaria alternata, Botrytis cinerea, Monilia fructicola, Verticillium dahliae, Colletotrichum gloeosporioides, Fusarium oxysporum fsp Radicis Lycopersici (FORL), and Fusarium solani strains to Cu-NPs, CuO-NPs, Ag-NPs and ZnO-NPs *in vitro*, and (b) comparing nanoparticle effectiveness against the above plant pathogens to bulk-size containing reagents and a reference fungicide containing Cu(OH)<sub>2</sub>.

#### 2. Materials and methods

#### 2.1. Nanoparticles, reagents and fungicide

NPs and reagents used in this study were purchased from Sigma Aldrich, MO, USA: silver [Ag-NPs] (<100 nm particle size), zinc oxide [ZnO-NPs] (particle size <50 nm), copper [Cu-NPs] (particle size 25 nm), copper oxide [CuO-NPs] (particle size <50 nm), copper sulphate [CuSO<sub>4</sub>], zinc sulphate [ZnSO<sub>4</sub>] and silver nitrate [AgNO<sub>3</sub>]. A commercial fungicide product (Copperblau-N 50 WP) containing the active ingredient copper hydroxide was purchased from NITROFARM (Hellas). All stock solutions of the antifungal compounds were prepared using sterilized distilled water as a solvent for all commercial or analytical grade active ingredients, and were added aseptically to sterilized growth medium prior to inoculation. Nanoparticle suspensions were subjected to sonication for 30 min using a Transonic 420 (Elma, Germany) sonicator prior to incorporation in growth media.

### 2.2. Fungal strains and culture conditions

Seven fungal strains from the fungal collection of the Pesticide Science Lab (Agricultural University of Athens) originating from various crop fields, located in regions of Southern and Central Greece (Table 1), were used to evaluate the fungitoxic activity of the NPs studied. All strains were grown on Potato Dextrose Agar (PDA) medium in order to obtain inoculum for the fungitoxicity assays and kept in growth chambers in the dark at 25 °C and 70% humidity. For each strain, for long-term storage purposes, four 5 mm mycelial plugs from the margin of rapidly growing fungal colonies were placed in 1.5 mL tubes containing 50% v/v of sterilized glycerol: water and kept at -20 °C.

# 2.3. In vitro mycelium growth inhibition tests

Sensitivity of fungal strains to NPs and fungicides was evaluated by measuring radial growth of strains on PDA. Fungitoxicity was expressed based on  $EC_{50}$  values (effective concentration causing 50% inhibition of mycelial growth) for each compound. PDA amended with 0, 1, 10, 100, 500, 1000, 5000 µg/mL Ag-NPs, 0, 1, 10, 100, 500, 1000 µg/mL CuSO<sub>4</sub>, ZnSO<sub>4</sub>, AgNO<sub>3</sub>, Cu-NPs, ZnO-NPs or CuO-NPs and 0, 50, 100, 500, 1000 µg/mL copper hydroxide was used to obtain fungitoxicity-curves for all strains. There were three replicate plates for each antifungal ingredient concentration – strain combination. Inoculum consisting of a 5-mm mycelial plug cut from the edge of 4-day old colony of each fungal strain grown on PDA was transferred to napoparticle or fungicide-amended and non-amended (control) media. Cultures were incubated at 25 °C in the dark for 4 days. The mean diameter of the colony on the fungicide-amended plates was expressed as the percentage of the

# Table 1

Fungal strains belonging to different species used in this study.

Fungal strain	Disease	Host	Origin (Region in Greece)
B. cinerea	Grey mold	Tomato	Crete
A. alternata	Black rot	Tomato	Crete
M. fructicola	Brown rot	Peach	Naousa
C. gloeosporioides	Anthracnose	Strawberry	Crete
F. solani	Fusarium root	Tomato	Kalamata
	rot		
F. oxysporum fsp Radicis Lycopersici	Crown root rot	Tomato	Larisa
V. dahliae	Verticillium wilt	Eggplant	Crete

mean diameter of the untreated control. Tests for each isolate were repeated twice for each concentration and fungicide.

#### 2.4. Inhibition of colony formation

The effect of NPs and fungicides on spore germination was assessed in vitro on PDA containing petri dishes. Each fungal strain was cultivated in appropriate medium in order to induce conidiation. In the case of A. alternata, B. cinerea, M. fructicola and C. gloeosporioides, a 5-mm mycelial plug cut from the edge of a rapidly growing colony was transferred on a 9-cm petri dish and incubated for 7 days at 25 °C in a growth chamber with a 12 h per day light-period. Conidia from each strain were harvested by scraping the surface of the plate, suspended in 10 mL sterilized-distilled water, and filtered using cheesecloth. In the case of V. dahliae, FORL and F. solani liquid cultures in 250 mL glass flasks containing Potato Dextrose Broth (PDB) were used to obtain conidia. Flasks were inoculated with four 5-mm mycelial plugs, cut from the edge of rapidly growing colonies from each fungal strain and incubated for 4 days in the dark at 25 °C in growth chambers under continuous shaking at 200 rpm. Conidia were then harvested by filtration of the liquid cultures using cheese cloth. The concentration of spores, in all cases, was determined using a haemocytometer (2 counts per replicate) and adjusted to 100 conidia/100 µL by serial dilutions in sterilized-distilled water.

Inhibition of colony formation assays was carried out on PDA petri dishes containing various nanoparticle and fungicide concentrations. PDA amended with 0, 1, 5, 10, 25, 50, 100, 250, 500, 1000, 2000 µg/mL Ag-NPs, ZnO or CuO; 0, 1, 2.5, 5, 10, 20, 50, 100 µg/mL Cu-NPs; and 0, 0.1, 1, 5, 10, 50, 100, 500 µg/mL copper hydroxide was used to obtain fungitoxicity-curves for all strains. There were three replicate plates of each compound concentration - strain combination. A volume of 100 µL from flasks containing a concentration of 10<sup>3</sup> conidia/mL from each fungal strain was transferred and spread on the surface of petri dishes containing PDA amended or not with the above-mentioned nanoparticle/fungicide concentrations. All dishes were incubated for 2 days in the dark at 25 °C, and then, the number of forming colonies was counted. The percent inhibition of colony formation was calculated by dividing the mean number of colonies from each antifungal compound treatment by the mean number of colonies formed in the untreated control, and multiplying the result with 100. EC<sub>50</sub> values based on relative percent inhibition were calculated for each compound/strain combination. The experiment was conducted twice.

# 2.5. Effectiveness of nanoparticles in vivo

The effectiveness of NPs in suppressing grey mold symptoms caused by B. cinerea in vivo was tested on plum fruit (Prunus domestica) of uniform shape, size, maturity and without any wound. Cu-NPs, Ag-NPs and ZnO-NPs were included in tests as well as the reference fungicide Copperblau-N containing copper hydroxide. Four plum fruits per treatment with antifungal agents were used while distilled water was used as the control treatment. Prior to treatment and inoculation, plum fruit were surface disinfected in a 1% sodium hypochlorite solution by dipping for 10 min. Following disinfection, fruit were rinsed three times with distilled-sterilized water and left to dry. Subsequently, fruit were sprayed until ran off using concentrations of 100 and 1000  $\mu$ g/mL for each NP/fungicide treatment. Plum fruit where air-dried for 2 h and then a sterile needle was used to remove fruit skin creating a  $4 \times 4 \times 2$  mm [length  $\times$  width  $\times$  depth] wound at the front face of each fruit. Wounds were inoculated using a pipette with a 100 µLdrop transferred from a suspension containing B. cinerea conidia at a concentration of 10<sup>4</sup> conidia/mL. Inoculated fruit were placed on top of wet sterilized paper inside plastic boxes  $24 \times 34 \times 10$  cm [length  $\times$  width  $\times$  height] covered by a lid and incubated in a growth chamber at 25 °C for 4 days. Symptom severity on NP/fungicide treated fruit was recorded by measuring the lesion diameter around each wound and expressed as a percent of the water-treated control. The experiment was repeated twice.

#### 2.6. Morphological examination of nanoparticle-treated conidia

Morphological examination of germinating conidia treated with NPs was conducted by spreading a 20-µL volume of a conidial suspension (10<sup>4</sup> conidia/mL) on a 20 × 20 mm coverslip subsequently covered with sterile cellophane. A PDA plug was then placed on top of the cellophane. Conidia were left for at least 6 h on PDA blocks in order to germinate. Subsequently, untreated PDA plugs were replaced by other PDA plugs treated with NPs in doses equal to the respective EC<sub>50</sub> values determined in the spore germination experiments. Cover slips were withdrawn and adhered conidia were examined with an x40 objective lens, on an OLYMPUS BX40 microscope.

### 3. Statistical analysis

The EC<sub>50</sub> values for each strain and antifungal compound were calculated by regressing the relative inhibition of mycelial growth/colony formation against the Log<sub>10</sub> compound concentrations. Cross sensitivity between antifungal compounds was evaluated using Pearson correlation coefficients. Data on efficacy of NPs were subjected to analysis of variance and mean separation using Tukey's HSD test ( $\alpha = 0.05$ ). All analyses were conducted using SPSS (SPSS Inc., Chicago, IL, USA).

#### 4. Results

#### 4.1. Effect of NPs on mycelial growth in vitro

A dose-dependent decrease in growth was observed in most fungal strains treated with NPs while the respective EC<sub>50</sub> values are shown in Table 2. Although sensitivity to tested NPs varied between strains, all fungal species were relative insensitive to CuO-NPs in terms of mycelial growth, exhibiting  $EC_{50}$  values >1000 µg/mL (see Table 2). This was also the case for Ag-NPs, which could not inhibit most of the fungal strains even at concentrations exceeding 5000 µg/mL. The only exception was B. cinerea, where mean inhibition by Ag-NPs was achieved at a concentration of 307 µg/mL (see Table 2). Among the four NPs, Cu-NPs exhibited the greater overall toxic activity against the 7 fungal species with EC<sub>50</sub> values ranging from 162 (V. dahliae) to 328 µg/mL for the FORL, followed by ZnO-NPs with respective EC<sub>50</sub> values ranging from 235 (A. alternata) to 847 µg/mL for the FORL strain (see Table 2). Copperblau-N, a protective broad-spectrum fungicide containing Cu (OH)<sub>2</sub>, was included in this study as a reference antifungal agent. In most cases, Cu-NPs were more effective than Cu(OH)<sub>2</sub>, while the rest of the NPs tested were equally or significantly less effective than the commercial fungicide except for ZnO-NPs which were more effective than Cu(OH)<sub>2</sub> against *M. fructicola*, *F. solani* and *V. dahliae* (see Table 2).

In order to investigate the effect of nanoparticle size to the observed mycelial growth inhibition, CuSO<sub>4</sub>, ZnSO<sub>4</sub> and AgNO<sub>3</sub> reagents containing the respective metals in bulk size were included in fungitoxicity experiments. For all fungal strain cases examined, ZnO-NPs were significantly more fungitoxic compared to ZnSO<sub>4</sub> (see Fig. 1). The most profound differences was observed in the cases of *M. fructicola* and *A. alternata*, where EC<sub>50</sub> values were equal to 628 and >2000  $\mu$ g/mL, 235 and 751  $\mu$ g/mL for ZnO-NPs and ZnSO<sub>4</sub>, respectively. A smaller but statistically significant difference in mycelial growth inhibition was observed in C. gloeosporioides between ZnO-NPs ( $EC_{50} = 551$  $\mu$ g/mL) and ZnSO<sub>4</sub> (EC<sub>50</sub> = 667  $\mu$ g/mL). Cu-NPs were significantly more fungitoxic than CuSO<sub>4</sub> towards V. dahliae, C. gloeosporioides, F. oxysporum (FORL) and F. solani, but that was not the case for the rest of the fungal species cases. Because silver NPs used in this study were hardly effective against mycelial growth in most of the strains studied, comparison with AgNO<sub>3</sub> was conducted only for the *B. cinerea* and C. gloeosporioides cases. In both cases AgNO<sub>3</sub> was more toxic to

#### Table 2

Effect of NPs on mycelial growth of seven plant pathogenic fungal strains.

Fungal strain	$EC_{50}^{a}$ (µg m/L) (mean $\pm$ SD <sup>b</sup> )					
	Ag-NPs	Cu-NPs	CuO-NPs	ZnO-NPs	Cu(OH) <sub>2</sub>	
B. cinerea	$306.95 \pm 4.75 \ a^d$	$310.23 \pm 2.34$ a	>1000	$670.29 \pm 16.32 \text{ c}$	$493.47 \pm 21.65$ b	
A. alternata	>5000	$296.56 \pm 8.72 \text{ c}$	>1000	$235.48 \pm 15.09 \text{ b}$	$210.07\pm3.15$ a	
M. fructicola	$4221.53 \pm 56.70 \text{ d}$	$219.46 \pm 10.12$ a	>1000	$628.22 \pm 24.05 \text{ b}$	$861.04 \pm 44.18 \text{ c}$	
C. gloeosporioides	$1099.90 \pm 80.43 \text{ c}$	$161.78 \pm 14.42$ a	>1000	$551.05 \pm 18.22 \text{ b}$	$563.44 \pm 9.82 \text{ b}$	
F. solani	>5000	$261.16 \pm 12.54$ a	>1000	$554.41 \pm 30.05 \text{ b}$	$765.61 \pm 10.62 \text{ c}$	
FORL <sup>c</sup>	>5000	$328.12 \pm 20.30$ a	>1000	$847.44 \pm 24.18 \text{ b}$	$856.96 \pm 73.82 \text{ b}$	
V. dahliae	>5000	$191.17 \pm 11.33$ a	>1000	$386.48 \pm 23.14 \ b$	$786.32 \pm 55.08 \ c$	

<sup>a</sup>  $EC_{50} =$  Effective concentration causing 50% reduction in mycelial growth rate.

<sup>b</sup> Standard deviation.

<sup>c</sup> FORL: F. oxysporum fsp Radicis Lycopersici.

<sup>d</sup> Within rows, values followed by the same letter do not differ significantly according to Tukey's HSD test ( $\alpha = 0.05$ ).

fungal growth than Ag-NPs (see Fig. 1a, d), suggesting the probability of an additional mode of toxic action for  $AgNO_3$ , which is different than ion metal toxicity.

#### *4.2.* Cross sensitivity profiles of fungal strains to antifungal compounds

Growth inhibition profiles of the fungal strains to NPs,  $AgNO_3$ ,  $CuSO_4$ ,  $ZnSO_4$  and  $Cu(OH)_2$  were analyzed using Pearson correlation

coefficients on  $EC_{50}$  or relative growth values. A statistically significant positive correlation between Cu-NPs and CuO-NPs was found (see Fig. 3a), while no significant correlation was found between Cu-NPs and other bulk-sized copper containing compounds (see Table 3). A positive correlation was observed between Ag-NPs and CuSO<sub>4</sub> or Cu (OH)<sub>2</sub> but not with AgNO<sub>3</sub> containing bulk sized silver (see Fig. 3b, c). No correlation was found between ZnO-NPs and any of the other antifungals used in this study (see Table 3).



**Fig. 1.** Sensitivity of fungal strains to NPs compared with bulk-sized metal containing reagents. Error lines represent the standard deviation of means (FORL: *F. oxysporum fsp Radicis Lycopersici*). Between treatments, bars marked by the same letter do not differ significantly according to Tukey's HSD test ( $\alpha = 0.05$ ). \* indicates values >2000 µg/mL.

# 4.3. Effect of NPs on colony formation

The ability of Cu, CuO, ZnO and Ag NPs to inhibit spore germination in the tested fungal species was assessed by colony formation inhibition experiments. Sensitivity of fungal strains to Cu(OH)<sub>2</sub> and NPs used in this study in terms of EC<sub>50</sub> (concentration causing 50% colony inhibition) is presented in Table 4. Cu-NPs were more toxic to all species compared to the reference commercial fungicide, except in the case of FORL where toxicities did not differ significantly (see Table 4). Similarly, ZnO-NPs were more effective than  $Cu(OH)_2$  in all cases but FORL (see Table 4). With the exception of the two Fusarium species tested, all other fungal strains were more sensitive to Ag-NPs than Cu(OH)<sub>2</sub> (see Table 4). Overall, NPs were significantly more effective against fungal strains at the spore germination level compared to mycelial growth. EC<sub>50</sub> values of Cu-NPs ranged from 3 (B. cinerea) to 29 (FORL) µg/mL, indicating a 10 to 100-fold increased spore sensitivity to these NPs, compared to the respective hyphal sensitivity revealed by mycelia growth assays (compare Tables 2, 4). A similar spore-inhibitory profile was observed for ZnO-NPs with EC<sub>50</sub> ranging from 5 (V. dahliae) to 165 µg/mL (FORL). All fungal strains were highly sensitive to Ag-NPs in terms of colony formation except for A. alternata and F. solani, where EC<sub>50</sub> values were 235 and >2000 µg/mL. Although significantly more toxic than in mycelial growth, CuO-NPs were less effective than both other NPs and the reference fungicide against fungal colony formation in all strain cases except for *M. fructicola* in which CuO-NPs were more effective than  $Cu(OH)_2$  (see Table 4).

#### 4.4. Effect of NPs on grey mold symptom suppression

Grey mold symptom suppression properties of selected NPs in comparison with the reference fungicide containing  $Cu(OH)_2$  are shown in Table 5. AgNPs were the most effective treatment exhibiting percent inhibition of *B. cinerea* disease symptoms equal with 85 and 100% at concentrations of 100 and 1000 µg/mL respectively. This was actually the only treatment that resulted in complete inhibition of symptoms. Cu-NPs treatment of plum fruit resulted in limited inhibition (16%) at the lower dose and a significantly higher inhibition (70%) compared to the reference fungicide at the higher dose (Table 5). ZnO-NPs showed inhibition rates which were not significantly different than the ones obtained by  $Cu(OH)_2$  applied at any concentration. Disease suppression on plum fruit did not increase significantly by increasing doses 10-fold in both ZnO-NPs and  $Cu(OH)_2$  cases (Table 5).

# 4.5. Effect of NPs on hyphal and spore morphology

Microscopic observations under an optical microscope of NPs treated fungal conidia at non-lethal concentrations (equal to the respective  $EC_{50}$  values) have revealed certain abnormalities in hyphae and germination tubes (see Fig. 2). Cu-NPs at a concentration of 3 µg/mL have resulted in deformation in hyphae, cytoplasm segmentation and exudates formation (see Fig. 2). Spores treated with NPs in  $EC_{50}$  value concentrations had abnormal tube elongation. Typical spore tube

elongation involves hyphal branching in all directions in a concentric manner (see Fig. 2a). *B. cinerea* spores treated with copper NPs had germinating tubes advancing in a constantly changing direction without any branching as if searching for a less toxic path (see Fig. 2b). This apparently indicates the involvement of a chemiotactic mechanism for avoiding xenobiotics in this fungal strain.

# 5. Discussion

In an attempt to evaluate the effectiveness of NPs against a number of important plant pathogenic fungi, the mean inhibitory concentrations of Cu, CuO, Ag and ZnO-NPs were determined in *in vitro* poison agar assays. Mycelial growth inhibition assays in vitro revealed significant variations in sensitivity between fungal strains belonging to different species and between the NPs tested. Overall, the toxicity of NPs to fungal strains was in the order of: Cu-NPs > ZnO-NPs > Ag-NPs > CuO-NPs, considering the total number of sensitive strains and the range of sensitivity of tested species. Ashajyothi et al. (2016) reported that Ag-NPs were the most toxic to strains of Aspergillus niger and Fusarium oxysporum among Ag, Cu, Au and ZnO-NPs in zone inhibition mycelial growth assays. In another study, Ag-NPs exerted stronger overall inhibition than Cu-NPs against Rhizoctonia solani, F. oxysporum and F. redolens (Aleksandrowicz-Trzcinska et al., 2018), indicating that species-specific sensitivity probably accounts -at least partially- for variations between nanoparticle effectiveness.

In the present study, Cu-NPs was the most effective nano-sized compound against mycelial growth exhibiting significant inhibition at relatively low concentrations for all fungal strains tested. Mean sensitivity of all tested fungal strains to Cu-NPs was significantly higher than that of a commercial product containing Cu(OH)<sub>2</sub>, except in the case of A. alternata that exhibited greater sensitivity to Cu(OH)<sub>2</sub>. Significant antifungal activity of Cu-NPs has been demonstrated in a number of fungal species including: Fusarium sp., A. niger, R. solani, Alternaria solani, A. alternata, Phoma destructiva (Pandey et al., 2018; Nemati et al., 2015; Aleksandrowicz-Trzcinska et al., 2018). Interestingly, coppercontaining CuO-NPs were significantly less effective in inhibiting mycelial growth in all pathogens tested in this study. This is in alignment with previous studies on comparative toxicity of copper nanomaterials, which report a higher toxic effect of Cu-NPs compared with CuO-NPs (Keller et al., 2017). CuO-NPs effectiveness against B. cinerea, A. alternata, A. fumigatus, F. solani and A. flavous mycelial growth in vitro has been reported to be limited, while B. cinerea has been proposed to detoxify CuO-NPs by biotransformation to Cu-oxalate and subsequent extracellular secretion (Hao et al., 2017; Kovačec et al., 2017; Magbool et al., 2017). ZnO-NPs were the second most effective NPs towards fungal strains used in this study. They exhibited satisfactory mycelial inhibition, which was greater or equal than that of  $Cu(OH)_2$  in most of the fungal species tested.

Various size zinc oxide NPs acquired with different preparation methods were successfully tested against many bacteria and pathogenic fungi and their efficiency seems to depend on particle size and concentration (Sun et al., 2018). Silver NPs used in this study were practically

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Correlation between sensitivity of fungal strains to NPs and their bulk counterparts.

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	Cu-NPs	Ag-NPs	ZnO-Nps	CuO-NPs	CuSO <sub>4</sub>	Cu(OH) <sub>2</sub>	ZnSO <sub>4</sub>	$AgNO_3$
Cu-NPs	1.0	$-0.08^{a}$	0.305	0.855*	0.287	-0.211	0.314	0.547
Ag-NPs	-	1.0	0.383	-0.016	$0.808^{*}$	0.888**	0.136	0.524
ZnO-Nps	-	-	1.0	0.28	0.478	0.618	0.537	0.646
CuO-NPs	-	-	-	1.0	0.274	-0.115	0.149	0.716
CuSO <sub>4</sub>	-	-	-	-	1.0	0.594	-0.08	0.485
$Cu(OH)_2$	-	-	-	-	-	1.0	0.403	0.433
ZnSO <sub>4</sub>	-	-	-	-	-	-	1.0	0.018
AgNO <sub>3</sub>	-	-	-	-	-	-	-	1.0

<sup>a</sup> Pearson correlation coefficient values.

\* Corresponds to a significance lever of P = 0.05.

\*\* Corresponds to a significance lever of P = 0.01.

#### Table 4

Effect of NPs on spore germination in terms of colony formation of seven plant pathogenic fungal strains.

Fungal strain	$EC_{50}^{a}$ (µg m/L) (mean ± SD <sup>b</sup> )	$\frac{EC_{50}^{a} (\mu g m/L)}{(mean \pm SD^{b})}$						
	Ag-NPs	Cu-NPs	CuO-NPs	ZnO-NPs	Cu(OH) <sub>2</sub>			
B. cinerea	$5.08\pm0.30~a^d$	$3.05\pm2.30$ a	$422.11 \pm 15.39  d$	$16.29\pm4.00~\mathrm{c}$	$10.35\pm0.10~\text{b}$			
A. alternata	235.55 ± 15.21 c	$7.69 \pm 1.00$ a	$678.20 \pm 26.56 \text{ e}$	$17.54 \pm 3.61 \text{ b}$	$255.82 \pm 1.20 \text{ d}$			
M. fructicola	$50.60 \pm 8.72$ c	$6.45 \pm 0.27$ a	$215.02 \pm 11.08 \text{ d}$	$20.32 \pm 2.10 \text{ b}$	$355.51 \pm 2.81$ e			
C. gloeosporioides	$7.79\pm1.14$ a	$17.44 \pm 3.18 \text{ c}$	178.20 ± 23.12 e	$10.36 \pm 1.27 \text{ b}$	$90.65 \pm 12.34 \text{ d}$			
F. solani	>2000	$18.84 \pm 2.44$ b	>2000	$6.81 \pm 0.29$ a	878.24 ± 30.17 c			
FORL <sup>c</sup>	390.20 ± 35.34 c	$29.04\pm4.32$ a	>2000	$164.50 \pm 24.31 \text{ b}$	$25.23 \pm 3.45$ a			
V. dahliae	$2.36\pm0.07~\mathrm{a}$	$13.32\pm0.81~c$	$252.12 \pm 10.33 \ d$	$4.66\pm0.14b$	$15.25\pm1.17~\mathrm{c}$			

<sup>a</sup>  $EC_{50} =$  effective concentration causing 50% reduction in number of colonies.

<sup>b</sup> Standard deviation.

<sup>c</sup> FORL: F. oxysporum fsp Radicis Lycopersici.

<sup>d</sup> Within rows, values followed by the same letter do not differ significantly according to Tukey's HSD test ( $\alpha = 0.05$ ).

effective against mycelial growth only in the case of *B. cinerea*. In fact, Ag-NPs and Cu-NPs were the most effective NPs against this fungus and performed better than the commercial fungicide containing copper hydroxide. Besides *B. cinerea*, several Ag-NPs studies have reported strong inhibition against a number of plant pathogenic fungi such as *C. gloeosporioides*, *Bipolaris sorokiniana*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *A. alternata and F. oxysporum* (Pandey et al., 2018). The larger particle sizes (<100 nm) of Ag-NPs used in the present study in comparison with the ones (4–21 nm) used in the above-mentioned studies could account for the lack of satisfactory mycelial inhibition of most of the fungal species tested.

Several mechanisms of antibacterial/antifungal action of NPs have been proposed including generation of Reactive Oxygen Species (ROS), disruption of microbial membranes by physical contact and the liberation of antimicrobial ions (Sun et al., 2018; Hoseinzadeh et al., 2017; Król et al., 2017). Whether NPs act at a similar way as their bulk counterparts has been the subject of debate between researchers. In an attempt to answer this question, cross sensitivity of NPs used in the present study with reagents containing the respective bulk-sized metals was evaluated. Despite the potential differences in sensitivity between different fungal species tested, a positive correlation was found between both Cu containing NPs, but not with any of the bulk Cu containing antifungal agents [CuSO<sub>4</sub>, Cu(OH)<sub>2</sub>], indicating that Cu-NPs toxicity to fungal strains is not proportionally similar with bulk copper containing fungicides. Absence of statistical correlation between silver and zinc oxide NPs and their respective bulk counterparts also indicate differences in toxicity and potentially an alternate or additional mode of action, besides ion-release. Further studies at the biochemical level are needed to validate such a claim.

Regardless of their efficacy in inhibiting mycelial growth, toxicity of all NPs to fungal spores increased dramatically. Although there were differences in sensitivity between fungal species, in the majority of fungal cases, Cu-NPs were the most effective NPs followed by Zn-NPs, while both antifungal agents were able to inhibit colony formation at lower doses than the commercial fungicide Cu(OH)<sub>2</sub>. Ag-NPs successfully inhibited spore germination/colony formation more effectively than Cu(OH)<sub>2</sub>, except for soil-borne pathogen F. solani, which proved to be relatively tolerant to these NPs. Even though their activity against spore germination was drastically increased compared with that in mycelial growth, CuO-NPs were the less effective of the four NPs tested. It has been reported in the literature that the activity of silver, zinc oxide and gold NPs against A. niger and F. oxysporum was higher in spore germination than mycelial growth (Ashajyothi et al., 2016). Nemati et al. (2015) has demonstrated a stronger fungicidal effect of copper Cu-NPs against F. expansum, A. solani and A. alternata compared to a broadspectrum fungicide containing copper (Bordeaux mixture) in terms of colony formation. Successful inhibition of colony formation, utilizing Ag-NPs against plant pathogenic fungi B. sorokiniana and M. grisea, was demonstrated by Jo et al. (2009). This increased toxic effect of NPs against fungal spores, compared to hyphal growth, could be attributed to structural differences between spore and vegetative fungal walls. Chitin content of many fungal species is significantly higher in hyphal walls, compared to spore walls, rendering the later more susceptible to heavy metals (Bartnicki-Garcia, 1968). Furthermore, during the

#### Table 5

Effect of NPs on	symptom s	everity cause	d by B.	cinerea spores	inoculated	on plum fruit.

Dose (µg/mL)	% inhibition <sup>a</sup> Mean $(\pm SD^b)$	% inhibition <sup>a</sup> Mean (±SD <sup>b</sup> )				
	Cu(OH) <sub>2</sub>	ZnONPs	AgNPs	CuNPs	Mean $(\pm SD^{D})$	
100	41.17 (±5.32) b <sup>c</sup>	41.17 (±7.56) b	85.29 (±4.25) a	16.64 (±3.22) c	17 (±2.02)	
1000	44.11 (±4.11) c	47.05 (±6.50) c	100 a	70.58 (±8.10) b		
		0				

<sup>a</sup> Calculated as the percent inhibition in terms of observed lesion diameter compared to the untreated control.

<sup>b</sup> Standard deviation of the mean.

<sup>c</sup> Within rows, values followed by the same letter do not differ significantly according to Tukey's HSD test ( $\alpha = 0.05$ ).



**Fig. 2.** Microscopic observation of *Botrytis cinerea* conidia (top) and hyphae (bottom) treated (b, d) or untreated with Cu-NPs (a, c). Black arrow indicates abnormal hyphal swelling with cytoplasm exudates.

process of spore germination, enzymes such as disulfide reductases and glucanases result in the softening of cell walls, in order to facilitate germ tube elongation, and thus create sensitive sites for toxic substances in contact with the fungal cell (Bartnicki-Garcia, 1968).

Potential suitability of NPs as alternative antifungal agents heavily depends on their effectiveness not only in vitro but primarily on their ability to exert their disease suppressing activity in vivo (Hao et al., 2017). Assessing nanoparticle disease suppressive properties in fieldscale experiments is hindered by practical difficulties mostly due to the lack of formulations of such substances. Detached fruit experiments provide an intermediate means of evaluating NPs effectiveness in vivo while very limited data is available in literature. In the present study, NPs spray applications on plum fruit resulted in significant disease suppression against B. cinerea. Ag-NPs and Cu-NPs were more effective than the reference fungicide at higher doses while Ag-NPs completely inhibited symptom development at the higher concentration. Hao et al. (2017) have reported successful B. cinerea symptom suppression on detached rose petals by carbon and copper nanoparticles while Ag-NPs were found to suppress disease symptoms of ryegrass pathogen B. sorokiniana (Jo et al., 2009). Although fungitoxic activity exerted by Cu-NPs in vitro against B. cinerea was similar to that of Ag-NPs, the later nanoparticles were dramatically more effective when applied in vivo. This observation demonstrates the importance of in vivo experiments for practical effectiveness evaluation of active substances. A possible explanation for such toxicity increase of Ag-NPs on plum fruit could be attributed to the differences between growth medium composition and the actual plant-tissue surface in contact with the fungal inoculum. PDA is a relatively rich growth medium and is expected to contain a higher protein content, rich in sulfide amino acids known to bind with [Ag<sup>+</sup>], hindering dissolution of Ag-NPs or directly react with Ag-NPs to form precipitates without undergoing dissolution and thus could result in a reduction in toxicity compared with the plum fruit surface, where such substrate is not readily available (Levard et al., 2012; Zhang et al., 2016). In any case, interactions between Ag-NPs and organic matter are complex and a number of factors including nanoparticle coating and aggregation are involved, underlying mechanisms that differentiate toxicity of Ag-NPs between growth media and plant tissue have yet to be elucidated. Different inhibition levels of Ag-NPs against B. cinerea depending on the growth medium used in fungitoxicity tests in vitro have been reported by Kim et al. (2012).



**Fig. 3.** Correlation between sensitivities of fungal strains to (a) Cu-NPs (EC<sub>50</sub> values) and CuO-NPs (relative growth at 1000 µg/mL), (b) Ag-NPs (relative growth at 5000 µg/mL) and Cu(OH)<sub>2</sub>, and (c) Ag-NPs (relative growth at 5000 µg/mL) and CuSO<sub>4</sub> (EC<sub>50</sub> values). Here, r is the Pearson correlation coefficient, and P the significance level.

All the above cases demonstrate a promising potential of NPs to be used as protective fungicides at early stages of disease initiation, by inhibiting spore germination of plant pathogenic fungi.

In summary, tested NPs were able to inhibit *in vitro* mycelial growth of fungal strains in a dose-response manner with the most effective being Cu-NPs and Zn-NPs. Although species dependent, the toxicity of NPs was not significantly correlated with the respective toxicity of their bulk counterparts, indicating potential differences in their mode of action. All NPs tested in this study were more toxic at the spore germination level than at mycelial growth and, in most cases, more effective than the commercial fungicide containing Cu(OH)<sub>2</sub>. Furthermore, their suppressive antifungal properties extend to actual plant level, as

indicated by their grey mold disease suppressive properties, making them excellent candidates for alternative, lower-dose, protective fungicides against both foliar and soil/seed-borne plant pathogenic fungi.

#### **Conflict of interest**

All three authors declare that they have no conflict of interest.

## **Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

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