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Evaluation nanoparticles of metals and metal oxides for antifungal effect on *Verticillium dahliae*

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Abstract

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The soil-borne fungus *Verticillium dahliae* is the causal agent of wilting disease and affects a wide range of plant species worldwide. Soil borne pathogens are significant contributors to plant yield loss globally. The constraints in early diagnosis, wide host range, longer persistence in soil makes their management cumbersome and difficult. The aim of this study is to evaluate the nanoparticles that have demonstrated activity in suppressing mycelia growth of *Verticillium dahliae*. The limiting effect on mycelial growth at a dose of 0.5 mg/l of the tested products against *Verticillium dahliae* was reported for Zinc with a particle size of 60–70 nm and 790 nm. A good effect for both isolates was reported with Zinc 18 nm, Mg micro powder 35 µm and Mg oxide 18 nm. The average tested nanoparticle concentration of 1.5 mg/l, the best limiting effect was observed for Zinc with a particle size of 60–70 nm during the entire reporting period. The high concentration (2.5 mg/l) of the products showed a high inhibitory effect on both tested isolates of the pathogen. A very good limiting effect is reported for almost all materials in the (3–12-day) reporting period.

Keywords: Verticillium dahliae; nanoparticles; *in vitro*; test

Introduction

Tomatoes (*Solanum lycopersicum* L.) are a valuable horticultural crop that are grown and consumed worldwide. Optimal production is hindered by several factors, among which *Verticillium dahliae*, the cause of Verticillium wilt, is considered a major biological constraint in temperate production regions. *V. dahliae* is difficult to mitigate, because it is a vascular pathogen, has a broad host range and worldwide distribution, and can persist in soil for years (Acharya et al., 2020). The soil-borne fungus *Verticillium dahliae* is the causal agent of wilting disease and affects a wide range of plant species worldwide (Witzel et al., 2017).

The development of effective and ecofriendly agrochemicals, including bactericides, fungicides, insecticides, and nematicides, to control pests and prevent plant diseases remains a key challenge. Nanotechnology has provided opportunities for the use of nanomaterials as components in the development of anti-phytopathogenic agents. Indeed, inorganic-based nanoparticles (INPs) are among the promising ones. They may play an effective role in targeting and killing microbes via diverse mechanisms, such as deposition on the microbe surface, destabilization of cell walls and membranes by released metal ions, and the induction of a toxic mechanism mediated by the production of reactive oxygen species. Considering the lack of new agrochemicals with novel mechanisms of action, it is of particular interest to determine and precisely depict which types of INPs can induce antimicrobial activity with no phytotoxicity effects, and which microbe species are affected (Kanakari & Dendrinou-Samara, 2023).

The emerging role of metal and metal oxides nanoparticles (NPs) in plant disease diagnostics to combat crop diseases has been described. These NPs constitute new weapons against plant pathogens and facilitate the early diagnosis/ management of crop diseases specifically in resource-poor conditions. The interactions between NPs, phytopathogens and plants showed great diversity and multiplicity which reduces chances of the development of resistant pathogen strains (Khan et al., 2022). The large-scale use of conventional pesticides and fertilizers has put tremendous pressure on agriculture and the environment. In recent years, nanoparticles (NPs) have become the focus of many fields due to their cost-effectiveness, environmental friendliness, and high performance, especially in sustainable agriculture. Traditional NPs manufacturing methods are energy-intensive and harmful to the environment. In contrast, synthesizing metal-based NPs using plants is like chemical synthesis, except the biological extracts replace the chemical reducing agent. This not only greatly reduces the use of traditional chemicals, but also produces NPs that are more economical, efficient, less toxic, and less polluting. Therefore, green synthesized metal nanoparticles (GS-MNPs) are widely used in agriculture to improve yields and quality (Jiang et al., 2022).

Engineered nanoparticles (NPs) (1–100 nm) that have demonstrated activity in suppressing plant diseases are metalloids, metallic oxides, nonmetals, and carbon nanomaterials. NPs have been integrated into disease management strategies as bactericides/fungicides and as nano fertilizers to enhance plant health. Although, there are reports of over 18 different NPs of single element and carbon nanomaterials affecting disease and/or plant pathogens, only Ag, Cu, and Zn have received much attention thus far. Some NPs act directly as antimicrobial agents, while others function more in altering the nutritional status of the host and thus activate defense mechanisms. For example, NPs of Ag and Cu can be directly toxic to microorganisms. Other NPs of B, Cu, Mn, Si, and Zn appear to function in host defense as fertilizers. As demand for food production increases against a warming climate, nanoparticles will play a role in mitigating the new challenges in disease management resulting in a reduction in active metals and other chemical inputs (Elmer et al., 2018a).

The aim of this study is to evaluate the nanoparticles that have demonstrated activity in suppressing mycelia growth of *Verticillium dahliae*.

Material and Methods

Isolation

Verticillium isolates were collected in 2022, from different varieties tomato from Maritsa Vegetable crops research Institute –Plovdiv, Bulgaria (MVCRI).

Pathogenicity

The pathogenicity tests for each isolation number were conducted on growth chamber to tomato cultivar Ideal. The seedlings were inoculated by 20 ml of conidial suspension (80×10^6 conidia per 1000 ml) of each isolate on top of the soilless medium in each pot. Non-inoculated seedlings were included as controls. Plants were watered daily starting immediately after inoculation.

DNA amplification

Total DNA was extracted from mycelia obtained from Potato dextrose agar (PDA) culture grown at 25°C for 7 days. The targeted region amplifies the ITS region comprising the ITS region and the 5.8S rDNA gene. The PCR reaction used the *Taq* DNA polymerase system: a 25-µL PCR mixture contained 1 µL (0.2 µg) of DNA template, 2.5 µL of buffer II solution (containing all the dNTPs and $MgCl_2$), 1 µL of each 10-µm primer (ITS 4 and ITS 5), 1µL of *Taq* DNA polymerase, and 18.5 µL of distilled water. PCR reactions were performed in a thermo cycler (Biorad T100 Thermal Cycler). PCR was performed at: 94°C-3'; and 35 cycles: 94°C – 45"; 57°C – 30"; 72°C – 1'. The PCR products were separated electrophoretically into 1% agarose gel in TBE buffer for 30 min at 100 V. The products are visualized under UV light. DNA 100 bp marker (Fermentas) was used. The DNA bands were visualized under UV illumination.

In vitro screening of metal ions to control mycelia grow Fusarium

The inhibiting effect of Iron oxide nano powder/ nanoparticles (Gamma high purity 99.55%, size 18 nm), Iron nano powder purity 99.55% (size 60–70 nm), Iron nano powder/ nanoparticles purity 99.55% (size 790 nm), Zinc nano powder (high purity 99.55%, size 60–70 nm), Zinc nano powder/ nanoparticles purity 99.55% (size 790 nm), Zinc oxide nano powder (purity 99.99%, size 18 nm), Magnesium micron powder (purity 99.95%, size 35 µm), Magnesium oxide nano powder (purity 99.95%, size 18 nm) on the mycelium growth of the two pathogenic species of *Verticillium dahliae* was tested using *in vitro* Thornberry's method (Thornberry, 1950). Each Petri dish was inoculated with 1 cm disc of 14-day old fungal culture in the center. Three dishes for each treatment were used as control. All the inoculated plates were incubated at 25°C in dark, until the mycelia growth reached the edge of the control plate. Three perpendicular measurements of the colony diameters were made, and the plug diameter was subtracted to determine the diameter growth rate (Batzer et al., 2005). We investigated 3 different concentrations of ions: 0.5 mg/l, 1.5 mg/l and 2.5 mg/l. The mycelia measurement was carried out on 3, 6, 9, 12 and 15 days from inoculation.

The percentages of the linear mycelia growth reduction of the pathogenic fungi were calculated using the following formula:

$$I\% = \frac{C - T}{C},$$

where: $I\%$ – index of fungal mycelia growth reduction C – mycelia diameter in the control T – mycelia growth in the treatment

The data were processed, and software products used for the investigation were "MS Excel Analysis ToolPak Add-Ins" (https://support.office. com) and "R-3.1.3" in combination with "RStudio-0.98" and install package "agricolae 1.2-2" (De Mendiburu, 2015).

Results and Discussion

The limiting effect on mycelial growth at a dose of 0.5 mg/l of the tested products against *Verticillium dahliae* was reported for Zinc, with a particle size of 60–70 nm and 790 nm. A good effect for both isolates was reported with Zinc 18 nm, Mg micro powder 35 µm and Mg oxide 18 nm. It was found that as the reporting period increased, the effect of the nanoparticles on the mycelial growth of the fungus decreased. The weakest effect was registered for iron and iron oxide, and an increasing effect was also found in the first days of the reading (Table 1).

Table 1. *In vitro* screening to nanopowder/nanoparticles of metals and metal oxides inhibits mycelia grow to *Verticillium dahliae*

Product	3 day		6 day		9 day		12 day		15 day	
0.5 mg/l										
	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%
Iron oxide 18 nm	5.00	8.11	-1.75	1.79	11.11	14.44	0.00	0.00	0.00	0.00
Iron size 60-70 nm	30.00	18.92	14.04	6.25	22.22	22.22	0.00	0.00	0.00	0.00
Iron size 790 nm	5.00	8.11	1.75	2.68	8.89	6.67	0.00	0.00	0.00	0.00
Zinc 60-70 nm	55.00	48.65	77.19	79.46	83.89	83.33	76.11	76.67	68.33	70.56
Zinc 790 nm	55.00	40.54	55.26	56.25	61.67	62.22	53.89	51.11	26.67	30.00
Zinc 18 nm	33.00	37.84	50.00	48.21	55.00	60.00	45.56	45.00	0.00	0.00
Mg MP 35µm	50.00	45.95	56.14	53.57	56.67	55.00	45.00	45.56	0.00	0.00
MgO 18 nm	25.00	16.22	21.05	19.64	27.78	30.00	14.44	16.11	0.00	0.00
Product	3 day		6 day		9 day		12 day		15 day	
1.5 mg/l										
	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%
Iron oxide 18 nm	-2.63	-2.50	0.85	0.00	7.78	9.44	0.00	0.00	0.00	0.00
Iron size 60-70nm	18.42	17.50	13.56	17.24	26.67	22.22	0.00	0.00	0.00	0.00
Iron size 790 nm	10.53	15.00	5.08	6.03	6.67	11.11	0.00	0.00	0.00	0.00
Zinc 60-70 nm	52.63	55.00	83.05	82.76	87.78	84.44	81.67	74.44	70.00	73.33
Zinc 790 nm	47.37	50.00	73.73	71.55	77.22	77.78	60.00	66.11	18.89	20.00
Zinc 18 nm	39.47	45.00	60.17	60.34	65.56	63.89	54.44	52.78	42.22	37.78
Mg MP 35µm	44.74	50.00	54.24	57.76	58.89	57.78	48.89	12.22	0.00	0.00
MgO 18 nm	36.84	42.50	34.75	42.24	40.00	47.78	28.89	34.44	0.00	0.00
Product	3 day		6 day		9 day		12 day		15 day	
2.5 mg/l										
	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%	V1 – I,%	V2 – I,%
Iron oxide 18 nm	0.00	7.69	0.83	5.08	8.89	11.67	0.00	0.00	0.00	0.00
Iron size 60-70nm	26.83	17.95	3.33	22.03	30.00	33.33	0.00	0.00	0.00	0.00
Iron size 790 nm	4.88	-2.56	31.67	2.54	43.33	38.89	0.00	0.00	0.00	0.00
Zinc 60-70 nm	58.54	53.85	83.33	82.20	82.22	83.89	77.78	79.44	67.78	68.33
Zinc 790 nm	53.66	51.28	73.33	75.42	75.00	77.22	70.56	68.33	12.22	52.22
Zinc 18 nm	48.78	43.59	65.00	61.02	68.89	71.67	61.11	60.56	39.44	40.00
Mg MP 35µm	56.10	48.72	57.50	57.63	57.78	57.22	45.56	37.78	0.00	0.00
MgO 18 nm	51.22	53.85	56.67	54.24	56.67	51.67	45.56	40.00	0.00	0.00

The average tested nanoparticle concentration of 1.5 mg/l, the best limiting effect was observed for Zinc with a particle size of 60–70 nm during the entire reporting period. A better effect was registered with the remaining nanoparticles compared to the lower concentration of 0.5 mg/l. Again, iron was found to have no effect on mycelial growth, and a stimulatory effect was also observed at the beginning of the reading.

The high concentration (2.5 mg/l) of the products showed a high inhibitory effect on both tested isolates of the pathogen. A very good limiting effect is reported for almost all materials in the 3–12-day reporting period. Again, the very good effect of Zinc as a material limiting mycelial growth is confirmed. It is important to note that at this dose, mycelial growth was also inhibited by iron (Table 1).

Sizes in the range of 60–70 nm, 790 nm and 35 µm of nanorods had a limiting effect on mycelial growth at the low application dose (Figure 1). The medium and high application concentration of the products proves the higher efficiency against the pathogen at particle sizes 60–70 nm, 790 nm, 18 nm for Zinc (Figures 2 and 3).

Approximately 22 000 species of pathogens and pests are known to cause various diseases in crop plants, and are associated with substantial qualitative and quantitative crop losses (Adisa et al., 2019; Zhao et al., 2020). It has been reported that about 14% of crops are lost to diseases, and in susceptible cultivars yield losses could be up to 40% amounting $220 billion worldwide (Savary et al., 2019; Farooq et al., 2021).

Soil borne pathogens are significant contributors to plant yield loss globally. The constraints in early diagnosis, wide host range, longer persistence in soil makes their management cumbersome and difficult. Therefore, it is crucial to devise innovative and effective management strategies to combat the losses caused by soil borne diseases. The use of chemical pesticides is the mainstay of current

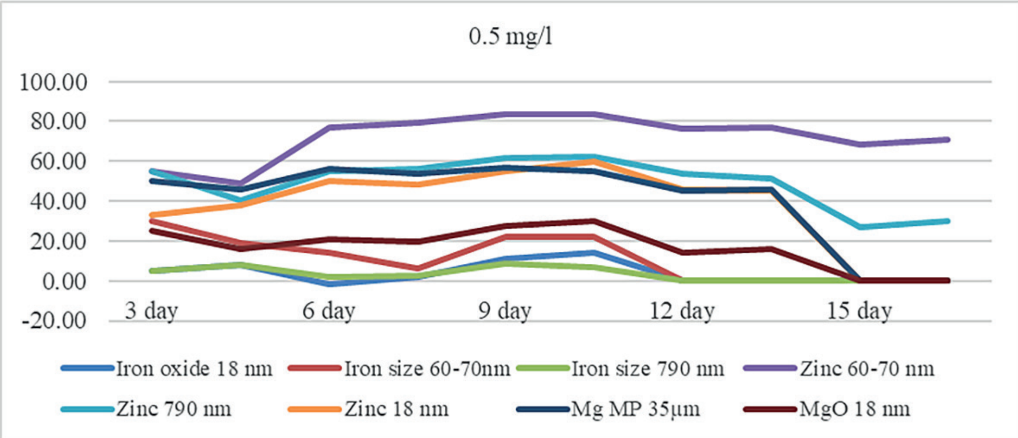

Fig. 1. Effect of nanoparticle size on mycelial growth of *Verticillium dahliae* isolates at a dose of 0.5 mg/l

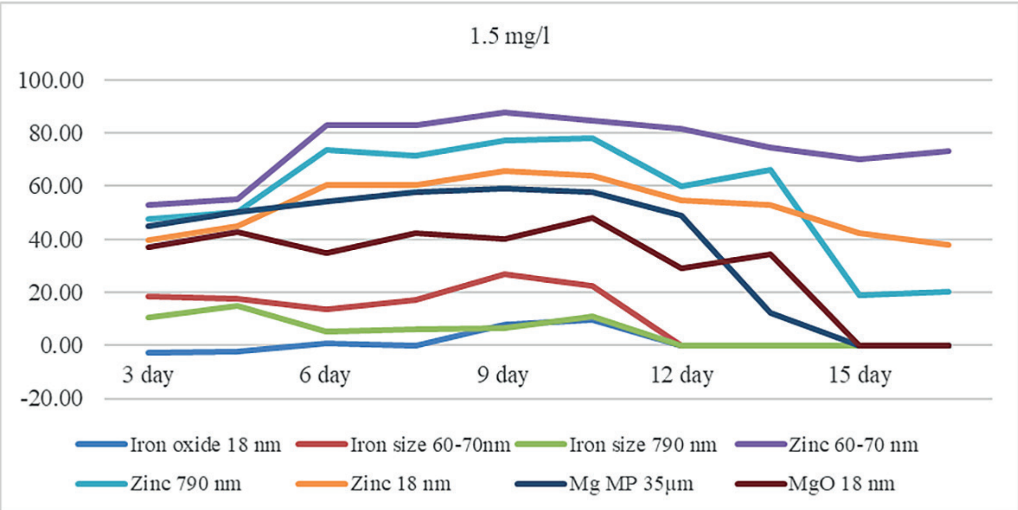

Fig. 2. Effect of nanoparticle size on mycelial growth of *Verticillium dahliae* isolates at a dose of 1.5 mg/l

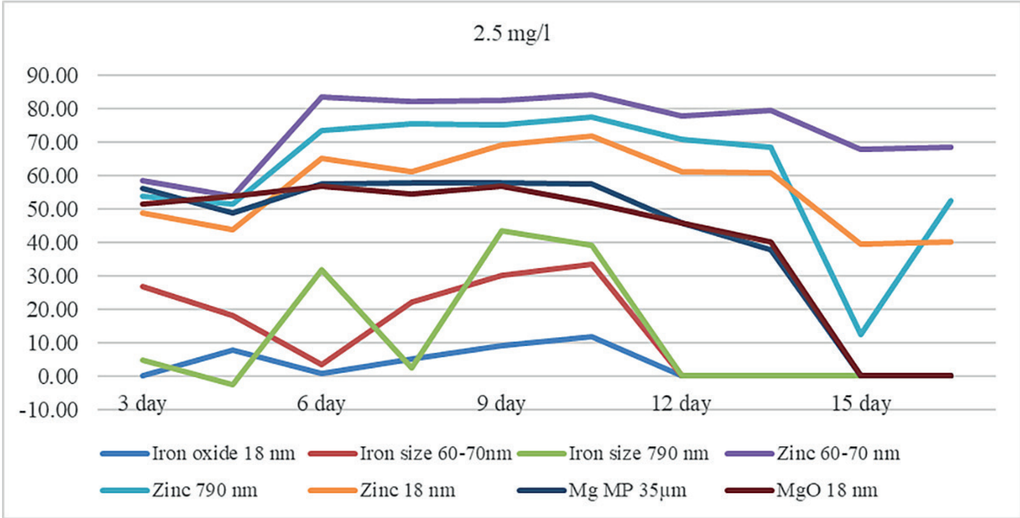

Fig. 3. Effect of nanoparticle size on mycelial growth of *Verticillium dahliae* isolates at a dose of 2.5 mg/l

plant disease management practices that potentially cause ecological imbalance. Nanotechnology presents a suitable alternative to overcome the challenges associated with diagnosis and management of soil-borne plant pathogens (Dutta et al., 2023).

The reduced efficacy of already available disease control compounds/formulations and the emergence of novel pesticide-resistant pathogen strains have increased the need for alternative measures, such as the application of NPs, to control crop diseases. The main aim of NPs application in plant disease management is to act as an agriculture amendment with greater ability to control the spread of crop diseases in a sustainable way. In contrast to conventional chemical products of similar composition, NPs are highly potent, require much lower application doses, and improve/maintain crop production (Adisa et al., 2019).

The antipathogenic potential of metal/metal oxide NPs against phytopathogens have been reported to depend on the size and dose of NPs (Kalia et al., 2020). Since metal/metal oxide NPs are applied at lesser rates as compared to their conventional equivalents, it can minimize the over application and environmental contamination.

Currently, most of the information related to the role of metal/metal oxide NPs in crop disease management has involved the use of Ag, Cu, Ti, & Zn based NPs (Elmer et al., 2018b; Elmer & White, 2018; Rajwade et al., 2020; Servin et al., 2015).

The anti-phytopathogenic potential of ZnO NPs have been reported in several studies (Graham et al., 2016; Hafez et al., 2014; Khan & Siddiqui, 2021; Khan & Siddiqui, 2018; Rajiv et al., 2013; Siddiqui et al., 2018, 2019; Wani & Shah, 2012).

These findings confirmed the previous reports that correlate Zn nutrition with enhanced biocontrol efficacy (Elmer & White, 2018). Hence ZnO NPs, along with other existing disease management strategies, have the potential to be used for improving plant health. Additionally, the recent advances in registering ZnO NPs products, such as Zinkicide™ for management of plant disease, are encouraging and emphasizes the recognition of metal/metal oxide NPs as a feasible alternative to conventional disease management approaches.

One of the key challenges in the agricultural industry is the need to address issues associated with pesticide use (environmental contamination, bioaccumulation, and increases in pest resistance), which demands a reduction in the quantity of pesticide applied for crop and stored product protection. Nanotechnology is emerging as a highly attractive tool to achieve this goal, by offering new methods for the formulation and delivery of pesticide active ingredients, as well as novel active ingredients, collectively referred to as nano pesticides (Hayles et al., 2017).

Conclusions

From all tested products, it can be concluded that Zinc has a very good effect limiting mycelial growth. A positive result was also observed with magnesium, while iron did not have such an effect, and even a stimulating effect was registered.

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