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Study of the antifungal effect of nanoparticles of metals and metal oxides on *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract

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Fusarium wilt is a systemic disease, as the fungus spreads inside the infected plant. The aim of this research is to investigate the antifungal effect of different metal ions on restricting the *Fusarium* mycelia growth.

The pathogenicity test of all isolates was confirmed on tomato variety Ideal. The isolates from *Fusarium oxysporum* f. sp. *lycopersici* were identified with a polymerase chain reaction (PCR)-based technique.

The inhibiting effect of iron oxide (Gamma high purity 99.55%, size 18 nm), iron purity 99.55% (size 60–70 nm), iron purity 99.55% (size 790 nm), zinc (high purity 99.55%, size 60–70 nm), zinc purity 99.55% (size 790 nm), zinc oxide (purity 99.99%, size 18 nm), magnesium micron powder (purity 99.95%, size 35 μm), magnesium oxide (purity 99.95%, size 18 nm) on the mycelium growth of the *Fusarium oxysporum* f. sp. *lycopersici* was tested. The nanoparticles which demonstrate the highest restriction level on the mycelia growth of *Fusarium* isolates in the 3 different concentrations were zinc (high purity 99.55%, size 60-70 nm).

Keywords: tomato; antifungal effect; metal ions; *Fusarium oxysporum*

Introduction

The *Fusarium* species are ubiquitous soil-borne pathogens of a wide range of horticultural and food crops which cause destructive vascular wilts, rots, and damping-off diseases (Bodah, 2017). In addition to the losses caused before or during harvest, some *Fusarium* species are capable of producing mycotoxins in food and agricultural commodities (Nayaka et al., 2008; 2009; Mudili et al., 2014).

Fusarium will on tomato *(Solanim lycopersicum*), caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen is an economically important disease, and it is a destructive disease on tomato crops worldwide (Jones et al., 1991). Three different host specific races of the pathogen (race 1, 2 and 3) have been identified (Cai et al., 2003). Race 1 was initially observed in variety I 886 (Booth, 1971) and race 2 was first reported in 1945 in Ohio (Alexander & Tucker, 1945). Race three of *F.o.* f. sp. *lycopersici* was identified in Australia in 1978 (Grattidge & O'Brien, 1982) and was subsequently reported in several U.S. States and Mexico (Davis et al.,1988).

Most commercial tomato varieties grown through the world are resistant to race 1 and 2, and a few are resistant to race 3 (Biju et al., 2017). Chemical treatments and soil solarization in fields usually fail to control the vascular wilt fungus. Developing resistant varieties involves crossing resistant wild-type plants and existing cultivars for their properties, such as color, shape, and good taste. Resistance genes liked to molecular markers would be beneficial for tomato development programs (Hanson et al., 2016).

Innovative solution for sustainable agriculture with high vegetation and crop yield is mandatory; novel nano materials

can improve plant immunity and restrain plant diseases. Iron is fundamental nutrient element; it plays vital role in enzyme activity and RNA synthesis; furthermore, it is involved in photosynthesis electron-transfer chains (Elbasuney et al., 2022). Plants' malnutrition is one of the most serious problems that threaten agricultural wealth; as it causes huge losses in agricultural production, reduction in the product quality, as well as the secretion of toxins that cause poisoning and multiple serious diseases affecting humans and animals that feed on this product (Powlson et al., 2011). The plant may be exposed to a series of oxidative explosions in the cells, and failure of enzymes to perform important chemical transformations to protect, may increase cell death and the susceptibility to pathogens infection with may increase (Chi et al., 2009).

Nanotechnology (polymer/inorganic nano composites) may bring potential benefits via improving plant immunity, disease resistance, and securing high crop yields (Pramanik et al., 2020), drug release (Xu et al., 2019), wastewater treatment (Abd Elkodous et al., 2021).

The aim of this research is to investigate the antifungal effect of different metal ions on restricting the *Fusarium* mycelia growth.

Material and Methods

Isolation

Fusarium isolates were collected in 2022 from different tomato varieties from Maritsa vegetable crops research Institute – Plovdiv, Bulgaria (MVCRI). One-centimeter segments were cut from the base of each stem and upper part of the taproot for each plant. Root and stem parts were washed free of soil, surface-disinfested for 2 min in 90% ethyl alcohol, rinsed with sterile distilled water, and plated onto water agar medium in 9 cm diameter Petri dishes. Mycelia colonies emerging from the plated segments were transferred to either potato dextrose agar, Czapek-Dox agar medium in 9 cm diameter Petri dishes for identification based on morphological characteristics and microscopic observations.

Pathogenicity

The pathogenicity tests were conducted for each isolation number. The ability of isolates to cause disease was tested in growth chamber on the tomato cultivar Ideal. The seedlings were inoculated by 20 ml of conidial suspension (72.3×10^6 conidia per 1000 ml). Non-inoculated seedlings were included as controls. Plants were irrigated daily starting immediately after inoculation.

DNA isolation and amplification of causative pathogen

Total DNA was extracted from mycelia obtained from PDA culture grown on at 25^0C for 7 days. Aerial mycelia were harvested from the culture plates using a sterile transfer needle, and placed in a sterile 1.5-ml micro centrifuge tube containing 300 µl of extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 25 mM EDTA, and 2% sodium dodecyl sulfate, pH 8.5). Uncapped tubes were placed in a boiling water bath for 5 min, and then cooled to 25°C. Two hundred µl of phenol that was equilibrated with extraction buffer (v/v) and 200 µl of chloroform was added. The tubes were vortexed for 4 min, and then centrifuged at 13000 *g* for 5 min. The supernatant was transferred to a new sterile 1.5-ml tube, and 200 µl of chloroform was added; the mixture was vortexed for 30 sec, and then centrifuged at 13000 *g* for 15 min. The supernatant was extracted with 200 µl of isopropanol, and centrifuged at 13000 *g* for 15 min. The nucleic acid pellet, after washing with 70% ethanol, followed by air-dried for 15 min, was resuspended in 50 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.5). DNA was finally treated with Ribonuclease A.

The targeted region amplifies the ITS region comprising 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 in 1% agarose gel in TBE buffer for 30 min at 100 V. 5 µl sample mixed with 2 µl dye were dripped. The products were 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 Fusarium mycelia growth

An *in vitro* screening was carried out under laboratory conditions. Two species *Fusarium oxysporum* f. sp. *lycopersici* were tested using Thornberry's method (Thornberry, 1950) to inhibiting mycelium growth effect using:

- Iron oxide nanoparticles (Gamma high purity 99.55%, size 18 nm);
- Iron nanoparticles purity 99.55% (size 60–70 nm);
- Iron nanoparticles purity 99.55% (size 790 nm);
- Zinc nanoparticles (high purity 99.55%, size 60–70 nm);

- Zinc nanoparticles purity 99.55% (size 790 nm);
- Zinc oxide nanoparticles (purity 99.99%, size 18 nm);
- Magnesium micron powder (purity 99.95%, size 35 μm);
- Magnesium oxide nanopowder (purity 99.95%, size 18 nm).

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. The 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/ml, 1.5 mg/ml and 2.5 mg/ml. 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:

$$I\% = \frac{C - T}{C} \times 100,$$

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

The data was statistically processed and using the software products "MS Excel Analysis ToolPak Add-Ins" (https://support.office. com) and "R-3.1.3" in combination with "RStudio-0.98" with installed package "agricolae 1.2-2" (Mendiburu, 2015).

Results

The *Fusarium* isolates were collected from tomato field in MVCRI, Plovdiv, Bulgaria. The pathogenicity of all isolates was confirmed on tomato variety Ideal. Symptoms in infected plants appeared 20 days after inoculation. Two isolates caused typical symptoms of *Fusarium* wilt – yellowing and drooping of lower leaves, exhibited stunting, dark brown vascular discoloration and death.

The isolates *Fusarium oxysporum* f. sp. *lycopersici* were identified based PCR technique. The primer pairs ITS 4 and ITS 5 were used for detection and discrimination of the species consisting of fragments with approximate length of 670–672bp fragment.

Fusarium oxysporum is an important, soil-inhabiting ubiquitous fungus, known for its phylogenetic diversity (Xiong & Zhan., 2018; Nicholas et al., 2017; Arpita et al., 2012). Certain molds produce toxic secondary metabolites called mycotoxins on a variety of plants and agricultural commodities that are closely connected to animal and human food chains (Ramana et al., 2012). Researchers found that plants treated with nano fertilizers and natural bio-stimulants tend to have more antioxidant enzyme activities (Yadav et al., 2019). Nanotechnology could play an important role in agriculture. The potential uses and benefits of nanotechnology are enormous and can be exploited to improve production and resistance to plant diseases (Rai & Ingle, 2012). Nanotechnology enables plants to exploit water, pesticides, and fertilizers more efficiently (Adisa et al., 2019).

The antifungal effect on nanoparticles and micron powders on the mycelia growth of *Fusarium oxysporum* f. sp. *lycopersici in vitro* tests was determined (Table 1).

On the third day of the reporting, in a concentration 0.5 mg/ml, the best pronounced effect of *Fusarium* isolates test with iron purity 99.55% (size 60–70 nm), iron purity 99.55% (size 790 nm) and zinc (high purity 99.55%, size 60–70 nm) (Figure 1). Our results are similar to Ashraf et al. (2022), reported that Fe3O4 NPs significantly reduced the disease severity in tomato plants infected with *F. oxysporum*.

On the 6th day, the antifungal activity is maintained for zinc (high purity 99.55%, size 60-70 nm) and magnesium nanopowder (purity 99.95%, size 35 μm). By the end of the 15-day reporting, the limitation of mycelia growth was visible with zinc (high purity 99.55%, size 60-70 nm) and magnesium nanopowder (purity 99.95%, size 35 μm) (Figure 2).

The concentration of 1.5 mg/ml of iron purity 99.55% (size 60–70 nm), zinc (high purity 99.55%, size 60–70 nm) and magnesium micron powder (purity 99.95%, size 35 μm) in the highest degree limit mycelia growth on the 3rd day after inoculation. During the entire reporting period, the best results were observed for zinc (high purity 99.55%, size 60–70 nm). This was confirmed at the highest concentration of 2.5 mg/ml (Figure 3).

Fusarium wilt is a systemic disease, as the fungus spreads inside the infected plant. It is difficult to combat *Fusarium* wilt disease chemically (Jain et al., 2019). This disease is very dangerous, especially in areas where the hot weather prevails during the planting season (Gressel et al., 2014). Members of *Fusarium* genus refuge biosynthetic machinery capable of producing interesting bioactive secondary metabolites, and produce antifungal, antibacterial and cytotoxic compounds, such as alkaloids, sesquiterpenes, polyketides, carotenoids, anthraquinone, cyclopentanone, and naphthoquinone derivatives (Manici et al., 2017). It is necessary investigate a new methods and techniques to control the disease.

Usually using soil solarization and chemical treatments in fields fail to control the vascular wilt fungus. *Fusarium* isolates treatment with different concentration of metal

Table 1. The impact of nanoparticles of metals and metal oxides on mycelia growth of *Fusarium oxysporum* f. sp. *lycopersici*

Product	3 day		6 day		9 day		12 day		15 day	
0.5 mg/l										
	F1 – I %	F2 – I %	F1 – I %	F2 – I %	F1 – I %	F2 – I %	F1 – I %	F2 – I %	F1 – I %	F2 – I %
Iron oxide	15.00	12.70	1.79	4.71	0.00	0.00	0.00	0.00	0.00	0.00
Iron size 60–70 nm	58.33	22.22	16.07	8.24	0.00	0.00	0.00	0.00	0.00	0.00
Iron size 790 nm	61.67	25.40	13.10	8.24	0.00	0.00	0.00	0.00	0.00	0.00
Zinc 60–70 nm	38.33	45.28	41.07	39.41	27.78	34.44	18.89	21.67	13.33	18.89
Zinc 790 nm	25.00	25.40	20.24	18.24	0.00	0.00	0.00	0.00	0.00	0.00
Zinc 18 nm	21.67	12.70	4.76	8.24	0.00	0.00	0.00	0.00	0.00	0.00
Mg MP 35 µm	33.33	38.10	31.55	39.41	19.44	24.44	14.44	16.67	0.00	0.00
MgO 18 nm	25.00	25.40	22.02	20.00	0.00	0.00	0.00	0.00	0.00	0.00
1.5 mg/l										
Iron oxide	8.62	-9.26	-3.53	-5.36	0.00	0.00	0.00	0.00	0.00	0.00
Iron size 60–70 nm	46.55	1.85	5.88	9.52	0.00	0.00	0.00	0.00	0.00	0.00
Iron size 790 nm	22.41	20.37	6.47	6.55	0.00	0.00	0.00	0.00	0.00	0.00
Zinc 60–70 nm	43.10	12.96	32.61	50.00	45.00	50.00	39.44	44.33	35.56	38.89
Zinc 790 nm	32.76	12.96	31.76	22.02	0.00	0.00	0.00	0.00	0.00	0.00
Zinc 18 nm	24.14	7.41	18.82	12.50	0.00	0.00	0.00	0.00	0.00	0.00
Mg MP 35 µm	39.66	25.93	35.88	38.69	25.00	27.22	13.89	13.33	0.00	0.00
MgO 18 nm	32.76	20.37	32.94	28.57	0.00	0.00	0.00	0.00	0.00	0.00
2.5 mg/l										
Iron oxide	15.52	1.67	6.47	-4.19	0.00	0.00	0.00	0.00	0.00	0.00
Iron size 60–70 nm	13.79	25.00	18.82	2.40	0.00	0.00	0.00	0.00	0.00	0.00
Iron size 790 nm	6.90	15.00	4.71	18.56	0.00	0.00	0.00	0.00	0.00	0.00
Zinc 60–70 nm	15.52	25.00	44.71	43.71	46.11	44.44	33.33	38.89	31.11	36.67
Zinc 790 nm	41.38	26.67	47.65	38.32	26.67	28.89	0.00	0.00	0.00	0.00
Zinc Oxide 18 nm	20.69	28.33	-5.88	23.35	0.00	0.00	14.44	7.78	0.00	0.00
Mg MP 35 µm	32.76	36.67	42.35	36.53	24.44	18.89	0.00	0.00	0.00	0.00
MgO 18 nm	55.17	51.67	40.59	38.92	13.33	11.67	0.00	0.00	0.00	0.00

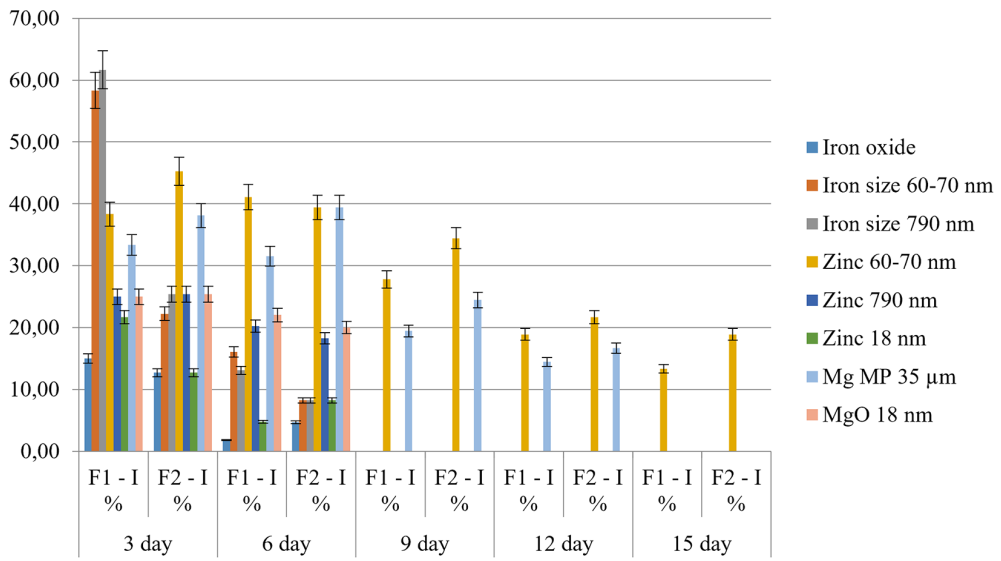

Fig. 1. The effect of concentration on 0.5 mg/l of different ions on mycelia growth of *Fusarium* isolates

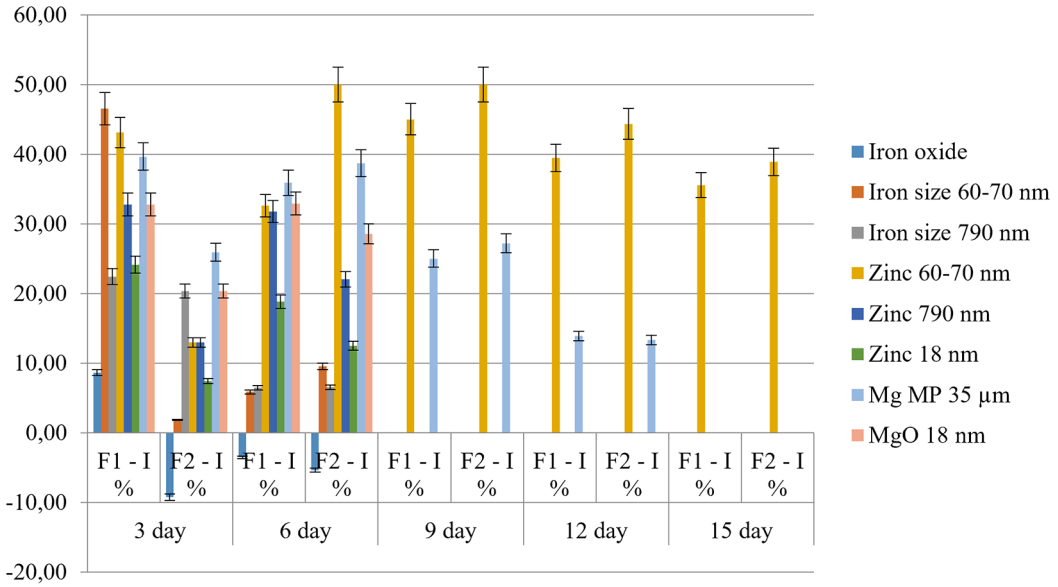

Fig. 2. The effect of concentration on 1.5 mg/l of different ions on mycelia growth of *Fusarium* isolates

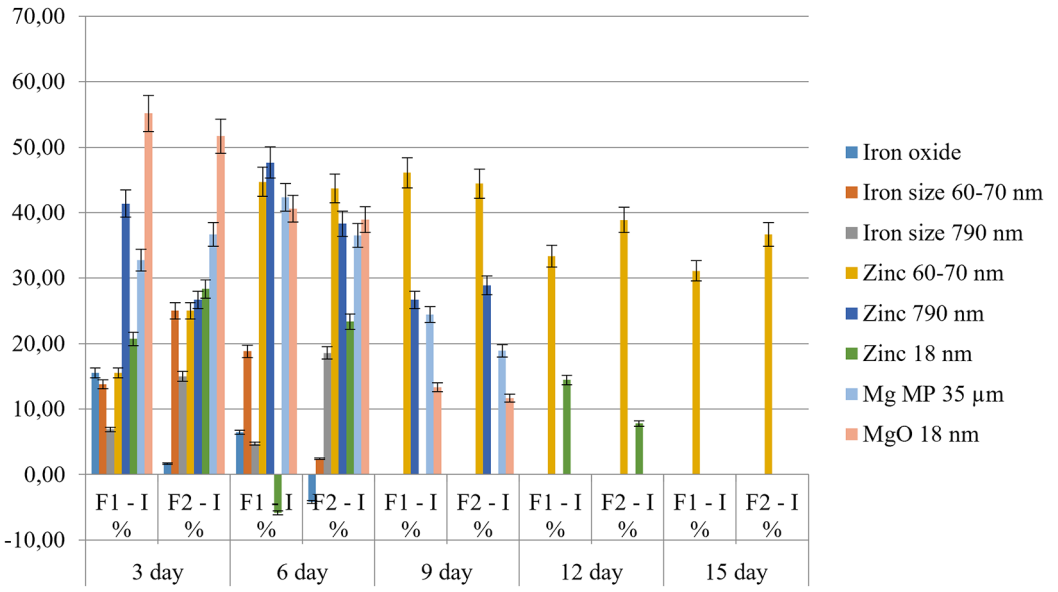

Fig. 3. The effect of concentration on 2.5 mg/l of different ions on mycelia growth of *Fusarium* isolates

nanoparticles and micropowder estimated the inhibitor effect of mycelia grow.

The nanoparticles which show the best effect to restrict the mycelia growth of *Fusarium* isolates were zinc (high purity 99.55%, size 60–70 nm) in the 3 different concentrations.

Conclusion

In vitro tests were used to determine the antifungal effect of nanoparticles on the mycelia growth of *Fusarium oxysporum* f. sp. *lycopersici*. The best pronounced effect in the direction of *Fusarium* isolates on the third day of the reporting was observed in a concentration of 0.5 mg/ml with iron purity 99.55% (size 60–70 nm), iron purity 99.55% (size 790 nm) and zinc (high purity 99.55%, size 60–70 nm). The activity was maintained for the zinc (high purity 99.55%, size 60-70 nm) and magnesium micron powder (purity 99.95%, size 35µm) on the 6th day. The concentration of 1.5mg/ml of iron purity 99.55% (size 60–70 nm), zinc (high purity 99.55%, size 60–70 nm) and magnesium micron powder (purity 99.95%, size 35 µm) limit mycelia growth to

the highest degree on the 3rd day. During the entire reporting period, the best results were observed for zinc (high purity 99.55%, size 60–70 nm) and confirmed at the highest concentration of 2.5 mg/ml.

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