STUDYING THE EFFICIENCY OF SOME NANOPARTICLES ON SOME PLANT PATHOGENIC FUNGI AND THEIR EFFECTS ON HYPHAL MORPHOLOGY

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ABSTRACT

This study was investigated the effect of iron NPs and essential oils of clove in normal and nanoemulsion forms for controlling *Fusarium oxysporum, Fusarium solani*, and *Alternaria solani* compared with Azoxystrobin fungicide under laboratory conditions. Data revealed that all tested compounds are capable of inhibiting the growth of mycelial of *F. oxysporum F. solani,* and *A. solani* from 0 to 84.4%, 0 to 88.9 %, and 0 to 61.1 %, respectively. There is relationship was found between the tested concentration of all treatments and their percentages of inhibition of mycelium. In addition, the use of Azoxystrobin, iron NPs and essential oils of clove in normal and nanoemulsion increased the activity of polyphenol oxidase (PPO). The fungal hyphae morphology was investigated by using scanning electron microscopy (SEM). The fungi hyphae without treatments are regular branching, linearly and normal morphology shaped, the surface of the hyphae is smooth and apical tapered. Treatments caused loss of linearity, irregular branching of the terminal hyphae, deformations of the hyphal shape, and the lysis cytoplasm of the hyphal.

Keywords: *Fusarium oxysporum, Fusarium solani*, *Alternaria solani*. Azoxystrobin, iron NPs, clove oil, clove oil NPs, PPO, SEM.

دراسة كفاءة بعض الجسيمات النانوية على بعض الفطريات المسببة للأمراض النباتية وتأثيرها على الشكل المظهري للهيفات.

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المستخلص:

تهدف الدراسة الى معرفة تاثير كلا من زيت القرنفل فى صورتة الطبيعية والنانوية والحديد النانوي والمبيد الفطري ازوكسيستروبين على فيوزاريوم أوكسيسبوروم و فيوزاريوم سولانى و التيرناريا سولانى فى المعمل. اظهرت النتائج ان جميع المركبات المختبرة قادرة على تثبيط الفطريات المختبرة. وكلما زاد تركيز المركبات المختبرة ادي الى زيادة نسبة التثبيط مقارنة بالكنترول. ومن خلال البيانات المتحصل عليها وجد أن فيوزاريوم سولانى هو الأكثر حساسية ، بينما أقلهم حساسية هو التيرناريا سولانى لجميع المركبات المختبرة. كما وجد أن مبيد ازوكسيستروبين أعطى أفضل النتائج ضد جميع الفطريات المختبرة. وايضا ادت المعاملات الى زيادة نشاط انزيم البولي فينول اوكسيديز مقارنة بالكنترول. كما اظهر استخدام الميكرسكوب الماسح للالكترونات ان هيفات الفطريات فى الكنترول ذات تفرعات منتظمة وشكلها طبيعى وملساء بينما هيفات الفطريات بعد المعاملة اصبحت ذات تفرعات غير منتظمة وادى الى حدوث تشوهات وتحلل الستيتوبلازم للهيفات الفطريات.

الكلمات المفتاحية: فيوزاريوم أوكسيسبوروم وفيوزاريوم سولانى والتيرناريا سولانى، ازوكسيستروبين، الحديد النانوي، زيت القرنفل

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INTRODUCTION

Due to the harmful effects the synthetic fungicides on the environment and humans. In addition to targeting pathogens, pesticides could kill various beneficial organisms, and their toxic forms can persist in soil (13) and their frequent use has led to the emergence of resistant strains. Additionally, in recent years public pressure to reduce the use of synthetic fungicides in agriculture has increased (2). The world begins to use plant-derived products as disease control agents as they had fewer environmental effects and low mammalian toxicity (7) and (6). Essential oils have a long history of use as natural microbial agents and are used in several foods, pharmaceuticals, and cosmetic products. Essential oils inhibited the growth of a wide range of microorganisms, with fewer side effects than synthetic fungicides (28). Clove (*Syzygium aromaticum* L) used for centuries for medicinal purposes or food preservatives. Flower buds had many medicinal properties as an antifungal, antimicrobial, antiviral, carminative, hypertensive aphrodisiac, light stomachic, and anesthetic (18) and (21). Clove essential oil had the antifungal properties to exhibit strong inhibitory effects against the mycelial growth of many fungi and is widely investigated due to its availability, popularity, and high essential oil content (27) and (22). Nowadays nano-biotechnology has emerged as one of the fastest-growing areas of research in different science and technology (35). The nanocapsules, prepared by interfacial deposition of the preformed polymer (nanoprecipitation), represent an effective method to obtain robust nanosystems suitable for applications in various fields, ranging from medicine, health, and agri-food to the environment **(9).** Nanoparticles have high efficiency against plant pathogens viz. nematodes, bacteria, viruses, and fungi, and easy application to soil, foliage, and seeds **(17).** The new trend of nanotechnology is safe eco-friendly antifungal agents such as plant-based oils. Essential oils (EOs) are lipophilic compounds, easily degradable by the effects of oxygen, light, moisture, and, temperature. Nanoencapsulation is a valid strategy to overcome these obstacles. This technology allows for the protection the essential oils from thermal and photodegradation phenomena, increasing their solubility in aqueous environments, masking their flavor, and improving their bioaccessibility and bioavailability **(12).** Nanoemulsion oil of Eugenol showed fungicidal activity against *F. oxysporum* **(3).** Nanoemulsion essential oil of citronella had high efficacy against *S. rolfsii* and *R. solani* **(5).** Nanoemulsions of clove, orange black seed, and lemon oils significantly decrease the mycelial growth of *F. solani, A. tenuissima, and G. candidum*. Nanoemulsion (25). The present study aimed to evaluate the antifungal activities of clove oil, clove oil (NPs), Fe (NPs), and Azoxystrobin fungicide for controlling *Fusarium oxysporum, Fusarium solani,* and *Alternaria solani*, as well as the determination PPO activities, and their effects on *F. oxysporum, F. solani, and R. solani* hyphae by using scan electronic microscope.

MATERIALS AND METHODS

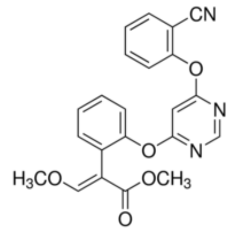
1. Treatments

Common name: Azoxystrobin :**Trade name:** Amistar 25 %SC

Chemical formula: $C_{22}H_{17}N_3O_5$

Chemical name: Methyl (E)-2-[2-(6-(2-cyanophenoxy) pyrimidin-4-yloxy) phenyl] =-3methoxyacrylate

Chemical structure



Clove oil: obtained from International Favors and Plant oils Inc., Giza., Egypt.

Clove oil (NPs): nanoemulsion for natural oil synthesized at College of Biotechnology, Misr University for Science and Technology, Egypt

Iron (NPs): obtained from the Agriculture Research Center of Kafr Eel-Sheikh

2. Fungi strains used

Fusarium solani (*F. solani*): Obtained from the Department of Plant Diseases, Faculty of Agriculture, Damanhur University

Alternaria solani (*A. solani*): obtained from the Department of Plant Pathology, Agricultural Research Station, Itay El Baroud

Fusarium oxysporum (*F. oxysporum*): brought from Plant Pathology Institute, Agricultural Research Center

2.1. *In vitro* antifungal activity assay

Used to essential clove oil at concentrations of 200 and 400 μg/ml, clove oil nanoemulsion (NPs) at concentrations of 50 and 100 μg/ml, iron NPs at concentrations of 50 and 100 μg/ml, and fungicide Azoxystrobin at 250 μg/ml were tested *in vitro* against *F. oxysporum, F. solani,* and *A. solani* by Poisoned Food Technique. Added separately to get the required concentrations then mixed with 50ml of sterilized PDA medium and transferred equally into three Petri dishes (9 cm). The media allowed solidifying. Control without tested compounds. These plates were inoculated with an added disc 5cm diameter of the culture of the test fungi. All dishes were incubated at 27±2ºC for 7 days and radial growth of the colony was measured when the mycelia of control were complete with the colonization of the fungi. Each treatment was performed in triplicate. The inhibition percentages were calculated according to the method of **(33)** by using the formula:

$$\mathbf{MGI\% = \frac{R-r}{R}\,x100}$$

MGI%: mycelium growth inhibition percentage

R: the growth of mycelium of fungi in the control plate

r: the growth of mycelium of fungi in the treatment plate

3. Determination of polyphenol oxidase activity (PPO).

The activity of polyphenol oxidase was assayed according to the method of **(10**) using a spectrophotometer as follows: The sample of each fungus was homogenized in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 4000 rpm for 10 min at 4˚C. The final mixture contained 1 mL enzyme, 2 mL 0.1 M phosphate buffer (pH 7.0), and 1 mL 0.1 M methyl catechol, and an increase in the absorbance was measured at 495 nm. The activity of PPO was expressed as units'/mg tissue.

4. Effects of different antifungal treatments on hyphae morphology: The changes in the morphology of fungal hyphae using different antifungal treatments were characterized by scanning electron microscopic (SEM). The SEM observation (JOEL, JSM 5300) with high resolution at an accelerating voltage of 120 Kev. An aliquot of each material was coated with a copper grid and scanned for its shape and size. The tested cells were subjected to X-ray Electron Dispersive Analysis (EDA) using an X-ray Oxford detector unit (model 6697, England)- Faculty of Science, Alexandria University.

5. Statically analysis

Data were analyzed using ANOVA. Analysis of variance (ANOVA) using software 1998 - 2005 cost at 6.3111 and Duncan, multiple range test at level $P < 0.05$ used method of Winer 1971.

RESULTS AND DISCUSSION

Comparing obtained data using Essential oil (EOs) of clove in normal and nanoemulsion, iron NPs and Azoxystrobin fungicide at used concentrations as μg/ml in Table (1). Through the data obtained, it was found that *F. solani* is the most sensitive, while the least sensitive is *A. solani* to all tested compounds. It also found that the fungicide Azoxystrobin gave the best results against all tested fungi. The highest level of inhibition was obtained using the fungicide Azoxystrobin, while the lowest level of inhibition was by iron NPs at 50 μg/ml. The growth of mycelium decreased with increasing the concentrations in all treatments. Data revealed that all tested were compounds capable of inhibiting the growth of mycelial of *F. oxysporum F. solani,* and *A. solani* from 0 to 84.4%, 0 to 88.9 %, and 0 to 61.1 %, respectively. The inhibition growth of mycelium increased with increasing the concentrations in all treatments.These results agree with (41) who found that the antifungal activities of Azoxystrobin fungicide and clove oil were able to inhibition of spore germination of the fusarium isolated. In addition, Azoxystrobin and Sarfun 500 μg/ml were regarded as a prospective means of limiting the development and inhibiting mycelium growth of *Colletotrichum gloeosporioides* and protecting *Hypericum perforatum* L. (11). Also, essential clove oil had stronger antifungal activities against *Penicillium citrinum* and *Aspergillus flavus* than *Rhizopus nigricans* (39). The previous research showed that microbial-synthesized iron nanoparticles by *Fusarium* *oxysporum* exhibited antimicrobial activity (32) and ZnO NPs enhanced the antimicrobial effect (30). The activity of Azoxystrobin fungicide compared with essential oils extracted from *Eberm linaloolifera, Eucalyptus staigeriana, Cinnamomum camphora, and Eucalyptus globulus L.* against *Alternaria solani* and all tested compounds controlled early blight disease *in vitro* assays (36). Essential oil clove had antifungal activity and strongly inhibited the mycelial growth of fungi (8). Essential oils play an important role in controlling plant diseases and pests. Nanoemulsion containing lemongrass and clove oils had antifungal agents against *F. oxysporum* and was rapidly affected as fungicidal (29). Many authors explained the antimicrobial activity of plant oils due to passing oils through the cell wall and cytoplasmic membrane of the fungus depending on partition and hydrophobicity in the plasma membrane. The clove oil exhibited inhibitory activity against the mycelial growth of *F. oxysporum* and *R. solani*. The concentrations used significantly reduced the mycelial growth compared with the control (34). The biosynthesized iron nanoparticles exhibited inhibitory activity against different microorganisms (22). The efficiency of nanoemulsions of clove, orange black seed, and lemon oils against *F. solani, A. tenuissima* and *G. candidum*. Nanoemulsion of clove and black seed oils showed a significant decrease in the mycelial growth of fungal isolates at 5000 ppm (25)

Table 1. Effects of Azoxystrobin, iron NPs and essential oil of clove in normal and nanoemulsion forms on mycelial growth (cm) and inhibition (%) of *F. oxysporum, F. solani,* and *A. solani* after 7 days of incubation at 27±2 ˚C

Treatments (µg/ml)	Mycelial growth (cm)			Inhibition (%)		
	F.o	F.s	A.s	F.o	F.s	A.s
*Control	9.0a	9.0a	9.0a	0.0	0.0	0.0
Clove oil 200	4.6d-f	3.7f-i	5.9c	48.9j	58.9gh	34.4 l
Clove oil 400	3.9e-h	3.1g-k	5.3cd	56.7hi	65.6e	41.1k
Clove oil (NPs) 50	4.1d-g	2.6h-k	5.1c-e	54.4i	71.1d	43.3k
Clove oil (NPs) 100	3.3f-j	2.2i-k	4.6d-f	63.3ef	75.6c	48.9j
Fe (NPs) 50	6.1 bc	4.1d-g	7.1b	32.2 l	54.4i	21.1m
Fe (NPs) 100	5.3cd	3.5f-j	8.1a	41.1k	61.1fg	10.0m
Azoxystrobin 250	1.4jk	1.0k	3.5f-j	84.4b	88.9a	61.1fg

F.o: *F. oxysporum*, F. s: *F. solani*, and A. s: *A. solani*
Each number represents the mean of 3 replicates.
***Control without active ingredient (medium free and discs were cut from the pathogen only on PDA).**
Different letters in Table (1) indicated significant differences according to Duncan's multiple range test at the level P ≤ 0.05

Determination of polyphenol oxidase enzyme (PPO).

The efficiency of Azoxystrobin, iron NPs and clove oils in normal and nanoemulsion on polyphenol oxidase enzyme showed in Figure (1). Treatment with Azoxystrobin fungicide and nanoparticles led to an increase in polyphenol oxidase enzyme in all tested fungi. The highest increase was when using the fungicide Azoxystrobin, while the lowest was when using Fe NPs at 50 µg/ml compared to the control. The results also showed an increase in polyphenol oxidase enzyme in *A. solani*, then followed by *F. oxysporum*, and the least of them *F. solani*. In addition, the increase in concentrations in all treatments increased the polyphenol oxidase enzyme.The more recent research on polyphenol oxidase reviewed fungi and plants and the main aspects considered properties, structure, location, distribution, and discovered inhibitors (PPO). Most fungi and plants have multiple forms of PPO and their function remains enigmatic. The function of PPO is probably different from that in plants and PPO plays an important role in defense against other pathogens (23). The activity of PPO in response to treatment with compounds indirectly helped in cell wall reinforcement to prevent the penetration and dissemination of compounds within the cell wall of fungi (19). The mechanism of definition against microbial pathogens initiates with the induction of defense enzymes such as PPO, deposition of the cell wall, and hypersensitive response (26). CuO NPs and MgO NPs had a great impact on increasing the enzyme activities of PPO (Ismail 2021). Treated with ZnO NPs increased the activities of PPO enzyme defense (16). The activity of polyphenol oxidase increased when treated with oil of Vitex agnus-castus L and zinc oxide nanoparticles compared with the untreated (4).

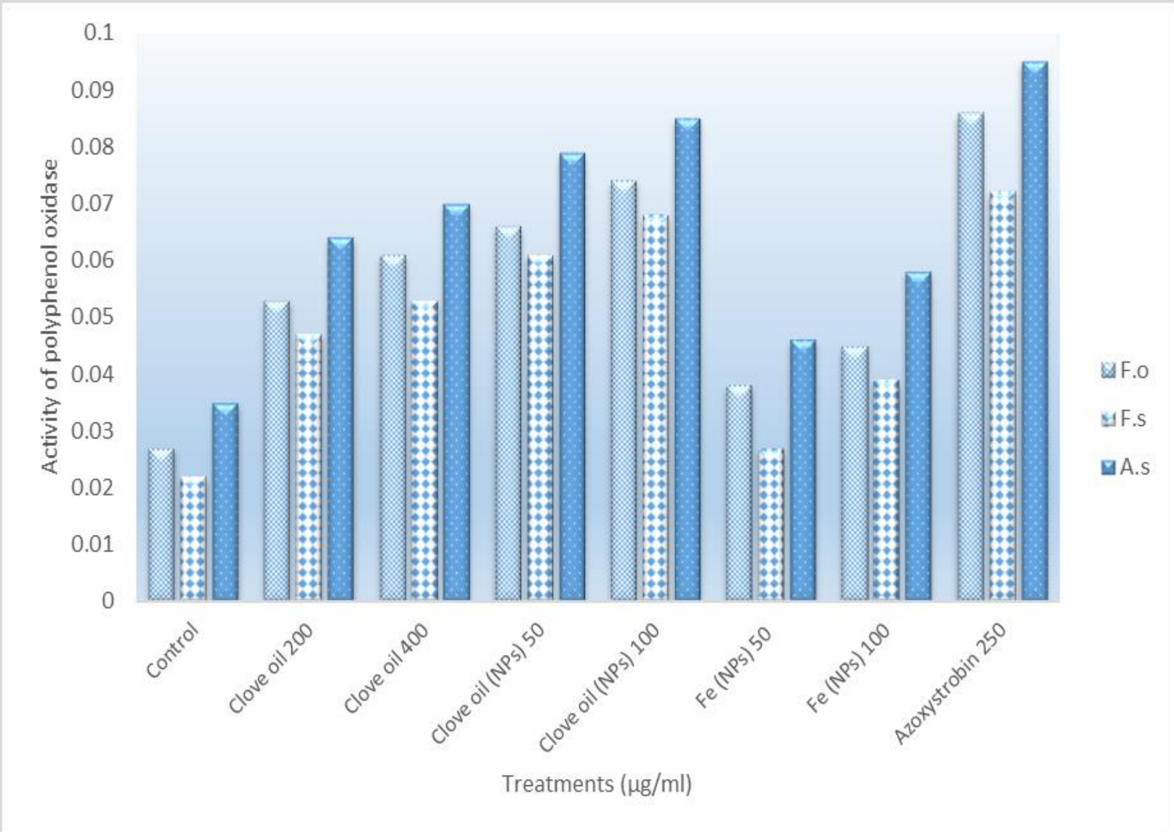


Figure 1. Effects of Azoxystrobin, iron NPs and essential oils of clove in normal and nanoemulsion forms on polyphenol oxidase enzyme (PPO) in *F. oxysporum, F. solani,* and *A. solani*

Effects of Azoxystrobin fungicide, iron NPs and clove oils in normal and nanoemulsion on Morphological fungi hyphae: Use the scanning electron microscope at 1500 magnification to know the effect of the Azoxystrobin fungicide, iron NPs and clove oils in normal and nanoemulsion forms on hyphae of *F. oxysporum*, *F. solani*, and *A. solani*. The information obtained from the SEM images referred to the effect on the hyphae of each fungus varies according to the type and concentration of each treatment. In addition, the shape of the hyphae for each fungus differs in control from the treatment. The data from obtained results by SEM revered significant morphological changes in the surface of the cell wall of each fungus. It found that the surface wall of the fungus *F. solani* was the highest affected, while the least affected was *A. solani*. The hyphae on control regular branching, linearly and normal morphology shaped, the surface of the hyphae is smooth and apical tapered. Treatments caused loss of linearity, irregular branching of the terminal hyphae, deformations of the hyphal shape, and lysis cytoplasm of the hyphal. The use of the pesticide Azoxystrobin caused the rupture of hyphae, irregular branching in the apical of hyphae, and the loss of linearity and morphological shape. The effect of the Azoxystrobin on the rupture and deformation of the hyphae in the fungus *F. solani*, while the fungus *A. solani* was the least deformation and rupture in its hyphae. It also found that clove oil in the nanoemulsion form caused more rupture and distortion of the hyphae than clove oil in its normal form. Iron nanoparticles cause less tearing and deformation of hyphae than Azoxystrobin and clove oil. Effects of Azoxystrobin, iron NPs and essential oils of clove in normal and nanoemulsion forms on *F. oxysporum* hyphae in Figure (2). Through the results obtained from the scanning electron microscope, these treatments affected the hyphae of the fungus and led to the occurrence of deformations and ruptures, and the most obvious of them were Azoxystrobin, then followed by nanoemulsion of clove oil, clove oil, and the least effect by iron NPs. Effects of Azoxystrobin, iron NPs and essential oils of clove in normal and nanoemulsion forms on the hyphae of *F. solani* in Figure (3). Treatment with these compounds leads to distortions and ruptures, and these distortions in *F. solani* hyphae were more than what happened to the hyphae of *A. solani* and *F. oxysporum*. The occurrence of distortions may be due to its sensitivity to the tested compounds over other fungi. Effects of Azoxystrobin, iron NPs and essential oils of clove in normal and nanoemulsion forms on *A. solani* hyphae in Figure (4). It observed that the hyphae of *A. solani* fungi were the least affected, perhaps due to the least sensitivity to the tested treatments over other tested fungi. Treatment with oil of clove on the *P. digitatum* caused irregular branching of hyphae, loss of linearity, and effects on the morphology of hyphae (40). Cavities and pores observed on the treated surface of hyphae with AgNPs and pits may be due to the creation of pores on the membranes of fungus. The reaction of AgNPs with sulfur and phosphorous-containing materials outside or inside of cells possible cause. In addition, positive charges of AgNPs maybe bind with the negative charge of fungal membranes destroying the lipid bilayer of the membrane, leading to inducing intracellular ion efflux resulting in the death of the cell (15). The treatment with AgNPs caused morphological changes in the cell wall surface of hyphae of A. solani compared to the control (2). The effect of clove oil on fungi morphological features showed disruption and conidial malformation as well and in the case of F. oxysporum, clove oil increased the production of chlamydospores but decreased the conidial numbers (34). AgNPs caused changes in the structure of a mycelial and deformation of the hyphal in *F. solani* (37). The hyphae of *R. solani* treated with ZnO NPs lost their smooth surface and formed unusual bulges on the surface of hyphae opposite of untreated hyphae with a smooth surface may be back to ZnO NPs deforming hyphae structure and inhibited *R. solani* growth (16).

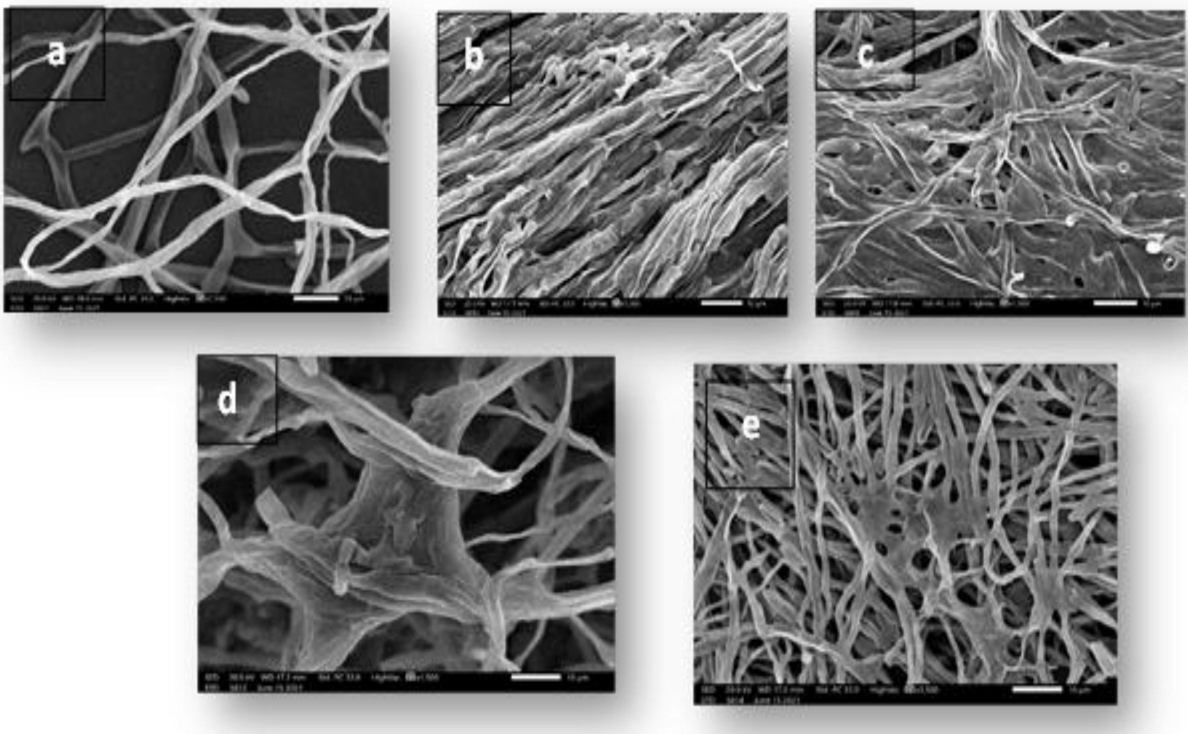


Figure 2. SEM of *F. oxysporum* hyphae after a week of incubation at 27 °C. (a) Control, (b) treated with Azoxystrobin, (c) treated with clove oil, (d) treated with clove oil nanoemulsion, (e) treated with iron NPs

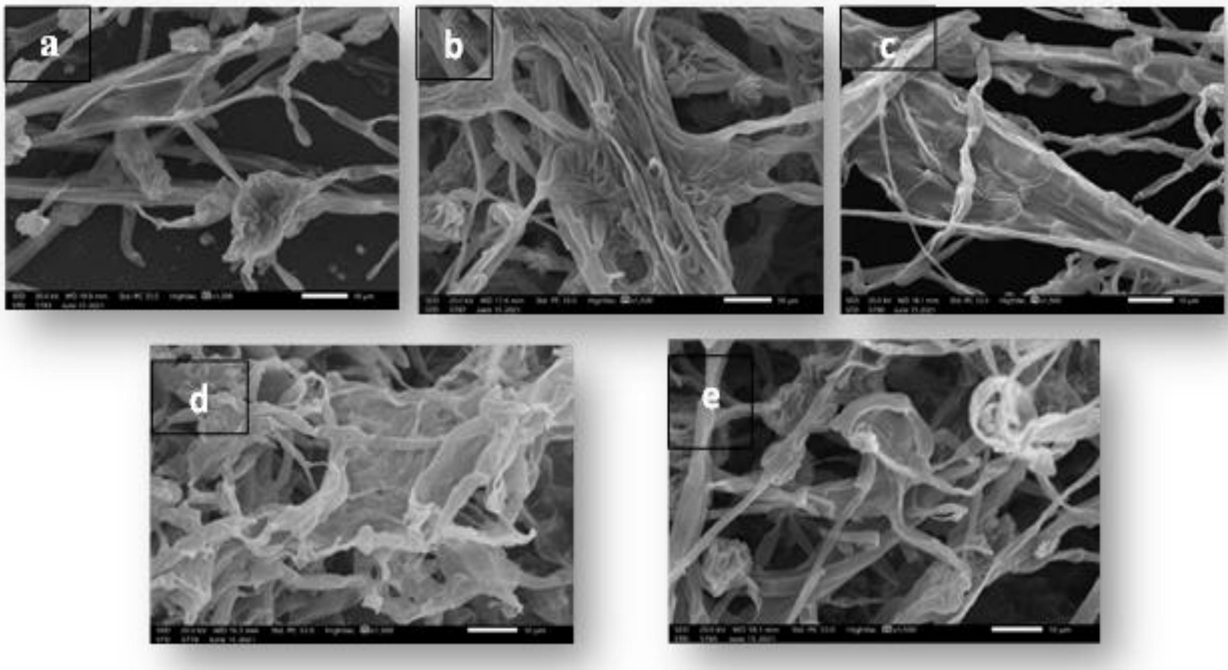


Figure 3. SEM of *F. solani* hyphae after a week of incubation at 27 °C. (a) Control, (b) treated with Azoxystrobin, (c) treated with clove oil, (d) treated with clove oil nanoemulsion, (e) treated with iron NPs

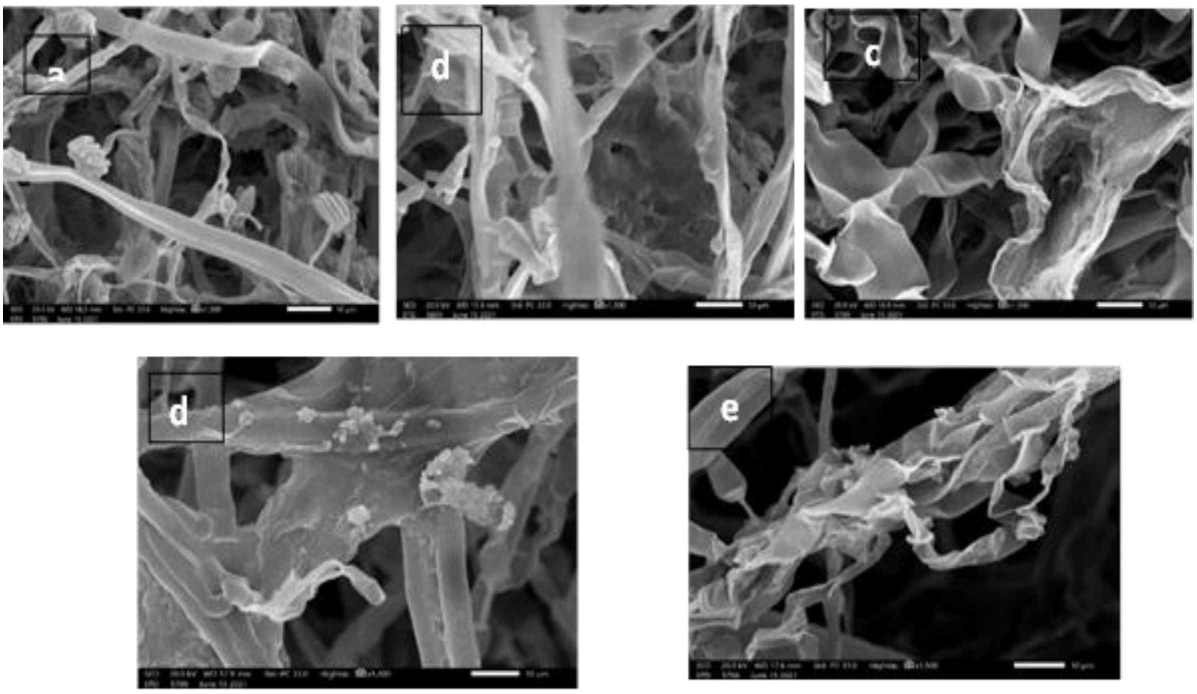


Figure 4. SEM of *A. solani* hyphae after a week of incubation at 27 °C. (a) Control, (b) treated with Azoxystrobin, (c) treated with clove oil, (d) treated with clove oil nanoemulsion, (e) treated with iron NPs

CONCLUSION

Azoxystrobin, iron NPs and essential oils of clove in normal and nanoemulsion forms had antifungal activities against *F. oxysporum, F. solani*, and *A. solani*. Treatments with tested compounds led to an increase in polyphenol oxidase enzyme in all tested fungi. SEM indicated the shape of the hyphae for each fungus differs in control from the treatment.

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