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Research Article
Fungal Biosynthesis of Silver Nanoparticles and Their Role in Control of *Fusarium* Wilt of Sweet Pepper and Soil-borne Fungi *in vitro*

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Abstract

Background and Objective: Use of silver nanoparticles (AgNPs) against phytopathogens is a rapidly growing area due to their unique physico-chemical properties. Therefore, the present investigation reports the biosynthesis of AgNPs by *Trichoderma harzianum* (*T. harzianum*), a safe fungus for human and plant. **Materials and Methods:** The synthesized AgNPs were subjected to physical characterization using UV-Visible spectra, fourier transform infrared spectroscopy (FT-IR) and scaninig electron microscopy (SEM) images. Antifungal activity of synthesized AgNPs with poisoned food technique was used against phytopathogenic fungi. **Results:** UV-Vis spectra with characteristic absorption peak at 415 nm. Biomolecules mediating the synthesis and stabilizing the nanobactericides was confirmed with fourier transform infrared spectroscopy (FTIR). Scanning electron microscopy (SEM) investigations confirmed that AgNPs were formed with 7.8 nm. Isolation trials from sweet pepper plants showing characteristic symptoms of wilt yielded one fungal isolate which purified and identified as *Fusarium oxysporum* (*F. oxysporum*). While two fungal species *Alternaria alternata* and *Trichoderma harzianum* were isolated from health plant (no symptoms of wilt). As the applied concentrations of the AgNPs increased, fungal colony formation decreased. AgNPS at concentrations of 20, 40 and 80 ppm inhibited the *F. oxysporum* growth by 12.5,12.5 and 61.11%, respectively. Concentrations at 80 ppm caused 100% growth inhibition of *T. harzianum* and *A. alternata* while concentrations at 100 ppm caused 100% growth inhibition of *F. oxysporum*. **Conclusion:** The present study demonstrated that it is possible to perform the biogenic synthesis of AgNPs that used as fungicide against pathogenic fungi.

Key words: Biosynthesis, silver nanoparticles, *Fusarium* wilt, sweet pepper, soil-borne fungi

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Till now, several physical and chemical methods were employed for the synthesis of silver nanoparticles (AgNPs)[1]. However, concern has been raised on the toxicity of chemical agents used in AgNPs synthesis. Thus, it is essential to develop a green approach for AgNPs production without using hazardous substances to the human health and environment. Compared with the traditional synthetic methods, biological strategies provide a novel idea for the production of nano-compounds[2,3].

Using fungi for biosynthesis of nanoparticles is advantageous as compared to plants because fungi produces more protein which results in high production of nanoparticles[4] and provides longer stability[5]. Green synthesis of AgNPs using a number of fungi such as the bioagent *Trichoderma* sp.[6], *Stachybotrys chartarum*[7] and *Penicillium citrinum*[8]. Other fungi such as *Trichoderma harzianum*, *T. virens*, *T. asperellum*, *T. pseudokoningii* and *T. longibrachiatum*[9,10] have been used for AgNPs synthesis. Newly the antifungal activity of silver nanoparticles has been studied by several researchers(Abdelghany *et al*.[3], Abdel Ghany[7], Xu *et al*.[11] and Abdel Ghany *et al*.[12]).

Akram *et al*.[13] stated that sweet pepper (*Capsicum annum* L.) is considered one of the most essential vegetable crops because of its high nutritional value including antioxidants, vitamins and other nutritional compounds. Therefore, improving the bio-production of this crop as well as vegetables is one of the objectives in agriculture in the world[13]. Sweet pepper is liable to be attack by microbial diseases including bacterial, fungal, viral and nematode diseases as well as physiological disorder. However, *Fusarium* wilt is considered the major devastative and destructive fungal disease affecting crop production of pepper[14,15].

Beside natural and biological control of phytopathogens[16-18], in recent years, engineered nanoparticles have achieved particular attention as a potential candidate for improving crop yield, resistance and disease management technologies[19,20]. Findings from Kim *et al*.[21] demonstrated that AgNPs with low toxicity and a broad spectrum of antimicrobial activity were also very effective against plant phytopathogenic fungi. Also, Min *et al*.[22] showed that silver nanoparticles strongly inhibited the fungal growth and sclerotial germination of *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *S. minor*. Elamawi and El-Shafey[23] reported that AgNPs reduced *Fusarium* disease incidence on tomato to 5% compared to 100% for the untreated plant (control). Objectives of the present study were to biosynthesis of AgNPs using fungal biomass of *Trichoderma harzianum* and to evaluate the efficacy of AgNPs for suppression of plant pathogenic fungi *in vitro*.

MATERIALS AND METHODS

Biosynthesis of silver nanoparticles: The *Trichoderma harzianum* RCMB 017002 (2) was obtained from culture collection of Regional Center for Mycology and Biotechnology (RCMB), Al- Azhar University Cairo, Egypt. *Trichoderma harzianum* RCMB 017002 (2) inoculated in potato dextrose broth media for 7 days at 28°C. The biomass was harvested after complete incubation by filtering through filter paper followed by repeated washing with distilled water to remove any medium component from the biomass for several times. Three grams of fungus biomass was brought in contact with 100 mL of sterilized double distilled water with concentration of 1 mM $AgNO_3$ and incubated at 25°C for 3 days. Control (without the silver ion) was also run along with the experimental flask.

UV-visible spectroscopic analysis: The reduction of silver ions was confirmed by qualitative testing of supernatant by UV-Visible spectrophotometer. One milliliter of sample supernatant was withdrawn after 24 h and absorbance was measured using UV-visible spectrophotometer between 300-800 nm at RCMB.

Fourier transform infrared spectroscopy and transmission electron microscopy analysis: The dried powder of AgNPs was subjected to fourier transform infrared spectroscopy (FTIR) analysis. Two milligrams of the sample was mixed with 200 mg KBr (FTIR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 450-500 cm^{-1} in FTIR spectroscopy at a resolution of 4 cm^{-1}. Finally, the AgNPs were characterized by Scanning Electron Microscopy (C Joel Jem-1200 EX II. Acc. Voltage 120 kV. MAG-medium) at RCMB.

Isolation of the pathogen and other fungi: Root samples of 5 of each pepper plants infected with wilting and health plants were collected from vegetable garden, Jazan, Saudi Arabia during June, 2016. The root samples were cut into small pieces up to 1.5 cm length and surface sterilized by 15% H_2O_2 for 30-45 sec, then rinsed with distilled water for 3 times. These surface sterilized roots were placed onto potato dextrose agar (PDA) medium in petri plates and incubated at

25°C for 6 days. The appearing fungal isolates on the root pieces were transferred to PDA medium petri plates for purification and identification according to Booth[24], Domschand Gams[25], Leslie and Summerell[26].

Antifungal activity of silver nanoparticles by using poisoned food technique: Potato dextrose agar medium (PDA) with different concentration (20, 40, 80 and 100 ppm) of AgNPs was used. About 25 mL of the growth medium was poured into each petri-dish and allowed to solidify. Five millimeters disc of 5-day-old culture of the test fungi was placed at the center of the Petri-dish and incubated at 27°C for 7 days, the growth was measured in millimeter. The PDA medium without the AgNPs served as control. The fungi toxicity of AgNPs in terms of percentage inhibition of mycelia growth was calculated by using the formula:

$$\text{Inhibition (\%)} = \frac{dc\text{-}dt}{dc} \times 100$$

Where:

dc = Average increase in mycelial growth in control

dt = Average increase at each treatment[27]

Statistical analysis: The results are reported as Mean±SD of three independent replicates. Statistical analyses of data were carried out by computer using SPSS version 22.0 software.

RESULTS AND DISCUSSION

Different fungal species of *Trichoderma harzianum* were tested for extracellular biosynthesis of AgNPs (Table 1). *T. harzianum* RCMB 017002 (2) was the strain able to synthesis AgNPs. According to previous studies AgNPs were successfully synthesized from agriculturally beneficial fungus *T. harzianum*[10,28].

Reduction of silver ions into AgNPs using *T. harzianum* RCMB 017002 (2) was evidenced by the visual color change of solution of silver nitrate turned dark brown on addition of fungal biomass under dark condition due to excitation of surface plasmon vibrations in AgNPs whereas, the control (without silver nitrate salt) did not exhibit any color change as shown in Fig. 1, it indicated the formation of AgNPs. The generation of dark brown color is due to the surface plasmon resonance exhibited by the AgNPs. Similar observation was made by several authors (Abdel Ghany[7], Vahabi *et al.*[29], Shelar and Chavan[30]). The synthesis of the AgNPs in aqueous solution was monitored by recording the absorption spectra at a wavelength range of 200-800 nm (Fig. 2). In the UV-Vis spectrum, surface plasmon resonance (SPR) peak was observed at 415 nm that confirmed the synthesis of AgNPs which are nearby similar to result of Jyoti *et al.*[31] reported an intense peak at 414 nm. Past studies suggested that a SPR peak located between 410 and 450 nm has been observed for AgNPs and might be attributed to spherical nanoparticles[30,32].

Size distribution of AgNPs in the aqueous solution was evaluated by SEM images. The size distributions of AgNPs were obtained by measuring nanoparticle diameter in the images (Fig. 3). The nanoparticles shape observed by SEM were almost of spherical shape and the average particle sizes 7.8 nm. In contrast , Devi *et al.*[9] reported that the average size of AgNPs synthesized by another species of *Trichoderma* was 8-60 nm. Also, Ahluwalia *et al.*[10] found that the size of

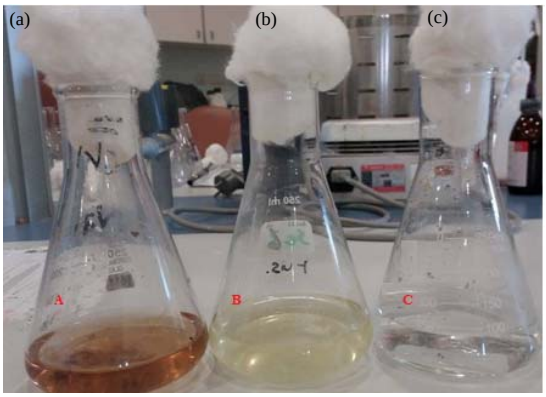

Fig. 1: Conversion silver nitrate to AgNPs by *T. harzianum* RCMB 017002 (2), (a) Distilled water inoculated with biomass and silver nitrate, (b) Metabolized medium without silver nitrate inoculated with biomass as a control and (c) Silver nitrate solution without biomass as a control

Table 1: Extracellular biosynthesis of AgNPs by different strains of *Trichoderma*

Fungal strain	Extracellular biosynthesis of AgNPs
Trichoderma harzianum RCMB 017002 (1)	-ve
Trichoderma harzianum RCMB 017002 (2)	+ve
Trichoderma viride RCMB 017001 (1)	-ve
Trichoderma viride RCMB 017001 (2)	-ve
Trichoderma longibrachtichum RCMB 017005	-ve
Trichoderma hamatum RCMB 017004	-ve

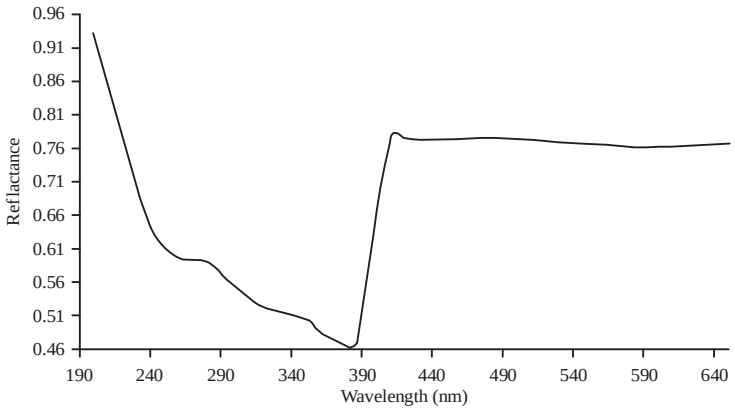

Fig. 2: UV-Vis spectrum of silver nanoparticles produced by *T. harzianum* RCMB 017002 (2)

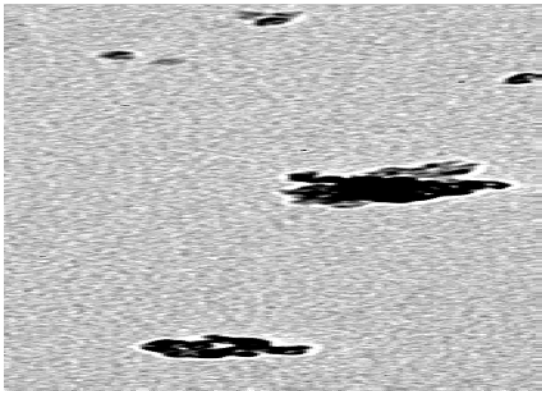

Fig. 3: Scanining electron microscope of detected AgNPs produced by *T. harzianum* RCMB 017002 (2)

AgNPs was 51.10 nm produced by *T. harzianum*. Agnihotri *et al.*[33] revealed that the shape and size of the green synthesized AgNPs depend on the environmental conditions including temperature and pH of the medium as well as the microorganisms, for example, *Aspergillus niger* (5-35 nm)[34] and *Aspergillus parasiticus* (less than 50 nm)[35]. According to Shelar and Chavan[30], the cell filtrate of *T. harzianum* was used as a producer of AgNPs, resulting the formation of it within 3 h and the TEM analysis showed polydisperse spherical and occasionally ellipsoid nanoparticles in the size range from 19-63 nm and average size 34.77 nm. The SEM measurements by Vahabi *et al.*[29] indicated that extracellular biosynthesis of silver nanoparticle by *Trichoderma reesei* produces AgNPs with the diameters in the range of 5-50 nm.

FTIR measurement was carried out to identify the possible biomolecules responsible for the reduction of the Ag^+ ions and capping the bioreduced AgNPs synthesized by *T. harzianum* RCMB 017002 (2). The FTIR spectrum of the freeze-dried powder of AgNPs reveal the presence of different functional groups (Fig. 4). The FTIR spectrum shows several absorption bands indicating the presence of capping agent with the nanoparticles. The peaks in the range of 3265.46-3353.92 cm^{-1} were assigned as -OH stretching in alcohols and phenolic compounds with strong hydrogen bonds. The presence of these groups is due to the stability of the nanoparticles. Bands at 2920.29 and 2855.63 cm^{-1} region arising from C-H stretching of aromatic compound were observed. These functional groups have role in stability/capping of AgNP as reported in many studies[7,12,31,36]. These groups may be between amino acid residues in protein and synthesized AgNPs. The FTIR spectrum supports the presence of proteins in the synthesis of AgNPs. Another peaks were detected by FTIR spectrum indicate that the AgNPs manifest absorption peaks at about 1053.89, 1412.95 and 1626.37 cm^{-1} which represent amide linkages groups[8]. According to MubarakAli *et al.*[37] these proteins were present as enzymes that could reduce $AgNO_3$ ions to form silver nanoparticles. Organic functional groups like OH, C-O linked to the surface of nanoparticles are found by FTIR[38].

Isolation trials from sweet pepper plants showing characteristic symptoms of wilt (Fig. 5) yielded one fungal isolate which purified and identified as *F. oxysporum*. While two fungal species related to *Alternariaalternata* and *Trichoderma harzianum* were isolated from health plant (no symptoms of wilt). The fungus *Fusarium oxysporum* is one of soil-borne plant pathogens and is widely distributed in various soil types worldwide. Shafique *et al.*[39] reported that *F. oxysporum* induced maximum characteristic symptoms of pepper wilt and plants died within few days of inoculation. Also, recently Murtza *et al.*[40] mentioned that Fusarium wilt caused by *F. oxysporum* f.sp. *capsici* is a potential risk to successful production of chilli in Pakistan and causes huge yield loss. In the current study the absence of *F. oxysporum*

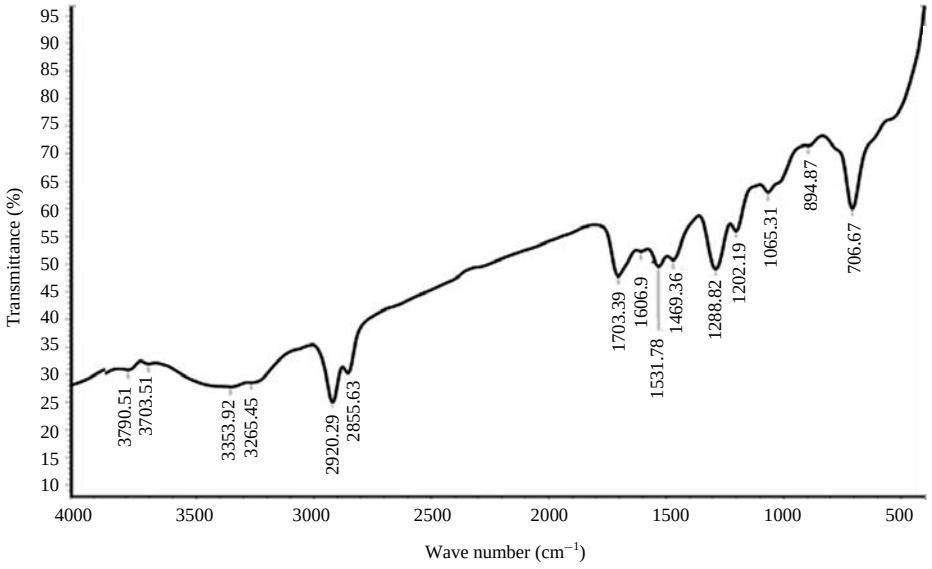

Fig. 4: FTIR spectrum of AgNPs formed after 72 h of incubation of the biomass of *T. harzianum* RCMB 017002 (2)

Fig. 5: Fungal isolation site from sweet pepper plants showing characteristic symptoms of wilt (inside red circular) and health pepper (inside blue circular)

Table 2: Antifungal activity of different concentrations of AgNPs

AgNPs concentrations (ppm)	*Fusarium oxysporum*		*Alternaria alternata*		*Trichoderma harzianum*	
	Colony radius (cm)	Growth inhibition (%)	Colony radius (cm)	Growth inhibition (%)	Colony radius (cm)	Growth inhibition (%)
Control	7.2±0.2	0.00	5.3±0.4	0.0	8.2±0.2	0.0
20	6.3±0.4	12.50	2.5±0.5	52.8	6.8±0.1	17.1
40	5.0±0.2	30.50	1.7±0.1	67.9	3.2±0.5	60.9
80	2.8±0.5	61.11	0.0	100.0	0.0	100.0
100	0.0	100.00	0.0	100.0	0.0	100.0

around the the roots of health pepper plants indicate that *F. oxysporum causes* the wilt of these plant. AgNPs showed various levels of inhibition on colony formation of *F. oxysporum*, *T. harzianum* and *A. alternata* (Fig. 6). As concentrations of the silver compounds increased, colony formation decreased. AgNPS at concentrations of 20, 40 and 80 ppm inhibited the *F. oxysporum* growth by 12.5, 30.5 and 61.11%, respectively. Concentrations at 80 ppm caused 100% growth inhibition of *T. harzianum* and *A. alternata* while concentrations at 100 ppm caused 100% growth inhibition *F. oxysporum* (Table 2). The obtained results was agreement with El-Argawy *et al.*[41], who showed that 25, 50

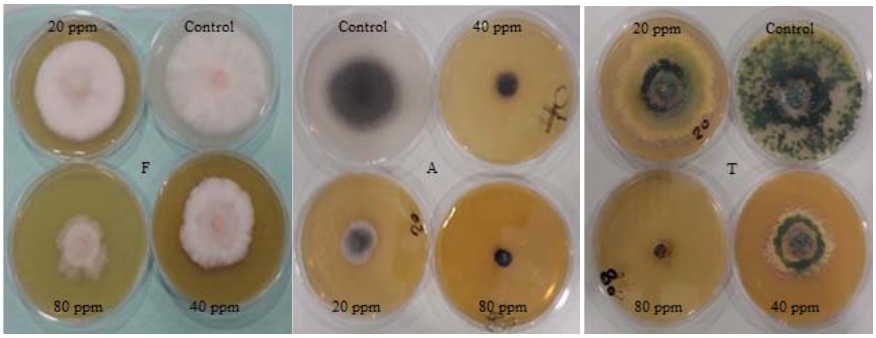

Fig. 6: Fungal growth (F: *F. oxysporum*, A: *A. alternata*, T: *T. harzianum*) at different concentrations 20, 40, 80 ppm of AgNPs

and 100 ppm of AgNPs increased the *in vitro* fungal growth inhibition of *Fusarium oxysporum* f.sp., betae, *Sclerotium rolfsii* and *Rhizoctonia solani* by reducing the radial fungal growth with the best effect was recorded with the highest concentration. In contrast, at 200 ppm of AgNPs the growth of *Magnaporthe grisea* was reduced but not completely inhibited[23]. Since silver nanoparticles have different modes of action including interfering with fungal cell membrane potential and causing cell death[42], its application for control of various plant pathogenic fungi is relatively safer compared to conventional synthetic fungicides[43]. Kim *et al.*[21] tested the efficacy of AgNPs for their antifungal activity against plant pathogenic fungi (*Alternaria alternata*, *A. brassicicola*, *A. solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *P. spinosum*, *Stemphylium lycopersici*) by poisoned food technique and found that AgNPs maximally inhibited the colony growth of different fungi mostly at 100 ppm concentration. Also, AgNPs were applied *in vivo*[44], it reduced the incidence of *F. oxysporum* infection as a seed borne pathogen.

CONCLUSION

The present study demonstrated that it is possible to perform the biogenic synthesis of silver nanoparticles using *T. harzianum* as appropriate safe fungus. It should be mentioned that *Trichoderma harzianum* is not known to be harmful to humans. AgNPS at concentrations of 20, 40 and 80 ppm inhibited the *F. oxysporum* growth *in vitro* causing wilt of sweet pepper.

SIGNIFICANCE STATEMENTS

This study discovers the safe fungus *T. harzianum* that can be beneficial for AgNPs biosynthesis. This study will help the researchers to uncover safer drugs used against antibiotic resistance bacteria as well as fungi. Thus, a new theory on these nanoparticles of *T. harzianum* as a novel compounds, may be arrived at novel structure of antifungal AgNPs discovery.

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