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Mycosynthesis of silver nanoparticles and their role in the control of *Fusarium* wilt of pepper

Amal MI El-Eraky[1], AH Moubasher[2,3,*], MA Ismail[2,3], AH El- Shaer[4], HA Gouda[4]

[1]Department of Plant Pathology, Faculty of Agriculture, Assiut University, Assiut, Egypt
[2]Department of Botany and Microbiology, Faculty of Science, Assiut University, 71526, Assiut, Egypt
[3]Assiut University Mycological Centre (AUMC), Assiut University, 71526, Assiut, Egypt
[4]Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt

***Corresponding author: e-mail: ahamaumc@yahoo.com**

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Abstract: For the aim of control *Fusarium* wilt of pepper, antifungal activities of silver nanoparticles (AgNPs) were investigated *in vitro* and under greenhouse conditions. Silver nanoparticles were synthesized and characterized from five fungal species. Under *in vitro* conditions, the four concentrations of silver nanoparticles (10, 25, 50 and 100 ppm) synthesized by fungi inhibited the mycelial growth of *F. oxysporum* f. sp. *capsici* (AUMC 11424) on PDA with sharp decline between 10 and 50 ppm, and a slight decline between 50 and 100 ppm. Under greenhouse conditions AgNPs synthesized by the five tested fungi at 50 ppm dose was more efficient than 100 ppm of AgNPs and the highest inhibition of wilt incidence was 84.6% at 50 ppm concentration synthesized by *Botryotrichum atrogriseum* (AUMC 11415) and *Albifimbria verrucaria* AUMC 11414 AgNPs. At 100 ppm from *B. atrogriseum* AUMC 11415), the highest inhibition was 81.5% of control (without AgNPs). The current results revealed also that smaller-sized particles of *B. atrogriseum* and *A. verrucaria* were more active than those of *T. harzianum* towards the fungal pathogen.

Key words: silver nanoparticles, biosynthesis, *F. oxysporum* f. sp. *capsici*, antifungal activity.

Introduction

Synthetic chemical fungicides are widely used in conventional agriculture to control plant diseases. Moreover, such chemicals can be lethal to beneficial microorganisms in the rhizosphere and useful soil insects, and they may also enter the food chain and accumulate in the human body as undesirable chemical residues (Bartlett *et al*. 2002). Therefore, scientists in the agricultural field are searching for alternative eco-friendly and less capital intensive approaches to control plant diseases. One of potential applications is the use of nanomaterials that is expanding and has been considered as an alternative solution to control plant pathogens (Kanhed *et al*. 2014, Sekhon 2014, Ahmed and Lee 2015). Some metal nanoparticles have been studied and proved for their antifungal properties (Lemire *et al*. 2013, Singh *et al*. 2013, Xu *et al*. 2013). In addition, micromolar concentrations of silver have no harmful effects on humans (Berger *et al*. 1976). Therefore, silver has been used for controlling spore-producing fungal plant pathogens and sclerotia-forming species of *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *S. minor* (Min *et al*. 2009), powdery mildew in cucumber and pumpkin (Lamsal *et al*. 2011b), rice blast disease, caused by *Magnaporthe grisea* (Elamawi and EL-Shafey, 2013) and *Fusarium* wilt in lettuce and tomato (Ahmed *et al*. 2016).

Silver nanoparticles (AgNPs) are between 1 nm and 100 nm in size and consist of about 20–15,000 Ag atoms (Demirors *et al*. 2010). Silver particles have attracted scientific interest because of their unusual properties compared to bulk metal such as excellent electrical and thermal conductivity, surface-enhanced scattering, catalytical activity, chemical stability and antimicrobial activity. These properties have led to their tremendous range of applications (Frattini *et al*. 2005, Warheit *et al*. 2007, Chen and Schluesener 2008, Zielinska *et al*. 2009, Wijnhoven *et al*. 2009, Fabrega *et al*. 2011, Ravindra and Rajasab 2015, Singh *et al*. 2016, Jiang *et al*. 2017).

The filamentous fungi possess some distinctive advantages over bacteria viz like ease of handling, can secrete larger amounts of proteins which directly are translated to higher productivity of nanoparticles, high tolerance towards metals, as well as intracellular metal uptake capabilities (Dias *et al*. 2002, Mohanpuria *et al*. 2008, Phanjom and Ahmed 2015).

In the case of fungi the nanoparticles are formed on the surface of the mycelia and not in the solution. It was then suggested that in the first step Ag^+ ions are adsorbed on the surface of the fungal cells due to electrostatic interaction between negatively charged carboxylate groups in enzymes present in the cell wall of mycelia and positively charged Ag ions. Finally, the silver ions are then reduced by the enzymes present in cell wall, leading to the formation of silver nuclei (Mukherjee *et al*. 2001).

The inhibitory effects of AgNPs on bacteria and fungi have been proposed to be due to several mechanisms. It is assumed that AgNPs possess high affinity towards phosphorous and

sulphur present in the cell. As silver ions (Ag+) from AgNPs interact with DNA containing phosphorous moieties, this results in inactivation of DNA replication. Interaction of AgNPs with sulphur-containing proteins present inside or outside the cell membrane leads to inhibition of enzyme functions and thus greater permeability of the membrane, causing cell death (Feng *et al.* 2000, Dorau *et al.* 2004).

Therefore, the objective of the present study was to synthesize and characterize silver nanoparticles from some fungal strains of high antagonistic effect against pepper wilt pathogen. Also, the antifungal effect of the synthesized silver nanoparticles against this pathogen *in vitro* and under greenhouse conditions was evaluated.

Materials and Methods

1- Strains used for biosynthesis of Ag-NPs

Botryotrichum atrogriseum AUMC 11415, *Penicillium oxalicum* AUMC 11419, *Clonostachys rosea* AUMC 11442, *Albifimbria verrucaria* AUMC 11414 and *Trichoderma harzianum* AUMC 11422 were used for synethesis of silver nanoparticles. These fungi were isolated from the rhizosphere of healthy pepper plants obtained from different locations in Assiut and Behera Governorates, Egypt and showed high antagonistic effect against *F. oxysporum* f.sp. *capsici* AUMC 11424 (Ismail *et al.*, 2017). Identification of the pathogen using morphological and molecular techniques was checked by Assiut University Mycological Centre (AUMC) specialists.

2- Biosynthesis of Ag-NPs

The fungal strains were inoculated each in 250-ml Erlenmeyer flasks containing 100 ml potato dextrose broth (PDB) and incubated at 28° C on a rotatory shaker at 120 rpm for 5 days. After incubation, mycelial biomass was separated by filtration, washed with sterilized distilled water to remove any medium component. Twenty g of fungal biomass (fresh weight) were mixed with 200 ml of deionized water in 500 ml Erlenmeyer flask and agitated for 5 days at 25°C. After the incubation, the culture filtrate was obtained by passing it through whatman No 1 filter paper. The filtrate was collected and used for nanoparticles synthesis. For synthesis, 50 ml of 1 mM AgNO3 solution was mixed with 50 ml of culture filtrate in 250 ml Erlenmeyer flask and agitated for 72 hr at 25°C (Basavaraja *et al.* 2008).

3-Characterization of Ag-NPs

After incubation of the above mixture, the preliminary detection of Ag-NPs was carried out by visual observation of color change of the culture filtrate where silver nitrate turned into brown due to its reduction to silver ions (Basavaraja *et al.* 2008). These samples were subjected to characterization using the following techniques:

(i) UV-Vis spectroscopy

The bioreduction of Ag+ in aqueous solution was monitored by periodic sampling of 0.2 ml aliquots of the suspension, then diluting with 2 ml deionized water and subsequently measuring UV–vis spectra (A Perkin Elmer Lambada 950) of the resulting diluents (Vahabi *et al.* 2011).

(ii) Transmission electron microscopy (TEM)

This was performed by fabricating a drop of suspension onto a clean electric stub and coated with gold and palladium. The prepared sample was examined using a Joel JSM- 4500 LV electron microscope operating at 15 KV in Assiut University, Electron Microscopy Unit and micrographs were taken.

(iii) X-ray diffraction measurements

X-Ray diffraction (XRD) measurements of the bioreduced silver nitrate solution drop-coated onto glass substrates was carried out by using X-ray diffractometer (Model PW 1710 control unit Philips Anode material Cu, 40 KV, 30 M.A, optics: Automatic divergence slit) with Cu Kα radiation λ=1.5405 °A over a wide range of Bragg angles ($30° \leq 2\theta \leq 80°$) (Prema and Rincy 2009).

4- Antifungal activity of AgNPs test using agar plate assay

Four concentrations, 10, 25, 50, and 100 ppm of each silver nanoparticles were prepared by diluting the original stock solution suspension ($AgNO_3$, 0.05N). All solutions were stored at 4°C until use. *In vitro*, assay was performed on PDA plates. One ml of each AgNPs concentration was mixed with the growth medium prior to plating in a Petri dish (90 × 15 mm). Medium containing silver nanoparticles was incubated at room temperature. After 48 hr of incubation, agar plugs of uniform size (5 mm diam.) containing *F. oxysporum* f.sp. *capsici* AUMC 11424 were inoculated simultaneously at the center of each Petri dish containing silver nanoparticles, followed by incubation at 28 ± 2℃ for 10 days. Agar plates without nanomaterial were used as control. Three replicates of each treatment were used. Radial growth of fungus was recorded. The growth inhibition rate was calculated when growth of the fungal mycelia in control plates reached the edge of the Petri dish (Kim *et al.* 2012). Inhibition rate was calculated by using the following formula:

$$\text{Growth inhibition} = \frac{H - h}{H} \times 100$$

Where (H) is the diameter of fungal mycelial growth in control plate and (h) is the diameter of fungal mycelial growth in the silver nanoparticles – treated plates.

Evaluation of silver nanoparticles on pepper wilt incidence under greenhouse conditions

The effect of silver nanoparticles on wilt severity was evaluated under greenhouse conditions. This experiment was carried out in 2016 pepper-growing season in the greenhouse of Plant Pathology Department, Faculty of Agriculture, Assiut University. Inocula of fungal pathogen (*F. oxysporum* f. sp. *capsici*) were prepared in 250 ml flasks containing potato dextrose broth (PDB) inoculated with 5-mm diameter agar plugs of 7-day-old culture. Flasks were incubated for 14 days at 28°C in a rotary shaker, then the suspension was blended at a low speed for 20 seconds to get the homogenous mass and the suspension was used. Sterilized pots (25 cm diam.) were filled each with 3 kg sterilized sandy loamy soil. Each pot was sown with two seedlings of balady pepper cultivar (land breed). 50 ml of the fungal suspension were added to the root zone of 20-day-old seedlings. After 2 days, pots were re-infested with 25 ml of 50 ppm or 100 ppm concentration of AgNPs prepared as described by Ahmed *et al.* (2016). Three replicate pots were used for each treatment. Plants inoculated with fungal pathogen only were used as control (refer to Fig. 6). Percentage of wilt incidence was recorded after 3 weeks from sowing date and severity of wilt was determined for each plant using the following scale:

0= no disease; 1= minor symptoms on a few leaves; 2= symptoms on majority of leaves, minor dwarfing; 3= significant dwarfing, yellowing, wilt, and defoliation; 4 = some shoots with severity and some shoots dead and 5= death of whole plant. Percentage of disease severity was calculated according to the following formula (Rajput *et al*. 2008):

$$\text{Disease severity} = \frac{(n \times 1) + (n \times 2) + \ldots}{tn} \times 100$$

Where n = number of plants in each group, tn= total number of plants

Results and Discussion

Synthesis of silver myconanoparticles

The appearance of a brownish colour indicates the formation of myco-AgNPs in the reaction mixture. Reduction of silver ions was reflected in the form of colour change of the culture filtrates, which varied from pale yellow to brown. When subjected to similar conditions, 1 mM AgNO3, used as a control, did not demonstrate any colour change. It is known that AgNPs exhibits brown color in aqueous solution due to the fungal supernatant which contains a high level of protein components, including the enzyme NADH reductase, a major enzyme used for the reduction of metals. Synthesis of myco-AgNPs was based on surface plasmon resonance (SPR) involving a change in colour (Weiping *et al*. 2001, Fatima *et al*. 2016).

Characterization of silver nanoparticles

Spectrophotometric analysis of fungal extract of all fungi tested showed an absorbance peak at 290 nm which is specific for the silver nanoparticles (Figure 1). That classic UV–Vis peak of silver nanoparticles shifted from 390 to 290 nm may be due to various mechanisms involved during their synthesis. Several mechanisms have been suggested either that the electrons of silver nanoparticles are excited in presence of UV light toward the surface and form a characteristic peak of silver nanoparticles at 290 nm (Luo *et al.* 2005, Khan *et al.* 2011, Raza *et al*. 2013, Søren *et al.* 2015) or the presence of the carboxyl group of amino acid residues and the amine of peptide chains along with reducing groups like aldehyde and ketone governs the bioreduction of silver ions to $[Ag(NH3)_2]^+$ group (Zhang *et al*. 2005).

TEM micrographs of myco-AgNPs were employed to visualize the size and shape of silver nanoparticle. The morphology of monodisperse AgNPs is almost spherical and the size of the myco-AgNPs were about 18.5 nm in case of *Botryotrichum atrogriseum* AUMC11415, 20.3 nm in case of *Albifimbria verrucaria* AUMC 11414 and 24.2 nm in case of *Trichoderma harzianum* AUMC 11422 (Fig. 2). The size variation was due to oxidation of metal salts into their respective nanoparticles in the presence of fungal enzymes (Fatima *et al.* 2016). These results are in accordance with those reported earlier giving an indication of synthesis of good myconanoparticles (Mouxing *et al.* 2006, Fatima *et al.* 2016).

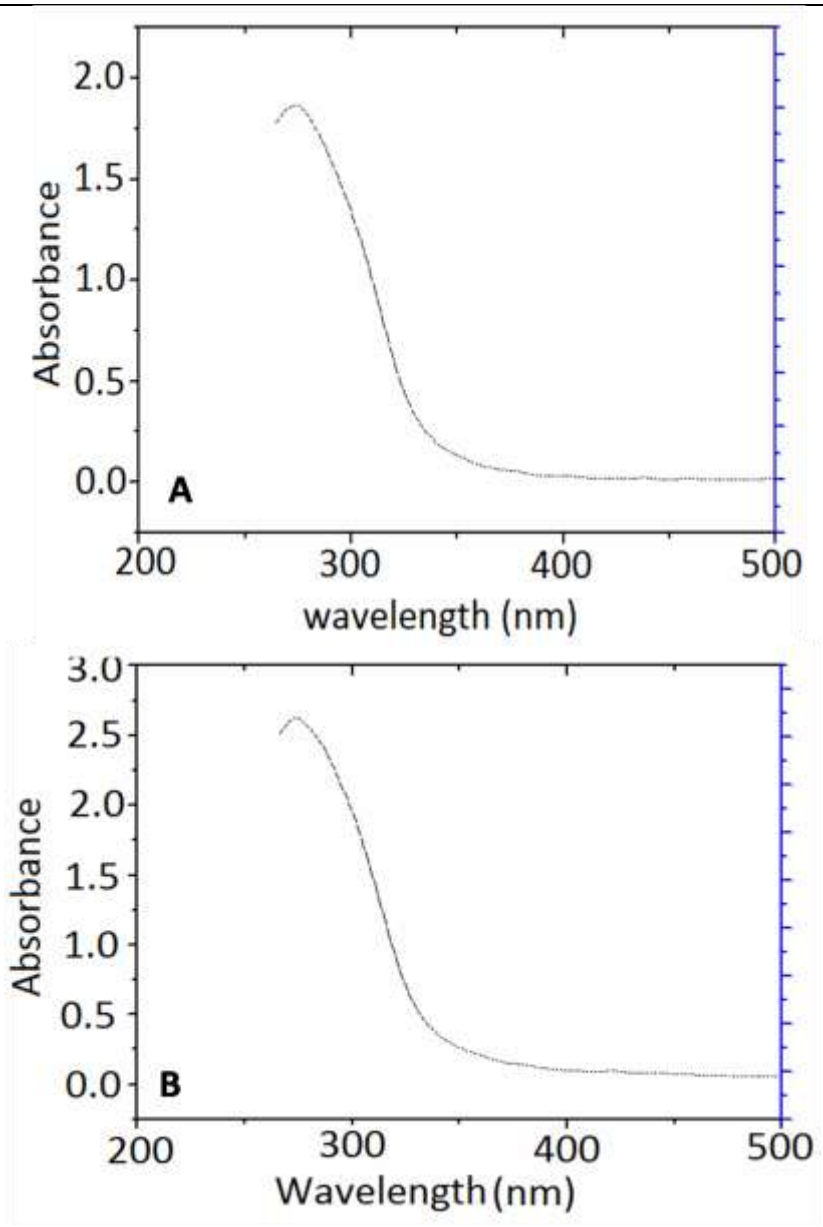

Figure 1: UV-Visible spectrum of silver nanoparticles synthesized from *Botryotrichum atrogriseum* AUMC11415 (**A**) and *Albifimbria verrucaria* AUMC 11414 (**B**).

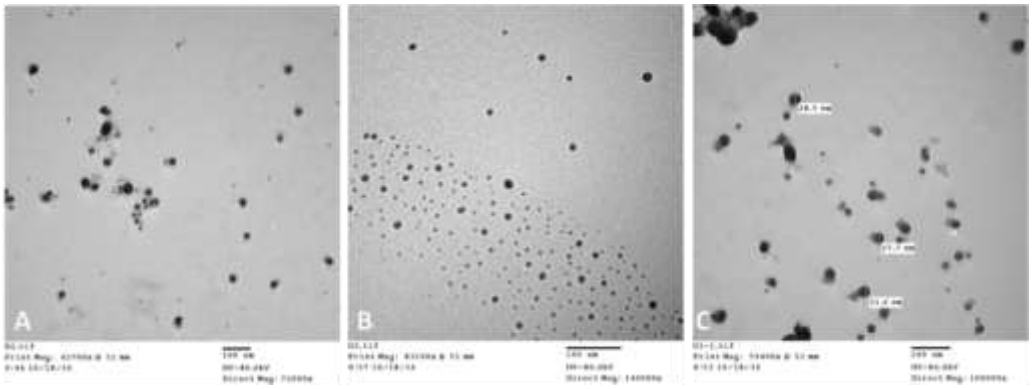

Figure 2: TEM micrographs of silver nanoparticles synthesized by *Botryotrichum atrogriseum* AUMC 11415 (A), *Albifimbria verrucaria* AUMC 11414 (B) and *Trichoderma harzianum* AUMC 11422 (C).

The XRD diffraction pattern is commonly used to determine the crystalline nature of Ag nanoparticles. The crystalline domain size was calculated by the width of the XRD peaks using Scherrer formula $D=0.96\ \lambda/\beta \cos\theta$, where D is crystalline domain size perpendicular to reflecting planes, λ is the x-ray wavelength, β is the full width at half maximum and θ is the diffraction angle. The average particle size was 20-40 nm.

The XRD patterns of dried sample obtained from colloid samples of the two fungal strains tested revealed the existence of sharp diffraction lines at low angles of 2θ values ranging from 10 to 80°. XRD pattern of crystalline silver nanoparticles synthesized by *Botryotrichum atrogriseum* AUMC 11415 showed peaks at 2θ values of 37.72, 45.9, 57.16 and 76.6 correspond to the (176), (236), (87) and (126) crystal planes of face-centered cubic silver respectively (Fig 3A). While those synthesized by *Albifimbria verrucaria* AUMC 11414 showed peaks at 2θ values of 37.84, 45.9, 64.2 and 76.6 correspond to the (349), (431), (132) and (172) crystal planes of face-centered cubic silver respectively (Fig. 3B). Average size of nanoparticles synthesized by both stains as revealed by XRD pattern was 14.8 and 16.5 nm respectively. Additional diffraction peaks were consistent with standard database files (JCPDS card No. 04-0783) which may be due to bio-organic compounds or metalloproteinase which are responsible for production and stabilization of resultant nanoparticles (Jena *et al.* 2012, Patil 2015) or the crystallization of bio-organic phase occurs on the surface of the silver nanoparticles (Sathyavathi *et al.* 2010, Rout *et al.* 2012). The line broadening of the peaks is primarily due to small particle size (Kirthika *et al.* 2014).

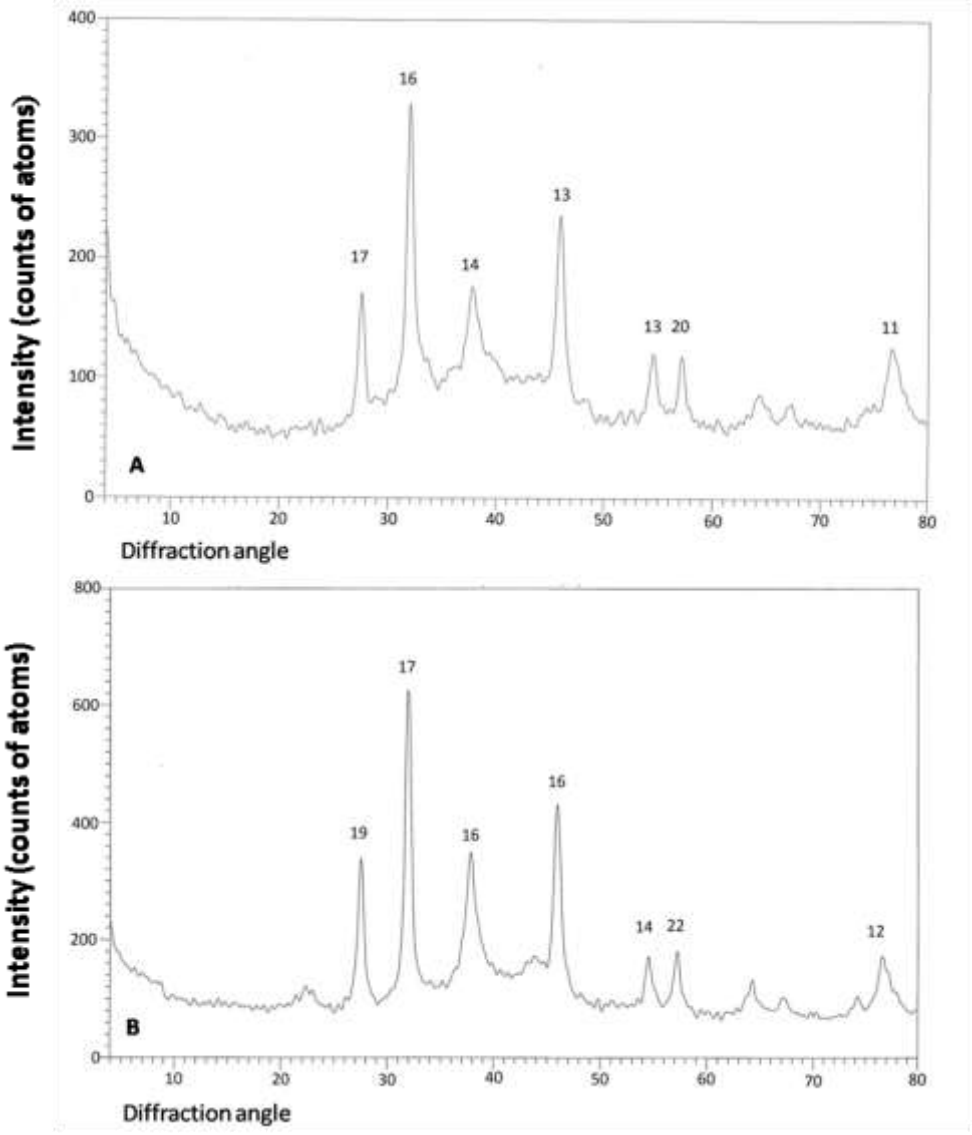

Figure 3: XRD pattern of crystalline silver nanoparticles synthesized by *Botryotrichum atrogriseum* AUMC 11415 (**A**) and *Albifimbria verrucaria* AUMC 11414 (**B**).

Effect of silver nanoparticles on the mycelial growth of pepper-wilt pathogen on agar plates

Results in Table (1) & Figure (4) showed that all concentrations of AgNPs synthesized by the five tested fungi inhibited the mycelial growth of the pathogenic fungus, with sharp decline between 10 and 50 ppm and a slight one between 50 and 100 ppm. Although the inhibition rates induced by all fungi were high, those of *Penicillium oxalicum* were relatively less efficient. These results are in agreement with those obtained previously by (Ahmed *et al.* 2016) using NiNPs which significanty inhibited the mycelial growth of both *Fusarium* wilt of lettuce and tomato. Kim *et al*. (2012) revealed also that $AgNP_3$ possessed antifungal activity the mycelial growth of *Fusarium* wilt pathogens of cucumber, tomato and potato. Morones *et al.* (2005) found also that AgNPs has an inhibitory effect on colony and spores of *F. oxysporum*. Silver nanoparticles disrupt transport systems, including ion efflux (Morones *et al.* 2005), which cause rapid accumulation of silver ions, which interrupts cellular processes and produces reactive oxygen species via their reaction with oxygen, which are detrimental to cells, causing damage to proteins, lipids, and nucleic acids (Storz and Imlay 1999, Hwang *et al.* 2008). The current results of TEM indicate that *B. atrogriseum* AUMC 11415 and *A. verrucaria* AUMC 11414 had smaller-sized particles than *T. harzianum* AUMC 11422, so they were more active towards the fungal pathogen. More importantly, the particles of different sizes showed different rates and extent of growth inhibition. Therefore, smaller particles seem to interact with cells and become more bioavailable either by dissolving in the close vicinity of the outer cell surface or inside the cells (Ivask *et al.* 2014). Previous studies showed also that as NP size decreases, the relative surface area rises, and a proportionately greater number of atoms are exposed at the surface (Oberdörster *et al.* 2005; Nel *et al.* 2006). Thus, smaller NPs, which cross the cell membrane more readily than do larger NP, probably provoke greater cytotoxicity because of greater interactions with cell surface components per square nanometer, producing more aggregation of adsorbed proteins (Mendoza *et al.* 2014).

Table 1: Inhibition percentages of growth of *Fusarium* wilt pathogen, *F. oxysporum* f. sp. *capsici* by different concentrations of silver nanoparticles synthesized by the 5 strains.

AgNPS type	AUMC No.	AgNPs concentration			
		10ppm	25ppm	50ppm	100ppm
Botryotrichum atrogriseum	11415	20±1	46±0.3	87±0.4	90±0.4
Clonostachys rosea	11442	19±2	40±0.3	78±0.4	90±0.3
Albifimbria verrucaria	11414	20±0.4	45±1	87±1	90±0.3
Penicillium oxalicum	11419	17±0.4	38±1	72±0.3	81±1
Trichoderma harzianum	11422	19±0.2	42±1	82±0.3	90±1
Control (no AgNPs)		0	0	0	0

* Data obtained as inhibition percentages after 10 days of inoculation in relation to control treatment.

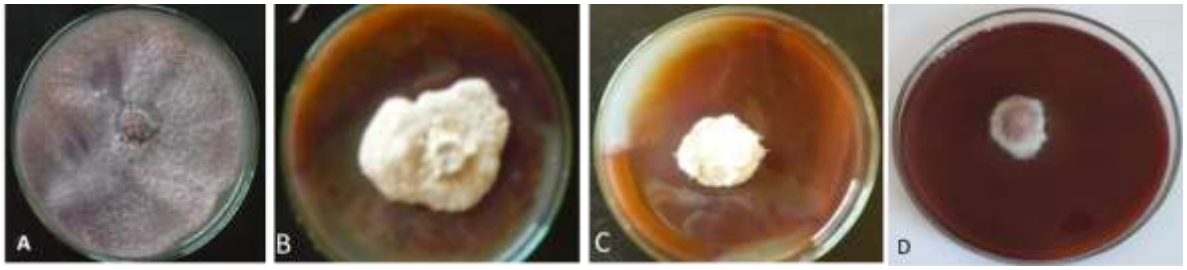

Figure 4: Effect of different concentrations of silver nanoparticles of *Botryotrichum atrogriseum* on *F. oxysporum*: control, 0 ppm (A), 25 ppm (B), 50 ppm (C) and 100 ppm conc. of AgNPs (D).

Assessment of silver nanoparticles effect on wilt incidence under greenhouse conditions

The present results showed that the soil treatment with 50 ppm concentration of nanoparticles synthesized by the five tested fungi regularly reduced wilt severity more than treatment with 100 ppm with a reduction rate of 84.6-53.8% and 81.5-47.7%, respectively (Table 2 & Figure 5). This result can be due to a toxic effect exerted by the higher dose on the vigor and shape of some plants that show some signs of wilt. The present results are in agreement with those recorded by Ahmed *et al.* (2016) who showed that *in vivo*, NiNPs at 50 ppm was better than 100 ppm in control of *F. oxysporum* f. sp. *lactucae* and *F. oxysporum* f. sp. *lycopersici*, the causal agents of lettuce and tomato, respectively. Also, Lamsal *et al.* (2011a) reported that the application of 50 ppm silver nanoparticles showed the highest inhibition rate of anthracnose disease of pepper caused by *Colletotrichum* sp. *in vivo*. This suggests that pepper wilt disease suppression can be achieved with a low concentration of silver nanoparticles when it is applied before disease outbreak in the field (Lamsal *et al.* 2011a).

Table 2: Inhibition percentages of *Fusarium* wilt severity under greenhouse conditions by silver nanoparticles synthesized by the five fungal species.

AgNPS type	AUMC No.	50ppm	100ppm
Botryotrichum atrogriseum	11415	84.6±0.2	81.5±1
Clonostachys rosea	11442	78.4±0.5	76.9±0.4
Albifimbria verrucaria	11414	84.6±0.2	80±0.2
Penicillium oxalicum	11419	53.8±0.4	47.7±1
Trichoderma harzianum	11422	69.2±0.4	66.1±0.2

* Data obtained as inhibition percentages in relation to control treatment.

Figure 6: Shows the scale of *Fusarium* wilt severity (A) and the effect of AgNPs synthesized by *B. atrogriseum* on the control treatment (treated with fungal pathogen only) (B), pots treated with fungal pathogen + 50 ppm AgNPs (C) and pots treated with fungal pathogen + 100 ppm AgNPs (D).

Conclusion

In the present study, *Botryotrichum atrogriseum* AUMC 11415, *Penicillium oxalicum* AUMC 11419, *Clonostachys rosea* AUMC 11442, *Albifimbria verrucaria* AUMC 11414 and *Trichoderma harzianum* AUMC 11422 were used for the synthesis of AgNPs. The myco-AgNPs generated showed promising antifungal activities *in vitro* and under greenhouse conditions against the causal agent of pepper wilt, *F. oxysporum* f. sp. *capsici*. The best effect of nanoparticles on mycelial growth of the pathogen was observed at 100 ppm concentration and the best reduction was 90% comparable to the control by AgNPs synthesized by *Botryotrichum atrogriseum*, *Clonostachys rosea*, *Albifimbria verrucaria* and *Trichoderma harzianum*. Under greenhouse conditions AgNPs, 50 ppm treatment was more effecient than 100 ppm of AgNPs and the highest reduction of wilt was 84.6% compared to control by both AgNPs generated from *Botryotrichum atrogriseum* and *Albifimbria verrucaria*.

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