

Bioproduction of silver-nano particles by *Fusarium oxysporum* and their antimicrobial activity against some plant pathogenic bacteria and fungi

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Abstract: In the present study *Fusarium oxysporum* was used for bioproduction of nano-silver particles. Electron microscopy observation showed particles with sizes ranged from 16-30 nm. Antimicrobial activity of these particles was investigated against plant pathogenic bacteria (*Erwina cartovra*, *E. amylovra*, *Dickya chransanthei*, *D. dianthical*, *Pectobacterium wasaibiae*, *P. atrosepticum chransanthei* and *P. wasabiae*) and pathogenic fungi (*Fusarium oxysporum*, *Alternaria alternata* and *Aspergillus flavus*). Results revealed that the nanoparticles have high antibacterial activity against the examined bacteria and fungi when compared with the generic antibiotics. Protein separation was applied for the nano-silver particles suspension using SDS-PAGE and a protein with molecular weight about 26kDa was observed. When this protein was characterized using TLC in comparing with four different types of Aflatoxins (P1, G1, G2 and B2), results showed that the protein is unique and has a high molecular weight more than the four examined toxins. We can conclude that the nano-silver particles have selectivity for this active protein that improves the antimicrobial activity against the pathogenic fungi and bacteria.

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1-Introduction

Plants are exposed to infection by a variety of pathogenic microorganisms that present in their environments and in soil rhizosphere. Plant diseases caused by pathogens, including bacteria, fungi, and viruses, which cause significant loss in crop yield worldwide (Shabana et al. 2008). Among these pathogens, *Erwina* spp members of the *Pectobacterium* genus are plant pathogens responsible for causing soft rot in many types of plants, including economically important carrot and potato crops (Pe´rombelon and Wolf 2002). Plant pathogenic fungus *Alternaria* spp. cause several diseases of citrus, including *Alternaria* brown spot of tangerine (*Citrus reticulata* Blanco), leaf and fruit spot of rough lemon. *Fusarium oxysporum* cause in plant diseases including *Fusarium* wilt and crown root rot disease (Kucharek et al. 2000)

Fusarium spp. are soil borne fungi which produce a number of different mycotoxins these mycotoxins (secondary metabolites) belong to the class of trichothecenes, zearalenone, nivalenol and fumonisins (Bhatnagar and Ehrlich, 2002). Mycotoxins are produced in response to different stress (environmental and/or fungus plant interaction) and these metabolites have a kind

biological activity (antibiosis, phytotoxicity and toxicity) (Biro 2003).

One of the most important defense strategies to control plant diseases is the use of nanotechnology by production of silver nanoparticles (AgNPs) (Bruchez et al. 1998 and Sastry 2003). It is well known that microorganisms such as bacteria, yeast and fungi play a vital role to diminish of the toxic metals by reduction of metal ions. Based on this theory, the *Fusarium* culture filtrate was considered as nanofactor by which the silver nitrate could be reduced into AgNPs (Fortin and Beveridge 2000). Moreover, Duran et al (2005) reported that the nanoparticles could also be stabilized directly in the process by proteins. *Fusarium oxysporum* was used for synthesis of nanoparticles on water and perfect stable gold/silver nanoparticles were obtained (Ahmed et al. 2003, Yazdi et al. 2011, Hafez et al. 2011). The antifungal activity of AgNPs produced by the bacterium *Boswellia ovalifoliolata* was recorded against *Aspergillus* and *Fusarium* (Leung and Foster 1996). The antibacterial activity of AgNPs produced by the fungus *Fusarium acuminatum* showed efficient antifungal activity against multidrug resistant and highly pathogenic bacteria,

Staphylococcus aureus, *Salmonella typhi*, *Staphylococcus epidermidis*, and *Escherichia coli* (Ingle et al. 2008).

The present study aimed to produce AgNPs using *F. oxysporum*, characterize the resulted AgNPs using Scanning Electron Microscope (SEM), separate the attached protein in the AgNPs suspension, and investigate the antimicrobial activity of the resulted AgNPs.

2. Materials and methods

2.1. Preparation of *F. oxysporum* filtrate

Erlenmeyer flasks (500 ml) contains 200 ml of sterile potato dextrose broth medium were sterilized then inoculated with 5 mm discs taken from a *F. oxysporum* plate (7days-old). The flasks were incubated without shaking in the dark at 25 °C for 1 week. Mycelial mats, on the surface of the medium were removed and the filtrate was stored at 4°C until use.

2.2. Bioproduction of Ag-NPs

For the Ag-NPs bioproduction, *F. oxysporum* filtrate was used to reduce the AgNO₃ into Ag-NPs. In a 250mL Erlenmeyer flask, 10 ml *F. oxysporum* filtrate was added to 100 ml of 1mM AgNO₃ solution in a 250 ml Erlenmeyer flask. The flasks were then incubated in a rotary shaker at 200 rpm in the dark at 27 °C then the filtrate centrifuged at 10.000rpm for 30min. The precipitate was collected, washed with sterile water 3 times and then dried in oven at 50°C for 30 min then weighted and dissolves in sterile distilled water until use.

2.3. UV-visible spectra analysis

The biosynthesis of Ag-NPs was monitored periodically in a UV-visible spectrophotometer. For the analysis, 0.1 ml of the sample was taken and diluted to 2ml with deionized water.

2.4. Tested organisms

The tested bacterial isolates (*E. cartovra*, *E. amylovra*, *P. wasabiae* Hf569027, *P. carotovorum atrosepticum* 1007, *D. chrysanthemi* Dsm4610, *P. wasabiae* 33 and *D. dadantii*) were cultured in Luria-Bertani (LB) Broth media (Becton-Dickinson and Co., USA). One ml aliquots of a 24 h- old broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37° C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, to produce a suspension containing about 10⁸- 10⁹ cfu/ ml. The suspension was stored in the refrigerator at 4° C till use.

The plant pathogenic fungi (*F. oxysporum*, *A. alternata* and *A. flavius*) were cultured on PDA plates for 7 days at 28°C. Then a disc of 0.5 cm (in

diameter) of each fungus was taken and inoculated into 250 ml Erlenmeyer flask then incubated at 30 °C for preparation of the fungal suspension.

2.5. Antibacterial and antifungal assay

The well diffusion method (Kavanagh 1972) was adopted with some minor modifications to assess the antibacterial and antifungal activity of the prepared Ag-NPs. Two ml of the standardized bacterial and fungal stock suspension were thoroughly mixed with 20ml of molten sterile nutrient agar which was maintained at 45 °C. Twenty milliliter aliquots of the inoculated nutrient agar were distributed into sterile Petri-dishes. The agar was left to set and in each of these plates 2 to 3 wells (10 mm in diameter) was made. The wells were filled with 0.1 ml sample of each of the Ag-NPs suspension using automatic microlitre pipette, and allowed to diffuse at room temperature for two hours. Three antibiotics (Ampicillin, Gentamicin and Streptomycin) were used as positive control. The plates were then incubated in the upright position at 30 °C for 18 hours for in case of bacteria and for 3 days for fungi. Three replicates were used against each tested organism. After the incubation periods the diameter of the resultant inhibition zones were measured, averaged and the mean values were calculated.

2.6. Characterization of Ag-NPs using the SEM

The SEM samples of the aqueous suspension of Ag-NPs were prepared by taking one drop of the suspension on glass slide and the films on the SEM grids were allowed to stand for 2 min, after which the extra solution was removed using a blotting paper and the grid was allowed to dry prior to measurement. SEM observations were performed on a HITACHI-JP/H7600 instrument (Japan) operated at an accelerating voltage of 100 kV. The size of the resulted Ag-NPs was estimated on the basis of SEM micrographs with the assistance of SigmaScan Pro software (SPSS Inc., Version 4.01.003). Energy dispersive X-ray (EDX) analyses were performed on a JEOL JSM-6400 microscope (Japan).

2.7. Extraction the protein from the produced Ag-NPs suspension

Protein extraction was done using SDS gel by adding (2ml of SDS (10%) and 100µl 2-mercaptoethanol) to 1g of freeze-dried Ag-NPs and mixed well followed by incubation for one hour at room temperature. Then the protein was separated on 12% SDS poly acrylamide gel according to (Maniatis et al. 1982).

2.8. TLC for the extracted protein

The extracted protein from the produced Ag-NPs was separated on TLC paper according to (Garel 1964, Honegger 1961, and Ritschard 1964).

Different aflatoxins (P1, G1, G2 and B2) were used as standard and also were separated on the same TLC paper as well for comparison.

3. Results and discussion

3.1. UV-vis spectroscopy

The *F. oxysporum* culture filtrate was a pale yellow color before the addition of silver nitrate and this color was changed into dark brown color due to the formation of Ag⁺ ions during the first 28 h of incubation. The appearance of a yellowish-brown color in solution of the fungus filtrate is a remarkable of the formation of Ag-NPs in the 3.2. SEM observations of Ag-NPs

SEM observations showed that the obtained Ag-NPs are roughly circular in shape with smooth edges (Fig. 1). The Ag-NPs are very small in size and their sizes ranged from 16 to 30 nm.

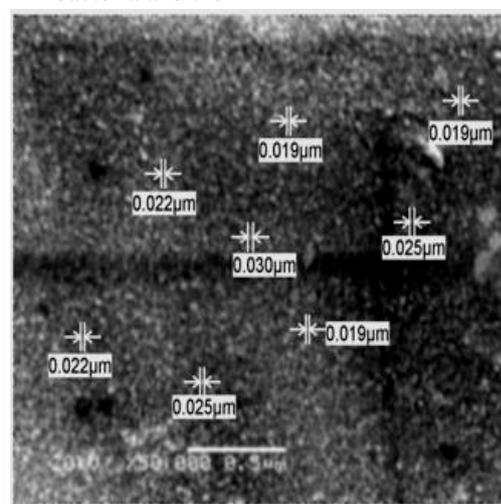
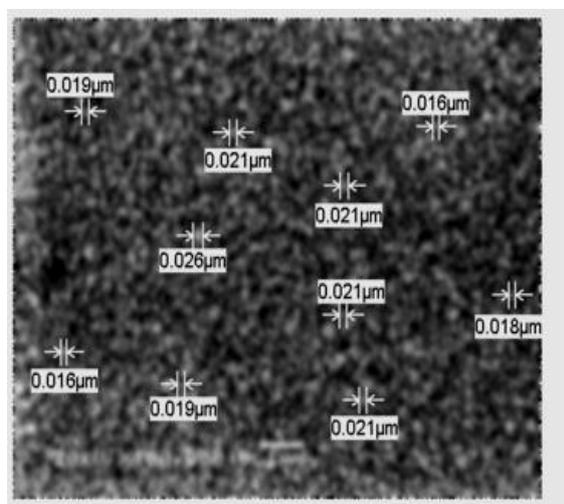


Fig. 1. Scanning Electron Microscope (SEM) images of Ag-NPs at 50X magnification power.

inhibition zones ranged between 10 – 20 mm when compared with the other antibiotics. The inhibition zones obtained by the three generic antibiotics were fluctuated between 11 to 15 mm. The antibacterial activity of Ag-NPs may exceed more than 100% when compared with ampicillin, 36% with gentamycin and was 32% with streptomycin (Fig. 2). These results are in agreement with that of Sarkar et al. (2007) whom reported that the Ag-NPs produced by *E. coli* (ATCC 10536) showed greater antibacterial efficiency compared to penicillin. The same observation was obtained by Li et al. (2005) whom proved that the *E. coli* nanoparticles showed high antibacterial activity more than amoxicillin. It was reported that Ag-NPs attach to the negatively charged bacterial cell wall and rupture it and cell death was deranged through the denaturation of cell protein (Lin et al. 1998, Zawrah et al. 2011). Moreover, Ag-NPs not only affect the bacterial cell wall but also the outer membrane and rupture of the

filtrate (Chan et al. 2005). UV-vis spectroscopy is an indirect method to examine the bioformation of Ag-NPs from aqueous AgNO₃ solution. Results presented in Table (1) revealed that the color intensity of the AgNO₃ solution increased with increasing the reaction time.

Table1. The color intensity (turbidity) of the AgNO₃ solution

O.D. ₄₆₀	1 st day	2 nd day	3 rd day	4 th day
Turbidity	0.092	0.530	0.860	0.923

3.3. Antimicrobial activity

The antibacterial activity of the obtained Ag-NPs was investigated against some pathogenic bacteria (Table 2). Results revealed that Ag-NPs have high antibacterial activity against all tested bacteria and the

plasma membrane, which leads to thereby depletion of cellular ATP (Lok 2006). Another mechanism by which complete blocking for the cell respiration as result of reaction between the Ag and SH group on the bacterial cell wall (Kumar et al. 2004). Morones et al. (2005) proposed the nanoparticles affect the defense system in the treated cells. Results presented in Table (3) revealed that AgNPs were effective against plant phytopathogenic fungi. The antifungal activity of the resultant AgNPs was investigated against the plant pathogenic fungi (*A. alternata*, *F. oxysporum* and *A. flavus*). Results revealed that Ag-NPs have potent antifungal activity against all tested fungi and the inhibition zones ranged between 10 – 26 mm when compared with the other antibiotics. The antifungal antibiotics Nystatin and Griseofulvin showed low inhibition with the tested fungi where the activity was 5, 8 and 5 mm with Griseofulvin and 4, 0 and 5 mm with Nystatin.

Table 2. antibacterial activity of the produced Ag-NPs against some pathogenic plant bacteria.

Treatment	Mean diameter of growth inhibition zones (mm)					
	<i>E. cartovra</i>	<i>E. amylovra</i>	<i>P. wasabiae</i>	<i>P. carotovorum atrosepticum</i>	<i>D. hrysanthemi</i>	<i>D. adantii</i>
Ag-NPs	20	14	18	19	10	18
Ampicillin	0	0	0	0	0	0
Gentamycin	11	11	0	0	0	15
<i>Streptomycin</i>	20	15	11	0	0	21

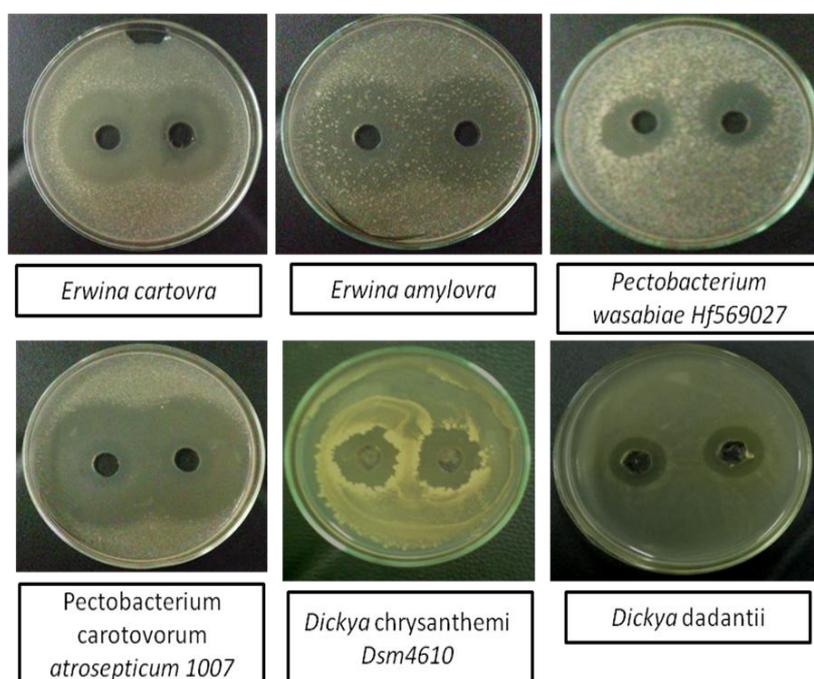


Fig.2. The antibacterial activity of AgNPs against some plant bacteria.

Table 3. Antifungal activity of Ag-NPs against some pathogenic plant fungi

Treatment	Mean diameter of growth inhibition zones (mm)		
	<i>A. alternata</i>	<i>F. oxysporum</i>	<i>A. flavus</i>
Ag-NPs	26	20	10
Griseofulvin	5	8	5
Nystatin	4	0	5

These results are in accordance with that of Huang et al. (2007) whom reported a successful reduction of sclerotium-forming fungi when AgNPs were used.

3.4. Protein separation using SDS-PAGE and TLC

The results presented in fig. (3) revealed that a single band was observed with molecular weight about 26KDa. The protein was purified from the gel and separated on TLC paper and the results proved that the isolated protein not related to any of the four examined aflatoxins. Nep1 derived from *F. oxysporum* is a member of a family of microbial proteins that are secreted by plant pathogenic oomycetes, fungi, and bacteria (Pemberton and Salmond 2004, Gijzen and

Nürnberg 2006, Kamoun 2006). Nep1-like proteins (NLPs) trigger plant defense responses and cell death. NLPs are small proteins of about 24 kD that exhibit a high degree of sequence conservation, including a pair of Cys residues. Cys residues predicted to form a disulfide bridge. Moreover, their necrosis and defense-inducing activity is heat-labile (Bailey 1995). Viet et al proved that NLPs can activate defense-associated responses, by the synthesis of phytoalexins and ethylene, the accumulation of defense-related transcripts, and cell death (Veit et al. 2001) and these ensures the fact that NLPs rapidly activate plant defense responses. These proteins have been shown to contribute to the virulence of

necrotrophic fungal and bacterial pathogens). NLPs act as positive virulence factors during infection of plants (Amsellem et al. 2002, Mattinen et al. 2004). For example, inactivation of the NLP-encoding genes (NLP) in different *Erwinia carotovora* strains resulted in significantly reduced levels of soft rot disease on potato (*Solanum tuberosum*).

It can be conclude that the bio-nanosilver particles can be used as an effective antimicrobial agent against a wide range of plant pathogens. Moreover, we will search about the suitable formula to applying the product on the infected plant both in green house and in the open field.

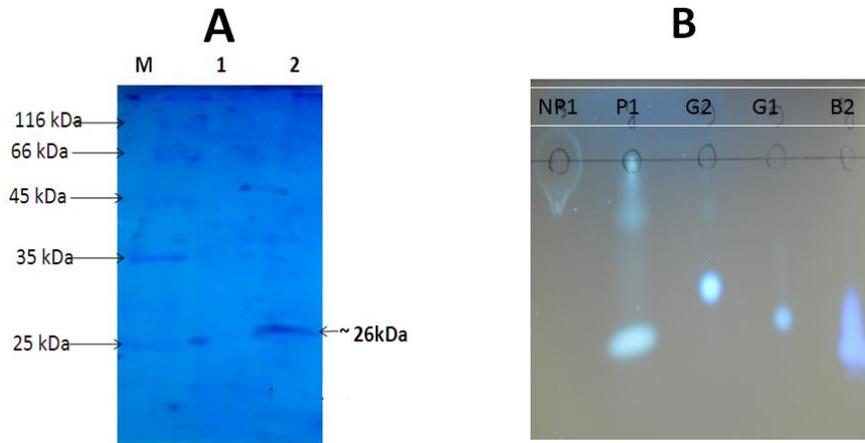


Fig. 3. SDS-PAGE and TLC-Paper separation for the extracted protein from the produced bionanosilver. A: 12% polyacrylamide gel; lanes: M; Mid range protein marker, 1: chemical synthesized nanosilver particles. Lane 2: the biosynthesized silver nano-particles. B: TLC-paper chromatography, lanes: NP1: the extracted protein from the bionanosilver. Lanes P1, G2, G1 and B2: The aflatoxins as standard.

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