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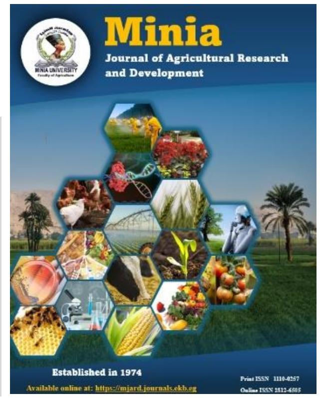

Impact of biosynthesized silver nanoparticles (AgNPs) on fusarium wilt disease in tomato: *In vitro* and *In vivo* studies

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ABSTRACT

An isolate of *Bacillus subtilis* was used as a bio-reducing agent to produce silver nanoparticles (AgNPs). in which the cell-free supernatant as mixed with silver nitrate ($AgNO_3$) in the reacting mixture (1:1). The biosynthesis was obviously observed by color change from white bale to dark brown and that was confirmed by UV-vis spectroscopy. Further examinations of AgNPs were made using SEM and TEM analysis. Nanoparticles showed a spherical shape with size in the range of 6-92 nm, and exhibition a slight aggregation. AgNPs have high potential to inhibit the growth: liner growth (mm), dry weight (mg) of two pathogenic isolates of *Fusarium oxysporum* grown on\in PDA media. Also, AgNPs gave significant protection against seed rot as well as the diseases incidence on tomato transplants. Moreover, the lower concentrations of AgNPs (12.5 and 25 ppm) have synergistic effect on seed germination and improve growth parameters of the roots and shoots growth.

Keywords: : *Bacillus subtilis*, AgNPs characterization, antifungal activity, *Fusarium oxysporum*, tomato wilt

1. INTRODUCTION

Losses in agriculture production due to plant pathogens and pests are estimated by 20-40% worldwide on annual basis (**Savary *et al.*, 2012**). To take outbreak of plant diseases extensive use of agrochemicals (i.e fungicide & pesticide) was implanted which consequently increases environmental pollution and health hazards. Moreover, frequent use of systemic fungicide for long time results in development of a resistant mutant of the pathogen to those chemicals (**Nyilasi *et al.*, 2010**). Thus, nanotechnology had a great a special attention as an alternative prospective approach to curb in plant diseases control as a sustainable, effective, and eco-friendly tool (**Jiang *et al.*,**

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2017). Engineered nanoparticles (NPs) possess the desired size and shape with special optical properties that enable them to be used for various agricultural applications including control of plant pathogens. Silver has been reported among the most widely used nanoparticles silver for control plant diseases. it also has a positive effect on plant performance growth. **Rastogi *et al*.**, **(2017)** stated that several factors affect activity of nanoparticles including size, shape, surface properties and methods of application.

Biologically synthesized is used an alternative method for traditional chemical process. Many bacterial species have been reported as biosynthesized agents for silver nanoparticles through secreting different enzymes in broth culture that include several reductases (**Anilkumar *et al.*, 2007**). **Saratale *et al.*, (2018)** indicated that bacterial cells act as a reducing bioagents, however they extracellularly induce nucleating and copping of nanoparticles by reducing metal ion (Ag^{+}) to metal AgNPs.

However, different *Bacillus* species have been reported as potential agent for biosynthesis of AgNPs (**Malarkodi *et al.*, 2013).** AgNPs have been showed antifungal properties include against aggressive fungal infections (**Rai *et al.*, 2014**), such as *Colletotrichum sp.*, *Fusarium oxysporum, Cephalosporium sp.* and *Rhizoctonia sp*. (**Balakumaran *et al.*, 2015, El-Gazzar and Rabie 2018**). **Ma and Hiradate. (2000)** indicated mechanism of AgNPs antifungal activity may be through damaging DNA which impairs the fungal culture's ability to proliferate. Also, by decreasing protein expression in the subunit as well as fatty enzymes and cellulase protein required for ATP generation (**Kim *et al.*, 2012**). Also, production of oxygen active species (ROS) can cause toxicity (**Xia *et al.*, 2016**), free radicals can cause damage cell wall, proteins, lipids and DNA.

Fusarium wilt of tomato is considered as one of the most important diseases of tomato both in the field and greenhouse-grown tomatoes worldwide. However, Fusarium wilt disease mainly caused by *Fusarium oxysporum* is affecting severe injury through all phases of plant growth. Lately, in Egypt, the injuries in tomato production due to *F. oxysporum* infection raised up to 67% of total planted area that makes severe damage during all stages of plant development (**Naeema *et al.*, 2022**).

Thus, the present study aimed to use *Bacillus subtilis* as biological agent to biosynthesized AgNPs, determine the characters of obtained AgNPs, evaluate the fungicide effect against *F. oxysporum,* and determine their role in tomato wilt development.

MATERIAL AND METHODS

Source of the microorganisms

Bacillus subtilis were kindly provided from Agric. Microbiology Dept., Fac. Agric., Minia Univ. Two isolates of *Fusarium oxysporum* were previously isolated, identified and its pathogenicity tested (**Hafez *et al,* 2024, In press**).

Bio- synthesizes AgNPs:

Green syntheses of AgNPs were prepared as described by (**Siddiqi and Husen, 2016**). Sixty ml of nutrient broth were prepared in 100 ml conical flask, inoculated with the *Bacillus subtilis*, then incubated at 37 °C. After3 days, supernatant was collected to use in the biosynthesis of AgNPs. An aqueous solution of silver nitrate (0.1 M) was added to the cell-free supernatant (1:1). The mixture was incubated on the rotary shaker at 37°C (150 rpm) for 24hours in dark conditions. The color change was observed and recorded each 12 h for 3 days using spectrophotometer (Milton Roy specrtronic 1201) at 430 nm (**Song and Kim, 2009**).

Characterization of biosynthesized AgNPs

Extracellular bio-reduced silver ions by the culture supernatant of the bacteria *Bacillus subtilis* were preliminarily examined by:

UV-Visible spectroscopy analysis

The UV-Vis spectrometry analyzed was carried out to characterize the synthesized of AgNPs. The reduction of pure silver ion was observed by pipetting 1 ml of the AgNPs solution into UV quartz cuvette cell and monitored in spectrophotometer in room temperature at wavelength ranged of 200 to 750 nm (**Kumar *et al.*, 2015**).

Scanning electron microscope (SEM)

SEM was used to determine the shape and size of nanoparticles in electron microscope unit, Central Lab., Minya University. A small drop of suspension of biosynthesis nanoparticles was applied on slides and left to dry under vacuum in desiccators before loading onto a specimen holder. The microscope operated at an accelerated voltage at 5-10 KV and different magnification (**Li *et al.*,2010**).

Transmission Electron Microscopy (TEM)

The size and morphology of the synthesized nanoparticles were recorded by using TEM model JEOL electron microscope JEM-100 CX (Tokyo, Japan). TEM studies were prepared by drop coating silver nanoparticles on to carbon-coated TEM grids. The film on the TEM grids were allowed to dry, the extra solution was removed using a blotting paper **(Ibrahim *et al.*, 2019).**

Purification of biosynthesized AgNPs

Silver colloids containing AgNPs were centrifuged at (5000 rpm, 25 minutes) after optimization of synthesis conditions, the supernatant was discarded, replaced with deionized distilled water, and washed three times by centrifugation**,** the residue billet was gathered, air dried, gently collected and stored at 4°C for other tests (**Gurunathan *et al.*, 2009**).

***In vitro* studies:**

Linear growth assays: The effect of AgNPs was evaluated against the linear growth of *F. oxysporum* isolates using the pour plate method **(Min *et al.*, 2009**). AgNPs at concentrations of 0.0, 12.5, 25, 50 and 75 ppm were added to PDA media before sterilization. Agar plugs (diameter, 5 mm) of fungal culture isolates (10-days old) individually were inoculated simultaneously at the center of each Petri dish containing AgNPs-media, incubated in darkness at 25 ± 2°C. After 7 days of incubation period, the mean of two perpendicular diameters was calculated. Three Petri dishes were used as replicates for each treatment.

Dry weight: The effect of biogenic AgNPs was evaluated against the growth biomass (dry weight) of *F. oxysporum.* Potato dextrose broth medium containing AgNPs 0.0, 12.5, 25, 50 and 75 ppm of AgNPs were singly prepared and autoclaved. The media were inoculated by disks of agar cultures, 10-days old (5 mm diameter) of each of isolates. The inoculated cultures were incubation in darkness at 25 ± 2 °C for 10 days. Three flasks were used as replicates for each treatment. After incubation period, the fungal growth was filtered through Watman filter paper No.1, dried at 70 °C for 12 hands then weighted.

Effect of AgNPs on tomato seeds germination of treated with spore suspension of *Fusarium oxysporum* and seedling growth parameters:

Effect on seed germination: Seeds of tomato (Genotype 023 F1) were disinfected by 5 % $NaClO_3$ solution for 3 min, rinsing with distilled water several times. seeds were treated individually with 50 ml of different concentrations of AgNPs in distilled water (0, 12.5, 25, 50 and 75 ppm) alone or with spore suspension at concentration 1.0 $x10^6$ microconidia/ml, AgNPs solution or fungal spores (positive control) or sterile distilled water (negative control). for 2 h. The treated seeds were plotted in Petri Dish (14 cm) containing wet filter paper (10 seeds/dish), placed in growth chamber (25±2°C, 12h light) for 10 days to estimate the germination percentage. the plates were examined for germination percentage, root length and shoot length (**Elamawi and Al-Harbi, 2014**).

***In vivo* studies:**

Effect of AgNPs on disease development

A pot experiment was performed under greenhouse conditions to determine the effect of green synthesized AgNPs on tomato wilt caused by *F. oxysporum*. The bioassay was developed by preparing fungal spore suspension (1 x 10^6 c.f.u./ml). Subsequently, seedlings of 30 days (Genotype 023 F1) were wounded at secondary roots, immersed in nanoparticles suspension at concentrations of 0.0, 12.5, 25, 50 and 75ppm for 4 h. Seedlings were transferred to spore suspension for 4 hours. The treated transplanted were cultivated in 25-cm-pots containing sterilized clay: sand: peat moss (1:1:1w:w:w). All agricultural practices were successfully applied according to recommendations of Ministry of Agricultural and Land Reclamation where plants were watered and fertilized with NPK 19:19:19 as well as necessary microelements. Each treatment was represented by five pots. After 40 days, disease incidence and severity were estimated as described by **(Weitang *et al.*, 2004).**

Effect on tomato transplants growth parameters:

The experiments were carried out under greenhouse of Pl. Path. Dept., Fac. Agric. Minia Univ. Root of tomato seedlings, 25 days-old (Genotype 023 F1) were immerged in AgNPs solution (0.0, 12.5, 25, 50, 75, ppm) for 2 h before transplanted in 25-cm-pots containing sterilized clay: sand: peat moss (1:1:1). All agricultural practices were successfully applied as abovementioned. After 30 days, some vegetative measurements were recorded on tomato plants i.e., fresh weight(mg), dry weight (mg), shoot length (cm) and root length (cm).

Statistical analysis

The protected least significant difference (L.S.D) values at 5 % ($P< 0.05$) were used to test the differences between treatments (**Gomez and Gomez, 1984**).

RESULTS

Biosynthesis of silver nanoparticles was carried out by *B. subtilus* as bioactive agents which may reduce $AgNO_3$ to Ag nanoparticles. The time course of AgNPs biosynthesis was determined visually by change in the color of the $AgNO_3$. As shown in Table 1 and Fig. 1. It is clearly shown that the pure aqueous solution of Ag NO_3as control with yellow color. The other screw bottle is colloid solutions of AgNPs (dark brown color). The color is increased with time. The maximum optical density (1.845) was detected after3 days of incubation.

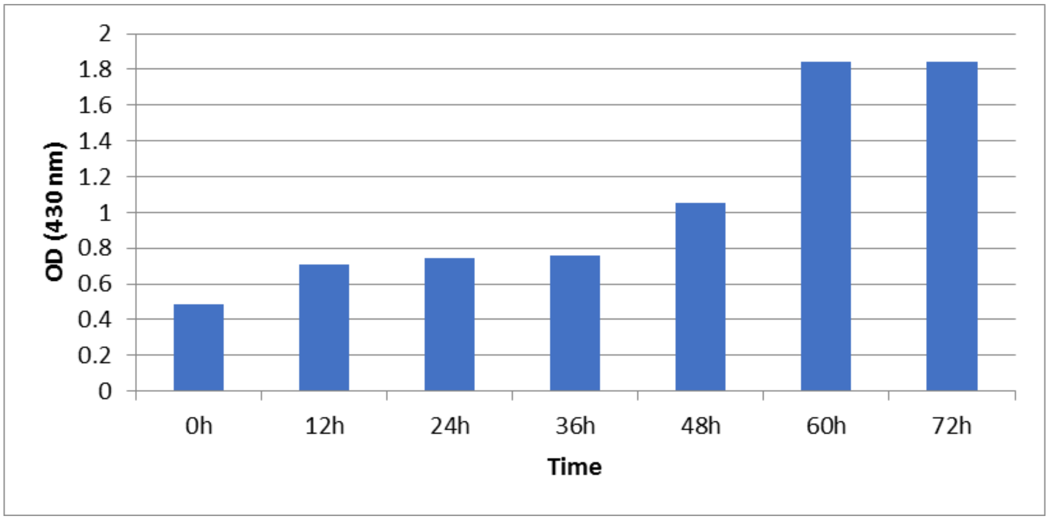

Fig. 1: Time course of AgNPs formation by *Bacillus subtilis* **expressed as OD at 430 nm.**

UV-vis spectral analysis

The formation and stabilization of AgNPs in aqueous solution of $AgNO_3$was monitored by using UV-vis spectrum analysis. The samples were scanned in wavelength between 200 to 750 nm. The maximum absorbance peak was detecting around 400 nm (Fig 2). The absorbance was 0.225.

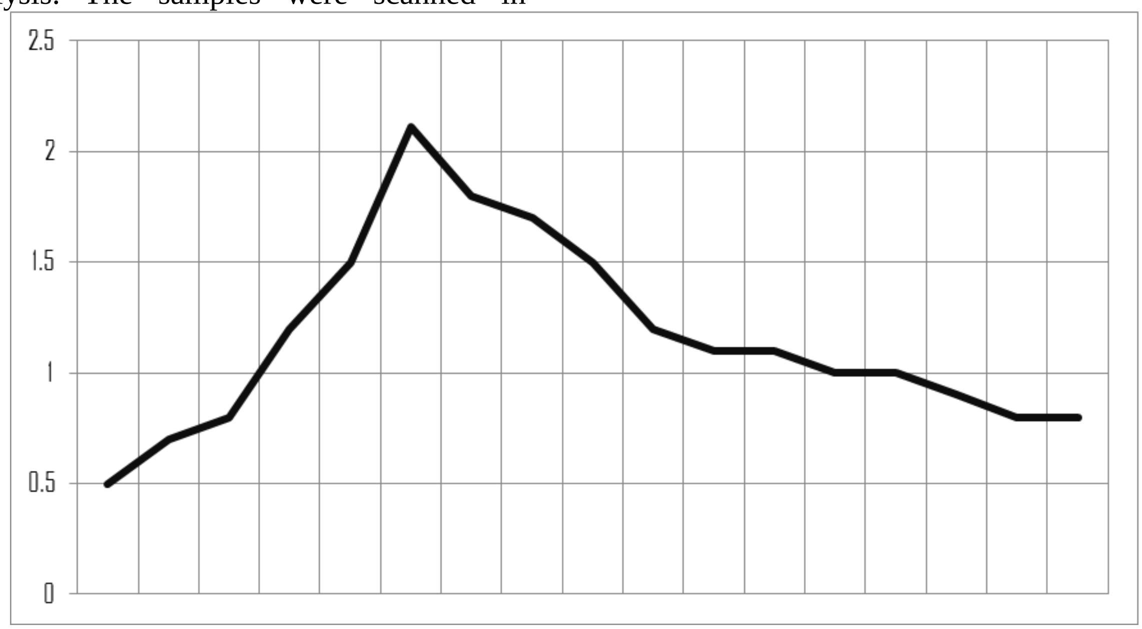

Fig (2): UV-Vis absorption spectrum of biogenic synthesized of AgNPs by free–cell culture filtrate of *B. subtilus*.

SEM analysis:

SEM micrograph of the examined samples is showing in Fig. (3). It can be seen that different shapes of silver nanoparticles were obtained, The SEM micrograph confirmed the formation of silver nanoparticles did not show a uniformed surface and almost shape with the diameter ranged between 6-92with average 36± 6.6nm.

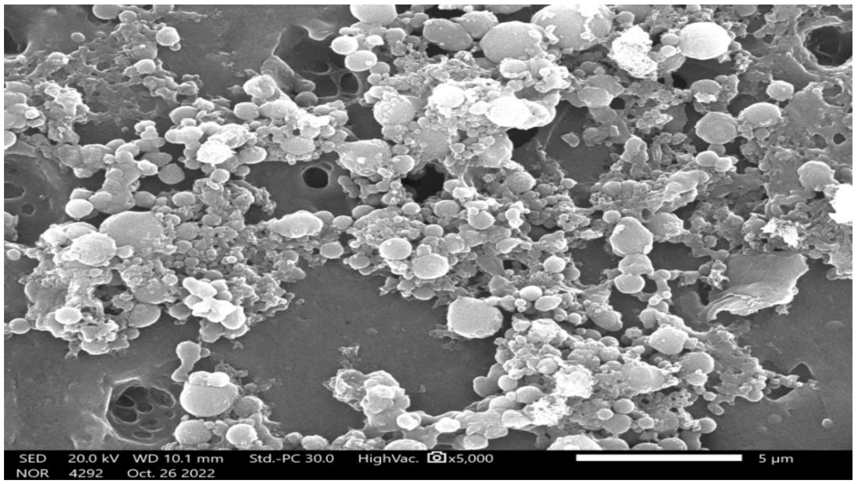

Fig. (3): SEM micrograph for AgNPs synthesized by the reduction of$AgNO_3$by *B. subtillus* culture filtrate

TEM analysis:

TEM analysis is represented in Fig. (4). TEM micrograph analysis showed smooth, spherical silver nanoparticles which showed slightly aggregated in certain location.

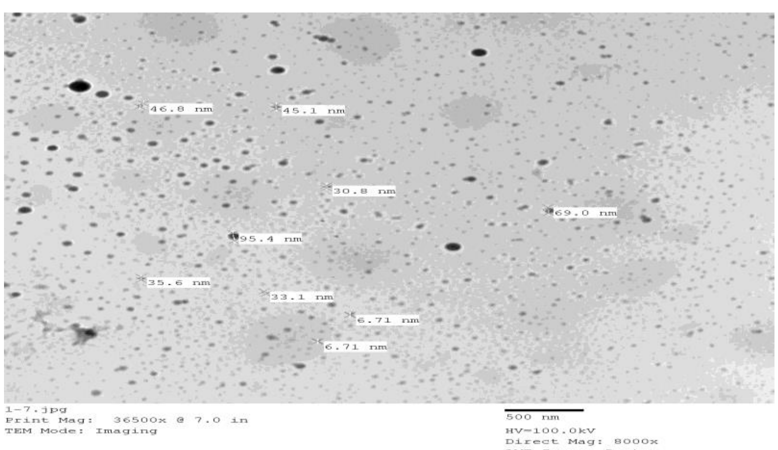

Fig. (4): TEM micrograph for AgNPs synthesized by the reduction of $AgNO_3$ by *B. subtillus* culture filtrate.

Effect of AgNPs on growth of *F. oxysporum*

The effect of biosynthesized silver nanoparticles on the growth of the fungus *F. oxysporum* grown was tested on PD medium at four tested concentrations (12.5, 25, 50 and 75 ppm). Results in Table (1) indicated that all tested concentrations significantly reduced the growth of the tested isolates in solid and broth PD medium compared to the control treatment. Moreover, the growth of both isolates as completely inhibited at 50 ppm of biosynthesized silver nanoparticles.

Table (1): Effect of AgNPs on *F. oxysporum* growth expressed as liner growth and dry weight

AgNPs Conc. (ppm)	Linear growth (mm)				Dry weight (mg)			
	Fo8	Fo2	Mean	Efficiency	Fo8	Fo2	Mean	efficiency
0.0	88.0	88.3	88.2	0.0	877.2	885.5	851	0.0
12.5	40.6	45.6	43.1	52.1	430.0	470.6	470.3	44.7
25	20.3	18.7	19.5	78.3	144.3	135.2	139.8	83.5
50	0.0	0.0	0.0	100	0.0	0.0	0.0	100
100	0.0	0.0	0.0	100	0.0	0.0	0.0	100
Mean	30.2	30.9	30.4	66.1	290.3	298.3	292.2	65.6

$LSD_{5\%}$ A 3.25 28.8
B 2.9 16.7
AB 7.2 42.8

Effect of AgNPs on tomato seed germination, seed decay, and seedling growth parameters:

Results in Table (2) show that concentrations of AgNPs (12.5, 25, 50 ppm) significantly increased tomato seed germination treated with spore suspension of both tested fusarium isolates in comparison with the control treatment. However, it was observed that inoculated seeds with 75 ppm AgNPs give non-significant difference in comparison with the control for both isolates. The same trend was noticed uninoculated seeds treated with the above-mentioned concentrations. AgNPs show a positive effect in shoot length at concentration of 25-50 ppm. An enhancement in root length was noticed treatment of 25 ppm in control seeds. In inoculated seeds, this enhancement was observed at concentration 25-50 ppm in case of F8 and 25 ppm in case of Fo2.

Table (2) Effect of silver nano particles on tomato seedling growth parameters: -

AgNPs Conc.	Treatments								
	Growth in control seeds			Growth in seed inoculated with F o 8			Growth in seed inoculated with Fo 2		
	Ger. %	Shoot Length	Root length	Ger. %	Shoot length	Root length	Ger. %	Shoot length	Root length
Zero	100	12.6	6.7	66.7	8.6	3.7	76.7	10.8	4.4
12.5	100	13.3	7.8	73.3	10.9	4.3	80	11.8	5.7
25	100	14.2	8.3	80	12.9	4,8	83.3	12.2	5.9
50	100	12.4	5.7	83.3	13,6	4,9	86.7	10.6	4.6
75	95	8.3	5.2	83.3	11,7	3.9	86.7	9.2	4.2
Mean	99	12.16	6.74	77.32	6.48	2.38	82.68	10.92	4.96
L.S.D 5%	4.3	1.7	1.6	2.16	1.2	1.0	1.38	1.2	1.3

Effect of AgNPs on tomato disease occurrence caused by *F. oxysporum*

Data in Table (3) summarized the. AgNPs at concentration of 12.5 and 25 had no significant effect on either DI % or Ds %. But the seedling treatment with 50 and 75 ppm of AgNPs both DI and Ds decreased to 26.3 and 16.7 and 23.6 and 15.6%, respectively.

Table (3): Disease incidence (DI) and disease severity (DS) caused by *F. oxysporum* as affected by AgNPs in tomato plants:

AgNPs Conc. (ppm))	Disease development					
	Fo8		Fo2		Mean	
	D I %	D S %	D I %	D S %	D I %	DS %
Zero	75.7	63.7	31.3	25.0	53.5	39.3
12.5	61.6	61.1	32.3	22.6	46.95	41.85
25	50.5	52.5	22.1	16.6	36.3	34.55
50	42.4	38.8	10.1	8.3	26.3	23.2
75	33.3	30.5	00	00	16.7	15.3

L.S.D $_{5\%}$ A 12.3
B 3.7
Ab 16.2

Effect on tomato transplants growth parameters:

Results in table (4) show the effect of different AgNPs concentrations (12.5, 25, 50, 75 ppm) on tomato plants. Data clarified that the inoculated plants by both tested isolates show decrease in all growth parameters. Isolate Fo8 is more effective than isolate Fo2. Treatments of tomato seedling by AgNPs caused enhancements in growth till 50 ppm. Concentration of 75 ppm

AgNPs caused a reduction in all growth parameters. The maximum growth was detected in case of 50 and 75 ppm.

Table (4) Effect of biosynthesized silver nanoparticles on tomato seed germination%, shoot and root length and fresh and dry weigh.

Inoculation by	AgNPs Conc.	Growth parameter			
		Shoot Length (cm)	Root length (cm)	Fresh weight (mg)	Dry weight (mg)
Fo8	0.0	18.6	4.5	152.6	48,2
	12.5	19.4	6.5	188.3	63.0
	25	22.3	8.6	223.5	85.4
	50	25.7	9.3	235.0	87.5
	75	15.2	3.9	148.8	42.3
Mean		20.2	6.6	189.7	69.6
Fo2	0.0	27.5	9.7	237.8	62.3
	12.5	29.6	11.3	243.5	76.8
	25	33.9	13.9	266.2	86.5
	50	34.2	15.2	272.4	89.4
	75	25.4	7.3	222.6	59.3
Mean		30.1	11.5	248.5	74.9
Control	0.0	31.6	13.6	260.7	80.3
	12.5	35.2	15.7	290.6	90.4
	25	37.6	17.9	310.3	100.5
	50	40.5	18.2	320.2	110.2
	75	28.6	14.3	240.0	70.4
Mean		34.7	16.0	284.4	90.4
L.S.D at $_5$ %		0.8	2.45	9.2	18.3

DISCUSSION

Nanotechnology offers an ecofriendly, sustainable, and effective way in management of plant disease through application of biological synthesis of nanoparticles. The process is simple in which bacteria, fungi, algae and plant extract are using a bio reducing agent ion met to metal nanoparticles. have a lot of interested because of their high potential antibacterial and antifungal effects. Bacteria are preferable microorganisms because of their easy cultivation and quick multiplication. However, several *Bacillus species* have been reported as biosynthesized agents for the production of silver nanoparticles **(Sunkar, and Nachiyar 2012, Saratale *et al,* 2018 and Das *et al,* (2014)**

In this study an isolate of *B. subtilis* was used as a bio reducing agent for the biosynthesis of silver nanoparticles from silver nitrate as a precursor for silver. **(Tariq *et al,* 2022**) suggested that a function group like polyphenols secreted as a secondary metabolite by bacterium, release electrons in the reaction mixture that interact with silver nitrate resulting into reduction of silver ions (Ag^{+}). That finding was confirmed to observation of the control (salt without the bacterium in which no change in color as observed and that certainty indicated that the bacterium as responsible for the reaction.

In this study UV-vis showed a clear sharp peak assigned between 200 – 800 nm due to surface plasmon response (SPR) value in the range of the spectrum of the tested solution. It is known that AgNPs acquire a unique optical property due to (SPR) that generated from electron movement (**Sadowski *et al.,* 2008), (Minaeian *et al.,* 2008).** Moreover, **Sunkar, and Nachiyar (2012**) reported that increasing SPR value toward high wavelength is proportional to intensity of AgNPs biosynthesis in the reacting mixture. Further characteristics about size, shape and distribution of formed silver nanoparticles were observed through SEM & TEM tools. It showed that most nanoparticle are spherical with diameter ranged between 6-92 with slight aggregation.

The potential of obtained AgNPs was tested against two isolates of *F. oxysporum* grown on\in PD medium *in vitro*. All tested concentration significantly inhibited the growth and the biomass of bio isolates compared with the control treat. There was complete inhibition for the growth of the fungus at 50 ppm for both isolates. Previous studies reported high antifungal activity against *F. oxysporum* treated with AgNPs particles (**Akpinar *et al.,* 2021., Elamawi and Al-Harbi, 2014., Ghojavand *et al.,*2020).** Many mechanisms were proposed for antifungal effect on phytopathogenic fungi such as damage of DNA, production of reactive oxygen species (ROS) and impairing of ATP generation. This study also, indicated that the tested concentrations gave a significant level of protection against infection of tomato seeds treated with fungal spores of *F. oxysporum* and silver nanoparticles. This finding agrees with that reported by (**Elamawi and Al-Harbi, 2014**). In addition, tested concentrations at the lower rate (12.5 and 25 PPM) stimulates the germination rate compare to the control these results were supported by the results reported by (**Elamawi and Al-Harbi, 2014., and Noshad *et al*., 2019**).

Although based metal silver nanoparticles reported to be toxic for different plant species when they applied at high concentration especially those with small sizes. In content, biosynthesized nanoparticles have mild toxic effect on plant since the obtained nanoparticles have different size and possess different penetration capacity. Results in this study indicated that using biosynthesized silver nanoparticles showed high potential in reducing wilt disease incidences with all tested concentrations in comparison with the control treatment. Moreover, results in this study indicated that using biosynthesized silver nanoparticles showed high potential in reducing wilt diseases incidences with all tested concentrations in comparison with the control treatment. Also, these as obvious increase in both root length, shoot length, fresh weight and dry weight compared with the control. In similar studies (**El-Gazzar and Rabie, 2018**) reported that bio-AgNPs have been used for inhibition of *Cephalosprium maydis* had a great potential to control the disease caused by the fungus. Other reports indicated the high inhibitory effect of AgNPs on some fungal diseases (**Lamsa *et al.,* 2011; Patel *et al.,* 2014 and Ahmed 2017**).

Concerning tomato diseases, previous studies indicated the potential of AgNPs to control some of bacterial diseases (**Noshad *et al.,*2020., Cheng *et al.,* 2020**), viral diseases **(Noha *et al.,* 2018, Shafie *et al.,* 2018**) and nematodes (**Hassan *et al.,* 2016, El-Deen and El-Deeb, 2018, Kalaiselvi *et al.,* 2019**). In tomato crop several.

CONCLUSION

In conclusion, AgNPs has a wide range of anti-fungal activity against pathogenic fungi as well as against other plant pathogens. However, their application under field condition is still limited. Challenges related to that fact may be due to their toxicity to plant species (**Rastogi *et al.*, 2017, Goswami and Mathur, 2019**), and expected accumulation in the soil (**Ahmed *et al.*, 2021)** which may lead to some environmental hazards. Further studies on regulation of AgNPs uptake by plants are needed.

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الملخص العربي

تأثير جزيئات الفضة النانوية المخلقة حيويا (AgNPs) على مرض ذبول الطماطم الفيوزاريومي: دراسات معملية و حقلية

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تم استخدام عزلة من البكتريا باسيلس ستلس كعامل للاختزال الحيوي للفضة لانتاج حبيبات فضة نانومترية, و امكن ملاحظة هذا التخليق الحيوي بالعين المجردة بتغيير لون نترات الفضة من اللون الابيض الي لون بني غامق و تاكيد هذا التخليق باستخدام جهاز القياس الضوئي بواسطة الاشعة فوق البنفسجية و اجريت مزيد من الدراسة باستخدام الميكروسكوب الاليكتروني الماسح و النافذ , و تتراوح حجم الاجسام النانومترية بين 6 – 92 نانوميتر و اظهرت قليل من التجمعات و الجزيئات الفضة النانوية قدرة كبيرة علي تثبيط نمو الفطر الفيوزاريوم اوكسيسبورم النامي علي بيئة البطاطس السائلة و الصلبة. ايضا اظهرت هذه الجزيئات قدرة معنوية علي حماية البذرة من العفن و كذلك البادرات من حدوث مرض الذبول, و علاوة علي ذلك اظهرت التركيزات القليلة منها (12.5, 25) جزء في المليون تأثير محفز لإنبات البذور و تحسين الصفات الخضرية و نمو الجذر و الساق.