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EFFECT OF BIOSYNTHESIZED SILVER NANOPARTICLES ON *Fusarium oxysporum* FUNGUS THE CAUSE OF SEED ROT DISEASE OF FABA BEAN, TOMATO AND BARLEY

Rabab M. Elamawi [1*] and Raida E. Al-Harbi [2]

[1] Rice Pathology Department, Plant Pathology Research Institute, BP 33717 Agricultural Research Center, Sakha, Egypt. *Corresponding author: rabab.elamawi@yahoo.com

[2] Microbiology Department, College of Science, PO Box 22452, King Saud University, Soudi Arabia,

ABSTRACT

Silver nanoparticles (AgNPs) was evaluated for its possible effects on the incidence of seed rot disease of Faba bean, Tomato and Barley caused by the seed and soil borne fungus *Fusarium oxysporium*. The Silver nanoparticles were biosynthesized by the fungus *Trichoderma longibrachiatum* and characterized by UV-Visible (UV-Vis) spectroscopy, Transmission Electron Microscope (TEM) and Dynamic Light Scattering (DLS) (Raid, 2013). Silver nanoparticles characterized as spherical shape, almost monodispersed at size of 1-20 nm. A preliminary study also performed in order to find out its suspected toxic effects, using different concentrations of Silver nanoparticles solutions on germination, vigour index and on the *Fusarium oxysporium* infection on the target crops. Silver nitrate solution or water treatment was used as positive and negative control respectively. Healthy and infected seeds were soaked in three different concentrations of Silver nanoparticles (0.5, 0.25 and 0.12 mM). At the higher concentration, silver nanoparticles alone or in combination with *Fusarium* spores showed adverse effect on seed germination and vigour index. The lower concentration of silver nanoparticles improved the seed germination percentage and vigour index, in addition to reduce the disease incidence (seed rot disease) caused by *Fusarium oxysporium*. The higher concentration of Silver nanoparticles 0.5 mM showed a slightly effects on seed germination percentage and vigour index in tomato seedlings. Silver nanoparticles at concentration of 0.12 mM were less toxic to the tomato, faba bean and barley seedlings. It also reduced the disease incidence. Therefore, it is recommended to consider Silver nanoparticles in further studies for possible controlling of seed-borne pathogens putting in mind its possible accumulation in the crop products and the consecutive in food chain.

Keywords: Bio-synthesized silver nanoparticles, *Trichoderma longibrachiatum,* seed borne diseases and *Fusarium oxysporium*

INTRODUCTION

Fusarium oxysporum Schlecht. is a common soil fungus found in the rhizosphere vicinity of many plant species. Most *F. oxysporum* strains lives as saprophyte on organic substrates. However, some strains cause root rots and wilt diseases.Other *F. oxysporum* strains are effectively used as biocontrol agents. *F. oxysporum* is a very complex group, divided into Formae specials and physiological races depending on their pathogenicity towards particular plant species or cultivars (Armstrong & Armstrong 1981). *F. oxysporum* produces chlamydospores, macroconidia and microconidia.

Currently, the treatment of seeds using chemical fungicides before planting is the common used method to protect tomato and other plants from *F. oxysporum* infection. The application of chemical fungicides show divers effects on other living organisms including the useful soil microorganisms (Khalifa *et al.,* 1995; Lewis *et al.,* 1996). The alternative methods of pest control can play roles in management systems (Sutton, 1996).

Positive or negative effects of nanoparticles on higher plants were presented by several reports. Nano-SiO_2 and nano- TiO_2 lead to an increase in nitrate reducatase in *Glycine max*, it also enhances the ability to absorb and utilize water, stimulate the antioxidant effects and accelerate the germination and growth (Lu *et al.,* 2002). Due to its variable shapes and sizes, it is difficult to predict their effects and mode of action in the environment (Holsapple *et al.,* 2005). Alumina nanoparticles displayed inhibitions to root elongations of corn, cucumber, soybean, cabbage and carrot (Yang and Watts,2005). ZnO nanoparticles also inhibit root elongations of ryegrass, radish and rape (Lin and Xing, 2007).

The adverse effects of silver nanoparticles were studied on seed germinations, root and shoot growth on three plant species (*Oryza sativa*, *Vigna radiata* and *Brassica campestris)* when seeds were soaked in different concentrations of nanoparticles. In additions, AgNPs and $AgNO_3$ significantly inhibited root growth of *Eutrochium fistulosum* and four *Carex* spp (Mazumdar and Ahmed, 2011). Silver nanoparticles were synthesized by chemical reduction methods (Yin *et al.*, 2012).

Silver nanoparticles were applied for the control of microorganisms and the prevention of deleterious infections. Studies reveal that antimicrobial activity of the silver nanoparticles is due to their positive charge that qualifies them in reacting with the negatively charged proteins on the cell membranes and thus contributing to their antimicrobial activities (Hamouda *et al.,* 2000 and Dragieva *et al.,* 1999).The antifungal effects of silver nanoparticles were measured against eighteen plant pathogenic fungi including genera of *Alternaria, Botrytis, Cladosporium*, *Corynespora*, *Cylindrocarpon*, *Fusarium*, *Pythium*, *Stemphylium* (Kim *et al.* 2012). The antifungal activity of the biosynthesized nanoparticles from olive seed extract had been tested against different fungal plant pathogens viz: *Aspergillus niger*, *Aspergillus falvus*, *Alternaria macrospora*, *Rhizoctonia bataticola* and *Sclerotium rolfsii* using agar diffusion method (Khadri*et al.,* 2013). The objectives of this research was carried out to evaluate the antimicrobial effects of bio-synthesized silver nanoparticles by *Trichoderma longibrachiatum* for controlling seed-borne pathogen *(Fusarium oxysporum*) which attack on tomato, faba bean and barley.

MATERIAL AND METHODS

Silver nanoparticles: *T. longibrachiatum* was isolated from cucumber leaves in Riyadh, Saudi Arabia and propagated according to Raida, 2013 methods. The silver nanoparticles were produced by *T. longibrachiatum* and characterized by different techniques including Ultraviolet-Visible

spectroscopy, Fourier-Transformed-InfraRed spectroscopy, Transmission Electron Microscopy and Dynamic Light Scattering (Raida, 2013). These techniques provide information about the formation, size and morphology of the particles, capping proteins and stability.

Fungal isolation and pathogenicity test: *F. oxysporum* Schlecht. isolated from tomato fields around Riyadh city, Saudi Arabia, grown on PDA medium and incubated at 28°C for 7 days. Under sterile conditions, spores were harvested. Microconidia were separated from the mycelium by filtration the conidia suspension through three layers of filter papers. Microconidia suspension was concentrated by centrifugation at 4000 rpm for 10 min and adjusted to 1.0 $x10^7$microconidia/ml. Virulence analysis of the *F. oxysporum* isolate was carried out on faba bean, tomato and barley seeds.

Effect of silver nanoparticles on colony formation of *Fusarium oxysporum*: 500 µl of the conidial suspension were mixed with serial concentrations of silver particles and adjusted to a final volume of one ml. Microconidial suspension was also prepared with sterile deionized water as control or mixed with the 1mM $AgNO_3$ solution which used as raw materials during nanoparticle synthesis. Treated conidia suspensions were incubated for 24 h at 28°C in an orbital shaker (120 rpm). 25-µl aliquot of the spore suspension of each treatment was spreaded on PDA medium. Three PDA plates for each treatment were used as replicate. The number of colonies formed on plates was counted after one and two days. The average number of colonies from silver-treated spore suspensions were compared with the number the $AgNO_3$ solution and with the water control as percent colony formation.

Effects of silver nanoparticles on seed germination and inhibition of *Fusarium oxysporum* infection: Fifty seeds from each of faba bean, tomato or barley were subjected to standard blotter method in which the seeds were incubated according to the standard procedures of ISTA (ISTA, 1996). Seeds were surface sterilized in 5 % sodium hypochlorite solution for 3 min and washed in three successive changes of sterile distilled water and left for air drying. Seeds were transferred onto filter paper in Petri dishes (10 seed per dish), soaked in different concentrations of AgNPs (0.5, 0.25 and 0.12mM) alone or with Spore suspension at concentration 1.0 $x10^7$microconidia/ml, 1mM $AgNO_3$ solution or fungal spores (positive control) or Sterile distilled water (negative control). After ten days of incubation period, the plates were examined for germination percentage, root length and shoot length. The infected seeds were also counted. Barley seedling was examined after 7 days for germination and growth rate. Germinated seed was considered when the radicle or plumule emerged from the seed coat.

Vigour Index was calculated by using the formula of Abdul Baki and Anderson (1973) as shown below:

Vigour Index (VI) = (Mean shoot length + mean root length) x Germination (%).

Statistical analysis: Differences between treatments for the different measured variables were tested using one-way analysis of variance (ANOVA) with used Statistical Package for Social Sciences (SPSS), followed

by Tukey's HSD post-hoc test when significant differences were found (p = < 0.05). P values lower than 0.05 were considered significant

RESULTS AND DISCUSSION

Silver nanoparticles activity against *Fusarium oxysporum in vitro*: Silver nanoparticles with size 1-20 nm at a concentration of 0.5mM was effective against *Fusarium oxysporum* and displayed 68.2% inhibition of colony formation comparing to the negative control (water) (Fig. 1) and (Table 1). Statistical analysis revealed a significant effect to the use of AgNPs and $AgNO_3$ compared with the control treatment. The P value shows 0.047 that they have significant effect so; the nanoparticles strongly inhibited the fungal growth.

Silvers nanoparticles possess different properties, which might come from morphological, structural and physiological changes (Nel *et al.,* 2003). Silver nanoparticles are highly reactive as they generate Ag^+ ions while metallic silver is relatively unreactive (Morones *et al.,* 2005). Also, the nanoparticles efficiently penetrate into microbial cell, which implies lower concentrations of nano-sized silvers sufficient for microbial control. A previous study indicates that silver nanoparticles disrupt transport systems including ion efflux (Morones *et al.,* 2005). Also, silver ions are known to produce active oxygen species (ROS) via their reaction with oxygen, causing damage to cells proteins, lipids, and nucleic acids (Storz & Imlay, 1999 and Hwang *et al.,* 2008).

Figure-1: Antifungal activity of biosynthesis silver nanoparticles against colonies formation of *Fusarium oxysporum*. (1) Control, (2) $AgNO_3$, (3) Silver NPs mixed with *F. oxysporum* spores for 24 h.

Table 1: Effect of AgNPs synthesized by *Trichoderma longibrachaitum* on colonies formation of *F. oxysporum*

Fungus	Number of formed-colonies /cm^2			
	Control	$AgNO_3$	Ag-NPs	P value <0.05
Fusarium oxysporum	13.92	5.92	4.42	0.047
%		57.5	68.2	

Effects of silver nanoparticles on *Fusarium oxysporum* infection, germination and viguor index: The disease incidence of *F. oxysporum* was shown on tomato, faba bean and barley seeds at percentage of 100, 80 and 50 % respectively, (Table 2, 3 and 4).

Different concentrations of silver nanoparticles (0.5, 0.25 and 0.12 mM) alone or incombination with spore suspension of *F. oxysporum*, 1mM silver nitrate, compared with spore suspension of *F. oxysporum* or sterilize distilled water were used to determine seed germinations(%), disease incidence (%), root length (cm),shoot length(cm) and vigour index on three different crops viz: fababean, tomato and barley. Data illustrated in Table (2) and Fig (2) indicate that the seed germination of tomato plant was not affected by silver nanoparticles concentration but vigour index was affected at the highest concentration of AgNPs. Whereas, negative control (water) showed highest vigour index value 870 but the positive control treatment with $AgNO_3$ reduced seed germination to 70% compared with 100 % of the negative control (water) while the low value of vigour index was 30.1. Treatment with *Fusarium* infection reduced the germination to 10 % and vigour index to 18.2. Correspondingly, the disease incidence (rot seed %) of infected tomato with *Fusarium* was 100%. The formulated treatment 'of AgNPs at different concentrations and *Fusarium* suspension showed a significant inhibition in diseases incidence (rot seed) to present 5% infection only. Germination percentage of tomato seeds was not affected by the treatments, but vigour index was affected by the mixture of AgNPs at 0.5 mM with *Fusarium* spores. Tomato roots were sensitive to all silver nanoparticle treatments. The 0.5 mM AgNPs and 1mM $AgNO_3$ had noticeable effected on the shoot length. $AgNO_3$ had stronger inhibitory effect than AgNPs on tomato shoot length. Negative effects in root and shoot length was observed as brown color on some parts of roots. Tomato seeds were affected by a number of diseases, among them; wilt disease caused by *F. oxysporum f. sp. Lycopersici* (Suarez-Estrella *et al.,* 2007 and Kim and Kim 2008). A new approach in crop protection is to reduce the disease damage level using silver nanoparticles. Data presented here suggest silver nanoparticles as a possible approach for controlling seed-borne pathogens.

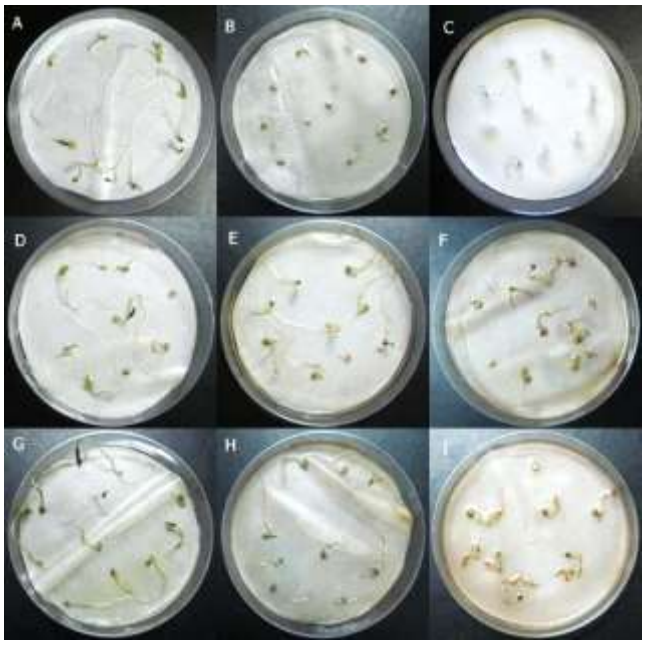

Figure-2: Effect of AgNPs on tomato seed germination after 10 days. A; Control (water), B; 1mM $AgNO_3$, C; *Fusarium oxysporum* spore suspension (SS), AgNPs concentrations, D; 0.12mM, E; 0.25 mM, F; 0.5mM, G; SS + 0.12mM AgNPs, (H) SS + 0.25mM AgNPs, and (I) SS + 0.5mM AgNPs.

Table 2:The effect of AgNPs on germination of tomato seeds, vigour index and *Fusarium oxysporum* infection

Treatments	Germination %	Disease Incidence %	Root length (cm)	P value = ≤ 0.05	Shoot length (cm)	P value = ≤ 0.05	Vigour index (VI)
Control (water)	100	-	6.8	0.000	1.9	0.000	870
$AgNO_3$ 1mM	70	-	0.43	0.000**	0	0.000**	30.1
AgNPs 0.12mM	90	-	4.6	0.078	0.9	0.210	495
AgNPs 0.25mM	100	-	3.25	0.000**	1.27	0.787	452
AgNPs 0.5mM	90	-	0.83	0.000**	0.47	0.046*	117
AgNPs 0.12mM+ SS	100	5	2.30	0.000**	2.33	0.971	463
AgNPs 0.25mM+ SS	100	5	1.75	0.000**	0.67	0.051*	242
AgNPs 0.5mM+ SS	90	5	1.75	0.000**	0.1	0.000**	166.5
F. oxysporum	10	100	1.6	0.000**	0.22	0.000**	18.2
SS = *F. oxysporum* spores suspension; * mean difference is significant at the 0.05 level							

In case of faba bean, concentrations of AgNPs at 0.12mM and 0.25mM alone or incombination with the *Fusarium* spore suspension did not show any significant differences in seed germination percentage, diseases incidence (rot seeds), and vigour index compared the control or 1mM $AgNO_3$. AgNPS at concentration of 0.25 mM significantly reduced the shoot length Fig. (3) and (Table 3). The concentration of 0.5mM of AgNPs alone or incombination with *Fusarium* spore suspension significantly reduced germination percentage by 60 and70%, and vigour index value by 72 and 97.3, respectively compared with the control (water), 1mM AgNO3 or *Fusarium* infection. Whereas, the P value less than 0.05 was considered significant. Furthermore, it was observed that the roots became colored with brown dark regions as result to AgNPs treatments. Treatment with seed borne pathogen *Fusarium* caused a reduction in faba bean seed germination as it reached 20%. The disease incidence of *Fusarium* infection recorded as rotted-seeds was 50%. But, incidence of *Fusarium* infection was reduced when the faba bean seeds treated with any of AgNPs concentration to record 5%. Faba bean seeds treated with low concentration of AgNPs showed a great impact on germination and reduced the disease incidence.

Figure 3: Effect of AgNPs on faba bean seed germination after 10 days. A; Control (water), B; 1mM $AgNO_3$, C; *Fusarium oxysporum* spore suspension (SS), AgNPs concentrations, D; 0.12mM, E; 0.25mM, F; 0.5mM, G; SS + 0.12mM AgNPs, (H) SS + 0.25mM AgNPs, and (I) SS + 0.5mM AgNPs

Table 3: The effect of AgNPs on germination of faba bean seeds, vigour index and *Fusarium oxysporum* infection.

Treatments	Germination %	Disease Incidence %	Root length (cm)	P value =≤ 0.05	Shoot length (cm)	P value = ≤ 0.05	Vigour index
Control (water)	100	0	2.67	0.000	1.52	0.000	419
$AgNO_3$ 1mM	90	0	2.6	1.000	0.82	0.258	307.8
AgNPs 0.12mM	100	0	2.33	0.999	1.45	1.000	378
AgNPs0.25mM	100	0	2.2	0.992	0.35	0.001**	255
AgNPs 0.5mM	60	0	0.9	0.026*	0.3	0.000**	72
AgNPs0.12mM+ SS	100	5	2.95	1.000	1	0.723	395
AgNPs0.25mM+ SS	90	5	2.03	0.966	0.67	0.063	243
AgNPs 0.5mM+ SS	70	5	1.04	0.055*	0.35	0.001**	97.3
F. oxysporum	20	50	0.15	0.000**	0.2	0.000**	7
SS = *F. oxysporum* spores suspension; * mean difference is significant at the 0.05 level							

In case of Barley seeds, Fig (4) and Table (4) illustrate that 0.5mM AgNPs alone or incombination with *Fusarium* spore suspension caused significant inhibition of seed germination by 70 and 60%, respectively. While, vigour index value was also decreased to 167.3 and 177, respectively compared to the control (water) which record 1450. Treatment with seed borne pathogen *Fusarium* caused a reduction in barley seed germination as it reached 60%. The incidence of *Fusarium* infection recorded as rotted-seeds was 80%. But, the incidence of *Fusarium* infection reduced when the barley seeds treated any of the different AgNPs concentrations to record 5% only.

Germination percentage and vigour index were measured to determine the effects of AgNPs, $AgNO_3$, in the presence of *Fusarium* infection on faba bean, tomato, and barley. Consistent with our expectations, the AgNPs were toxic to seedling, and both vegetative growth and root elongation were inhibited at higher concentrations. This phenomenon was also reported in aquatic (*Lemna minor*) and terrestrial (*Lolium multiflorum*) plants, algae, fungi, vertebrates (zebra fish), invertebrates (*Caenorhabditis elegans*), reviewed by Levard *et al*., 2012.

Figure 4: Effect of AgNPs on barley seed germination after 10 days. A; Control (water), B; 1mM $AgNO_3$, C; *Fusarium oxysporum* spore suspension (SS).AgNPs concentrations, D; 0.12mM, E; 0.25mM , F; 0.5mM, G; SS + 0.12mM AgNPs, (H) SS + 0.25mM AgNPs, and (I) SS + 0.5mM AgNPs

Table 4: Effect of AgNPs on the germination of barley seeds, vigour index and *Fusarium oxysporum* infection.

Treatments	Germination %	Disease incidence %	Root length (cm)	P value = ≤ 0.05	Shoot length (cm)	P value = ≤ 0.05	Vigour index
Control (water)	100	-	5.8	0.000	8.7	0.000	1450
$AgNO_3$ 1mM	70	-	3.25	0.360	0.33	0.000**	250.6
AgNPs 0.12mM	100	-	6.77	0.994	7.53	0.968	1430
AgNPs 0.25mM	100	-	2.8	0.166	5.72	0.112	852
AgNPs 0.5mM	70	-	0.23	0.000**	2.16	0.000**	167.3
AgNPs0.12mM+ SS	100	5	6.79	0.366	7.17	1.000	1396
AgNPs 0.25mM+ SS	90	5	3.37	0.427	4.91	0.013	745.2
AgNPs 0.5mM+ SS	60	5	1.18	0.003**	1.77	0.000**	177
F. oxysporum	60	80	1.65	0.010*	2.77	0.000**	265.2
SS = *F. oxysporum* spores suspension. * mean difference is significant at the 0.05 level							

The current study showed that the inhibitory effect of $AgNO_3$ on seedling growth is higher than that of AgNPs. This results is different from the finding of Yin *et al*., (2011) who illustrated that AgNPs has stronger inhibitory effect on *Lolium multiflorum* growth than $AgNO_3$. Results also showed that tomato, faba beans and barley plants were differ in their tolerant to AgNPs and $AgNO_3$. AgNPs' toxicity on seedling growth under normal growth conditions was partially consistent in the pure culture experiments. Therefore, the increase in the release of AgNPs into the environment may show adverse effects on wetland plants.

AgNPs reduced the incidence of *F. oxysporum* infection as a seed borne pathogen in all used concentrations and on seeds of plant species viz: tomato, faba bean and barley with no significant differences in disease incidence as it recorded 5 % only. Silver ions produce active oxygen species (ROS) via their reaction with oxygen, causing damage to cells proteins, lipids, and nucleic acids (Storz and Imlay, 1999; Wang *et al.,* 2008). *F. oxysporum* can live in the soil for long period of time; therefore the rotational cropping is not a useful control method. The fungus also spreads through infected dead plant materials and that makes cleaning up at the end of the season is important practice. However, the control methods of *F. oxysporum* as a damping-off pathogen could be carried out by planting resistant varieties, and using chemical reagent as antifungal (Khalifa *et al.,* 1995; Lewis *et al.,* 1996 and Sutton, 1996). However, the study suggests the possible use of silver nanoparticles as a new approach for the eradication of phytopathogens even though there are some restrictions of using the nanoparticles in horticulture. These may involve the evaluation of phytotoxicity and antimicrobial effects in hosts, and development of delivery systems of silver nanoparticles into host tissues colonized by phytopathogens.

CONCLUSION

A high concentration of AgNPs showed a negative effect on faba bean and barley seedlings, whereas the germination percentage, vigour index were significantly decreased. The low concentration of AgNPs improved the germination percentage; vigour index in addition to reducing the disease incidence (rotted seeds) resulted from the inoculation with *Fusarium spores*. On the other hand, a high concentration of AgNPs did not show a significant effect on tomato seedling whereas the germination percentage, vigour index were slightly decreased. Silver nanoparticles, at a concentration 0.12 mM were less toxic to the seedlings of tomato, faba beans and barley; also it reduced the growth of *Fusarium* infection. These preliminary studies propose the use silver particles for controling seed-borne pathogens. Further studies are recommended to study the divers effect on nanoparticles used in the formulated pesticides on the environment as well as their passing through the food chain and subsequently their possible accumulation in the human bodies.

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تاثير جزيئات الفضه المتناهية الصغر (النانونيه) والمخلقه حيويا علي مرض عفن البذور المتسبب عن فطر فيوزاريوم اوكسيسبوريوم فى الفول البلدى، الطماطم والشعير

رباب ممدوح أحمد العماوي[1] و رائدة بنت إبراهيم بن عبدالله الحربي [2]

[1] قسم بحوث أمراض الأرز - معهد بحوث أمراض النباتات - مركز البحوث الزراعية – مصر

[2] قسم الأحياء الدقيقة- كلية العلوم- جامعة الملك سعود

أجرى تقييم للتأثير العكسى لسمية جزيئات الفضة النانونية بإستخدام إختبار إنبات البذور وقوة البادرة على بذور نباتات الطماطم و الفول و الشعير بالاضافه الي مدى تأثيرها فى تقليل حدوث اعفان للبذور نتيجه للعدوي الفطريه بالفيوزاريوم. وجزيئات الفضه النانونيه التى تم تخليقها بواسطه الفطر تريكوديرما لونجيبراكتيوم طبقا ل (رائده , 2013), جزيئات الفضه النانونيه وصفت باستخدام جهاز التحليل المطيافية فوق البنفسجية والمرئية والميكروسكوب الالكتروني النافذ و جهاز التحليل الحجمي للجزئيات وقياس الجهد الكهربائي زيتا. تراوح حجمها بين1- 25 نانوميتر وذات شكل كروي وغالبا مفرد. وقد اجريت دراسات اوليه من اجل معرفه الاثار السامه المحتمله للتركيزات المختلفه من جزيئات الفضه النانونيه علي نسبه الانبات و مؤشر قوه الانبات و العدوي الفطريه بالفيوزاريوم. بالاضافه الي استخدام نترات الفضه التي استخدمت كماده خام لانتاج جزيئات الفضه النانونيه. أما البذور السليمه و المعداه فقد نقعت في محلول من تركيزات الفضه النانونيه (0,5 , 0,25 و 0.12 مليمولر). محلول جزيئات الفضه النانونيه مفردا او خلطا مع جراثيم الفيوزاريوم لتوضح تاثيراتها العكسيه علي الانبات و مؤشر قوه الانبات فى التركيز العالي (0,5 مليمولر). في حين ان التركيز المنخفض (0,12) مليمولر لم يؤثر علي الانبات و مؤشر قوه الانبات بالاضافه الي تقليل حدوث المرض (البذور المتحللة) كنتيجه للعدوي بالفيوزاريوم وذلك في بذور الفول و الشعير. بينما كان محلول جزيئات الفضه النانونيه ذو تاثير طفيف علي نسبه الانبات و مؤشر قوه الانبات لبذور الطماطم, بينما عند تركيز 0,12 مليمولر كان اقل سميه علي بذور الطماطم و الفول و الشعير بالاضافه الي تقليل للبذور المتحللة. ويخلص البحث إلى إمكانية إستخدام الجزيئات المتناهية الصغر (النانونية) للفضة فى مكافحة الأمراض النباتية مع الوضع فى الإعتبار دراسة تأثيره على البيئة ودرجة إمتصاصه فى النبات وإنتقاله إلى سلسلة الغذاء ثم تراكمه فى النهاية فى جسم الإنسان وما قد ينجم عن ذلك من آثار سلبية.

قام بتحكيم البحث

أ.د / محمد عبد الرحمن الوكيل — كلية الزراعة – جامعة المنصورة

أ.د / محمد رشدى سحلى — مركز البحوث الزراعية