Evaluation of Antifungal Effect of Silver Nanoparticles against the Phytopathogenic Fungi *Rhizoctonia solani*

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Abstract: Silver nanoparticles (AgNPs) are known to have antimicrobial effects, why it is being used in agriculture, water treatment and health sector. In this context, this study aimed to evaluate the *in vitro* antifungal activity of AgNPs against the phytopathogenic fungi *Rhizoctonia solani* isolated from infected tomato plants. The antifungal effect of AgNPs was compared to two conventional pesticides namely, thyophenate-methyl (Topsin-M) and ethylene-bis-dithiocarbamate (Mancozeb).Furthermore, the synergistic effect of AgNPs was evaluated when mixed with Topsin-M and Mancozeb. Comparable results for inhibition of mycelial growth were reported for AgNPs and Topsin-M with percentages of 92.2% and 94.1%, respectively. AgNPs were more efficient in inhibiting mycelial growth of *R. solani* compared to Mancozeb. Synergistic effects were observed when AgNPs were combined with the conventional fungicides. This study highlighted the promising efficiency of AgNPs in controlling phytopathogenic fungus alone or in combination with other conventional pesticides.

Keywords: Silver nanoparticles, Topsin-M, Mancozeb, antifungal effect, synergistic effect

INTRODUCTION

Control of plant diseases is one of the most challenging aspects of crop production. Fungal diseases cause serious damage to various types of plants which decrease their yield and cause economic loss (Peng *et al*., 2021). *Rhizoctonia solani* is an important soil-borne plant pathogenic fungus with a global distribution and a wide host range (Ajayi-Oyetunde and Bradley, 2018). The pathogen is best known to cause “damping-off” and can survive in the soil without a host for many years(Georgiou *et al*., 2000).The broad host-range and the ability to form sclerotia make this pathogen very difficult to control(Georgiou *et al*., 2000).

Various pesticides have been used to control *Rhizoctonia solani*, however, resistance to pesticides available in the market has increased and became a serious problem (Chattopadhyay *et al*., 2017). Therefore, it is necessary to look for alternative disease control measures that are efficient and safe for the environment.

The use of nanomaterials is expanding and is considered an alternative solution to control plant pathogens. Some metallic nanoparticles such as zinc, copper and Silver nanoparticles (AgNPs) have been studied and tested for their antifungal properties for pathogens such as *Klebsiella pneumonia, Staphylococcus aureus* and *Candida albicans* and *Penicillium notatum* (Janaki *et al*., 2015; Medda *et al*., 2015; Shaikh *et al*., 2019; Kamel *et al*., 2022). AgNPs have received special attention as a possible antimicrobial agent in several studies (Sondi and Salopek-Sondi, 2004; Baker *et al*., 2005; Melaiye *et al*., 2005). In addition, the efficiency of AgNPs against phytopathogenic microorganisms have been demonstrated by several researchers (Kim *et al*., 2009; Min *et al*., 2009; Lamsal *et al*., 2011; Peng *et al*., 2021; Jian *et al*., 2022).

In this context, the aim of this study was to evaluate the antifungal activity of AgNPs on the phytopathogenic fungus *R. solani*. Furthermore, the antifungal activity of AgNPs was compared to that of two conventional fungicides, thyophenate-methyl (Topsin-M) and ethylene-bis-dithiocarbamate (Mancozeb). Finally, the synergistic inhibition effect was tested for AgNPs and the two conventional fungicides at different concentrations.

MATERIALS AND METHODS

Synthesis of AgNPs

The chemical reduction method was used to prepare AgNPs, using ice-cold sodium borohydride ($NaBH_4$) to reduce silver nitrate ($AgNO_3$). $AgNO_3$ and $NaBH_4$ were obtained from Sigma Ltd. $AgNO_3$ solution (0.001 M) was utilized as a metal salt precursor, whereas $NaBH_4$ (0.002 M) was used as a reducing and stabilizing agent.

$AgNO_3$was dripped at 1 drop per second into the stirring solution of $NaBH_4$, the stirring was stopped when all the $AgNO_3$was added. The color of the mixture changed to yellow which designate the formation of yellow AgNPs. The formed AgNPs were purified by centrifugation (Hettich Instruments LP, Beverly, MA 01915, USA) at 10000 rpm for10 min and any insoluble material was discarded. To remove excess ionic silver (Ag^+), the silver colloids were rinsed four times with deionized water. A powder of AgNPs was obtained after drying the sample in an oven at 60°C for 24 h.

Characterization of AgNPs

Ultraviolet- visible spectroscopy (UV-Vis)

UV-Vis spectroscopy was used to determine the plasmonic surface resonance of AgNPs which is indicated by absorbance at wavelength range of 200–800 nm. About 300 μL of colloidal AgNPs was added to a quartz cuvette and the absorbance was measured using a JASCO V-570 UV/VIS/NIR double beam spectrophotometer.

Transmission Electron Microscopy (TEM)

The surface morphology and particles size of AgNPs were characterized by a Transmission Electron Microscope (TEM) Model, (JEOL-JEM-1230). The TEM operated at 80-100 kV.

Zeta potential

Surface charges of nanoparticles were detected by using a HORIBA, Zeta sizer nano series, dynamic light scattering (DLS) apparatus. Aliquot of nanoparticles was diluted with deionized water, and then sonicated for 10 min before the measurements.

Isolation and identification of fungus

Fungi were isolated from the roots of infected tomato plant. The isolated fungi were identified morphologically based on colony shape, color, mycelial character and spore shape using an optical microscope.

Determination of the Antifungal Activity of AgNPs and traditional fungicide

The antifungal effects of AgNPs and two conventional fungicides namely, thyophenate-methyl 70% wettable powder (Topsin-M) and ethylene-bis-dithiocarbamate wettawble powder (Mancozeb) was evaluated against the phytopathogenic fungus *Rhizoctonia* sp. Different concentrations (5, 10, 25, 50 and 100 ppm) of the AgNPs and the conventional fungicides were prepared in sterile potato dextrose agar media at 45°C.After that, the media were poured in Petri dishes. Subsequently, the pathogenic fungus were inoculated from a culture of 7 days old and placed in the center of the Petri dish. They were incubated at 25°C for 7 days. Potato dextrose agar media without the addition of the AgNPs was used as a control treatment. Each treatment was performed in triplicate. Diameter of mycelial growth *in vitro* was measured at 3, 5 and 7days after the start of the experiment. Radial growth was converted into percent inhibition by using following formula given by (Da Silva Bomfim *et al*., 2015).

$$\text{Percent growth inhibition (I)} = \frac{C - T}{C} \times 100$$

Where,

C = Radial growth in control plate (mm)

T = Radial growth in the treated plate (mm)

Toxicological studies on the joint action.

The joint action between AgNPs and the two fungicides was studied at two concentrations equivalent to EC_{10} and EC_{25}.

The joint action was calculated using Limpel's formula(Richer, 1987).

$$E = X + Y - XY/100$$

Where: E = expected additive effect of the components A and B;

X = effect due to component A.

Y = effect due to component B.

The co-toxicity factor was calculated according to (Mansour *et al*., 1966):

$$\text{Co-toxicity factor} = \frac{\text{Observed effect (\%) - Expected effect (\%)}}{\text{Expected effect (\%)}} \times 100$$

A co-toxicity factor value of 20 or more is considered potentiation or synergism, values of -20 or less are considered antagonism and values more than (-20 and less than +20 indicate additive effect.

Statistical analysis

Percentages inhibition of mycelial growth were plotted against concentrations as log/probit regression lines, the values of EC_{50} , EC_{25} and EC_{10} values as well as the slope of the toxicity lines were calculated using Ld-p Line® software. Duncan's multiple range tests ($p \leq 0.05$) was used to compare the means of different treatments using COSTAT program.

RESULTS AND DISCUSSION

Characterization of the synthesized AgNPs

UV-Vis spectroscopy

Figure (1) shows the absorbance spectrum of AgNPs stabilized with borohydride (BH_4). The spectrum shows a sharp absorption peak at the wavelength of 425 nm, which is distinctive for AgNPs (Solomon *et al*., 2007). This peak was caused by collective excitation of all the free electrons in the particles (Yusuf, 2019). This is consistent with the presence of only nanoscale particles which are smaller than the wavelength of the incident light (Solomon *et al*., 2007; Yusuf, 2019). The observed peak showed widening which suggested the formation of AgNPs with different sizes. This was also observed for AgNPs synthesized by Chemical reduction method (Quintero-quiroz *et al.* 2019).

TEM analysis

To obtain more information about the shape and size of the synthesized AgNPs TEM images were captured. TEM micrographs showed that the AgNPs were spherical with regular edges (Fig. 2). Other studies obtained the same spherical shape of nanoparticles synthesized by the means of chemical reduction methods (Besenhard *et al*., 2018; Quintero-Quiroz *et al*., 2019). The size distribution ranged from 6 to 18 nm with an average of 8.5±2 nm (Fig. 3). This variability in size distribution was also seen in UV-Vis spectrum.

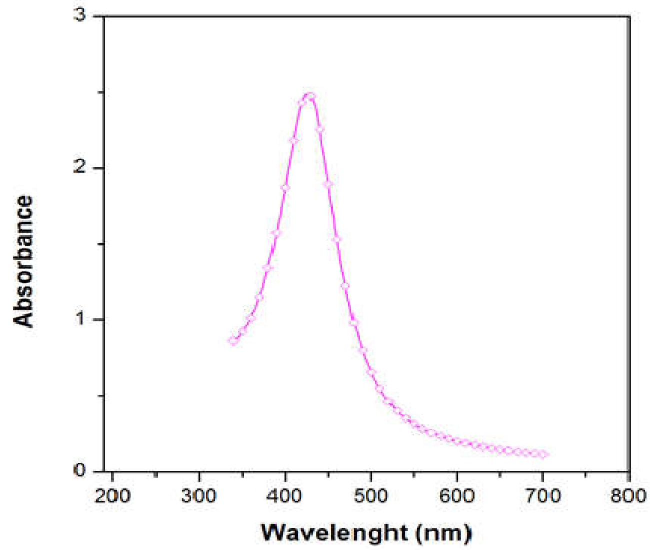

Fig. (1): UV-Vis Absorption spectrum of AgNPs solution

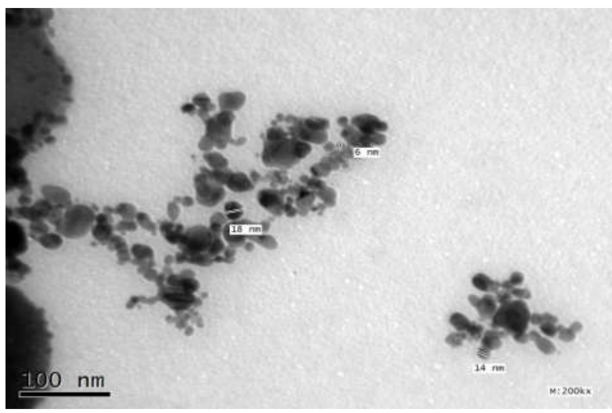

Fig. (2): TEM micrograph showing AgNPs at 200 Kx magnification.

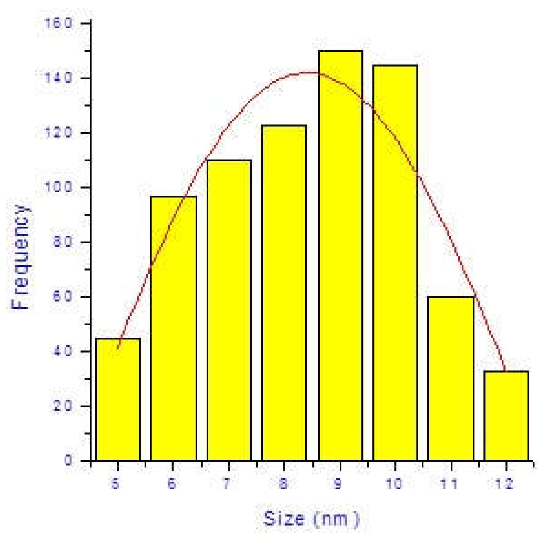

Fig. (3): Size distribution of AgNPs

Zeta (ζ)-potential

Zeta (ζ)-potential was used to describe the surface charge, colloidal stability and dispersion of the synthesized AgNPs when suspended in a solution (Saxena and Shaikh, 2021). Fig. (4) Shows the value of zeta potential of AgNPs which was equal to -11.1 mV at pH=7.The value of zeta potential reported in the present study is moderate which indicated a relatively stable colloidal suspension of the synthesized AgNPs (Feng *et al*., 2022). This negative value may be ascribed to the presence of borohydride on the surface of AgNPs.

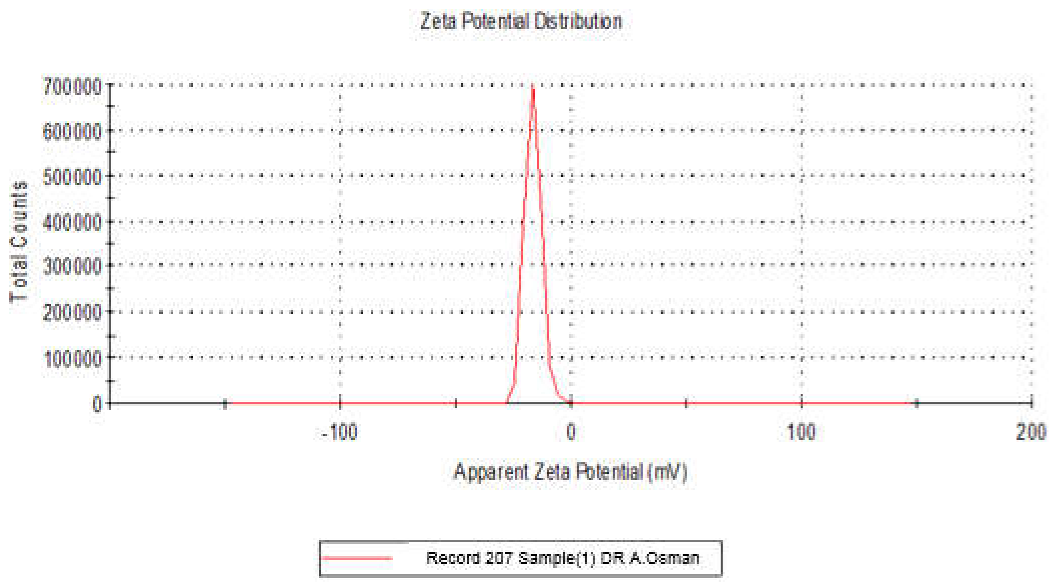

Fig. (4): Zeta potential

Fungicidal effect of AgNPs and the conventional fungicides Topsin-M and Mancozebon the radial growth of *R. solani.*

Table (1) shows the percentage inhibition of radial growth of *R. solani* as a function of concentration of AgNPs, Topsin-M and Mancozeb. All treatments significantly inhibited the radial growth of *R. solani* compared to the control (Table 1). Percentage inhibition of the radial growth of *R. solani* increased as a function of concentration and exposure time with both tested pesticides. The highest levels of inhibition were observed at concentration of 100 ppm after 7 days for Topsin-M followed by AgNPs with percentages of 94.1% and 92.2%, respectively. However, these percentages inhibition of Topsin-M and AgNPs was not statistically significantly different. The lowest percentage of inhibition of 22.1% was observed for Mancozeb at a concentration of 5 ppm after 3 days. The efficiencies of AgNPs to inhibit mycelial growth at all the studied concentrations and time intervals were close to those observed for Topsin-M. The high efficiency of AgNPs is probably due to the high intensity at which the solution is capable to saturate and adhere to the fungal hyphae and to control the plant diseases. Results from the present study agree with those obtained from (Kim *et al*., 2009; Min *et al*., 2009; Lamsal *et al*., 2011; Akpinar *et al*., 2021; Jian *et al*., 2022)who found that AgNPs had significant inhibitory effects on plant pathogenic fungi such as *Bipolaris sorokiniana, Colletotrichum* sp., *Fusarium graminearum* and *Fusarium oxysporum*.

Several hypothesis exist about the mode of action of AgNPs, in some studies it was reported that Ag^+ stopped DNA duplication, leading to a deactivated expression of ribosomal subunit proteins and to the synthesis of disabled enzymes and cellular proteins, which intern affect adenosine triphosphate production (Chaud *et al*., 2021; Jian *et al*., 2022). Min *et al*. (2009) observed inhibition of the hyphal growth of *R. solani* causing deformities of the hyphal walls which were then prone to collapse at very low concentration (7 mg L^{-1}) of AgNPs. Elgorban *et al*., (2015) reported that AgNPs antifungal agents caused destruction of the cell membrane. Furthermore, Oktarina *et al*., (2021)reported morphological changes of *R. solani* colonies when treated with AgNPs, which decreased radial growth significantly.

Table (1): Inhibition percentage of *R. solani* mycelial growth under different concentrations of AgNPs and the two conventional fungicides after 3, 5 and 7 days

Treatment	Inhibition rate (%)														
	3 days					5 days					7 days				
	Concentration ppm														
	5	10	25	50	100	5	10	25	50	100	5	10	25	50	100
AgNps	43.1^{kl}	49.5^{ij}	51.3^{hij}	70.1^{d}	82.2^{bc}	48.9^{ij}	55.7^{gh}	59.9^{fg}	76.8^{c}	89.9^{ab}	54.9^{gh}	58.7^{fg}	69.2^{d}	81.5^{bc}	92.2^{a}
Topsin-M® M-70%WP	36.9^{lm}	46.5^{jk}	54^{gh}	62.1^{ef}	79.6^{bc}	46.6^{jk}	49.2^{ij}	56.1^{fgh}	69.3^{d}	82.9^{bc}	49.1^{ij}	56.9^{fgh}	65.5^{de}	77.8^{bc}	94.1^{a}
Mancozeb® 80% WP	22.1^{q}	23.1^{q}	30.8^{op}	35.5^{mn}	55.9^{gh}	23.8^{pq}	25.4^{pq}	33.1^{mno}	46.7^{jk}	58.6^{fg}	26.5^{pq}	31.1^{op}	38.7^{lm}	43.4^{kl}	67.8^{d}
Control	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}	0^{r}

Means followed by the same letter(s) are not significantly different according to Duncan's multiple range tests ($p \leq 0.05$)
Means followed by the different letter(s) are significantly different according to Duncan's multiple range tests ($p \leq 0.05$)

Comparative toxicity of AgNPs,Topsin-Mand Mancozeb against *R. solani*

Results presented in (Table 2) shows that all the treatments inhibited the radial growth of *Rhizoctonia* spp. with the increase in exposure time. The calculated EC_{50} (4.92 ppm) for AgNPs after 7 days of exposure was 1.35and 9 times lower than that for Topsin-Mand Mancozeb, respectively. These results confirmed that AgNPs was more effective in inhibiting the radial growth of *Rhizoctonia solani* than the two conventional pesticides used in this study. In accordance with the results reported in the current study, Li *et al*. (2022) reported that the EC_{50} for inhibiting mycelial growth of *Alternaria alternata, Pestalotiopsis microspora, Diaporthe actinidiae*, and *Botryosphaeria dothidea* ranged between 2.31 to 14.74 ppm. Results from this study suggested that AgNPs could be used as an excellent antifungal pesticide.

Table (2): Comparative toxicity of AgNPs, Topsin-Mand Mancozeb against *R. solani* after 3, 5 and 7day of treatment

Compound	Exposure time(Days)	Line equation Regression of probit (y) on log concentration (x)	Slope (b)	Concentration ppm			
				LC_{50}	LC_{50} limit	LC_{25}	LC_{25} limit
AgNPs	3	y = 4.148 + 0.816 x	0.791 +/-0.12	11.02	6.77-15.57	1.55	0.44-3.09
Topsin-M® M-70%WP	3	y = 4.048 + 0.818 x	0.733 +/-0.12	14.64	9.73-20.26	2.12	0.82-4.68
Mancozeb	3	y = 3.663 + 0.653 x	0.675 +/-0.12	105.05	62.59-239.06	10.52	5.13-16.18
AgNPs	5	y = 4.183 + 0.952 x	0.954 +/-0.13	7.06	4.11-10.12	1.25	0.39-2.44
Topsin-M® M-70%WP	5	y = 4.24 + 0.778 x	0.749 +/-0.12	9.96	6.25-13.67	2.18	1.26-3.57
Mancozeb	5	y = 3.665 + 0.733 x	0.743 +/-0.12	64.83	43.48-88.01	8.05	3.92-12.32
AgNPs	7	y = 4.301 + 0.978 x	0.952 +/-0.13	4.92	2.58-7.37	0.91	0.25-1.89
Topsin-M® M-70%WP	7	y = 4.044 + 1.127 x	1.019 +/-.013	6.68	4.14-9.27	1.45	0.56-2.60
Mancozeb	7	y = 3.759 + 0.748x	0.750 +/-0.12	46.11	32.18-77.12	5.81	2.56-9.32

Synergistic antifungal activity of AgNPs and the two conventional fungicides, Topsin-M and Mancozeb on *R. solani* growth inhibition:

In real life, more than one type of pesticide could be used against a specific microorganism in an attempt to overcome pesticides resistance, increase efficiency and use fewer pesticides. Table (3) shows that all treatments have synergistic effects except the combined treatment between Topsin-M and Mancozeb at the concentrations of EC_{10}+EC_{25} and EC_{25}+EC_{25} which had an additive effect with co-toxicity factor of 8.6 and 17.9, respectively. No antagonistic effects were observed in the tested mixed proportions of all the three pesticides.

The highest co-toxicity factors of 49.4 and 48.8 were observed when AgNPs and Topsin-M were mixed at their EC_{25} and when AgNPs at EC_{25} were mixed with Topsin-M at $EC_{10,}$ respectively. In an earlier study, AgNPs combined with the pesticides tebuconazole orpropineb showed a synergistic effect against *Bipolaria maydis* (Huang *et al.,* 2018). Furthermore, Huang *et al*., (2020)reported synergistic effect of AgNPs and epoxiconazole against *Setosphaeria turcica*.

Table (3): Joint action effect between AgNPs and two commercial fungicides (Topsin-Mand Mancozeb) on *R. solani* radial growth inhibition

Treatment	Concentration	Observed	Expected	Co-T	J.AT
AgNPs +Top	EC_{10}+EC_{25}	42.2	32.5	29.8	**synergism**
	EC_{10}+EC_{10}	28.2	19	48.4	**synergism**
	EC_{25}+EC_{25}	65.4	43.75	49.4	**synergism**
	EC_{25}+EC_{10}	48.2	32.5	48.3	**synergism**
AgNPs +Man	EC_{10}+EC_{25}	43.5	32.5	33.8	**synergism**
	EC_{10}+EC_{10}	24.5	19	28.9	**synergism**
	EC_{25}+EC_{25}	57.8	43.75	32.1	**synergism**
	EC_{25}+EC_{10}	39.4	32.5	21.2	**synergism**
Top +Man	EC_{10}+EC_{25}	35.3	32.5	8.6	**Additive**
	EC_{10}+EC_{10}	24.7	19	30	**synergism**
	EC_{25}+EC_{25}	51.6	43.75	17.9	**Additive**
	EC_{25}+EC_{10}	40.1	32.5	23.3	**synergism**

Co-T: Toxicity coefficient factor
J.AT: Joint action type

CONCLUSION

In this study, AgNPs were synthesized using $NaBH_4$ as a reducing and stabilizing agent. The synthesized AgNPs had a spherical shape with regular edges and their average size were 8.5 ± 2 nm. AgNPs significantly inhibited the mycelial growth of *R. solani* at all the studied concentrations and time intervals with percentages ranging from 43.1-92.1 %. This suggests that it could be used as an alternative for traditional fungicides. Synergistic antifungal effects were observed for AgNPs when mixed with the two conventional fungicides at EC_{10} and EC_{25} in different combinations. These results provide a prelude to use less amounts of pesticides and reduce resistance to fungicides.

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تقدير الفاعلية الإبادية لجزيئات الفضة النانوية ضد فطر ريزوكتونيا سولانى الممرض للنبات

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من المعروف أن جزيئات الفضة النانوية (AgNPs) لها تأثيرات مضادة للميكروبات وهو ما جعلها تستخدم في العديد من المجالات مثل الزراعة والصحة ومعالجة المياه وفى هذا السياق تهدف هذه الدراسة إلى تقييم النشاط المضاد للفطريات في المختبر لـ AgNPs ضد أحد الفطريات الممرضة للنبات *Rhizoctonia solani* والمعزوله من نباتات الطماطم المصابة. تمت مقارنة التأثير المضاد للفطريات لـ AgNPs مع اثنين من مبيدات الآفات التقليدية وهما ثيوفينات - ميثيل (توبسين-ام) وإيثيلين - بيس - ديثيوكاربامات (مانكوزيب). علاوة على ذلك، تم تقييم التأثير التآزري لـ AgNPs عند مزجه مع توبسين - امومانكوزيب. أظهرت النتائج أن التأثير التثبيطى لنمو الفطر لجزيئات الفضه النانوية AgNPs وتوبسين كانت بنسب ٩٢.٢٪ و ٩٤.١٪ على التوالي. وقد كانت جزيئات الفضة النانوية AgNPs أكثر كفاءة في تثبيط النمو الفطري لـ *R. solani* مقارنة بمانكوزيب. كما أظهرت جزيئات الفضة النانوية AgNPs تأثيرات تآزرية عندما تم دمجها مع مبيدات الفطريات التقليدية. هذه الدراسة سلطت الضوء على الكفاءة الواعدة لـ AgNPs في مكافحة الفطريات الممرضة للنبات بمفردها أو بالاشتراك مع مبيدات الآفات التقليدية الأخرى.