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Chitosan and Silver Nanoparticles as Control Agents of Some Faba Bean Spot Diseases

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Abstract

Faba bean (*Vicia fabae* L.) is one of the most economic legume crops in Egypt. The antifungal effects of different concentrations of Chitosan and silver nanoparticles (20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm) were studied against aggressive isolates of *Botrytis fabae* and *Alternaria alternata* causing *chocolate* spot disease and *Alternaria* leaf spot disease respectively. Under laboratory conditions, the application of five concentrations of Chitosan nanoparticles and silver nanoparticles to the cultures of *B. fabae* and *A. alternata* showed significant inhibition of mycelial growth. Number of spores/ml decreased with increasing of nanoparticle concentration. Mycelial weight of fungal pathogens also decreased. The lesion growth was suppressed by increasing in the concentration of nanoparticles with significantly different relative to control using detached leaf test. Under greenhouse conditions, chitosan and silver nanoparticles were sprayed in concentrations 60 ppm, 80 ppm, and 100 ppm on faba bean plants. The tested nanoparticles showed significant effect against *B. fabae* and *A. alternata* relative to control. The obtained results indicated that the highest reduction of chocolate spot severity was obtained with treatment by 100 ppm of silver nanoparticles (52.94%) followed by chitosan nanoparticles 80 ppm (50.59%). While the application of 100 ppm of chitosan nanoparticles was highly efficient against Alternaria leaf spot where the reduction in disease severity (67.13%) followed by 100 ppm silver nanoparticles which caused reduction rate (61.5%). The obtained results indicated the possibility of using chitosan and silver nanoparticles as a substance in the manufacturing of fungicides to minimize the impact of chocolate spot and Alternaria leaf spot diseases in faba bean. However, further experimental trials under field conditions and safety evaluation studies are needed before the nanoparticles types and concentrations can be used as potential antifungal agents.

Keywords: Antifungal effects; Nanosilver; Chitosan nanoparticles; Disease severity; Control agent

Introduction

Faba bean is one of the most important economic winter crops in Egypt. Global production of Faba bean reached 4,139,972 ton in 2014. Faba bean (*Vicia faba* L.) is an important staple food crop for population in Middle East. In Egypt, faba bean is annually grown in more than 37000 ha, the cultivated area in 2014 season was 37677 ha that produced about 134175 tons. This created an average yield of about 3.56 tons/hectare [1]. In addition, improving the production of faba bean is one of the main objectives in agriculture in many countries. Therefore, this crop helps to improve the soil fertility through nitrogen fixation [2]. Faba bean is belonging to family *Fabaceae*, which is native to North and Southwest of Africa. Faba bean plants are attacked by the fungal pathogens such as chocolate spot (*Botrytis fabae*) and Alternaria leaf spot (*Alternaria alternata*) which are responsible to cause considerable losses in the yield and its component [3]. Chocolate spot disease is the severe problem of faba bean plants. Alternaria leaf spot disease comes predominant on faba bean during the last years as a consequence of global climate change especially temperature in Egypt [4-6]. However, faba bean diseases especially chocolate spot and leaf spot diseases with rust and downy mildew can reduce yield production by about 22-34% and 61% yield losses on tolerant and susceptible faba bean varieties respectively [7,8]. Chocolate spot is a concern in most faba bean growing regions around the world. *Botrytis fabae* is one of the major disease causal agents with *Botrytis cinerea* and it is the most aggressive under moderately warm temperatures where faba bean is well established as a winter crop and humid conditions, particularly at flowering time [9-11]. The disease is currently managed using resistant cultivars, fungicides, and cultural practices. Most of the faba bean cultivars are susceptible to different fungus races. The pathogen is also highly variable so, breeding for durable resistance to chocolate spot a major challenge [12,13]. The symptoms of the disease appear in the form of small chocolate spots on the leaves. Leaves are the main part of the plant affected, but under favorable conditions for the disease it also spreads to stems, flowers and pods. The chocolate colored lesions start small, but can start to expand if moisture is available under warmer temperatures, eventually merging so that the whole leaf turns brown. After two or three weeks, the larger lesions will turn gray. Symptoms are varied and range from small spots on the leaves to complete spotting of the entire plant [14].

Alternaria leaf spot is caused by *Alternaria alternata*. This is a minor disease of faba beans occurring late in the growing season as the plants start to mature and is sometimes integrated and confused with chocolate spot disease. The main symptoms of this disease occurring as dark brown spots on the leaves which have a zoned brown ring with dark margins, the fungus probably survives on other hosts and crop residues [15]. Chemical control has good results against this pathogen but use of fungicides often leads to resistance of *A. alternata* in addition to environmental pollution effects [16,17]. Therefore, in recent decades, researchers focused attention to find economical and environmentally friendly strategies in integrated plant disease control. Nanotechnology helps agricultural fields to reduce the impact of plant diseases by production of alternative pesticides by using nanoparticles with ability to control the pathogens [17]. A lot of works have been achieved on the development of methods for controlling phytopathogens using different

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types of nanoparticles [17-20]. In recent years, great considerable attention is paid to antifungal effects of chitosan and silver nanoparticles due to their antifungal properties. Chitosan has demonstrated antifungal properties and has been explored for many agricultural uses. It has been utilized to control of some diseases, reduce their spread and enhance plant defenses [21,22]. Silver NPs have been used against different fungal plant pathogens due to their suppressive effects on structures and growth of fungi [23,24]. There are few studies on the applicability of silver to control plant diseases [25]. The antifungal activity of chitosan has been reported and developed in several studies both *in vitro* and *in vivo*, although chitosan activity against fungi has been shown to be less efficient as compared with its activity against bacteria [21].

The present study was carried out to evaluate the efficacy of Chitosan (ChNPs) and silver (AgNPs) in nanoparticle sizes at different concentrations for controlling *Botrytis fabae* and *Alternaria alternata*. *In vitro* studies deal with the effect of tested nanoparticles on radial growth, number of spores, spore germination, mycelial weight of pathogens and lesion size of diseases under different concentrations also the effects of ChNPs and AgNPs against chocolate spot and Alternaria leaf spot diseases on faba bean were studied under greenhouse conditions.

Materials and Methods

Tested nanoparticles

Chitosan nanoparticles (ChNPs) and silver nanoparticles (AgNPs) were purchased from Nanoway-Co, Egypt. AgNPs were prepared as liquid at the initial concentration of 1000 ppm while ChNPs were prepared as powder. The colloidal nanoparticles solution was adjusted to different concentrations of (20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm) using sterilized distilled water at room temperature 24°C. The nanoparticles have shape with an average size of 20-30 nm for AgNPs and 30-50 nm for ChNPs. The prepared solutions were stored at 4°C for experimental use. Sterilized distilled water was used as control treatments.

Isolation and purification of pathogenic fungi

Diseased plant leaf samples showed typical symptoms of *Alternaria* leaf spot and chocolate spot diseases were collected from different faba bean growing fields from Baloza (North Sinai Governorate), El-Nubaria (Beheira Governorate) during 2016 season, to isolate the fungal pathogens. Leaves were washed by running tap water, cut into small pieces and sterilized using sodium hypochlorite solution for 3 minutes, then washed several times by sterilized distilled water and blotted between sterilized filter papers. The sterilized pieces were transferred into Petri dishes contained Potato Dextrose Agar medium (PDA).

Virulence and identification of the fungal isolates

The pathogenicity of purified isolates of *Botrytis fabae* and *Alternaria alternata* were tested and proved by Koch's Postulates conducted of faba bean plants. The plants were grown in pots under greenhouse conditions. Three replicates were maintained for each isolate. The conidial suspension prepared in sterile distilled water at concentration (5×10^5 spore/mL) from 10-day old PDA cultures of fungal isolates. The spore suspensions were sprayed on the faba bean plants 25-day old plants. The plants sprayed with sterile water served as control. The symptoms were observed on 12 days after inoculation and the intensity of diseases was recorded. The symptom was observed and compared with the original symptoms. The fungal isolates were reisolated from artificial inoculated faba bean plants and compared with original culture isolates. The most virulent isolates were selected to laboratory and greenhouse experiments. The isolated fungi were purified using hyphal tip technique [26], The isolated fungi were identified based on morphological and cultural characters as described by Barnett and Hunter [27]. The isolates were identified as *Botrytis fabae* and *Alternaria alternata* at the Plant Pathology Unit, Plant Protection Department, Desert Research Center. The pure cultures of each isolate were maintained on PDA slants at 20°C for further experiments.

Growth media for fungal pathogens

Two different media were used in this study i.e. Faba bean leaf agar (FBLA) medium were used for *B. fabae* and prepared (Extract of 250 g leaves of faba bean, 20 g sodium chloride, 30 g sucrose and 20 g agar in 1 liter distilled water) according to Leach and Moore [28]. While Potato dextrose agar PDA medium were used for *A. alternata* and prepared according to Riker and Riker [29] (Extract of 200 g of peeled potato, 20 g dextrose and 20 g agar in 1-liter distilled water).

In vitro screening of tested nanoparticles against pathogenic fungi

Effects of different concentrations of Chitosan and Silver nanoparticles were tested against *B. fabae* and *A. alternata in vitro*. The concentrations of nanoparticles were prepared by adding the amounts of the stock solutions of nanoparticles to 100 ml of FBLA and PDA media cooled to 40°C-50°C. Four replicates (Petri dishes 9 cm in diameter) were used for each concentration. 5 mm in diameter agar plugs were obtained from the actively growing edge of fungal cultures (7 old day cultures) inoculated in the center of plates supplemented with different concentrations of nanoparticles with four replicates. The plates were incubated at 28°C for 12 days. Colony diameters were measured every 72 h until full growth in control. The percentages of growth inhibition were calculated relative to control using the following formula:

$$Inhibition\ rate(\%) = (R - r) / \mathrm{R} \times 100$$

Where R is radial growth of fungi in control and r is the radial growth of fungi in treated plates.

The sporulation was estimated after 15 days by adding 10 ml distilled water to each plate, then exposed to electric shaker to separate the conidiospores out their conidiophores by falling in the water. The spore suspension was filtered using clean sterilized cheesecloth and the filtrate was received in the test tubes. Number of spores was counted using the haemacytometer slide. The spore suspensions in different concentrations of tested nanoparticles were cultured on concave slides at 26°C. After 10 h, the spore germination rate was observed under a microscope and calculated according to the following formula

$$Total\ germination\ rate\ \% = \frac{\text{Germinated spores (spores / mL)}}{\text{Total spores (spores / mL)}} \times 100$$

While the germination inhibition rate was calculated using the following formula

$$Germination\ inhibition\ rate\ \% = \frac{\text{Germinated spores in control} - \text{germinated spores in treatments}}{\text{Germinated spores in control}} \times 100$$

Effect of AgNPs and ChNPs concentrations on mycelial weight of pathogens

Potato Dextrose Broth (PDB) was the culture medium for the growth experiment. The medium was dispensed into 250 ml conical flasks at the rate of 100 ml per flask and were sterilized by autoclaving at 121°C for 20 min. Nanoparticle concentrations were introduced separately into PDB inside flasks at the concentrations of 60 ppm and 100 ppm. The media were allowed to cool down (30°C–40°C) before chloraphenicol (1%) was added aseptically to suppress bacterial growth.

Each of the fungal isolate of *A. alternata* and *B. fabae* were introduced directly from the inoculated plates. The flasks were incubated at 30°C for seven days, then the mycelial weights were recorded.

Detached leaf test (plant material, inoculums and inoculation)

The plant materials were prepared by growing the faba bean seeds in 16 cm diameter pots for 30 days. Disease assessment for detached leaf test was assessed to confirm the virulence of fungal isolates and select the most virulent isolate in the experiments of this study. Also detached leaf test was created out to know the effects of nanoparticles on the growth of the pathogen and development of chocolate spot and Alternaria leaf spot on leaflets of faba bean *in vitro* where spore suspensions of fungal pathogens were treated with different concentrations of nanoparticles. 250 µL of the spore suspensions of *B. fabae* and *A. alternata* were mixed with 300 µL of Nanoparticles solution at different concentrations and incubated for 24 h, while the spore suspension mixed with sterile distilled water were used as control. The number of germinated spores was examined by optical microscopy. The concentration of spore suspension was adjusted to 2×10^5 spore/ml by suspension in sterilized water, then 25 µL of the spore suspension were dropped on sterile faba bean leaflets. The spore suspension was inoculated at different places on the upper leaflet surface. The leaflets were kept moist. Each concentration contained four replicates. The development of infection was assessed by measuring the expansion of the lesions from third to sixth day after incubation (DAI). The diameter of the lesions was recorded. The average lesion size was calculated for each leaflet at each evaluation time as the mean of the sizes of lesions of the three leaflets measured according to Terefe et al. [30]. The average measurements of the lesion sizes on the three inoculated leaflets in each Petri dish were used for statistical data analysis.

Effect of tested nanoparticles on infection with *B. fabae* and *A. alternata* under greenhouse conditions

The efficacy of chitosan and silver nanoparticles against *B. fabae* and *A. alternata* were determined at open greenhouse. Faba bean seeds of Giza 3 cv. (susceptible cultivar to leaf spot diseases) used in these experiments were obtained from the Department of Legume Crop Research, Department of Field Crops Research Institute, Agricultural Research Center, Giza, were grown in pots (16 cm), four pots were randomly arranged for each treatment. Based on the obtained data from laboratory experiments, three effective concentrations of Chitosan nanoparticles and silver nanoparticles solution (60 ppm, 80 ppm and 100 ppm) were applied on the leaves of plants for 3 weeks before and after the disease outbreak. For pre-treatment of inoculation, the chitosan and silver nanoparticle preparations were sprayed on 30 days old plants. The plants were inoculated with conidial suspensions (10^5 conidia/ml^{-1}) where the most aggressive isolates of pathogenic fungi of *A. alternata* and *B. fabae* were used throughout this experiment, the inoculated plants were covered with plastic for 10 h. The distilled water was used as a control. Ten days after the final inoculation the reaction was recorded using 0-9 scale of Bernier et al. [31].

Disease assessment

The number of infected leaves of 30 randomly selected leaves per treatment, the samples were included the fully expanded leaves from the upper and middle thirds of the plant. Then the obtained data from three age-group of leaves were averaged. The disease severity (D. S%) was calculated based on following equation

$$D.S.\,\% = \left(T / C\right) \times 100$$

Where T=Disease severity of treatment, and C=Disease severity of control. While disease inhibition calculated in basis of the equation:

$$DR = \left[\left(A - B\right) / A\right] \times 100$$

Where, DR: disease reduction, A: Disease severity of control, B: Disease severity of treatment.

Statistical analysis

The obtained data were analyzed by analysis of variance ANOVA, the mean values were compared using the Duncan's multiple test range test at 0.05 probability level [32]. The analysis was done using (SAS ver. 9.2).

Results and Discussion

This study was carried out to evaluate different concentrations of chitosan nanoparticles (ChNPs) and silver nanoparticles (AgNPs) against *Botrytis fabae* and *Alternaria alternata* under laboratory and greenhouse conditions. The main concept of this study depends on the comparison between the effectiveness of nanosilver and nanochitosan concentrations to control chocolate spot and *Alternaria* leaf spot diseases on faba bean.

Effect of chitosan and silver NPs on radial growth of *B. fabae* and *A. alternata*

Antifungal effects of five concentrations (20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm) of each AgNPs and ChNPs were evaluated against *B. fabae* and *A. alternata*. The nanoparticles inhibited the radial growth of pathogens at different concentration levels where all the tested cultures showed clear significant effect compared with control treatment. The most effective treatments were (100 ppm) of AgNPs where inhibited the fungal growth by (79% and 70%) in *B. fabae* and *A. alternata* respectively, followed by (80 ppm) of AgNPs with inhibition rate (77% and 67%) in radial growth of *B. fabae* and *A. alternata*. Also, the Chitosan nanoparticles were effective, ChNPs 80 ppm recorded the inhibition rate (69%) of *B. fabae* and (63%) of *A. alternata* respectively. While 100 ppm recorded (72% and 68%) as inhibition rate of *B. fabae* and *A. alternata* radial growth respectively (Table 1).

A maximum 92.97% spore germination of *A. alternata* was inhibited by AgNPs 100 ppm and 83.57% was inhibited by ChNPs 100 ppm concentration, followed by AgNPs 80 ppm (89.19%) and ChNPs (78.38%). Figure 1 illustrates the germination spore rate. Taken as a whole, all the examined nanoparticle concentrations were found effective in controlling spore germination of *A. alternata*. ChNPs 20 ppm concentration was found less effective for inhibition of mycelial growth and spore germination. This respect with Lamsal et al. [18] who reported that silver nanoparticles inhibited growth of *Colletotrichum* spp. With 50 ppm and 100 ppm by 84.56% and 93.5% respectively. Paulkumar et al. [33] found that the silver nanoparticles have excellent antimicrobial activity against plant pathogens *Citrobacter freundii* and *Erwinia cacticida*, they concluded that the silver nanoparticles have a beneficial application in field of plant disease control. Kim et al. [34] studied the antimicrobial activity of silver nanoparticles against *Acidovorax citrulli* and they found the growth of five strains of *A. citrulli* was inhibited by AgNPs. The extrapolation of these findings to more case is limited because the mechanism of chitosan and silver nanoparticles effects for control the tested fungi still not clear, but several mechanisms through some previous studies have been suggested for antifungal property of nanoparticles, one of them was that nanoparticles able to occupy the cell surface of the pathogen and degrade the lipopolysaccharide molecules and caused increases in permeability of the membrane [7,35]. Previous studies also suggested that silver ions are produce reactive oxygen species by their reaction with oxygen, which is causing damage to protein and nucleic acid of

No.	Treatments	*B. fabae*		*A. alternata*	
		Growth inhibition rate (%)	No. of spores/ml × 10^5	Growth inhibition rate (%)	No. of spores/ml × 10^5
1	ChNPs 20 ppm	18 g*	3.3 ab	15 g	1.31ab
2	ChNPs 40 ppm	28 f	3 bc	25 f	1.16 ab
3	ChNPs 60 ppm	45 d	2.6 cd	42 d	1.04 bc
4	ChNPs 80 ppm	69 b	1.6 fg	63 b	0.95 bc
5	ChNPs 100 ppm	72 b	1.3 g	68 a	0.53 de
6	AgNPs 20 ppm	26 f	3.1 ab	23 f	1.25 ab
7	AgNPs 40 ppm	33 e	2.9 bc	30 e	0.97 bc
8	AgNPs 60 ppm	58 c	2.2 de	52 c	0.72 cd
9	AgNPs 80 ppm	77 a	1.5 fg	67 a	0.68 cde
10	AgNPs 100 ppm	79 a	1.9 ef	70 a	0.36 e
11	Control	0 h	3.6 a	0 h	1.44 a

*(a-h) Values with the same letter in column are not significantly different.

Table 1: Effect of AgNPs and ChNPs on growth and number of spores of *B. fabae* and *A. alternate*.

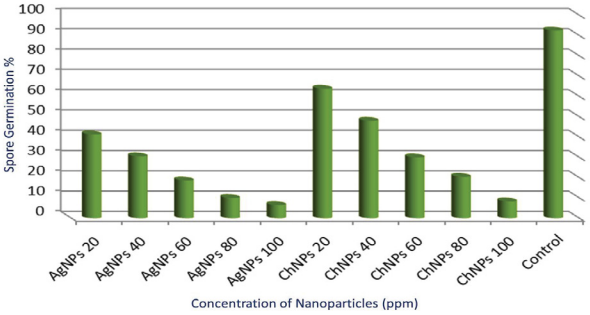


Figure 1: Effects of different concentrations of chitosan and silver nanoparticles on the spore germination of *Alternaria alternate*.

No.	Concentration of NPs	*Sporulation (× 10^3/ml)^2							
		B. fabae				*A. alternata*			
		Length		Width		Length		Width	
		Range 3	mean	Range 3	mean	Range 3	mean	Range 3	mean
1	ChNPs 40 ppm	22.2-28.4	25.3 a	12.1-18.3	15.2 a	36.4-42.7	39.55 a	15.2-18.4	16.8 a
2	ChNPs 60 ppm	18.1-23.8	20.95 c	6.5-17.9	12.2 bcd	33.5-40.7	37.1 abc	11.2-18.1	14.65 a
3	ChNPs 80 ppm	14.4-23.8	19.1 b	6.2-16.8	11.5 d	30.7-36.2	33.45 bc	11.4-16.2	13.8 a
4	ChNPs 100 ppm	14.4-21.2	17.8 bc	6.4-16.1	11.25 d	30.3-33.9	32.1 c	11.3-14.7	13 a
5	AgNPs 40 ppm	18.3-21.5	19.9 b	9.6-16.8	13.2 ab	33.7-41.1	37.4 ab	13.2-18.6	15.9 a
6	AgNPs 60 ppm	16.9-22.9	19.9 b	10.9-17.3	14.1 ab	32.4-41.2	36.8 abc	11.7-14.2	12.95 a
7	AgNPs 80 ppm	14.2-21.5	17.85 bc	7.1-16.4	11.75 cd	30.6-40.4	35.5 abc	11.5-17.3	14.4 a
8	AgNPs 100 ppm	13.5-22.7	18.1 bc	6.5-17.2	11.85 cd	30.3-36.8	33.55 bc	10.7-13.9	12.3 a
9	Control	18.6-28.4	23.5 a	9.5-17.8	13.65 abc	34.6-41.8	38.2 a	14.7-16.9	15.8 a

*(a-d) Values with the same letter in column are not significantly different.

Table 2: *In vitro* effect of AgNPs and ChNPs concentrations on conidial size (µm) of *B. fabae* and *A. alternate*.

the cells [18,36,37]. Other reports proposed that the mechanism of nanoparticles is related to their penetration into the fungal cell [38-40]. Morones et al. [40] observed that silver nanoparticles disrupt transport

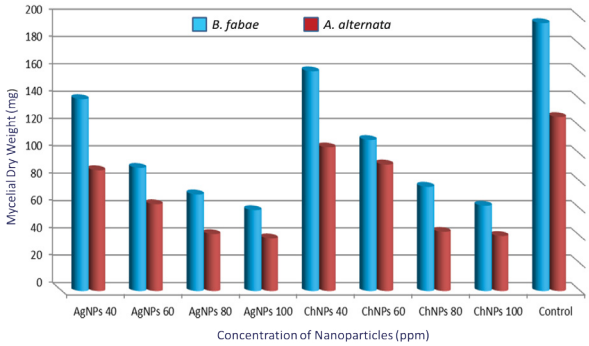


Figure 2: Effect of AgNPs and ChNPs on Mycelial dry weight of *B. fabae* and *A. alternate*.

systems including ion efflux and caused rapid accumulation of silver ions, in addition to interrupting cellular processes such as respiration and metabolism due to reacting with molecules. Moreover, using effective concentrations of ChNPs and AgNPs were effective against the tested fungal pathogens relative to control and were agreed with findings of some investigators who studied a successful antagonism of various fungal pathogens by silver nanoparticles at different concentrations [41-43]. Darsef and Suhartono [44] suggested that the mechanism, which chitosan affects the growth of fungi, may be due to its ability to interfere with the negatively charged residues of macromolecules exposed on fungal surfaces forming polyelectrolytic complexes, and affecting membrane permeability as well as causing leakage of intracellular electrolytes and proteinaceous constituents. The obtained results of this study indicated that the inhibition of fungi can be achieved by increasing of nanoparticle concentration. The highest antifungal properties were observed in the case of treatment with silver nanoparticles followed by Chitosan nanoparticles. However, inhibition effect of silver and chitosan nanoparticles against *B. fabae* and *A. alternata* at different concentrations it may lead to some findings in various fields of fungal pathogens control but there are some queries still remaining related to the mechanism of interaction of nanoparticles with fungal cell and the pass way of these nanoparticles inside the structures of fungi in addition to the impact of these alternative control agent on metabolites of pathogens.

Effect of nanoparticle concentrations on mycelial weight and conidial size of *B. fabae* and *A. alternata*

The influence of ChNPs and AgNPs on mycelial weight and conidial size of *B. fabae* and *A. alternata* was tested in various concentrations (40 ppm, 60 ppm, 80 ppm and 100 ppm). Results of these experiments showed the antifungal effects of chitosan and silver nanoparticles on the inhibition of mycelial growth weight. The concentrations AgNPs (60 ppm, 80 ppm and 100 ppm) inhibited the mycelial growth of the *B. fabae* where recorded (84.61 mg; 63.88 mg and 56.37 mg) respectively, also the high concentration of ChNPs were reduced the mycelial weight by (107.25 mg; 67.41 mg and 59.82 mg) with 60 ppm, 80 ppm and 100 ppm respectively compare to control which recorded (192.82 mg), whereas the lowest inhibitory effects were shown by 40 ppm of both ChNPs and AgNPs but also with inhibition rates compared with control. *Alternaria alternata* was more sensitive to increasing of nanoparticle concentration. The mycelial growth was inhibited to 36.3 mg by AgNPs 100 ppm compared to 122.51 mg in control (Figure 2). Application of the ChNPs and AgNPs concentrations had significant effect on growth of *B. fabae* and *A. alternata* in this study. The tested nanoparticles were able to reduce the weight of mycelium at all used concentrations. As shown in Table 2, the chitosan and silver

No.	Treatments	*Average lesion size (mm) / time									
		B. fabae					*A. alternata*				
		3th day	4th day	5th day	6th day	Mean	3th day	4th day	5th day	6th day	Mean
1	ChNPs 40 ppm	10.12 a	11.57 a	14.29 b	14.64 b	12.66	4.5 ab	5.2 b	6.4 b	7.9 b	6.00
2	ChNPs 60 ppm	7.74 b	9.37 b	12.21 b	12.98 b	10.58	4.0 bc	4.8 b	5 c	5.5 c	4.83
3	ChNPs 80 ppm	4.54 c	6.13 d	8.53 c	9.16 c	7.09	3.5 cd	3.8 c	4.3 d	4.9 c	4.13
4	ChNPs 100 ppm	3.18 d	3.64 e	4.99 de	5.74 d	4.39	0 e	4.2 c	5 c	5.3 c	3.63
5	AgNPs 40 ppm	7.62 b	11.75 a	14.17 b	14.67 b	12.05	4.0 bc	4.2 c	4.8 a	5.3 c	4.58
6	AgNPs 60 ppm	5.25 c	7.64 c	7.93 c	9.83 c	7.66	3.2 cd	3.9 c	4.1 d	4.7 cd	3.98
7	AgNPs 80 ppm	0 e	3.98 e	5.15 de	5.73 d	3.72	2.8 d	3 d	3 e	3.4 e	3.05
8	AgNPs 100 ppm	0 e	0 f	2.52	3.49 d	1.50	0 e	2.6 d	2.8	2.8 e	2.05
9	Control	10.53 a	11.78 a	17.47 a	18.52 a	14.58	4.9 a	6.8 a	8.4 a	9.5 a	7.40

*(a-f) Values with the same letter in column are not significantly different.

Table 3: *In vitro* effect of nanoparticle concentrations (ppm) on average lesion size (mm) caused by *B. fabae* and *A. Alternata* using detached leaf test.

No.	Treatments	*Disease severity after days %									
		Chocolate spot disease					*Alternaria* spot disease				
		3	6	9	Mean	Reduction%	3	6	9	Mean	Reduction %
1	ChNPs 60 ppm	30 a	28 b	55 b	37.67	33.53	34 b	38 bc	40.4 bc	37.33	48.15
2	ChNPs 80 ppm	19 c	27 b	38 cd	28.00	50.59	27 cd	33 cd	36.3 cd	32.00	55.56
3	ChNPs 100 ppm	23 bc	35 b	42 c	33.33	41.19	18 e	25 e	28.4 e	23.67	67.13
4	AgNPs 60 ppm	25 b	33 b	51 b	36.33	35.89	30 bc	42 b	46.3 b	39.33	45.38
5	AgNPs 80 ppm	23 bc	27 b	35 de	28.33	50.01	22 de	34 bc	37.5 cd	30.67	57.40
6	AgNPs 100 ppm	21 bc	28 b	31 e	26.67	52.94	23 de	28 de	32.2 de	27.67	61.57
7	Control	35 a	55 a	80 a	56.67	0	56 a	72 a	88.4 a	72.00	0

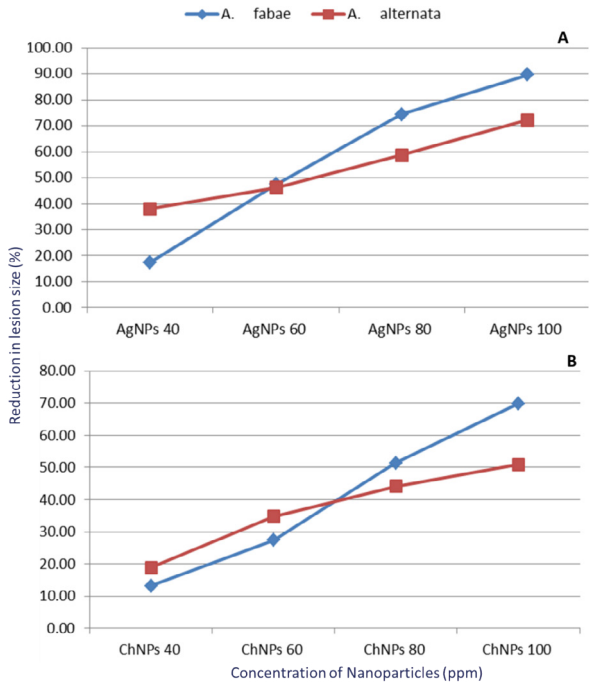
*(a-e) Values with the same letter in column are not significantly different.

Table 4: Effect of tested nanoparticles on *Alternaria* spot and chocolate spot disease severity on treated plants under greenhouse condition.

nanoparticles inhibited of conidial size of each *B. fabae* and *A. alternata* declined when the concentration increased. Different concentrations of the nanoparticles had different inhibition effect against conidial size of fungi. At greater concentrations, the spores were sparse and short, also the mean size of conidia varied in length and width. The conidial length of the *B. fabae* ranged from 13.5 to 22.7 µm while conidial width ranged from 6.5 to 17.2 µm at 100 ppm of AgNPs The ChNPs also were effective at highest concentration where the conidial length was ranged from 14.4 µm to 21.2 µm and the conidial width ranged from 6.4 µm to 16.1 µm at 100 ppm compared with the conidial length (18.6 µm-28.4 µm) and width (9.5 µm-17.8 µm) as conidial thickness from the control. In *A. alternata*, the conidial length ranged from 30.3 µm to 36.8 µm while conidial width ranged from 10.7 µm to 13.9 µm at AgNPs 100 ppm. With ChNPs 100 ppm, the conidial length was ranged from 30.3 µm to 33.9 µm and the conidial width ranged from 11.3 µm to 14.7 µm compared to the conidial length (34.6 µm-41.8 µm) and the width (14.7 µm-16.9 µm) in control (Table 2). The fungal spores were generally more sensitive to silver nanoparticles than chitosan nanoparticle; the *B. faba* spores were more sensitive to ChNPs and AgNPs than *A. alternata*. The sporulation test showed that the number of spores formed by mycelium increased in the culture after treatment with ChNPs at 40 ppm relative to control samples also the length and width of conidia were increased. While ChNPs and AgNPs at 60 ppm, 80 ppm and 100 ppm concentrations were reduced the length and width of conidia relative to the control. The *B. fabae* and *A. alternata* conidia were able to germinate at 40 ppm and 60 ppm of ChNPs but the conidia germinated poorly at 80 ppm and 100 ppm with reduction in both length and width. It is assumed that conidial size could be another factor involved in conidial germination and mycelial growth in fungi. It was found that conidial size was reduced in higher concentration of nanoparticles compared to control. The obtained results are agreed with the findings of some previous studies. Jafari et al. [45] in their research used AgNps with the size of 50 nm and observed inhibitory effect against *Penicillium chrysogenum*. They found that the particles have the toxicity effects on cytoplasma membrane. Angelova et al. [46] studied the inhibitory effect of silver nanoparticles on *Penicillium chrysogenum*, they reported that the silver nanoparticles lead to restraining impact on fungi. Some researchers have concluded that the chitosan nanoparticles can be decreased the fungal growth rate and sporulation at high concentration in high and low molecular weight and small nanoparticle size, in addition to other physical properties which can convert the long chains of chitosan into small pieces and prevent the formation of agglomerates and making the nanoparticles pass through the fungal cells [47-50]. The results of the present study are in appropriateness with these researches and provide the inhibitory effect of investigated nanomaterials against *B. fabae* and *A. alternata*. It may be referred to the formation of free radicals from the surface of AgNPs, uncontrolled generation of free radicals can attack membrane and conidia of the fungi then lead to a breakdown of membrane function as physical damage, thus affect the fungal structures [51-54].

Effect of ChNPs and AgNPs on chocolate spot and *Alternaria* leaf spot

Detached leaf test: Lesion size caused by both fungal pathogens *B. fabae* and *A. alternata* showed differences based on the time. The rate of lesion growth increased slowly than the lesion size of control in both of two tested pathogens. On the sixth day of inoculation, the average rate of lesion expansions caused by *B. fabae* varied from 3.49 mm at AgNPs 100 ppm to 14.67 mm at AgNPs 40 ppm, while the ChNPs recorded average rate of lesion size by 5.74 mm in 100 ppm and 14.64 mm in 40 ppm of chitosan nanoparticles compared with control (18.52 mm) (Table 3) (Figures 3A and 3B). Alternaria leaf spot lesions were also significantly different from their respective controls. The highest inhibition was scored with AgNPs 100 ppm (2.8 mm) as mean of lesion size, and the lowest (4.83 mm) was at ChNPs 40 ppm compared with control (7.4 mm). In all the nanoparticle concentrations studied, the lesion growth increased slowly with increasing in the concentration with significantly different from their control.



$$\text{Reduction in lesion size \%} = \frac{\text{mean of lesion size in control} - \text{mean of lesion size in treatment}}{\text{mean of lesion size in control}} \times 100$$

Figure 3: Effect of nanoparticle concentrations, A) AgNPs; B) ChNPs on lesion size (mm) of chocolate spot and *Alternaria* leaf spot of faba bean using detached leaf test.

Disease severity under greenhouse conditions: The tested nanoparticle action against *B. faba* and *A. alternata* was evaluated under greenhouse conditions (Table 4). Faba bean plants inoculated with conidia of fungal pathogens and maintained at different concentrations (60 ppm, 80 ppm and 100 ppm) of AgNPs and ChNPs to determine the developing of chocolate spot symptoms. All infected plants showed typical symptoms of small necrotic flecks clearly visible on leaves by the second and third day after inoculation. AgNPs 100 ppm was also the most effective one against *B. fabae* with reduction of disease severity (52.94%) followed by ChNPs 80 ppm (50.01%), while the most effective concentration of nanoparticles was 100 ppm of both silver chitosan and silver nanoparticles against *A. alternata* followed by the concentration of 80 ppm for each ChNPs and AgNPs. The tested nanoparticles showed significant effect against *B. fabae* and *A. alternata* relative to control. The results agree with the findings of Kasprowicz et al. [55] who reported that silver nanoparticles have been used to prevent some plant pathogens. Derbalah et al. [7] found that the silver nanoparticles have antimicrobial activity against *B. fabae*. Morones et al. [40] observed that silver nanoparticles are highly reactive due to generate Ag+ ions and have antifungal affect against various pathogens and AgNPs disrupt transport systems including ion efflux. Young et al. [42] suggested that, direct contact of silver with fungal spores or germ tubes is critical in inhibiting disease development. Chitosan nanoparticles are effective elicitors of plant resistance to many pathogens infections [56]. Liu et al. [57] found that chitosan possesses the natural antifungal role which increases the permeability of the outer membrane and inner membrane thus disrupts bacterial cell membrane with the release of cellular metabolites. Darsef and Suhartone [44] suggested that the inhibitory efficacy of chitosan against pathogenic fungi could also be related to its ability to increase the production of antioxidant activity (e.g., polyphenol oxidase and peroxidase) and defense-related enzymes (e.g., chitinase and β-1,3-glucanase) in chitosan. It could be concluded that, chitosan and silver nanoparticles can be used effectively in control of chocolate spot and Alternaria leaf spot of faba bean and prevention of deleterious infections. These nanoparticle types may be less toxic to human and environment. The results of this study focus on extended applicability of ChNPs and AgNPs for control of *B. fabae* and *A. alternata*.

Conclusion

Chitosan nanoparticles (ChNPs) and silver nanoparticles (AgNPs) were used to evaluate their antifungal effects against *Botrytis fabae* and *A. alternata* the causal agents of chocolate spot and Alternaria leaf spot diseases of faba bean. The antifungal activity of five concentrations of each nanoparticles type showed inhibition rate of fungal growth and number of spores in treated cultures of fungi by nanoparticle concentrations. These effects were increased with increasing nanoparticles. In all concentrations of studied NPs, the lesion growth was suppressed by increasing in the concentration with significantly different from their control using detached leaf test. Under greenhouse conditions, the tested nanoparticles at high concentrations showed significant effect against *B. fabae* and *A. alternata* relative to control. The efficacy of chitosan and silver based nanoparticles could further be tested on other plant pathogenic fungi *in vitro* and *in vivo*. The obtained results indicated the possibility of using chitosan and silver nanoparticles as a substance in the manufacture of fungicides to minimize the impact of chocolate spot and Alternaria leaf spot diseases in faba bean. However, further experimental trials under field conditions and safety evaluation studies are needed before these nanoparticles types and concentrations can be used as potential antifungal agents.

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