Efficacy of different Conidial concentrations, fungal Filtrations and silver nanoparticles Mycosynthesis using two isolates of *Metarhizium anisoplae* against Two Stored-grain Insects

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Abstract

The current study examined the effectiveness of conidial concentration, fungal filtrate of *M. anisoplae*, and biologically manufactured silver nanoparticles in preventing infection by the rust-red flour beetle and the hairy grain beetle in flour sacks and jute bags. One commercial and one local *M. anisoplae* isolates were utilized. The synthesized silver nano-particles were characterized using, UV-vis spectrophotometer, Fourier Transform Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), Scanning Electron Microscope (SEM) and Atomic Force Microscope (AFM) analysis. The size of the nanoparticles ranged between (37.22 - 75.93) nm for local fungus, with an average of 53.82 nm, and (6.176 - 22.25) nm for commercial fungus, with an average of 12.372 nm. The efficiency of three types of treatments for local and commercial isolates was evaluated: conidial suspension (1×10^8 conidia/ml), fungal filtrate (100%), and silver nanoparticles (20%). The results demonstrated that the silver nanoparticle treatment outperformed the other treatments in providing protection against infection with *T. castaneum* and *T. granarium* after an 8-week storage period, with averages of 26.7-36 insects/bag and 47.7-66 insects/bag. The number of individual insects per bag in the remaining treatments was significantly higher than the silver nanoparticle treatment. In the control treatment, the insect densities were 366.7 and 586 insects/bag, respectively. This research indicates persuasively that biologically manufactured silver nanoparticles can be employed as a biological control agent in the treatment of grain bags and their products. It was resistant to both the rust-red flour beetle and the hairy grain beetle, which commonly infect stored products.

Keywords: Spore suspension, fungal filtrate, silver nanoparticles, *Metarhizium anisoplae* , *Tribolium Castaneum*.

Introduction

Stored grains are grains that have been harvested and then stored under proper conditions for several months, including their products and foods prepared from them (11). Because the stored grains and their products are the primary worldwide source of food, insects that infect stored grains are among the most important economic pests (14). These harmful insects infest and contaminate many stored grains, including rice, lentils, wheat, corn, and others (18 and 5). Coleoptera insects are among the most significant pests affecting stored grains, accounting for 75% of all pests, the rust-red flour beetle and the hairy grain beetle are the most well-known pests of them. (1 and 15).

According to some study estimations, direct and indirect losses of grains and their products , the damage incidence reached 10% in temperate regions and 50% in tropical regions(22). The most insect damages are the high cost which may reach thousands of dollars per year due to the destruction of large amounts of infected grain., and medical issues such as digestive disorders and allergic diseases (16).

Despite the use of physical and chemical insect control techniques, the extensive and repeated application of chemical pesticides resulted a resistant insect strains as well as the harmful to other living organisms (non-target) and the environment. Insect pathogenic fungi are cosmopolitan in nature and also non-virulent to humans and other beneficial organisms. Simultaneously, entomopathogenic fungi have shown to be more effective with minimal dosages to coleopteran pests (8 and 6). *Metarhizium anisopliae* is one of the most common entomopathogenic fungi; it is soil-borne and infects mainly to agricultural insect pests that are found in the soil (20 and 14). Eco-friendly synthesized nanoparticles using entomopathogenic fungi are cost effective, and target specific for insect management. Silver nanoparticles have a major impact on insect antioxidants and detoxifying enzyme systems. They affect enzymes that are connected to oxidative stress resulting in cell death in insects. Several studies have been reported using biosynthesis of silver nanoparticles mediated by entomopathogenic fungi. (19) reported that silver nanoparticles mediated by *Chrysosporium tropicum* caused . In addition, In this research study, we evaluated the efficacy of conidial concentration, fungal filtrate of *M. anisoplae* and acute toxicity of Silver nanoparticles synthesized with *M. anisoplae* against the most important stored product insect pests (*T. granarium* and *T. castaneum*) under laboratory and storage conditions..

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Materials and Methods

The culture medium used:

Potato Dextrose Agar (PDA) culture media

The medium was prepared utilizing 200 g of peeled potato tubers chopped into small pieces. It was boiled for 20-30 minutes in a glass flask with 500 ml of distilled water. After the end of the boiling period, the mixture was filtered with a piece of gauze

fabric to extract the filtrate. In a further 500 ml, 20 gm of dextrose and 17 mg of agar were dissolved, then the potato filtrate was added to raise the total volume to 1 litre. As needed, the medium was poured into glass flasks and plugged with cotton stoppers before being autoclaved for 20 minutes at 121°C and 15 pounds per inch2 pressure. The flasks were allowed to cool after they had been sterilized. Then 250 mg of chloramphenicol per litre of water was added. The medium was then placed into the Petri dishes in accordance with the experiment, and a portion of it was refrigerated until use. *M. anisopliae* isolates were cultured in a medium. This medium was utilized to nurture two isolates of the fungus *M. anisopliae* that were currently being studied.

Potato Dextrose Broth Media (PDB)

It was prepared in the same fashion as indicated in the preceding section, but without the addition of agar. This medium was utilized to grow fungi in order to acquire fungal secretions.

Source of *M. anisopliae* fungus

M. anisopliae isolates have been obtained from the University of Babylon/ College of Agriculture. The diagnosis was verified by Prof. Dr. Jamal Hussein Kazem. While Dr. Akram Ali Mohammed obtained the commercial isolated Met-52-EC bioinsecticide, which was then activated and proliferated on PDA medium.

***T. castaneum* and *T. granarium* collection and identification**

A pure colony of rust-red flour beetle and hairy grain beetle was gathered from the insect laboratory at the College of Agriculture / University of Kufa. While, Dr. Hanaa H. Al-Saffar, an academic and researcher at the University of Baghdad/ Museum of Natural History, identified the two insects. Insects were nurtured on flour and sterilized wheat and incubated at 30±1 temperature and 60±5% relative humidity.

***M. anisopliae* spore suspension Preparation**

The conidial concentrations of *M. anisoplae* were prepared using the method describte by (13and 14). Aerial conidia were harvested from 14- day-old cultures by adding 15 ml of 0.01% Tween 80 to culture agar Petri dishes and gently scraping the surface of the cultures with a sterile inoculating loop to dislodge the conidia from the surface of the agar plates. The conidial suspension was pipetted from the Petri dish and filtered through 3 layers of cheesecloth. The number of conidia in the suspension was estimated, using a haemocytometer (Neubauer improved, Superior Marienfeld, Germany). The resulted suspension was diluted to the desired concentrations with 0.01% Tween 80 as required.. While the commercial isolate suspension was prepared by taking 10 g of loaded fungus with 1 liter of sterile distilled water containing 0.02% tween-20. Consequently, 1×10^{10} was obtained as the first dilution, and 1 mL of it was collected and mixed with 9 mL of sterilized distilled water. As a result, the second dilution was 1×10^{8} conidia/ml.(7)

***M. anisopliae* filtrate preparation**

This method utilized 250 ml wide-bottom glass flasks. In these flasks, 150 ml of previously prepared PDB culture media was added. After cooling the medium, the flasks were inoculated with 0.5 cm discs of *M. anisopliae* colonies cultured on PDA-containing dishes at a rate of 1-3 discs/flask. The flasks were maintained in an incubator at a temperature of 25±2 °C for 28 days, with the flasks being shaken every three days to divide the hypha and separate the spores. At the end of the experiment, the fungus cultured were filtered utilizing a glass funnel and Whatman No.1 filter paper. The filtrate was maintained in glass containers in the incubator at 25±2 °C until use (10).

Preparation of *M. anisopliae* biomass

The *M. anisopliae* biomass of both isolates was obtained after 7 days of growing in petri dishes containing solid PDA. Following that, two 5-8 mm pieces were taken with a corkscrew and placed in a beaker containing 200 ml of sterile liquid culture media (P.D.B). The beaker was subsequently covered and placed in an incubator at 25±2 °C for 21 days, with daily shaking, to collect biomass from *M. anisopliae* fungal isolates. The biomass was acquired after 21 days of incubation by purifying and filtering it with a funnel and filter paper. The fungus biomass was then washed three times with distilled water, followed by three washes with deionized water, to eliminate nutritional media residues. 13 g of biomass and 100 ml of deionized water were placed in a 500 ml glass beaker. The flasks were placed in an incubator for 5 days at a temperature of 25±2 °C with daily shaking. To obtain the fungal biomass filtrate, the biomass solution was filtered utilizing a funnel and Whatman No.1 filter paper. The fungus biomass extract was collected in a glass beaker and stored in an incubator at 25±2 °C until use (9).

Preparation of silver nanoparticles using an aqueous extract of two isolates of the fungus *M. anisopliae* biomass

To prepare the synthesized silver nanoparticles using each fungal isolate , Fifteen mL of fungal culture filtrate was transferred to 85 mL of silver nitrates solution mixed properly and heated at 60ºC using a hot plate magnetic stirrer (LabFriend India Private Limited, New Delhi, India). After this process, the solution was incubated in dark conditions at 28_± 2ºC for 72 h and the color changed into a dark brown (12). Then, the solution was filtered with Whatman no. 1 filter paper, and the solution is washed with double distilled water using centrifugation (Kesar Control Systems, Gujarat, India) with a range at 11,000 rpm for 8 min. The washing measures were repeated many times until the unwanted particles were removed. Finally, the pellet was dried in room temperature for two days and pellets were used for all the experiments and all spectroscopic studies.

Characteristics of silver nanoparticles

Fourier Transform Infrared Spectroscopy (FTIR) device was employed to identify functional groups in generated nanomaterials that act as inhibitors, reducing agents, and coating silver nanoparticles. The shape and size of the nanoparticles

generated were determined utilizing a scanning electron microscope (SEM). The chemical components of the sample were identified through Energy Dispersive Spectroscopy (EDS). Because each substance has a distinct atomic composition, it has an individual collection of peaks in the X-ray spectrum.

Field experiment

A field experiment to evaluate the treatment of flour and jute bags with spore suspension, fungal filtrate, and nanoparticles to protectflour and wheat from *T. castaneum*. *T. granirum* infection during storage.

The red-rust flour beetle experiment was carried out in one of the Najaf Mill storages affiliated with the Ministry of Industry and Minerals, which contains bags of infected grain. The hairy grain beetle experiment was carried out in a small aluminum storage at the faculty of Agriculture/University of Kufa. The infestation was created artificially by releasing insects into the store. This is to assess the efficacy of the biologically generated spore suspension, fungal filtrate, and silver nanoparticles in preventing flour and wheat from infection by these two insects via treating the bags with the treatments mentioned above. For assistance in the periodic inspection process, the 1 kg container bags include a top aperture that can be simply opened and closed. The spore suspension treatment was applied to each of the three duplicates at a concentration of 1×10^8 1 spore / ml, whereas the control treatment was (water + Tween-20). The fungal filtrate treatment required 100% concentration, while the control treatment simply required distilled water. In contrast to a silver nitrate solution, a silver nanoparticle concentration of 20% was utilized. The bags were then allowed to dry before being filled with flour and wheat that were previously sterilized at less than -20°C for 48 hours. The bags were examined every seven days to determine the presence or absence of infection. After 45 days of treatment, the number of beetles in each bag was manually counted.

Statistical analysis

The field experiments were carried out utilizing completely Random Block Design (CRBD) with many factors. The mean values were compared utilizing the least significant difference (L.S.D.) and a confidence level of 0.01. The data was analyzed implementing the GenStat7 statistical software.

Results and Discussion

Identification of active aggregates that contribute to the preparation of silver nanoparticles

Spectroscopy of silver nanoparticles from local and commercial fungus revealed several peaks of energy units, reflecting a complicated chemical composition of the fungus. The existence of groups of C-H aromatic compounds and C=C alkenes was detected, as well as N-O aromatic compounds, C-N aliphatic amines, and the amines group in N-H alkanes. The presence of O-H carboxylic acid groups and C-O alcohol groups was also detected utilizing spectroscopy (Figs. 1 and 2). These are distinguished through their strong affinity of silver ions (17) .

The spectroscopic examination demonstrated that aromatic compounds contain the most recorded regions, suggesting that there could represent reducing agents or nanoparticle stabilizers, followed by the alkenes group, carboxylic acids group, and organic compounds in the second rank. Furthermore, the carboxyl group, amines, and alkenes identified in proteins may perform an essential part in the biological reduction of Ag+ silver nitrate to Ag° silver nanoparticles. Additionally, they are essential for preventing nanoparticle agglomeration and stabilizing them (4 and 3), and alcohol aggregates can perform the similar effect.

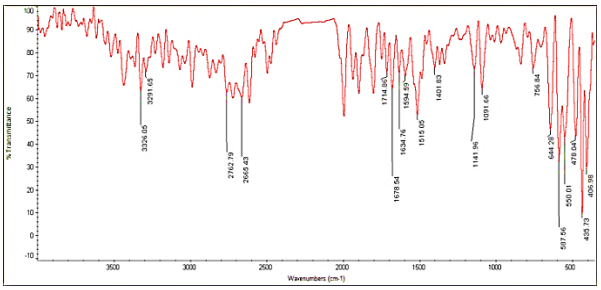

Figure 1. Illustrates Fourier transform infrared spectroscopy of silver nanoparticles prepared by the indigenous fungus *M. anisopliae*.

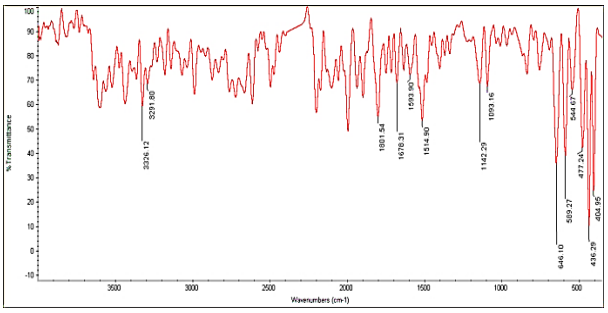

Figure 2. Demonstrates the Fourier transform infrared spectroscopy of silver nanoparticles prepared by the commercial fungus *M. anisopliae*.

Determination of the shape and size of the prepared silver nanoparticles

SEM analysis clearly shows an external morphology and crystalline structure of Ag NPs . The images demonstrated that the nanoparticles produced by the aqueous extract of *M. anisopliae* fungus exhibited spherical and oval shapes. As demonstrated in Figures 3 and 4, the size of the nanoparticles ranged between (37.22 - 75.93) nm for local fungus, with an average of 53.82 nm, and (6.176 - 22.25) nm for commercial fungus, with an average of 12.372 nm.

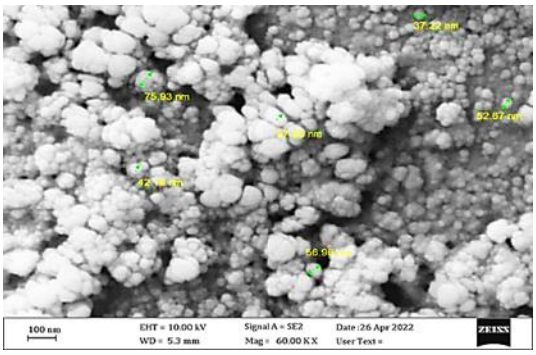

Figure 3. Illustrates a scanning electron microscope image of silver nanoparticles prepared by the aqueous extract of the indigenous fungus *M. anisopliae*.

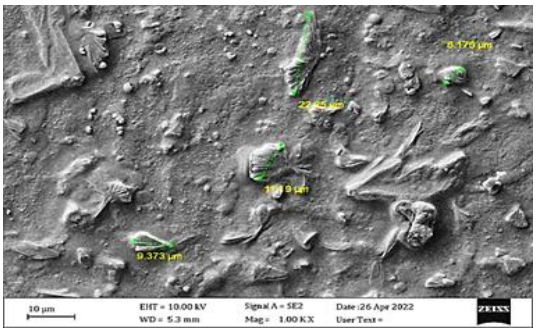

Figure 4. Demonstrates a scanning electron microscope image of silver nanoparticles prepared by the aqueous extract of the commercial fungus *M. anisopliae*.

Identification of chemical components in a sample utilizing energy dispersive spectroscopy (EDS)

Energ-dispersive micro analysis to gain further insight into the Ag NPs analysis of the sample was performed using the EDS techniqu . The peaks rangd from 0.85 and 1.04% indicating the presenc of Ag NPs(Figs. 5 and 6).

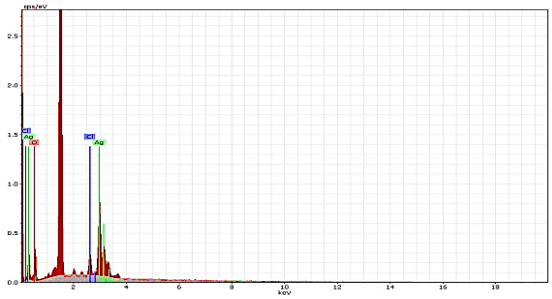

Figure 5. Demonstrates an X-ray scattering spectroscopy of silver nanoparticles formed by local fungus *M. anisopliae*.

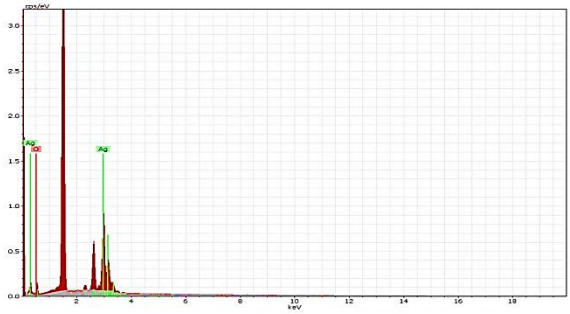

Figure 6. Illustrates an X-ray scattering spectrometry of silver nanoparticles formed from the commercial fungus *M. anisopliae*.

Field experiment evaluation of the treatment of flour bags with spore suspension, fungal filtrate, and nanoparticles in preventing *T. castaneum* infestation in storage. Table (1) shows the number of red-rust flour beetle infestations determined after treating flour bags with a spore suspension at a concentration of (1×10^8), fungal filtrate at a concentration of (100%), and silver nanoparticles at a concentration of (20%) for

local and commercial isolates of the fungus *M. anisoplae* and storing for a period of eight weeks in the storage facility infested with the red-rust flour beetle. The results showed that when silver nanoparticles were employed, the expected rate of maturity was the slowest (18.15). After 8 weeks of treatment, the average of the determined adults for the fungal filtrate and the spore suspension were (109.8 and 62.34) adults\bag, respectively, compared to the control treatment, which was (145.2)adult \bag. The lowest rate was determined to be (13.4) adult \bag after one week of treatment, while the highest rate was reported to be (180.8) adult \bag after eight weeks of treatment. The commercial isolate exhibited the lowest predicted adult rate in the spore suspension, fungal filtrate, and silver nanoparticles after 8 weeks of treatment, with (60.85, 98.09, and 15.6) adult \bag, respectively. The local isolate, on the other hand, exhibited the highest calculated adult rate, reaching (63.83, 121.5, and 20.7) adult \bag. This is consistent with the conclusion of (2) that the silver nanoparticle suspension had a repellent effect in various concentrations on adult insect *T. castaneum*, especially at concentrations (0.25, 0.5 , 1) ppm, reaching (46.67 , 66.67 , 63.33 ,76.67) % respectively.

Table 1. Treating flour bags with spore suspension, fungal filtrate, and nanoparticles in preventing *T. castaneum* infections in storage

Treatment	Isolate	Beetle Number/Week							
		1	2	3	4	5	6	7	8
Spore Suspension	Commercial	9.7	26	36	41.7	51.7	71.7	100	150
	Local	9.7	32.3	41	46	60.3	78.3	100	143
Fungal Filtrate	Commercial	21	35.7	42.3	50.7	75	133.3	186.7	240
	Local	25	52	56.7	71.7	93.3	143.3	226.7	303.3
Silver Nanoparticles	Commercial	1	5.3	13.3	15	17.7	21.7	23.7	26.7
	Local	1.3	10.7	12.3	18.3	22.7	30.7	33.3	36
Control		26	55	62.3	71.7	103.3	196.7	280	366.7
Rate Time Effect		13.4	31	37.7	45.01	60.6	96.5	135.8	180.8
LSD for isolate = 5.89; treatment = 9.11; Time = 8.66									

Field experiment evaluation of the treatment of jute bags with spore suspension, fungal filtrate, and nanoparticles in preventing *T. granirum* infestation in storage

Table (2) demonstrates the calculated number of adults of the hairy grain beetle treated with spore suspension (1×10^8 conidia / ml), fungal filtrate (100%), and silver nanoparticles (20%) after storing local and commercial isolates of the fungus *M. anisoplae* in jute bags. It was infected with the hairy grain beetle in storage for eight weeks. The results revealed that when silver nanoparticles were employed, the predicted rate of adults was the lowest, equivalent to (26.9) adults. After 8 weeks of treatment, the average calculated adults for the fungal filtrate and the spore suspension were (361.8 and 100.5) adults per bag, respectively, compared to the control treatment, which was (405.7) adult \bag. The lowest rate was determined after one week of treatment (14.5) adult \bag, while the highest rate was determined after eight weeks of treatment (311 adults). Commercial isolate provided the lowest rate in spore suspension, fungal filtrate, and silver nanoparticles in terms of the effect of isolation. After 8 weeks of treatment, it reached (74.5, 353.8, and 22.3) adult \bag, compared to the local isolate, which reached (126.5, 369.8, and 31.5) adult \bag. This is consistent with the conclusion of (2) that the silver nanoparticle suspension had a repellent effect in various concentrations on Adult insect T. castaneum, especially at concentrations (0.25, 0.5 , 1) ppm, reaching (46.67 , 66.67 , 63.33 ,76.67) % respectively.

Table 2. Treating jute bags with spore suspension, fungal filtrate, and nanoparticles in preventing *T. granirum* infections during storage.

Treatment	Isolate	Beetle Number/Week							
		1	2	3	4	5	6	7	8
Spore Suspension	Commercial	3.3	29	48.3	62.3	76.7	93.3	112.7	170
	Local	11.7	19.3	88.3	123.3	167.7	186.7	198.3	216.7
Fungal Filtrate	Commercial	15	110	259	433.3	460	493.3	523.3	536.7
	Local	26.7	11.3	393.3	436.7	465	515.7	550	560
Silver Nanoparticles	Commercial	2	2	10.7	11	28	36.3	41.7	47
	Local	2.7	5	16.7	18.7	43	50.7	55	60.3
Control		40	196	420	440	483.3	520	560	586
Rate Time Effect		14.5	53.2	176.6	217.9	246.2	270.9	291.6	311
LSD for isolate = 8.34; treatment = 10.44; Time = 9.38									

According to the results, silver nanoparticles were more effective in reducing the density of red-rust flour beetle and hairy grain beetleworking as a deterrence against adult invasion. The results additionally demonstrated that infection with *S. oryzae* beetles decreased promptly after storage with silver nanoparticles formed by *M. anisopliae* fungus (20). Al-Taee (2) discovered that when the bags were coated with nanoparticles, marjoram oil applied on the nanoparticles provided protection from infection by the rust-red flour beetle in the storage .

conclusion

conclusion environmental safety is of paramount importance in protection against storage insect pests. The green synthesized nanoparticles have several advantages such as easily degradable, cheaper and eco-friendly, not toxic to non-target organisms and they are plant-derived. SNPs can be easily synthesized and used as an effective nano-insecticide for mosquito control. In the present research, entomopathogenic fungal-derived SNPs have been found highly useful for controlling both *T. granarium* and *T. castaneum* in the storage... From this study we concluded that *M. anisoplae* -derived silver nanoparticles (SNPs) were safer and not toxic to non-target organisms.

Conflict of interest

The authors declare no conflict of interest.

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