Egyptian Journal of Plant
Protection Research Institute
www.ejppri.eg.net

Myco- silver nanoparticles synthesized using *Beauveria bassiana* and *Metarhizium brunneum* as a smart pest control

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ARTICLE INFO
Article History
Received:15/5/2018
Accepted: 20/7/2018

Keywords
Nanotechnology,
silver nanoparticles,
Beauveria bassiana ,
Metarhizium brunneum and
Tetranychus urticae.

Abstract:

Nanotechnology has recently been considered as a modern potential tool for crop protection at the nanoscale level. Biosynthesis of silver nanoparticles by using the entomopathogenic fungi, *Beauveria bassiana* (*Bb*) and *Metarhizium brunneum* (*Mb*) is ~~as~~ an eco-friendly and cost effective production system. The produced nanoparticles revealed a brownish color that is characteristic for silver nanoparticles. *Bb*-synthesized AgNPs and *Mb*-synthesized AgNPs were produced at various concentrations and characterized by UV-Vis spectrophotometer and Dynamic Light Scattering (DLS). The variation of hydrodynamic diameter (D_h) of silver particles at various concentration of culture in conjunction with UV-Vis spectra showed that production of AgNPs was maximized when using 15% of culture for both fungi and the size of particles was around 87 nm for both. The efficacy of *Bb*-synthesized AgNPs and *Mb*-synthesized AgNPs against the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) were tested. Results demonstrated that the treatments of either *Bb*- or *Mb*-synthesized AgNPs were found to be highly significantly virulent toward newly emerged adult female of *T. urticae*.

Introduction

Nanotechnology has increasingly developed as an effective, novel tool, generating various promising applications in different active fields at the nanoscale level. Such areas include: electrochemical sensors, biosensors, pharmacology, medicines, agriculture, food industry and pest management (Kim *et al*., 2007; Bhattacharyya *et al*., 2010; Rai and Ingle, 2012; Tarafdar *et al*., 2013; Bengalli, 2016 and Sayed *et al*., 2017a). Nanoparticles are materials at nanoscale levels that usually range in dimension from 1-100 nanometers (nm). They possess unique physico-chemical, optical and biological properties which can be manipulated suitably for desired applications (ISO, 2010). There is a growing interest in the synthesis of metallic

nanoparticles such as silver that are involved in several applications (Wei *et al*., 2015). Silver is known to have been used by the Persians, by ancient Phoenicians, Greeks, Romans and Egyptians for treatment related to bacterial infections (21-22) (Alexander, 2009).

Recently, the use of silver in nano-particulate form (AgNPs) is one of the most vital nanomaterials among several metallic nanoparticles. It has gained more importance due to their potential properties, as well as the increase in the development of products that contain nano-silver in the fields of biology, biotechnology, medicine, chemistry and agriculture (Sharma *et al.*, 2009; Wei *et al*., 2015 and Sandhu *et al*., 2017).

Various chemical and physical methods have been used for synthesis of metal nanoparticles. However, these methods are more expensive, highly energy-consuming and potentially toxic to the environment. It is necessary to develop alternative, eco-friendly methods. Therefore, biological synthesis method that are based on bacteria, fungi, yeast, actinomycetes, algae, viruses, bioderived chemicals and plant extracts have the ability to synthesis various types of nanoparticles and could be more advanced than other methods. Biological synthesis methods are cost effective, biocompatible, environmentally friendly approaches for nanoparticles synthesis without using hazardous materials, easily scaled up for large scale synthesis and there is no need to use high pressure, energy, temperature and toxic chemicals (Thakkar *et al*., 2010; Castro-Longoria *et al*., 2011; Sathya and Ambikapathy, 2012 and Sunkar and Nachiyar, 2013).

Microbial synthesis using fungi provide wide advantages over other methods using plants or bacteria. Fungi are easy to handle in the laboratory, require simple nutrients and possess high wall-binding capacity. In addition, they could be used as a source for the production of large amounts of nanoparticles. Various fungal species are gaining more attention in synthesizing different kinds of metal nanoparticles. These species show great potential, since they secrete large amounts of enzymes and are easy to use in the laboratory, which lead to higher yields of nanoparticles (Ahmed *et al*., 2003; Bhainsa and D'Souza, 2006; Mohanpuria *et al*., 2008; Dhillon *et al*., 2012; Banu and Balasubramanian, 2014; Soni and Prakash, 2012; Roy *et al*.,2014; Wei *et al*., 2014 and Amersan *et al*., 2016).The mechanism of synthesis of metal nanoparticles by microbes is not clearly explored.

Among these fungi are the anamorphic entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium brunneum* (Metschnikoff) Sorokin. Both fungi belong to the order Hypocreales (Ascomycota), infecting a wide range of pests and having cosmopolitan distributions (Roberts and St. Leger, 2004 and Rehner, 2005). Much effort has been put into research on the development of *B. bassiana* and *M. brunneum* as biological control agents to be applied in agriculture and forestry.

Indeed, nanotechnology has offered potential solutions for many problems to revolutionize a wide array of applications in the fields of biomedicine and pest management (Benelli and Lukehart, 2017 and Athanassiou *et al*., 2018). Rai and Ingle (2012) concluded that nanotechnology can provide green and eco-friendly alternatives for pest management without harming nature. Nano-pesticides, nanofungicides and nanoherbicides are being used in agriculture (Owolade *et al*., 2008 and Athanassiou *et al*., 2018). The mycosynthesis of metal NPs has also revealed interesting prospects for the management of certain insect pest species (Amerasan *et al*., 2016).

However, this technology has been studied particularly in the research of larvicides with potential focus on the protection against mosquito vectors or ticks of veterinary importance (Salunkhe *et al.*, 2011; Amerasan *et al.*, 2016; Prabakaran *et*

al., 2017 and Benelli, 2016). Little information is available about the toxicity of nanoparticles against other pests such as phytophagous mites.

In this research, we have made an attempt to present a rapid and eco-friendly approach of silver nanopartes production using two fungal species: free cell filtrates of *B. bassian*a and *M. brunneum*. In addition, we are submitting the preliminary results of toxicity of biosynthesized silver nanoparticles against the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae).

Materials and methods

1. Materials:

Beauveria bassiana strain GHA (ARSEF6444) was originally isolated from *Diabrotica undeciumpunctata* Barber (Coleoptera: Chrysomellidae) in Corvallis, Oregon, USA, and was obtained from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) in Ithaca, NY. The GHA strain is the active ingredient of a commercial product marketed by LAM International (BotaniGard® ES, Butte, MT, USA). *Metarhizium brunneum* (Petch) strain F52 was first cultivated from the codling moth *Cydia pomonella* in Austria. F52 has been incorporated into a commercial product Met52® (ATCC 90448, Novozyme Biologicals, Salem, VA, USA).

Silver nitrate ($AgNO_3$, 99%) was obtained from Fisher Scientific Co., Fair Lawn, NJ, USA. Commercial silver nanoparticles were purchased from Sigma-Aldrich as silver, dispersion nanoparticles, 10 nm particle size, 0.02 mg mL^{-1} mass concentration, in aqueous buffer containing sodium citrate as stabilizer. All chemicals used were purchased from Sigma-Aldrich unless otherwise stated. All suspensions were prepared in either sterilized deionized water or Milli-Q water.

2. *Beauveria bassiana* and *Metarhizium brunneum* cultures:

Fungal growth and media preparations were carried out based on the method previously described by Sayed and Behle (2017b) and Behle and Jackson (2014). Briefly, stock cultures of both fungi were grown on potato dextrose agar (PDA) media (Difco, Detroit, MI, USA) in Petri dishes for 3 weeks at 25 ± 2 ºC with a 12:12 h (L:D) photoperiod until sporulation. Each fungal conidia were harvested by scraping plates with 10 mL of sterile aqueous solution of 0.04% polyoxyethylene sorbitan mono-oleate (Tween 80, Sigma, St. Louis, US) using a fine sterile loop (Fisherbrand™ Disposable Inoculating Loops, Fischer Scientific, Pittsburg, PA, USA). Stock culture each was used to inoculate the culture medium at an initial concentration of 1.4×10^5 conidia mL^{-1} for *B. bassiana* and 1.1×10^5 conidia mL^{-1} for *M. brunneum*. Conidia concentrations were measured light microscopically (400 magnification) with Nomarski optics (BH2, Nikon America, Center Valley, PA, USA) using a hemacytometer (Bright-line, Hausser Scientific, Horsham, PA). Each whole culture fungi (1 L) was grown in 5 L baffled Erlenmeyer flasks (Bellco Glass, Vineland, NJ, USA) with 200 mL of the liquid media. This liquid media was incubated in a rotary shaker incubator (INNOVA 4000, NewBrunswick Scientific, Edison,NJ) for 4 days at 28º C and 350 rpm.

The liquid media used for *B. bassiana* and *M. brunneum* cultures contained basal salts as described by Jackson *et al.* (1997) which were supplemented with glucose (Fisher Scientific) at 80 g L^{-1} (40%) carbon (C) and acid hydrolyzed casein (derived from bovine milk, Hy-case™ MSF, Kerry Bioscience, New York, NY, USA) at 25 g L^{-1} (8.5% nitrogen (N) and 53% C), which produced a medium with a carbon-to-nitrogen ratio (C:N) of 23:1 and ~~had~~ an initial pH of 5.3. The basal salts per liter were as follows: KH_2PO_4, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CoCl_2 \cdot 6H_2O$, 37 mg; $FeSO_4 \cdot 7H_2O$, 50 mg; $MnSO4 \cdot H_2O$, 16 mg; $ZnSO_4 \cdot 7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thioctic acid, 500 mg each; folic acid, biotin, vitamin B12, 50 mg each. Glucose stock solutions (20% w/v) were

autoclaved separately and added prior to inoculation. Sterilization of liquid cultures and glucose stock solutions were performed at 121ºC for 20 min.

Both cultures were inoculated with conidial suspensions in the liquid culture medium. Flasks were hand-shaken frequently during the fermentation process to minimize mycelial growth and sporulation on the flask walls. For quality assurance, the fermentation broth was streaked onto nutrient agar plates, incubated for 48 hours at 30 ºC, and visually evaluated for bacterial contamination. In all experiments, pH was uncontrolled during culture growth. The final 4-d-old fermentation product of 1 L was expected to contain 35.3×10^8 conidia mL^{-1} for *B. bassiana*, 6.3×10^8 conidia mL^{-1} for *M. brunneum* with 20-25 g solids L^{-1} each. The whole fungal cultures were each stored at 4°C until used.

3. Biosynthesis of silver nanoparticles:

After growing *B. bassiana* and *M. brunneum* for 4 days, the whole fungal products were blended in a blender (Kitchen-Aid, St. Joseph, MI, USA) at high speed for one minute and filtered through Whatman #1 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK). Then, they were washed thrice in sterile distilled deionized water to remove any nutrient media that might interact with the silver ions. The resulting filter cake from each cultural fungal product was spread over baking sheets and allowed to air dry under an air drying chamber with lateral air inflow and controlled RH atmosphere (RH 50–60%) for 24 h to achieve moisture less than 4% (w/w) (Jackson and Payne, 2007). Water activity of theses dried conidia were measured with a water activity analyzer (Aqua Lab Model Series 3, 4TEV, Decagon Devices, Inc., Pullman, WA, USA). Dried conidia preparations were stored in 50 mL conical tubes at 4 °C until used. Approximately 10 g of each fresh fungal biomass was transferred to a 500-mL baffled Erlenmeyer shaker flasks containing 100 mL milli-Q water or sterilized deionized water. These flasks were incubated in a rotary shaker incubator for 3 days at 25º C and 200 rpm. The whole fungal products were filtered through Whatman #1 filter paper to obtain cell-free filtrates.

In a typical procedure, stock solution of silver nitrate ($AgNO_3$) was dissolved in sterilized deionized water at a concentration of 0.01 *M* (1.69 g L^{-1}). Various desired volume concentrations of the cell-free filtrates of each fungus were added to 250 mL baffled Erlenmeyer shaker flasks (each containing 5 mL of 0.01 *M* $AgNO_3$ solution) to provide 50 mL total volume of the whole mixture suspension. The final twelve treatment concentrations of 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, and 80% of each fungal cell-free filtrate were obtained with the final constant concentration of silver nitrate at 1 m*M* (0.169 g L^{-1}). Flasks containing the whole mixture suspensions of both fungi were incubated at 25 ºC in a rotary shaker incubator (INNOVA 4000, New Brunswick Scientific Co., Enfield, CT, USA) at 200 rpm for 5 d in ~~a~~ the dark to avoid any photochemical reactions during the experiment and until complete bioreduction of silver ions was achieved. Simultaneously, two 250 mL baffled Erlenmeyer shaker flasks, one containing only broth medium (50 mL) without silver nitrate solution, and the other consisting of only silver nitrate solution (50 mL), were maintained under similar experimental conditions as a control. These experiments were done with both fungi in triplicates.

According to visual observation, the cell-free filtrates of each fungus maintained in the presence of silver nitrate showed a color change from yellow to brown, whereas no color change could be observed in broth culture of fungi without silver nitrate and silver nitrate solution without the cultures. These control experiments indicate that the Ag^+ ions' reduction is not just a thermal process. The bioreduction of the Ag^+ ions in the samples and color change of the resulting solution were monitored, thus indicating the formation of silver nanoparticles. The

filtrates of *B. bassiana* and *M. brunneum* derived silver nanoparticles were named as *Bb*-AgNPs and *Mb*-AgNPs, respectively. The presence of *Bb*-synthesized silver nanoparticles (AgNPs) and *Mb*-synthesized silver nanoparticles was confirmed by UV–vis spectra, at the wavelength of 300–800 nm in the UV–vis spectrophotometer. The difference in color depends on size and shape of the nanoparticles formed as reported previously by Wiley *et al.*, 2006; Soni and Prakash, 2012 and Prabakaran *et al.*, 2017) .

After incubation, the silver nanoparticles obtained by these treatments were purified by centrifugation at 10,000 × g for 10 min, and then freeze-dried. The obtained AgNPs were weighed and stored at 4 ºC until used as described by Ingle *et al.*, 2008 and Soni and Prakash, 2012). Sterilized deionized water or Milli-Q water was used to suspend the *Bb*-AgNP or Mb-AgNP powders for the characterization and bioassay of prepared silver nanoparticle. Dynamic Light Scattering (DLS) experiment was performed for the measurement of particle size of *Bb*- and *Mb*-synthesized AgNPs. The concentration of silver nanoparticles was calculated according to the equation of Liu *et al.* (2007).

4. Characterization of AgNPs:

The nanoparticles were characterized using UV-Vis, Dynamic Light Scattering (DLS), and Fourier-transform infrared (FT-IR) spectroscopy. The synthesized silver nanoparticle suspensions at different percent concentrations were recorded via UV-Vis spectrum analysis. The change in spectra of these solutions was monitored in the range of 300-800 nm. UV-Vis spectra were obtained using a spectrophotometer (UV-2600, Shimadzu Scientific Instruments, Kyoto, Japan). Since each of *Bb* or *Mb* showed very strong absorption in the range of 200-500 nm, the UV absorption peaks of silver nanoparticles were isolated through deconvolution of the obtained spectra. Otherwise, the color changes of reaction mixtures were used as evidence for AgNP formation. The size distribution and average size of the synthesized AgNPs was measured by DLS (NanoBrook Omni Particle Size Analyzer, Brookhaven Instruments Corp., Holtsville, NY, USA).

The characterization of functional groups on the surface of AgNPs was performed by Fourier-Transform Infra-Red (FT-IR spectrum) spectroscopy. Measurements of the samples were monitored using a FT-IR spectrometer (Varian Escalibur 3100, Varian Inc., Randolph, MA) with diffuse reflectance mode (DRS-800) attachment. The bio-transformed products were diluted with potassium bromide in the ratio of 1:100. All measurements were carried out in the range of 400–4,000 cm-1 at a spectral resolution of 4 cm-1 (Prabakaran *et al*., 2016 and Amerasan *et al*., 2016). Unless otherwise stated, the synthesized silver nanoparticle was measured for more than three independent runs for all experiments using the cultures of *B. bassiana* and *M. brunneum* with the same procedures.

5. Mite colonies:

Mitcidial activities of the treatments were determined. Mite mortality was determined using adult female of mite *T. urticae* from laboratory colonies maintained at the Agricultural Research Center, Ismailia Agricultural Research Station, Ismailia, Egypt. The batches of mite colony were reared in the laboratory under constant conditions at 25 ± 2 °C, 60 ± 5% RH, and a photoperiod of 16: 8 (L:D) h away from any pesticide contaminations according to the method adapted by El-Esnawy *et al*. , 2012. Sweet potato cuttings (*Ipomoea batatas* (L.) Lam, family convolvulaceae, cv. 195A) with about 7 leaves each were used as a source of food. These cuttings were kept in 250 mL glass jars filled with tap water; four cuttings were placed in each jar. These cuttings were changed twice weekly in summer and once every week in winter. The old infested cuttings were placed on top of the new cuttings for a couple of days to facilitate mite transfer onto the new plant and avoid losing

selected mites in the process. The colony was established with five jars, and kept in special cages (60 X 60 X 60 cm) covered with cheese cloth (muslin) and provided with fluorescent light tubes of 40 watts to give constant illumination (16 hours/day).

6. Bioassay:

Leaf disk bioassays were performed to estimate the median lethal concentrations (LC_{50}) for the obtained *Bb*-AgNPs and *Mb*-AgNPs against newly emerged adult females of *T. urticae*. To obtain fixed-age females for the bioassay, quiescent deutonymphs were collected from the mite colony and isolated on fresh leaf discs. Sterilized deionized water or Milli-Q water was used to dissolve the samples of *Bb*-AgNPs or *Mb*-AgNPs to desired concentrations. For the freshly made *Bb*-AgNPs and *Mb*-AgNPs, six suspensions of 150, 125, 100, 75, 50 and ~~50~~ 25 ppm were prepared. The untreated control consisted of four solutions of 20, 10, 5, and 2.5 ppm of commercial silver nanoparticles (Sigma-Aldrich); four dosage dilutions of 170, 17, 1.7 and 0.17 ppm of $AgNO_3$ solution; and five concentrations of conidial serial suspensions of each *Bb* and *Mb* only ranging from 10^8 to 10^4 conidia mL^{-1}. Only liquid broth media of *Bb* and *Mb* and sterilized deionized water served to assess mortality. Leaf disks (3 cm diameter) were excised with a cork borer from 8-10 week old field-grown sweet potato. Disks were placed top-side up on water-saturated cotton pads in a Petri dish (90 mm diameter). Each dish contained 2 disks and each dish represented a replicate. Each leaf disk was immersed individually for 5 seconds with gentile agitation and allowed to air dry. Once treatments were dry, each disk was infested with 10 newly emerged adult females using a fine brush (Pelikn brush No. 000) and incubated (WTC binder, 7200 Tuttlingen, Germany) in the dark at 25^o C and 60-70% relative humidity for the desired exposure period. After incubation, the numbers of live and dead adult female mites were counted to calculate the activity of each treatment. For both exposure nanoparticles, adult females were considered dead if they did not respond when touched with a fine brush. The entire experiment was repeated three times on different dates using different mite cohorts.

7. Data analysis:

Data of bioassay mortalities was log transformed prior to analysis of variance to meet the normality assumptions and provided the best fit due to its lowest deviance. Dose response results obtained in bioassays were subjected to probit analysis using Probit Analysis-MSChart 2009 software (Chi, 2009) to calculate the median lethal concentrations of LC_{50} with its corresponding fiducial limits (95% FL) and slope for each. Mortalities due to *Bb*-AgNP and *Mb*-AgNP treatments were based on the mean of four replicates of dishes with 80 newly emerged adult females each, which were carried out three times on different dates using different mite cohorts with a total of 240 females for each treatment. Controls were included with each assay to indicate handling mortality, and thus data were not corrected for control mortality. Analysis of variance was used to determine the efficacy of each *Bb*-AgNP and *Bb*-AgNP treatments on the newly adult female stage of *T. urtiacea.* Treatment means were separated using the least significant difference (LSD) test at *p-value* 0.05 with the statistical software SPC for Excel (Knoware International, Inc., Denver, CO, USA).

Results and Discussion

1. Synthesis of *Beauveria bassiana and Metarhizium brunneum*-silver nano-particles (*Bb* and *Mb*-AgNPs):

Based on visual observation, when the free-cell fungal filtrates of *Bb* and *Mb* were mixed in the aqueous solution of silver nitrate $AgNO_3$, the color of the reaction mixture solutions changed from pale yellow to brownish due to reduction of silver ion, which indicated formation of silver nanoparticle *B. bassiana* - synthesized silver nanoparticles (*Bb*- AgNPs) and *M. brunneum* -synthesized silver nanoparticles

(*Mb*- AgNPs) as illustrated in Figure (1). Figure (1) highlights the change in color intensity of synthesized *Bb* or *Mb* –AgNPs, which increased with duration of incubation. The color of the solution

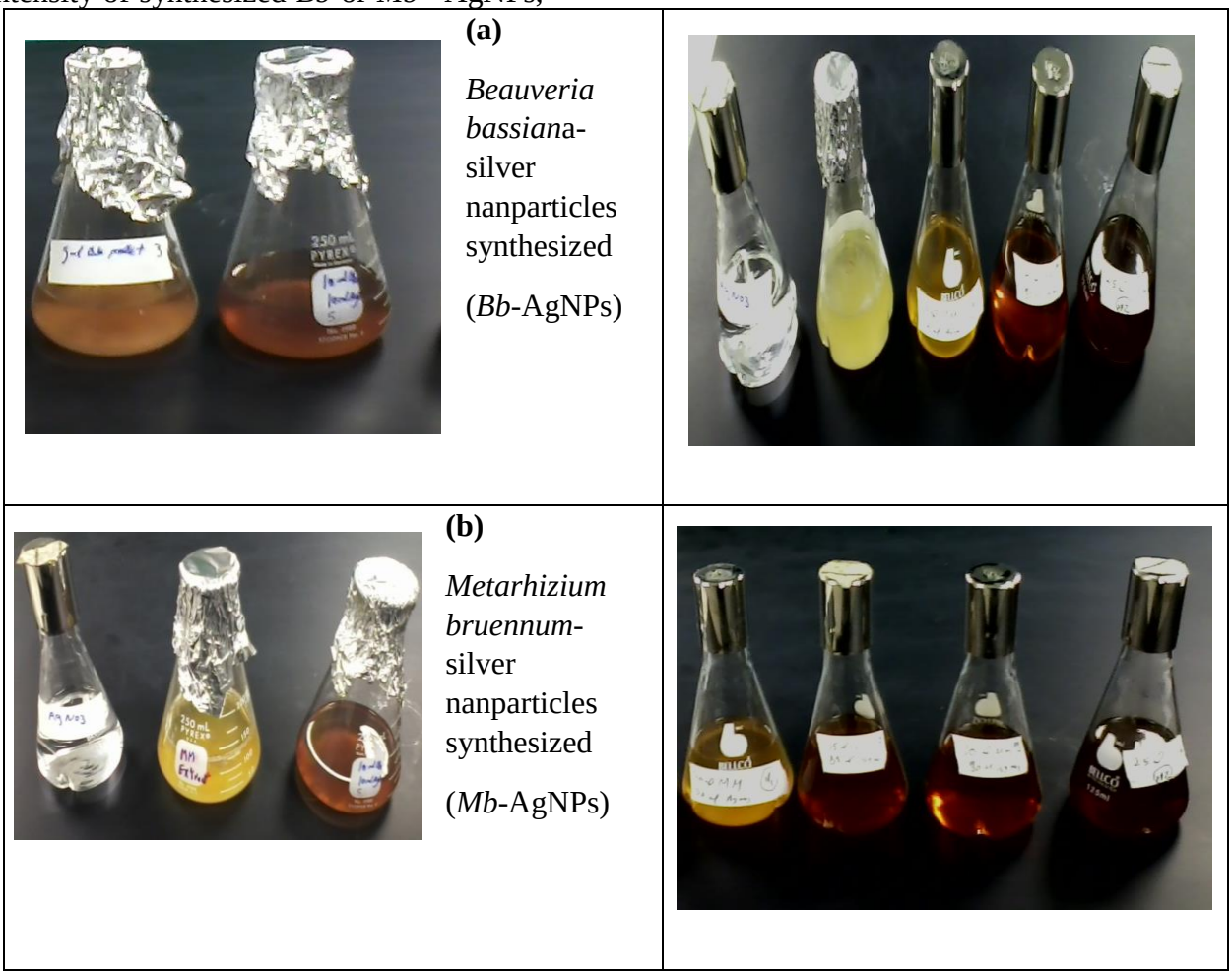

Figure (1): a, b, The change in color intensity in cell free filtrate of *Beauveria bassiana* and *Metarhizium bruennum* after exposure to silver nitrate of synthesized a=*Bb* - AgNPs or b=*Mb* -AgNPs increased with duration of incubation.

changed to dark brown after 5 d of incubation for the synthesis of nanoparticles as the concentration of *Bb* or *Mb* filtrates increased to 10% in the solution, then faded as the concentration of *Bb or Mb* exceeded 15%. In the case of the samples of fungal cell free filtrates without $AgNO_3$ or broth media of Bb and Mb or $AgNO_3$ solution alone, no color development was observed. Silver nanoparticle concentrations were characterized by UV spectrophotometry. The measurement of UV-Vis spectra for the silver nanoparticles was performed in the range of 300-700 nm, as illustrated in Figures 2 and 3. The analysis of UV-Vis spectra showed an appearance of a surface plasmon resonance peak (SPR) at 300-700 nm wavelength range, which corresponds to silver nanoparticles formation for both *Bb*-AgNPs and *Mb*- AgNPs. As the amounts of fungal cell free filtrates of *Bb* and *Mb* increased in the solution, higher absorption peaks were observed, indicating that there were increases in the reduction of silver precursor to silver nanoparticles. However, further increase in the amounts of fungal filtrates of *Bb* and *Mb* lowered the production of silver nanoparticles. In order to see the overview of this behavior, the signal intensity of UV-Vis spectra for each sample was integrated for the 300-700 nm range and plotted against the amount of Bb

and Mb. These data indicate that the development of color is maximized when the concentration of Bb or Mb was 10-15%. The size of the silver particles in each sample was analyzed by DLS measurement (Figures 4 and 5). This result illustrates the relationship between the hydrodynamic diameter (Dh) of produced silver nanoparticles and amount of Bb or Mb. As shown in Figures (4 and 5), the amount of silver nanoparticles increased until the concentration of fungal filtrates increased to around 15%. Therefore, combining the UV spectrophotometer and DLS data sets shows that the production of silver nanoparticles increased until the amount of Bb or Mb increased to around 15% without significant change in the size of silver nanoparticles. Further increase in the concentration of fungal filtrates caused a rapid increase in the size of particles in the solution. Since UV data showed that the concentration of silver nanoparticle decreased with higher fungal filtrate concentrations, the increased size of particles indicated that the produced silver nanoparticles were bound together, yielding aggregated nanoparticle product.

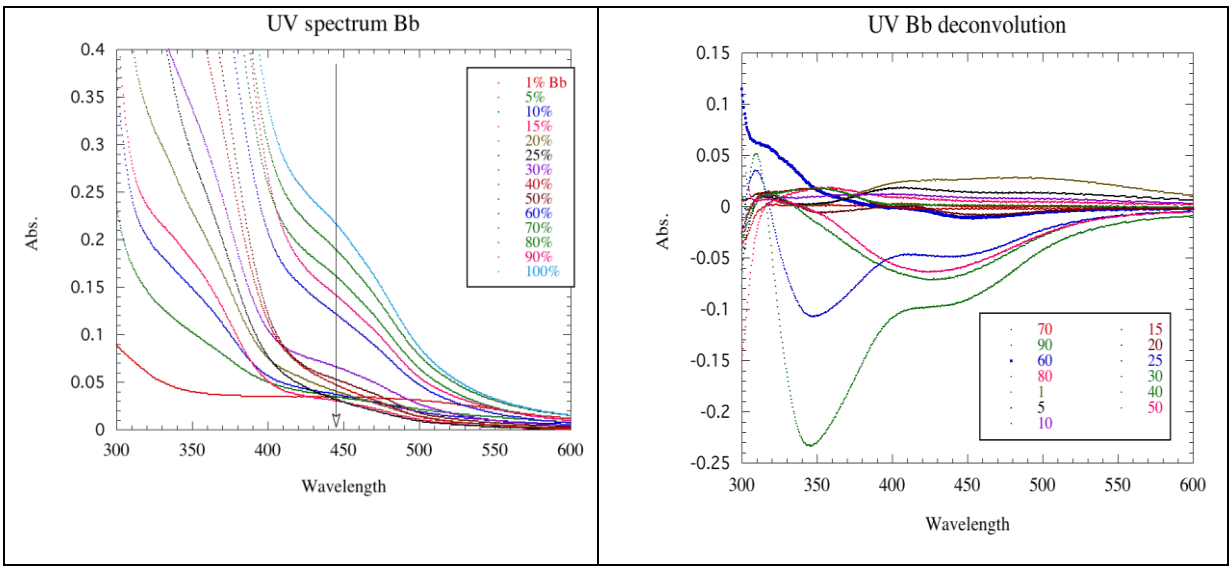

Figure (2): UV-Vis spectra response indicating the development of silver nanoparticles by *Beauveria bassiana* .

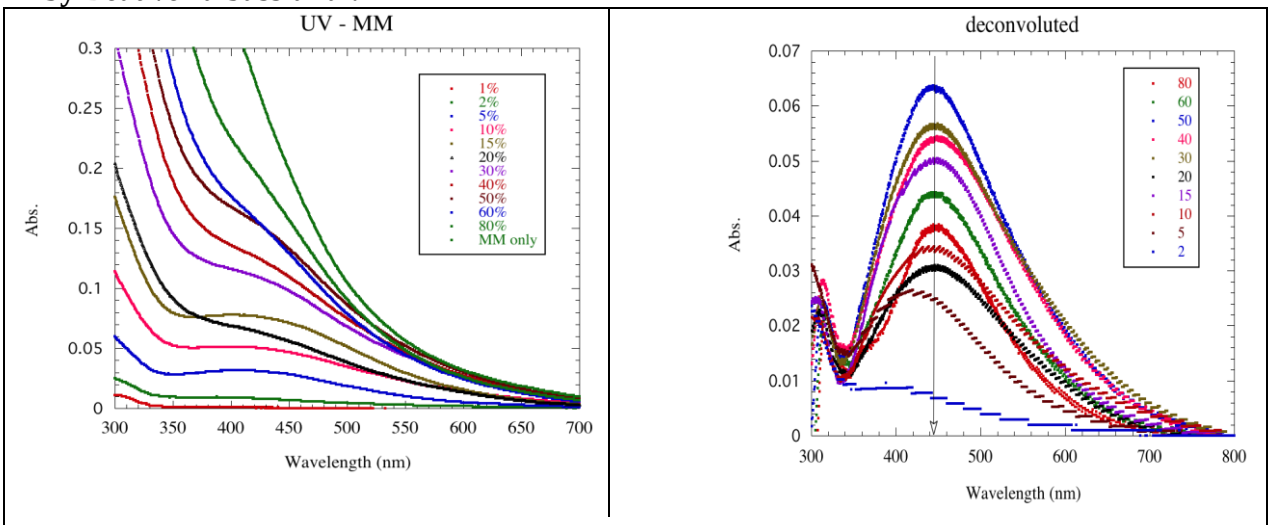

Figure (3): UV-Vis s **of silver nanoparticles by *Metarhizium brunneum***

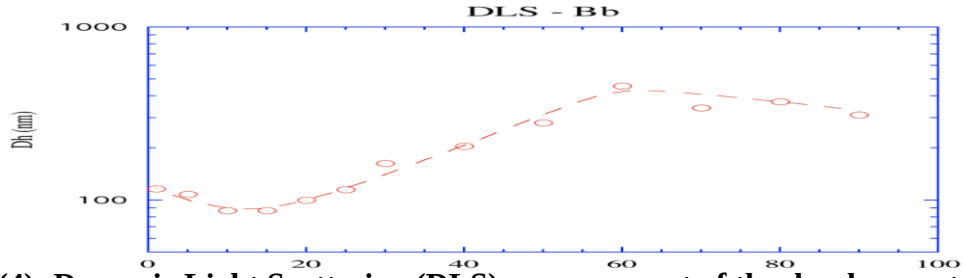

Figure (4): Dynamic Light Scattering (DLS) measurement of the development of silver nanoparticles by *Beauveria bassiana.*

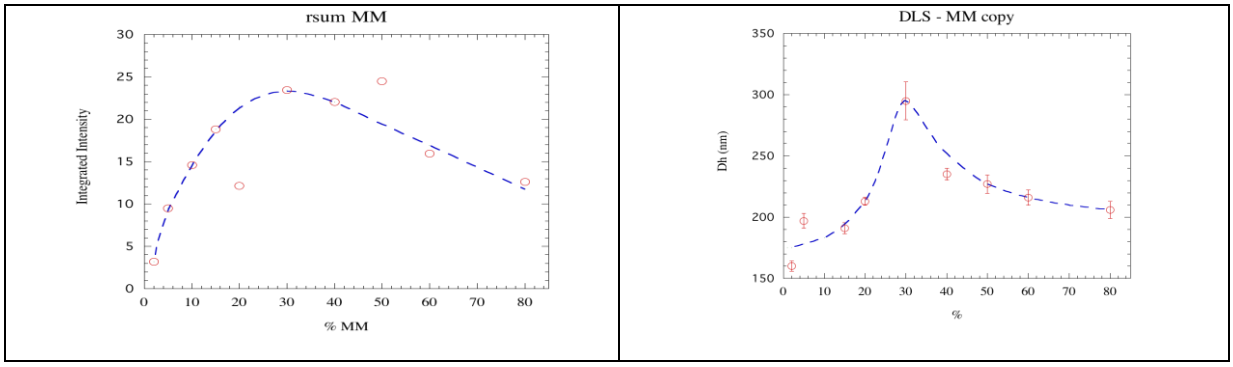

Figure (5): Dynamic Light Scattering (DLS) measurement of the development of silver nanoparticles by *Metarhizium brunneum* .

The presence of *Bb*-AgNPs or *M*b-AgNPs was confirmed and measured by UV–vis and DLS. As the concentration of fungal free cell filtrates increased, the observed particle size decreased and the UV absorption increased. These findings reveal that the average particle size of sample decreased as more silver nanoparticles were produced. The trend continued until 15% of *Bb* or *Mb* filtrates was added to the sample. The size of particles in silver nitrate/*Bb* filtrate was similar to that in silver nitrate/*Mb* filtrate sample. At higher concentrations of *Bb* filtrates, the overall behavior of *Mb* filtrates was similar to that of silver nitrate/*Bb* filtrate, i.e., the produced nanoparticles were bound together to form larger particles (Figure, 6).

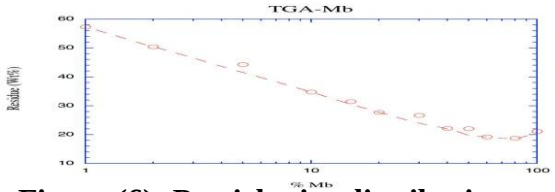

Figure (6): Particle size distribution curve of silver nanoparticles obtained by *Metarhizium brunneum.*

Although the DLS data for Bb and Mb systems looked quite different, both data showed the inflection point at the same concentration of 15% and the size of particle was around 87 nm for both. FT-IR spectroscopy analysis was carried out to identify the potential functional groups present in the bioactive molecules which may play a role in the formation and stability of AgNPs synthesized using the fungi Bb and Mb. The absorption spectra peaks of the Bb-AgNPs and Mb-AgNPs synthesized were elucidated in Figures (7 and 8). Figure (7) shows the absorbance bands analysis in bio-reduction of AgNPs synthesized by fungal cell filtrate of B. bassiana were mainly located at 3304.05 cm−1 for O–H hydroxy group, 2358.49 cm−1 for methylene C–H stretch, 1641.42 cm−1 for stretching carbonyl modes of –C–O and –C–O–C, 1396.46 cm−1 for –CH3 nitro compounds, and 1076.28 cm−1 for C-N stretching vibrations of aromatic and aliphatic amines. The band values at 729.24, 680.87, 594.01, and 534.81 cm−1 (aliphatic iodo-compounds, –C–H alkynes, and C–I stretch, respectively) were related to fungi-borne functional groups. On the other hand, the FT-IR spectrum of Mb-AgNPs indicated

absorption peaks located at positions 3437, 1635, 1376, and 1053 cm−1 (Figure, 8). Absorption bands are due to vibration of chemical bonds, and can be assigned to hydroxyl, carbonyl, aliphatic amine, or carboxylic acid groups involved in the reduction of AgNO3 to Ag+. These bending peaks were entirely different from corresponding control samples and may describe the presence of protein molecules involved in the reduction of AgNO3

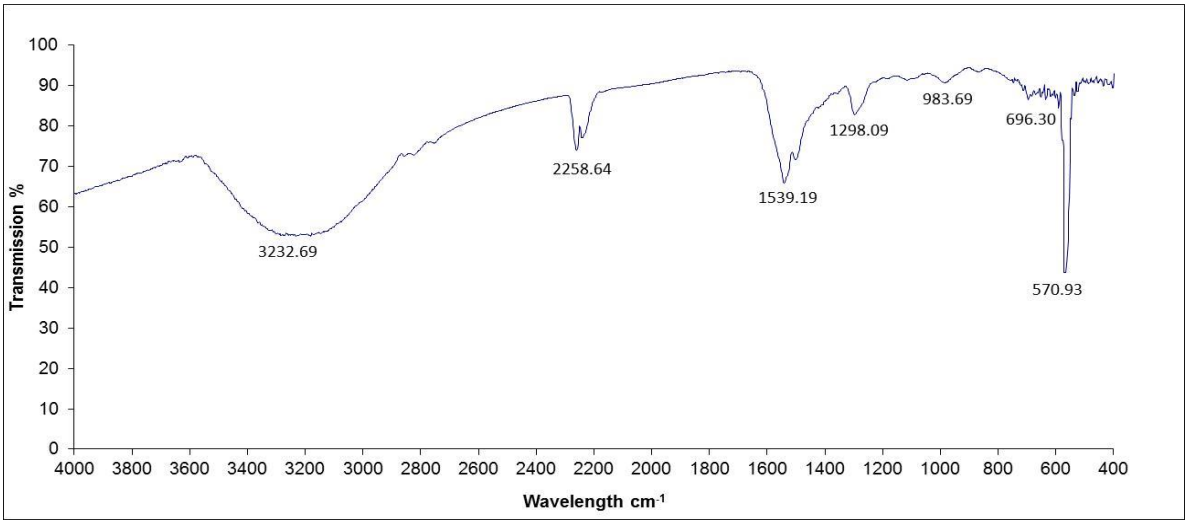

Figure (7): Fourier transform infrared spectrum (FT-IR) of vacuum-dried powder of myco-synthesized silver nanoparticles using *Beauveria bassiana* filtrate solution.

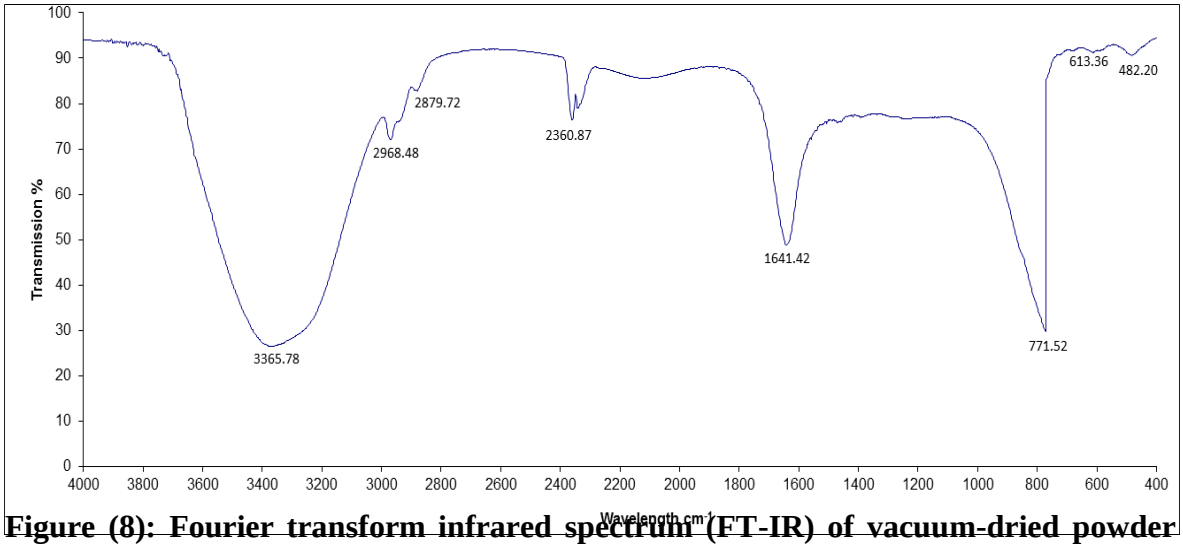

Figure (8): Fourier transform infrared spectrum (FT-IR) of vacuum-dried powder of myco-synthesized silver nanoparticles using *Metarhizium bruennun* filtrate solution.

2. Mitcidal activity of *Bb*-AgNPs *and Mb*-AgNPs:

The efficacy of the myco-synthesized *Bt*-AgNPs and *Bt*-AgNPs treatments against newly adult females of the two-spotted spider mite, (*T. urticae*) were tested based on dosage response data to assess relative LC_{50} values. Experimental conditions and LC_{50} values are noted in Table (1). The LC_{50} values of *Bb*-AgNPs and *Mb*-AgNPs compared after 24 h were estimated to be 35.45 and 41.22 ppm for *T. urticae*, respectively. There were greatly significant differences between adult female mortalities of *T. urticae* at various concentrations of *Bb*-AgNPs produced using *B. bassiana* ($F_{5,71}$ = 16.46, P < 0.0001*)* and *Mb*-AgNPs produced with *M. brunneum* ($F_{5,71}$ = 16.04, P < 0.0001*)*. There were no significant differences between *Bb*-AgNPs and *Mb*-AgNPs ($F_{1,10}$ = 0.16, P < 0.7*)*. However, it is noted that silver nanoparticle treatments using *M. brunneum* were more toxic to the adult female of *T. urticae* than in the case of *Bb*-AgNPs made using *B. bassiana*. The commercially purchased silver nanoparticles (AgNPs) did not kill the tested insects at the applied dosages up to 20 ppm. Furthermore, the highest concentration of 170 ppm of $AgNO_3$ had little or no effect on *T. urticae*. Therefore, it obvious that the efficacy of *Bb*-AgNPs or *Mb*-AgNP*s* against new emerged adult females of *T. urticae* are much more toxic than treatment with *Bb* or *Mb* only.

We desired to create biosynthesis silver nanoparticles with entomopathogenic fungi to investigate the potential use to enhance pest control. As was shown during visual observation, cell free fungal filtrates of Bb and Mb incubated with silver nitrate (AgNO3) showed a color change from yellow to reddish/dark brown. It is noted that the addition of silver nitrate (AgNO3) caused precipitation both inside and outside the microbial cells. We observed reduction of silver ion to form silver particles when silver nitrate was exposed to both fungal species as indicated by the color change of mixtures. Silver nanoparticles exhibit dark brownish color in aqueous suspensions due to the surface plasmon resonance phenomenon, and this color variation confirms formation of the silver nanoparticles. Several studies using different fungal species reported similar trends (Wiley *et al*., 2006; Riddin *et al*., 2006; Ingle *et al*., 2008; Birla *et al*.,2009; Soni and Prakash, 2012; Banu and Balasubramanian, 2014; Amerasan *et al*., 2016 and Kamil *et al*., 2017).

In this finding, formation of silver nanoparticles was investigated by treating silver nitrate with various concentrations of the cell free filtrates of *Bb* and *Mb* to find optimum conditions for the preparation of silver nanoparticles. The relative amount of silver nanoparticles formed in each culture concentration was quantified by using UV-Vis spectroscopy. The optimal production of nanoparticles could be estimated from the UV-Vis spectra that showed the highest integrated intensity (Figures 2 and 3). However, it should be noted that these data contain errors associated with the deconvolution process of raw spectra from the sample solutions. Each spectrum shown in Figures (4 and 5) was obtained by subtracting the spectrum for the culture solution from that for the mixture of culture and silver nitrate solutions. Since the absorbance of the former is much larger than that of the latter, the individual spectrum, especially the spectrum for silver nitrate/*Bt* pellet, was not smooth. As a result, the plot for integrated intensity of spectra showed roughness. DLS data clearly revealed the inflection point, and the physical meaning of this point could be explained by comparing DLS data with UV-Vis data.

The present study emphasizes the use of entomopathogenic fungi for the synthesis of silver nanoparticles with potent biological effect. Several species of fungi have been widely used for the biosynthesis of silver nanoparticle (AgNPs) production, including *Aspergillus fumigates* (Bhainsa and D'Souza, 2006), *Aspergillus niger* (Gade *et al.*, 2008), *Aspergillus oryzae* (Binupriya *et al*., 2010),

Table (1): The insecticidal activity of synthesized of *Metarhizium brunneum* and *Beauveria bassiana* –silver nanoparticles (*Mb*-AgNPs and *Bb*-AgNPs), ideal (standard) silver nanoparticles (AgNPs), and silver nitrate ($AgNO_3$) against newly adult female stage of *Tetranychus urticae*.

Material used	Concentration Ppm	Mortality[a] ±SD	LC_{50}[b] ppm	Fiducial limits		Slope[c]± SD	Chi-square
				Upper	Lower		
***Mb*-AgNP**	**150**	**81.3 ± 3.5**	**35.45**	**24.51**	**51.04**	**1.20 ± 0.13**	**6.60**
	125	**76.3 ± 3.4**					
	100	**67.1 ± 3.7**					
	75	**61.7 ± 2.2**					
	50	**55.4 ± 3.3**					
	25	**46.3 ± 3.1**					
***Mb*-AgNP**	**150**	**77.9 ± 3.7**	**41.22**	**31.01**	**54.65**	**1.17 ± 0.13**	**4.85**
	125	**71.3 ± 2.8**					
	100	**67.5 ± 3.8**					
	75	**56.7 ± 2.9**					
	50	**54.6 ± 2.7**					
	25	**42.1 ± 3.8**					
***Mb* only**	**spores mL^{-1}**		**1.49 E+04**	**1.82 E+01**	**9.43 E+06**	**0.252 ± 0.91**	**1.03**
***Bb* only**	**spores mL^{-1}**		**3.17 E+04**	**1.01 E+01**	**8.00 E+06**	**0.254 ± 0.07**	**1.03**
AgNPs Standard	**20**	**2.9 ± 0.7**					
	10	**1.3 ± 0.4**					
	5	**00**					
	2.5	**00**					
Silver nitrate ($AgNO_3$)	**170**	**7.1 ± 1.4**					
	17.0	**5.0 ± 1.7**					
	1.70	**3.8 ± 1.1**					
	0.17	**2.1 ± 0.7**					
Water		**0.4 ± 0.4**					

[a]Total number of adult female *Tetranychus urticae* tested 240 (three repetitions / four replicates per concentration).

[b]Delivered median lethal concentration (LC_{50}) expressed by *Mb*- or *Bb*-AgNPs ppm and estimated by the logistic model. Mortality censored after one-day application.

[c]Slope for mortality represents regression of proportion of adult female mortality versus $\log_{10}$ of *Mb*- *Bb* -AgNPs or *Bb* -AgNPs ppm.

*Chrysosporium tropicum (*Soni and Prakash, 2012*), Cladosporium cladosporioides (Balaji et al*., 2009), *Coriolus versicolor* (Sanghi and Verma, 2009), *Fusarium solani* (Ingle *et al.* , 2009), *Fusarium oxysporum* (Ahmad *et al*., 2003), *Penicillium* species (Sadowski *et al*., 2008), *Phaenerochaete chrysoporium* (Vigneshwaran *et al.*, 2006), *Phoma glomerata* (Birla *et al*., 2009), *Phytophthora infestans*, *Pleurotus sajor caju* (Nithya and Ragunathan, 2009), *Streptomyces hygroscopicus*, *Trichosporon beigelii* (Ghodake *et al*., 2011), *Trichoderma reesei* (Vahabi *et al.*, 2011), *Trichoderma viride* (Thakkar *et al*., 2010) and *Verticillium* species (Mukherjee *et al*., 2001).

However, the mechanism of myco-synthesis of the intercellular or extracellular synthesis of silver nanoparticles is represented by two main steps: trapping of Ag^+ ions on the surface of fungal cells and subsequent reduction of silver ions by the enzymes present in the fungal biomass (Mukherjee *et al*., 2001). Promising synthesis of nanoparticles appears by the use of specific enzymes or proteins secreted by fungi, *F. moniliformae* (Duran *et al*., 2005) and *F. oxysporum* (Kumar *et al.*, 2015? and Mohanpuria *et al*., 2008). Balaji *et al*. (2009) hypothesized that proteins, polysaccharides and organic acids released by the fungus *C. cladosporioides* were responsible for formation of spherical crystalline silver nanoparticles. The bioreduction of silver ions occurring on the surface of the cells and proteins might have a critical role in formation and stabilization of the synthesized nanoparticles. FT-IR spectra is an important tool for identifying types of chemical bonds in a molecule by making an infrared absorption spectrum that is like a molecular "fingerprint" (Senapat *et al*., 2004), which are responsible for the reduction of the ag+ ions and capping of the bio-reduced silver nanoparticles using the fungal filtrates. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in Figures (7 and 8). The results confirmed the presence of OH/COO– , -C-O-C- and -C=C- functional groups, which may indicate the presence of possible proteins. The proteins could most possibly form a coating which covers the metal nanoparticles, acting as a capping of silver nanoparticles to prevent agglomeration of the particles and providing stability in the medium.

Overall, our FT-IR spectrum indicates that the biomolecules (possibly proteins or enzymes) present in the fungal filtrates are responsible fr synthesis and stabilization of silver nanparticles (Ganesh Babu and Gunasekaran, 2009 and Dhanasekaran and Thangaraj, 2013).

The lethal activity of *Mb*-AgNPs was significantly greater than *Bb*-AgNPs for *T. urticae*, and the mortality rate was dosage dependent at higher concentrations. The functionality of nanoparticles produced by *Mb* is slightly better than that of nanoparticles produced by *Bb* filtrates. Other studies found that acute toxicity using *B. bassiana* and *M. anisopliae* synthesized silver nanoparticles were effective as mosquitocides (Banu and Balasubramanian, 2014; Amerasan *et al*., 2016 and Prabakaran *et al.*, 2016). Therefore, previous studies confirmed the metal nanoparticles are effective against plant pathogens and insect pests. Thus, nanoparticles could be included in the preparation of new formulations of biopesticides (Goswami *et al*., 2010; Bhattacharyya *et al*., 2010; Rai and Ingle, 2012; Kah and Hofmann, 2014; Roni *et al*., 2015; Benelli, 2017; Sayed *et al*., 2017a and Athanassiou *et al*., 2018).

Our research points out that use of *B. bassiana* and *M. brennum* to synthesize silver nanoparticles is a rapid, eco-friendly and easy approach and the produced myco-synthesized nanoparticles can be useful agents to enhance efficacy of pest control programs. Nanotechnology has potential to provide efficient alternatives for the management of pests in agriculture without harming the environment. However, in-depth studies on toxicological impacts of AgNPs to the environment and assorted life forms need to be performed to verify AgNPs as sustainable

biocontrol agents. There is now increased interest to consider potential issues relating to the use of nanotechnology for crop protection by developing nanopesticides that are less harmful to the environment than conventional formulations, in terms of both cost and performance (Kah and Hofmann, 2014). These novel products may present far more effective control of pests with lower quantities of pesticides, reducing the spray application times and promising improved human and environmental safety. Consequently, they could contribute to enhancement of agricultural productivity involving integrated pest management.

Acknowledgment

The authors thank Dr. Robert Behle for his valuable assistance in this research. This cooperative research was made possible by the Egyptian Ministry for Scientific Research, Science & Technology Development Fund, Agricultural Research Center (ARC), Plant Protection Research Institute (PPRI), Egypt and United States Department of Agriculture (USDA), Agricultural Research Service (ARS), National Center for Agricultural Utilization Research (NCAUR), USA.

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