Palestine Technical University Kadoorie(PTUK)

Nanoparticles: Agricultural applications and their role in disease management

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Abbreviations

Symbol	Description
AgNPs	Sliver nanoparticles
B.b	Beauveria bassiana
M.n	Metarhizium anisopliae
A.s	Aspergillus niger
V.l	Verticillium
PDA	Potato dextrose agar
SDA	Sabouraud Dextrose Agar
SEM	Scanning electron microscope
SAXS	Small angle X-ray scattering
DLS	Dynamic light scattering

Acknowledgements

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I wish to express my sincere thanks to the JCNS-2 institute at FZJ for providing me with all the necessary facilities for the research.
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Abstract

Nanotechnology has recently been considered a potential modern tool for crop protection at the nanoscale level. Biosynthesis of silver nanoparticles using living cells such as entomopathogenic fungi, Beauveria bassiana, Metarhizium anisopliae, Aspergillus niger, and verticillium is a promising and novelty tool in bio-nanotechnology. The biological synthesis methods are preferred over chemical and physical methods due to their eco-friendly, clean, safe, easy, and cost-effective sources for high productivity and purity. Detection and characterization of the biosynthesized silver nanoparticles are conducted using different techniques such as scanning electron microscope **(SEM)** and globally using small-angle x-ray scattering **(SAXS)**, and dynamic light scattering **(DLS)**. The particle sizes were measured through SEM imaging ranging from 10 - 30 nm in diameter. SEM results revealed particle size in the range of 10 -30 nm in diameter. The size of the silver nanoparticles mainly depends on the shaking water bath temperature and concentration of entomopathogenic fungi. Temperatures range between 10 and 25 °C in the air where used for the biosynthesis of the silver nanoparticles. For commercial use, additional justifications are needed for the NP synthesis mechanism in order to scale up nanoparticle production. Furthermore, the green synthesis of nanomaterials requires collaboration across basic science, chemical engineering, and industrial media to create novel commercial materials.

1 Introduction

1.1 Nanotechnology

Nanotechnology refers to the branch of science, engineering, and technology that deals with the understanding and control of matter at dimensions in the nanometer scale, approximately 1 and 100 nanometers, where unique phenomena enable novel applications due to the very large surface area to volume ratios experienced at these dimensions and with quantum effects that are not seen with larger sizes.

1.1.1 Nanotechnology in Agriculture

Nanoagriculture refers to the infusion of nanotechnology concepts and principles in agricultural sciences so as to develop processes and products that precisely deliver inputs and promote productivity without associated environmental harm. Nanotechnology provides new agrochemical agents and new delivery mechanisms to increase agricultural production, to improve crop productivity, and it promises to reduce pesticide applications. Nanotechnology includes several applications in agriculture: (1) *nanoformulations of agrochemicals for applying pesticides and fertilizers for crop improvement*; (2) *the application of nanosensors in crop protection for the identification of diseases and residues of agrochemicals*; (3) *nanodevices for the genetic engineering of plants*; (4) *plant disease diagnostics*; (5) *animal health, animal breeding, poultry production*; and (6) *postharvest management* [3]. These days, sustainable agriculture is needed. The development of nano chemicals has appeared as promising agents for plant growth, fertilizers, and pesticides. Recently, nanomaterials have been considered an alternative solution to control plant pests including insects, fungi, and weeds. Several nanomaterials are used as antimicrobial agents in food packing in which several nanoparticles such as silver nanomaterials are of great interest. Many nanoparticles (Ag, Fe, Cu, Si, Al, Zn, ZnO, TiO_2, CeO_2, $Al2O_3$, and carbon nanotubes) have been reported to have some adverse effects on plant growth apart from the antimicrobial properties. In food industries, nanoparticles are leading in forming food with high quality and good nutritive value. There is a need for an alternative approach than pesticides to control pests and pathogens. The application of nanotechnology in crop protection holds a significant promise in managing insects and pathogens, by controlled and targeted delivery of agrochemicals and providing diagnostic tools for early detection [3].

1.1.2 Nanopesticides in Agriculture

1.2 Nanoparticles

Nanoparticles (NPs) are tiny materials having sizes ranging from 1 to 100 nm. They can be of different shapes, sizes, and structures. They can be spherical, cylindrical, conical, tubular, hollow core, spiral, etc., or irregular [5]. The different groups of NPs include fullerenes, metal NPs, ceramic NPs, magnetic NPs and polymeric NPs NPs possess unique physical and chemical properties due to their high surface area and nanoscale size. NPs properties are dependent on their unique size, shape, and structure. Due to these characteristics, they are suitable candidates for various commercial and domestic applications, including catalysis, imaging, medical applications, energy-based research, and environmental and cultural applications [10].

1.2.1 Silver nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) are one of the most vital and fascinating nanomaterials among several metallic nanoparticles that play an important role in nanoscience and nanotechnology, particularly in agriculture. AgNPs have several unique physical and chemical properties which include optical, electrical, and thermal, high electrical conductivity, and biological properties. Due to their peculiar properties, they have been used for several applications, including as antibacterial agents, in industrial, household, and healthcare-related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopedics, drug delivery, as anticancer agents, and have ultimately enhanced the tumor-killing effects of anticancer drugs [2].
AgNPs is the most studied and utilized metallic nanoparticle for bio-system. They have been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities. Furthermore, it has a high surface area and a high fraction of surface atoms, has a high antimicrobial effect as compared to bulk silver. Additionally, Ag NPs have well-known recognition for their antioxidant, antibacterial, anti fungal, anti-viral and anti-inflammatory properties.
AgNPs have been experimented as pesticides to reduce the burden of pests from crops. It has a demand for pest protection and nutritional enrichment. This reduces the frequent use of chemical fertilizers in conventional farming. It can destroy unwanted microorganisms in soils and hydroponics systems [16].

1.3 Eco-friendly approach for biosynthesis of AgNPs

Metal nanoparticles have been created using a variety of chemical and physical processes. These techniques, however, are more expensive, require a lot of energy, and may be environmentally hazardous. Alternative, environmentally good, economical, novel, simple, cost-effective, eco-friendly and affordable methods must be developed. As a result, biological synthesis techniques (also known as "green synthesis") based on fungus, algae, bacteria, yeast, viruses, and plant extracts (such as essential oil) have the capacity to produce a variety of nanoparticles and hence may be more advanced than other techniques. Without the use of hazardous materials, high pressure, energy, high temperature, or toxic chemicals, biological synthesis techniques are economical, biocompatible, ecologically benign, and easily scaled up for large-scale synthesis [15]. The formation of NPs by the oxidation/reduction of metallic ions by secreted biomolecules such as enzymes, proteins, sugars, and carbohydrates is reflected in the green synthesis of NPs, which takes a bottom-up approach.
Fungi-based green synthesis of AgNPs has many advantages over approaches that involve plants, viruses, or bacteria. Fungi are simple to manage in the lab, require basic nutrients, and have a high capacity for adhering to walls. They might also be utilized as a source for the manufacture of large numbers of nanoparticles, which would in turn cause fungus to generate large numbers of proteins, increasing the yield of nanoparticles.[15]
Through the use of reducing enzymes either extracellularly or intracellularly as well as the biomimetic mineralization process, fungi are able to synthesize metal NPs in a variety of meso- and nanostructures.[4] Aspergillus niger, Beauveria bassiana, Metarhizium anisopliae, and verticillium sp. are a few examples of entomopathogenic fungal strains that can be exploited to produce AgNPs extracellularly.

1.4 Entomopathogenic fungi

In agricultural production systems and the natural environment, entomopathogenic fungi play a significant role in controlling pest-insect populations. These fungi contain enzymatic machinery that involves spore adhesion and recognition in their hosts, which results in infection and the demise of these insects. It is possible to manage pests with entomopathogenic fungi rather than risky, expensive chemical insecticides. Beauveria bassiana and Metarhizium anisopliae, for instance, The generation of virulent strains for target pests and their usage as biological control agents have attracted a lot of research attention.[8]. When the body of an insect host is in contact with these mi-

totic (asexual) spores of the fungus, known as conidia, they germinate, and the hyphae that arise pierce the cuticle. Following this, the fungus grows inside the insect's body and finally kills it after a few days; the development of insecticidal cyclic peptides is likely a contributing factor in this lethal outcome.[6]

Figure 1: Entomopathogenic fungi as a biological control agent

1.4.1 Metarhizium anisopliae

A fungus called M. anisopliae naturally develops in soils all over the world and acts as a parasitoid to afflict different insects with sickness. Due to the green hue of the fungus's spores, the sickness it causes is commonly referred to as "green muscardine disease." When the body of an insect host is in contact with these mitotic (asexual) spores of the fungus, known as conidia, they germinate, and the hyphae that arise pierce the cuticle. Then, after a few days, the fungus grows inside the insect's body and kills it; the development of insecticidal cyclic peptides is most likely what causes this lethal outcome (destruction). Dead bodies frequently have crimson cuticles. If the humidity level in the air is high enough, a white mold will develop on surfaces[7][6].

Figure 2: Metarhizium anisopliae as a biological control agent

1.4.2 Beauveria bassiana

Beauveria bassiana is a fungus that grows naturally in soils worldwide and acts as a parasite on various arthropod species, causing white muscardine disease. The time required for the fungi to enter the insect's body and begin to grow and eliminate it in the natural situation is approximately 48 hours, 4-5 days are needed to kill the pests and the life span of 6 months. It cannot stay alive at the high-temperature, therefore, the good time to apply it is during late afternoon or evening. The best temperature range for Beauveria bassiana is between 19 to 30 ^{o}C. Where the spores grow at a slow pace at temperatures below 17 ^{o}C and the become inactive above 33 ^{o}C.

Figure 3: Beauveria bassiana as a biological control agent

1.4.3 Verticillium

Figure 4: verticillium as a biological control agent

The soil-borne ascomycete fungi of the genus Verticillium can infect and kill insects, other arthropods, nematodes, and other invertebrates. The insects become infected when they come into contact with the sticky fungus spores, which then spread and penetrate the body. This process is known as parthenogenesis, and it results in the consumption of the insect's internal organs and eventual death. Brassinolide, a cyclodepsipeptide toxin produced by this fungus's mycelium, as well as other insecticidal toxins such as dipicolinic acid, are capable of killing the host when they infect aphids, whiteflies, rust fungi, spider mites, russet mites, wide mites, and scale insects.

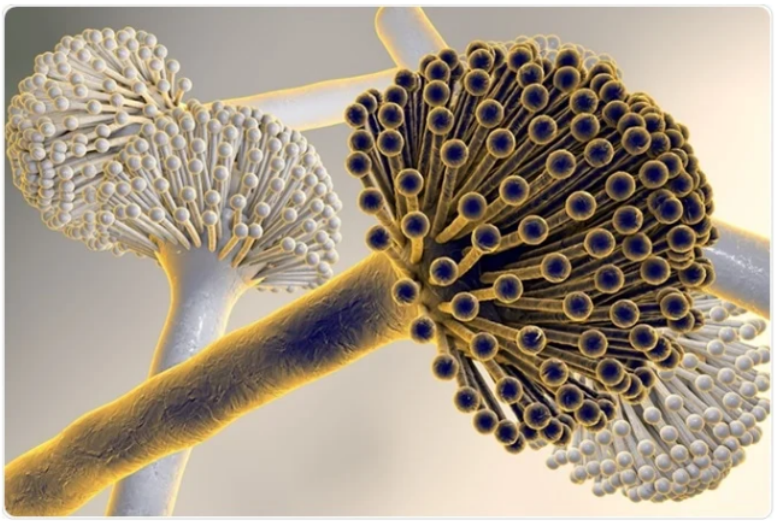

Figure 5: Fungi Aspergillus niger, black mold, which produces aflatoxins, cause pulmonary infection aspergillosis

1.4.4 Aspergillus niger

The fungus Aspergillus niger is a type of mold, which can sometimes be attributed to the cause of some cases of pneumonia. It is also the causative agent of 'black mold' on the outsides of certain foods, such as apricots, onions, grapes, etc - therefore making Aspergillus niger a food 'spoilage' organism. Aspergillus niger has a plethora of strains. The size of different strains of Aspergillus niger, as individual organisms, can range from anywhere between 900-1,600µm in length, with the rough, spherical conidia measuring 3-5µm. By affecting the mortality of second-stage juveniles and the prevention of egg hatching, a culture filtrate of Aspergillus niger was very effective against the root-knot nematode Meloidogyne incognita. Later, the Korean market registered the A. niger strain as a natural nematicidal agent [12].

1.5 Nanoparticles as a pesticide

The difficulty, though, is keeping the shelf life and viability of the fungus spores when they are subjected to high temperatures, UV light, and humidity. Extracellular surface proteins from fungi can bind to nanoparticles via free amine groups or cysteine residues in the proteins and electrostatic attraction of negatively charged carboxylate groups in enzymes found in the fungi's cell wall. Functional groups like -C-O-C-, -C-O-, and -C-C- are derived from heterocyclic compounds like proteins, which are present in the fungal extract and are the capping ligands of the metallic nano[13]. The extracellular medium, where the NP that covers the fungi spore is maintained, blocks the detoxifying system from accessing it and may result in higher fungi adhesion to insect bodies. This exposure duration to the biologically active molecule is increased.
The size, shape, and morphology of the produced NPs are ultimately influenced by the biochemical processing and interaction activities of a particular microbe as well as by environmental factors like temperature and pH [1].It was discovered that the pH has a significant impact on the synthesis of AgNPs in Beauveria bassiana. Protein structure is impacted by a decreased pH, which causes the protein to become denatured and lose its activity. As a result, large-sized nanoparticle NPs were seen.[14]
In the present study, we have isolated, a pure fungal strain for the biosynthesis of silver nanoparticles at four different temperatures to find the best temperature used to produce the best size of nanoparticles and the best coating of fungal spores at the same time, this temperature does not affect the fungus and keeps it alive, and the synthesized AgNPs were characterized using a Scanning Electron Microscope (SEM) (Fig. 20), Small Angle X-ray Scattering (SAXS)(Fig. ??), and Dynamic Light Scattering (DLS) (Fig. ??).

2 Materials and methods

2.1 Chemicals

In this research, we used Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) from France(Becton, Dickinson). Also, silver nitrate was used for the purpose of biosynthesis of the silver nanoparticles.

2.2 Media preparation and fungal growth

The four fungi used in this study (Beauveria bassiana, Metarhizium anisopliae, Aspergillus niger, and Verticillium sp) were cultured on Potato Dex-

trose Agar (PDA) media, which was prepared by suspending 15.6 grams of PDA powder in 400 ml distilled water (39g/L). Then the solution was heated to boiling to dissolve the medium completely. The media solution was then sterilized by autoclave at 15 lbs pressure (121°C) for 15 minutes. After that 20-25 ml was dispensed into sterile 9 cm diameter Petri dishes and stored at 4°C ready for use, finally, the fungi were cultured and grown on these Petri-dishes media after the media solidified. Furthermore, fungi were cultured on Sabouraud Dextrose Agar (SDA)media, which was prepared by suspending 26 grams SDA powder in 400 ml distilled water (65.0g/L). Then the solution was heated to boiling to dissolve the medium completely. The media solution was then sterilized by autoclave at 15 lbs pressure (121°C) for 15 minutes. After that 20-25 ml was dispensed into sterile 9 cm diameter Petri dishes and stored at 4 °C ready for use, finally, the fungi were cultured and grown on these Petri-dishes media after the media solidified.

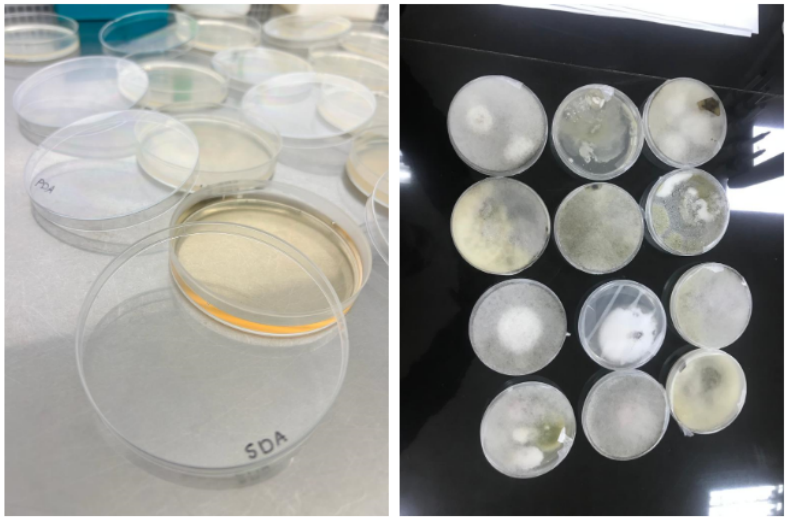

Figure 6: Left: Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) petri dish before fungal growth. Right: Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) Petri dishes after fungal growth.

2.3 Fungal isolates

Four newly isolated fungal strains, Aspergillus niger, Beauveria bassiana, Metarhizium anisopliae, and Verticillium sp carried out by transferring fungal mycelium from dead weevils and cultivated on potato dextrose agar medium and Sabouraud Dextrose Agar and incubated for 7-10 days at 28 ± 2 ^{o}C. The inoculated plates were checked daily. then sub-cultured on a new PDA

media to obtain a pure culture. The subculture was done every 2-3 weeks to maintain a new fresh and pure culture. Then after obtaining pure culture, the subculture was done on Sabouraud Dextrose Agar in order SDA yielded a significantly higher fungal count than PDA [11].

2.4 Identification of entomopathogenic fungi

2.4.1 Morphological examination method

Those strains were identified based on cultural characteristics and morphological examination. In a culture, Beauveria bassiana grows as a white mold. With the most common cultural media, it produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells having a short avoid and terminates in a narrow apical extension called a rachis. The rachis elongates each conidium is produced, resulting in a long zig-zag extension. The conidia are single-celled, haploid, and hydrophobic.[13] verticillium, the colony was off-white in color with a felt-like surface. On PDA, the underside of the colony was dark only in the central area with radiating ridges.[9]. For Metarhizium anisopliae having yellowish green on PDA, the underside of the colony was white. For Aspergillus niger having yellowish green on PDA, the underside of the colony was yellow.

Figure 7: Metarhizium anisopliae culture growth. Left: from the top and right: from the bottom.

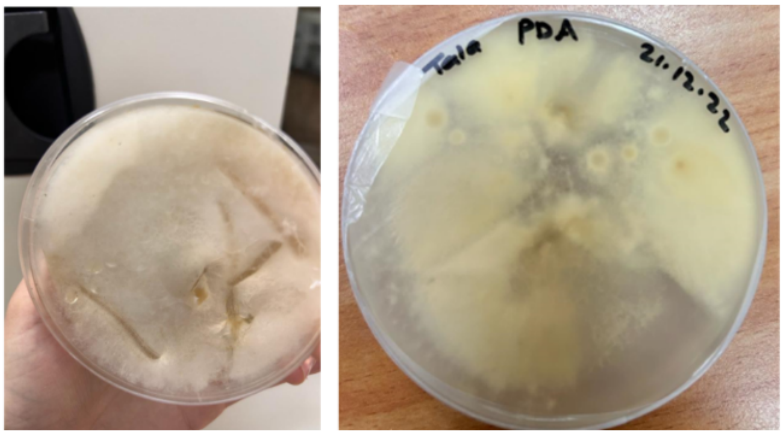

Figure 8: Beauveria bassiana culture growth. Left: from the top and right: from the bottom.

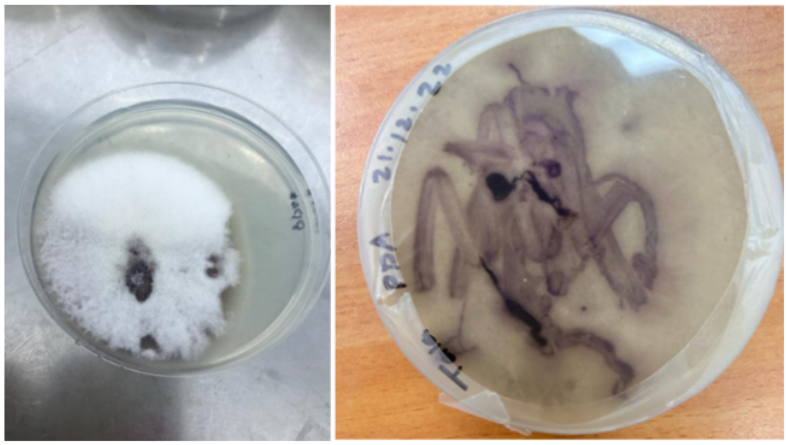

Figure 9: verticillium culture growth. Left: from the top and right: from the bottom.

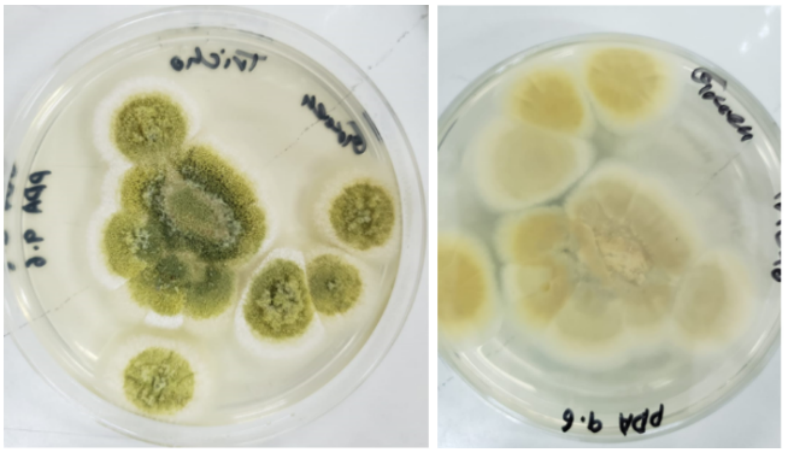

Figure 10: Aspergillus. niger culture growth. Left: from the top and right: from the bottom.

Figure 11: Left: Crystal violet dye and right: Fungal sample stained with Crystal violet dye.

Figure 12: Light Microscope

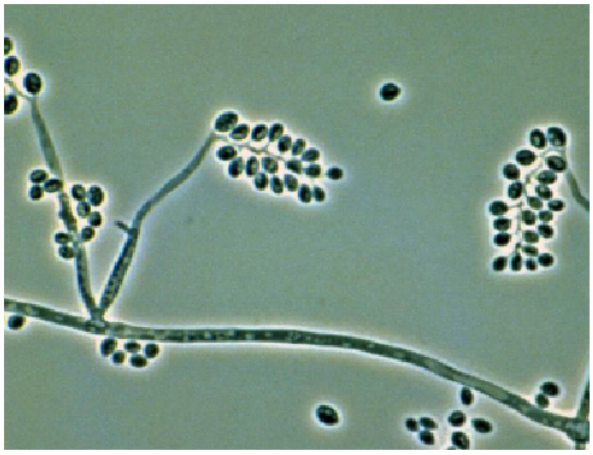

Figure 13: Spore-bearing structures of Beauveria bassiana in laboratory culture. The conidia typically develop in zigzag-like chains on long conidiophores.

2.4.2 Slide culture method

A small amount of entomopathogenic fungal culture was used with a loop or needle and spread out evenly on the middle surface of the microscope glass slide with water droplets. Then, the slide was stained with one micro. of 10 crystal violet dye (Fig. 11) covered with a cover glass and observed under a light microscope (Optika XDS-2 Trinocular AIPTEK international GmbH, Italy) (Fig. 12). Each type of fungi has a different conidia shape, Metarhizium anisopliae conidia oval, Beauveria bassiana conidia pointed from above, verticillium conidia bacillary shape, Aspergillus niger conidia like a ring.

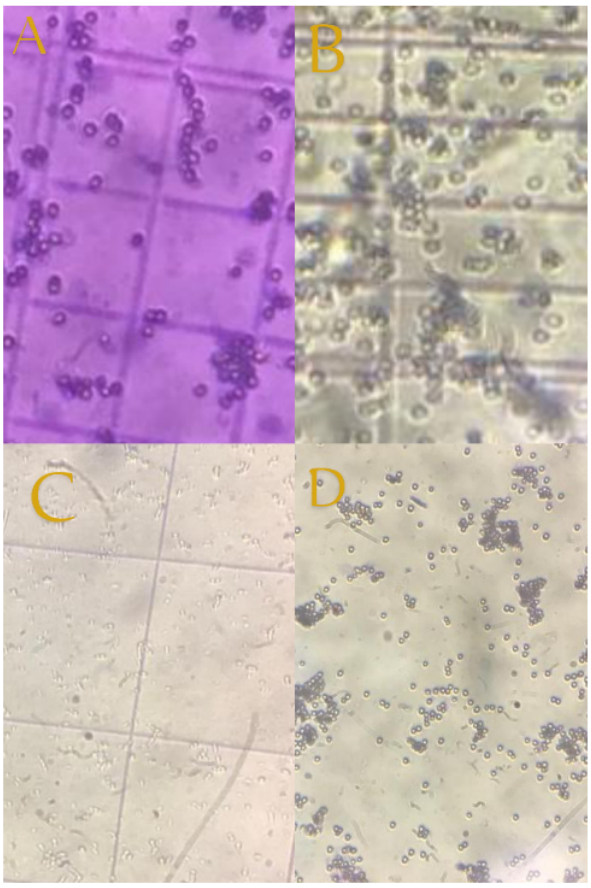

Figure 14: Morphological characteristics by light microscope (x1000) of (A) conidia of Metarhizium anisopliae, (B) conidia of Aspergillus niger, (C) conidia of verticillium, (D) conidia of Beauveria bassiana.

2.5 Biosynthesis of AgNPs

2.5.1 Preparation of cell-free filtrate

An amount of pre-planted fungi Beauveria bassiana, Aspergillus niger, verticillium, and Metarhizium anisopliae were taken conidia and spores were harvested in dry conditions by scraping the surface of agar plate about one gram with a sterile loop, each one was put in a sterile conical flask containing 100 ml of sterile D.W, one drop of tween twenty was added, stirred very well by vortex mixer to completely homogenized for 15min, then filtered through two layers of cheesecloth to obtain pure suspension without any debris, large particles, or hyphae. After that, flasks were completely covered with aluminum foil and incubated at 25°C,150 rpm for 120 h in an agitated condition with an orbital shaker with a water bath. Cell-free filtrates were then obtained by separating the fungal biomass by filtration using cheesecloth.

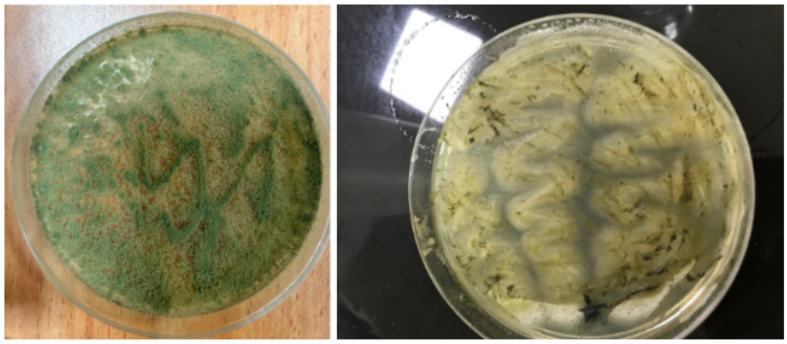

Figure 15: Fungi Petri-dish Left: before scarping and Right: after scarping

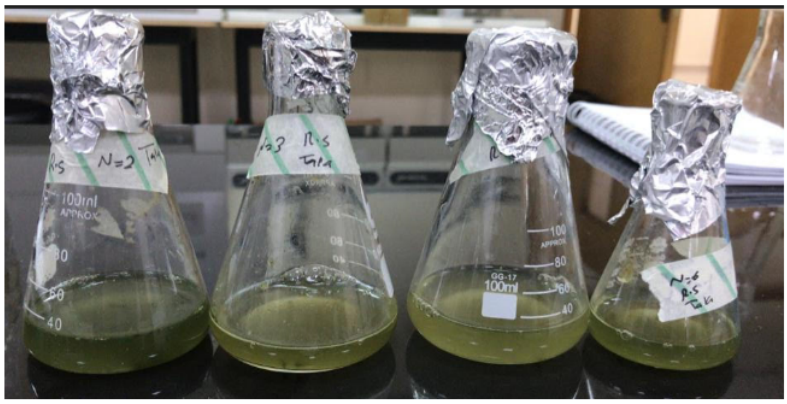

Figure 16: Biomass of fungi

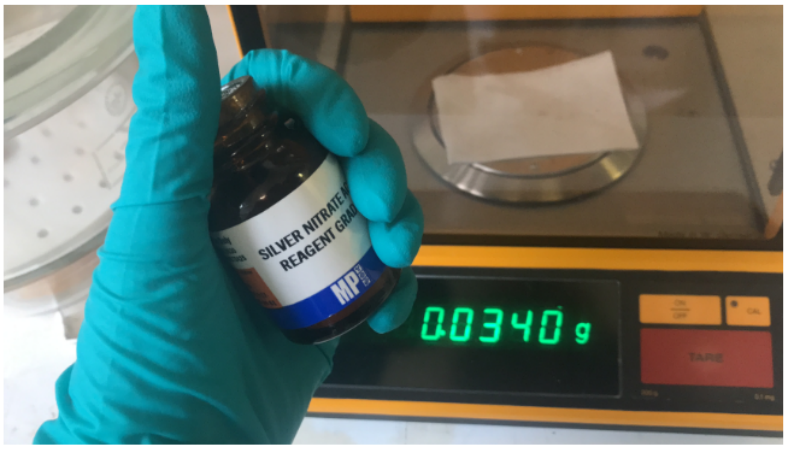

Figure 17: $AgNO_3$ as a powder

Figure 18: Fungi sample on an orbital shaker with a water bath.

2.5.2 Extracellular biosynthesis of AgNPs using cell-free filtrate

After the fungus was filtrated (Fig. 18), sixteen flasks from each type of fungi at 4 different temperatures were prepared by mixing 100 ml of fungi samples and 0.034 g of silver nitrate ($AgNO_3$) (Fig. 17). The flasks were completely covered with aluminum foil and incubated at different temperatures of (10°C, 15°C, 20°C, and 25 °C) in the dark for 72 hours on agitation with an orbital shaker with a water bath (Fig. 18). The control without adding $AgNO_3$ was maintained under the same conditions as reference samples as shown in Fig. 19.

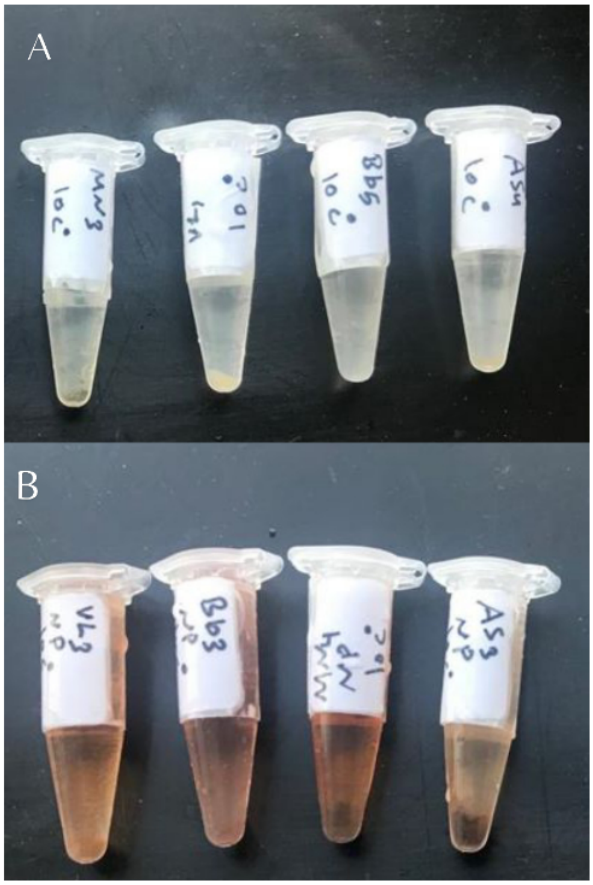

Figure 19: Fungal cell filtrate. (A) without $AgNO_3$(control) and (B) Fungal with $AgNO_3$.

3 Characterization Techniques

3.1 Scanning Electron Microscope (SEM)

Using scanning electron microscopy (SEM), information about AgNPs size and size distribution can be obtained. SEM (Fig. 20) uses electrons instead of light to form a largely magnified image. SEM as an instrument use an electron gun equipped with a tungsten filament cathode that thermionically emits an electron beam. Since the specimen chamber for SEM is at a high vacuum, a specimen is typically needed to be absolutely dry because if there is air or gas in the chamber, the electron will collide with the gas molecules and never reach the sample, leading to a lower image quality. Inelastic scattering interactions with beam electrons cause low-energy secondary electrons (50 eV) to be expelled from the conduction or valence bands of the specimen atoms. This is how the most popular imaging mode captures these electrons. The samples are prepared as follows to be measured with the SEM, 35 micro-liter of solvent are dropped into a cleaned 10 * 10 mm^2 silicon wafer as shown in Fig (21). The wafers were first cleaned with ethanol for 5 minutes on a sonicator and dried with nitrogen gas. The wafers are cleaned to remove dust from their surface. After drying overnight, Agin samples were cleaned using nitrogen gas to get rid of any dust before being put into the SEM chamber. Two samples can be put together into the holder as shown in Fig. 22

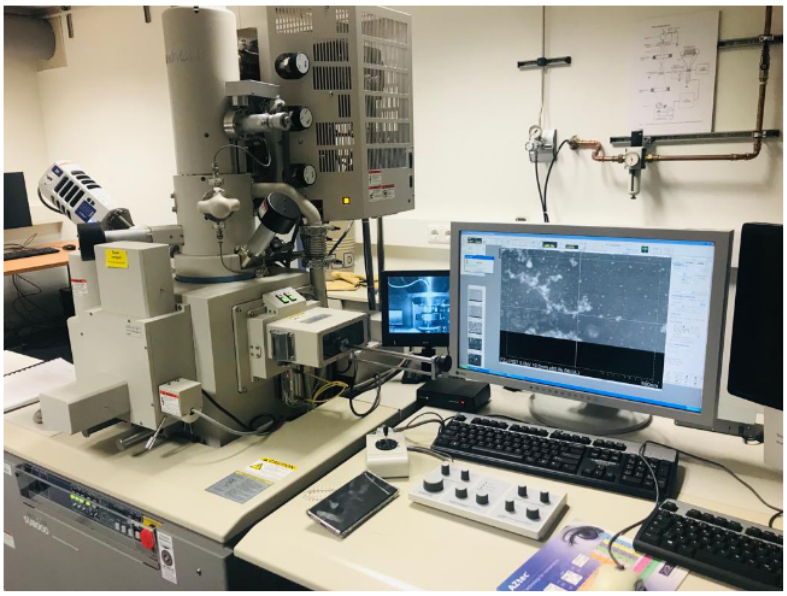

Figure 20: Scanning Electron Microscopy

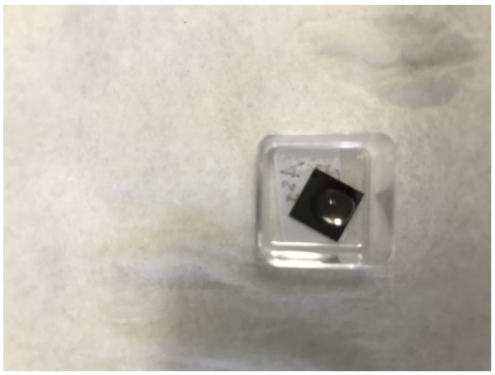

Figure 21: The sample after dropping solvent

Figure 22: SEM holder with fungus sample. The right sample is for the fungus with $AgNO_3$ NPs and the right sample is for funges without $AgNO_3$ NPs.

3.1.1 SEM results

In this section, the SEM images of the fungus (Beauveria Bassiana, Metarhizium Anisopliae, Verticillium and Aspergillus) as well as of the biosynthesized AgNPs at 10°C, 15°C, and 25 °C are shown.

Beauveria Bassiana

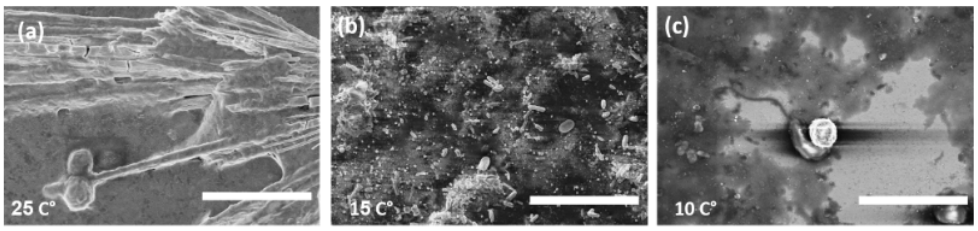

Figure 23: SEM images of Beauveria Bassiana at (A) 25 °C, (B) 15 °C, and (C) 10 °C. Scale bar 20.0um

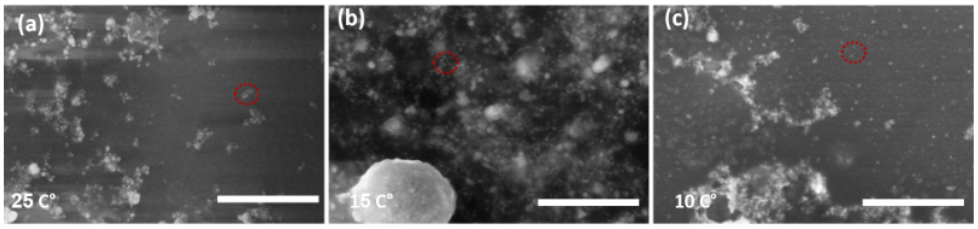

Figure 24: SEM images of Beauveria Bassiana spore and the biosynthesized AgNPs (red circle) at (A) 25 °C, (B) 15 °C, and (C) 10 °C. Scale bar 500nm

Metarhizium Anisopliae

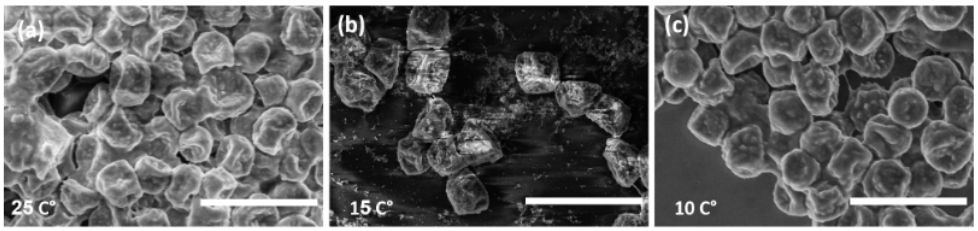

Figure 25: SEM images of Metarhizium Anisopliae at (A) 25 °C, (B) 15 °C, and (C) 10 °C. Scale bar 10.0um

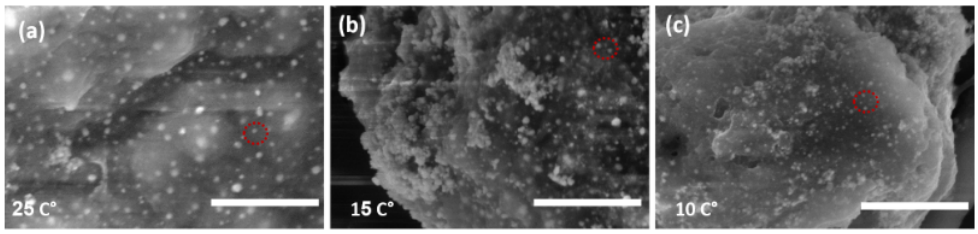

Figure 26: SEM images of Metarhizium Anisopliae spore and the biosynthesized AgNPs (red circle) at (A) 25 °C, (B) 15 °C, and (C) 10 °C. Scale bar 500nm

Verticillium

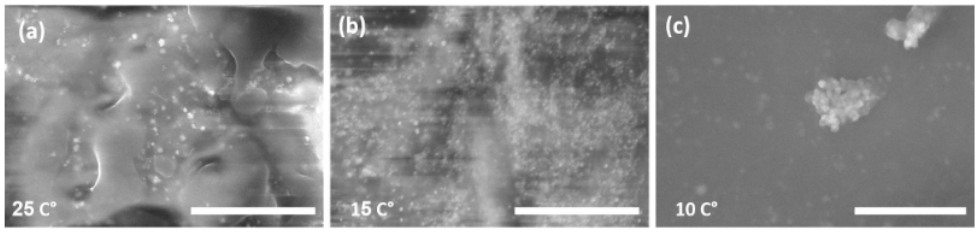

Figure 27: SEM images of Verticillium at (A) 25 °C, (B) 15 °C, and (C) 10 °C. Scale bar 1.00um

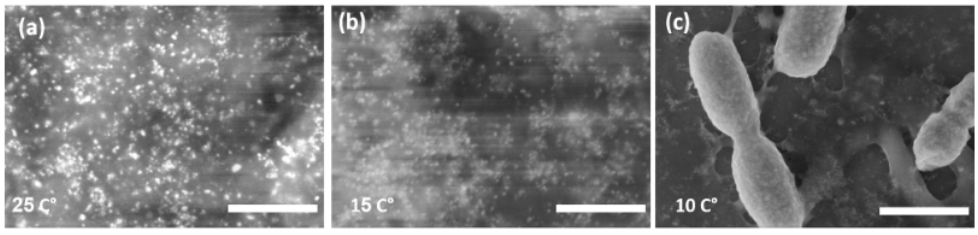

Figure 28: SEM images of Verticillium spore and the biosynthesized AgNPs at (A) 25 °C, (B) 15 °C, and (C) 10 °C. (a), (b) Scale bar 500nm and (C)1.00um

Aspergillus Niger

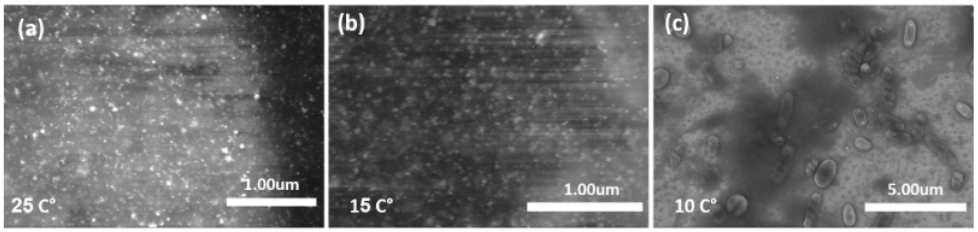

Figure 29: SEM images of Aspergillus Niger at (A) 25 °C, (B) 15 °C, and (C) 10 °C.

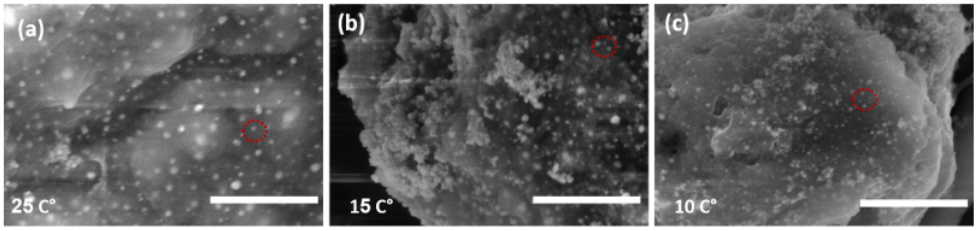

Figure 30: SEM images of Aspergillus Niger spore and the biosynthesized AgNPs (red circle) at (A) 25 °C, (B) 15 °C, and (C) 10 °C. Scale bar 500nm

Conclusion

In this study, AgNPs were synthesized extracellularly by Beauveria Bassiana, Metarhizium Anisopliae, Verticillium, and Aspergillus Niger at four different temperatures(10 °C,15 °C,20 °C,25 °C). The filtrate was treated with $AgNO_3$ where the protein, enzyme, and other compounds in the fungal liquid work as reducing agents and are responsible for converting silver nitrate to silver nanoparticles. The reaction started after 2 minutes of shaking the solvent by hand, with a change in color of the filtrate from pale yellow to brownish yellow. This is an indication of the formation of silver nanoparticles due to the excitation of surface plasmon vibration in the silver nanoparticle. Nanoparticles exhibit large specific surface areas causing a higher description of fungi spores causing full coverage of insect cuticles with fungi spores. The size, shape, and size distribution of the biosynthesized AgNPs were investigated locally with SEM. The obtained particle size depends on the type of fungi and the temperature.

The outcome result of the present study shows that the fungi Beauveria

Bassiana, Metarhizium Anisopliae, Verticillium, and Aspergillus Niger can be used as effective reducing agents for the synthesis of AgNPs. This biological reduction of metal would be advantageous for the development of clean, cost-effective, nontoxic, and environmentally acceptable metal nanoparticles. The synthesized AgNPs with 20 nm in diameter can cross the larva's cell wall easily and this leads to higher mortality.

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