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Synthesis of fungus mediate silver nanoparticles (AgNP) its characterization and study the efficacy against inoculam, biomass and protein content of *Fusarium oxysporum*

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Abstract
Nanotechnology is the art and science of manipulating matter less than 100 nm in size called nanoparticles (NPs) with some noble properties. Nanoparticles such as gold, silver, copper, zinc possess unique physiological properties due to its shape, size and increased surface area. Silver nanoparticles (AgNPs) possess antimicrobial properties against bacteria, fungi and viruses etc. In the present study, AgNPs were synthesized from the indigenous strain of biological control agent, *Trichoderma asperellum* and were characterized using UV-Vis-spectrophotometer, Dynamic Light Scattering (DLS) and Zeta-Sizer. The efficacy of bio synthesized AgNP was then tested against the pathogen *F. oxysporum* by agar well method and poison food technique at 100% concentration and mycelial growth inhibition was recorded. The effect of NPs on the inoculum of the pathogen, biomass and its protein content was also studied. Significant mycelial growth inhibition of the pathogen was recorded at the tested concentration of AgNP. Light microscopic study of the AgNP treated *F. oxysporum* showed cellular deformities and lysis of the mycelium after exposure to these NPs. When the effect of silver nanoparticle on the protein content and biomass production of *F. oxysporum* was studied, it was found that AgNP cause reduction in the protein content and biomass content of the fungus. Thus, the study showed encouraging result for using silver nanoparticles as an effective measure for controlling *F. oxysporum*.

Keywords: Conidia, *Fusarium oxysporum*, mycelium, nanotechnology, protein, silver nanoparticles

Introduction
Nanotechnology is the art and science of manipulating matter, design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale that is 1-100 nm beimg nanoparticles as the building block. Previous studies confirmed that metal nanoparticles are effective against plant pathogens and insects pests (Choudhury *et al.*, 2010) [6]. For example, an eco-friendly fungicide is under development that uses nanomaterials to liberate its pathogen-killing properties only when it is inside the targeted pathogen (Liu, 2006) [14]. However, the field application of nanoparticles in disease management, antimicrobial mechanisms and nano toxicity on plant ecosystem is yet to be known (Alghuthaymi *et al.*, 2015) [4].

Fungi become the best choice for the nanotechnologists for synthesis of nanoparticles, due to wide variety of advantages they provide over bacteria, yeast, plants and others (Dias *et al.*, 2002; Sanghi and Verma, 2009) [9, 20]. Moreover, they are easy to handle, needs simple nutrient, possess high wall binding capacity, as well as intracellular metal uptake capabilities (Dutta *et al.*, 2015) [10].

Fusarium oxysporum f. sp. *cubense* is a highly adaptive ascomycete fungi belonging to family Nectriaceae. They are found in soils of desert, grassland, soils of tundra etc and are ubiquitous soil inhabitant having ability to exist as saprophytes. Although the sexual means of reproduction is unknown, they produces three types of asexual spores *viz*., microconidia, macroconidia, and chlamydospores (Agrios, 1988) [2]. Their predominant role in native soils may be harmless or even beneficial plant endophytes or soil saprophytes but many strains are also pathogenic to plants. They are known to degrade lignin and complex carbohydrates.

Association of pathogenic form of *F. oxysporum* with plant roots, a form that is able to grow beyond the cortex and into xylem could exploit this ability and gains advantage over other fungi that are restricted to the cortex. The progression of fungus into vascular tissue may elicit an immediate host response, successfully restricting the invader, or an otherwise ineffective or delayed response, reduces the vital water conducting capacity and induces wilting resulting in generation of disease.

Though several chemical and biological approaches are available for the management of Fusarium but the rate of success is very poor. A number of chemicals that were used as effective agent for the management of Fusarium have been prohibited recently (Anonymous, 2020) [1]. Moreover, indiscriminate use of chemical creates problem of environmental pollution, leaves residues in the end products and many more. There is a possibility of using nanoparticles for management of plant pathogens, which can be effective even at much lower dosage and may reduce the pesticidal load in the environment. So, the present study was carried out with an aim to synthesize and characterize AgNP from biological sources and to study its effect against inoculum, biomass and protein content of *Fusarium oxysporum.*

Materials and methods

Isolation of *F. oxysporum*

The pathogenic fungi, *Fusarium oxysporum* was isolated from the root and stem tissues of wilt infected tomato plants (*Lycopersicon esculentum* L.) collected from the Horticultuarl Orchard of Assam Agricultural University, Jorhat, Assam. For isolation of the pathogen, root and stem tissues were washed under running tap water and were sectioned to 5-7 mm. Sections were then surface sterilized in 1.0% NaOCl solution for 1 minute, rinsed twice in distilled water and dried on sterile filter papers. Disinfected tissues were then transferred to petri plates containing Potato dextrose agar (PDA) medium and incubated at 27 ± 2 ^{0} C. After 7 days of incubation, fungal colonies were transferred to fresh PDA plates.

Characterization of *Fusarium oxysporum* f. sp. *lycopersici* isolates

The pathogen was grown on PDA medium at 27±2^{0}C for 10 days. For morphological and cultural characterization, observations were made on colony colour, radial growth, mycelia growth pattern, sporulation, size of macroconidia, micro conidia and chlamydospores and compared with the relevant literature.

Pathogenicity test

The pathogen, *Fusarium oxysporum* was grown in a 250 ml erlenmeyer flask containing 100 ml Potato dextrose broth (PDB) for mass multiplication of the inoculum. The flasks were incubated at 27±2^{0}C, 120 rpm for seven days into an incubator shaker. After seven days, the fungus was harvested and filtered through muslin cloth for separating mycelium with spores and the concentration was adjusted to X10^6 conidia per ml of water. For pathogenicity test, twenty one days old tomato seedlings were inoculated by standard root dip method. The roots of seedlings were washed thoroughly under running tap water to remove adhering soil particles. The roots were trimmed with a sterile scissor and were dipped in the spore suspension for 30 minutes. The inoculated seedlings were then transplanted in sterilized pots containing soil and sand in 1:1 ratio and were kept in net house condition to assess the symptoms produced by *Fusarium* sp.

Biosynthesis of silver nanoparticles (AgNPs) using culture supernatant of *Trichoderma asperallum*

In the present study AgNP was synthesized by mediating the *T. asperallum* and $AgNO_3$ as precursor. Mycelial biomass of *T. asperallum* was prepared in potato dextrose broth (PDB) amended with 0.1% peptone (Hi Media Laboratory Ltd.) and the organism was allowed to grow for 7 days at 100 rpm in 12 hrs light and 12 hrs dark condition in an shaker incubator (REICO) at 25 ± 1^{o}C. After 7 days of culture, biomass of *T. asperallum* was harvested and centrifuged at 5000 rpm for 10 minutes at 4^{o}C. Supernatant of *T. asperallum* was collected and treated with aqueous solution of 1 mM $AgNO_3$ solution at 1:1 ratio in a 250 mL erlenmeyer flask and allowed the reaction to proceed at pH 10 in a rotary shaker (REICO, Horizontal shaker) for 120 hrs. During the reaction process the erlenmeyer's flasks were wrapped with aluminum foil for maintaining dark condition. Controls were maintained without any precursor (Kaman and Dutta, 2019) [13].

Characterization of biosynthesized Silver nanoparticles

Characterization of AgNP was done by different equipment's such as UV-Vis Spectrophotometer, DLS and Zeta Sizer.

In vitro study of bio synthesized silver nanoparticles (AgNPs) against *Fusarium oxysporum*

An *in vitro* assay was performed to test the efficacy of synthesized AgNPs against *F. oxysporum* at 100% concentration by Poison Food Technique (Nene and Thapliyal, 1978). For this, PDA medium was poured in sterilized petriplate and was allowed to solidify. After the solidification well was formed with the help of cork borer (5 mm dia.) and AgNPs were poured into the well. Agar plugs cut with a sterilized cork borer containing the fungi was inoculated at the centre and a control was taken with a normal PDA medium. All the plate were incubated at 28 ± 1°C in a BOD incubator (REICO) till full growth was observed in the control. Mycelial growth inhibition was calculated when growth of mycelia in the control plate reached the edge of the petridish. The following formula used for calculation of the inhibition rate (%).

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

Where, R is the radial growth of fungal mycelia on the control plate and r is the radial growth of fungal mycelia on the plate treated with AgNPs.

Estimation of protein content in AgNP treated inoculum of *F. oxysporum*

For estimation of protein content in the inoculum of *F. oxysporum*, the pathogen wsa allowed to grow in PBD amended with AgNP. Two mycelial discs (5 mm) of freshly grown *F. oxysporum* were inoculated in the PDB and incubated at 25±2 ^{0}C in a BOD incubator. Control was kept without addition of AgNPs. The experiment were replicated thrice. Estimation of protein was done after 10 days growth in the media by Lowry method (Lowry *et al*., 1951) [15]. The reagents required for estimation of protein were as follows-

- Solution A: 2% Na_2CO_3 in 0.1 N NaOH.
- Solution B: 1% $CuSO_4.5H_2O$ solution.
- Working solution of B was prepared freshly before use by mixing equal volume of solution B (a) and B (b).
- Solution C was prepared by mixing solution A and working solution B. Freshly prepared before use by

mixing 50 ml of solution A and 1 ml of working solution B.
- Solution D: Folin-Ciocalteau reagent (1N).: ratio of water and reagent should be 1:1
- Protein solution(stock standard): 25µg per 0.1 ml(use albumin)
- Phosphate buffer (0.1 M,pH 7.6)
- 0.1N NaOH

The different steps involved in protein estimation are mentioned below

Extraction of protein from sample: 1 g of the sample was taken and grinded well with pestle and mortar in 5 ml of phosphate buffer (0.1 M, pH 7.6). Homogenate was centrifuged at 10,000 rpm for 10 minutes and the supernatant was directly used for protein estimation.

For preparation of standard curve and estimation of protein, BSA stock solution of volume 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml was pipetted out into a series of test tubes. In another set of test tubes 0.2 ml of the sample extract was pipetted. The volume was made upto 1 ml in all the test tubes with water. A test tube with 1 ml of water served as a blank. 5 ml of solution C was added and mixed well by vortexing and allowed to stand at room temperature for 10 minutes. 0.5 ml of solution D was added, mixed thoroughly with vortex mixture and kept at room temperature for another 30 minutes. UV-Vis spectrophotometer reading was taken at 660 nm. Then standard curve was drawn and the amount of protein was calculated as mg/g or 100 g sample.

Results and discussion

Identification of the isolate

The colonies of the isolated pathogen exhibiting the taxonomic features of *Fusarium oxysporum* were identified based on the work of Nelson *et al*. (1983) [17]. Morphological identification of the fungus was done based on macroconidia, microconidia, chlamydospores and colony growth traits. The colony colour and pigmentation of the fungus on PDA medium was recorded as white to light pink and finally turned to light purple. Also, fluffy mycelial growth of the fungus was observed on PDA medium which is evident from the image (Plate 1). Microscopic analysis of *Fusarium* sp. revealed presence of all three asexual spores i.e. macroconidia, microconidia and chlamydospores (Plate 2 and 3). Macroconidia were fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate with size 23-54 X 3-4.5 µm. Microconidia were abundant, never in chains, mostly non-septate, ellipsoidal or cylindrical, straight or curved, 5-12 X 2.3-3.5 µm. Chlamydospores were terminal or intercalary, hyaline, smooth or rough-walled, 5-13 µm. *F. oxysporum* produces three types of asexual spores: microconidia, macroconidia, and chlamydospores (Agrios, 1988) [2]. Further, Pathogenicity testing helped separation of the species into formae specials which has an important diagnostic implication (Brett *et al*., 2003) [5]. Thus, the isolate was identified as *F. oxysporum* f.sp. *lycopersici.*

Pathogenicity testing of *Fusarium oxysporum* f.sp. *lycopersici.*

The symptoms on tomato plants infected with *F. oxysporum* f.sp. *lycopersici* varied considerably with age as recorded during its isolation from the diseased sample. At an early stage, symptoms appeared as yellowing of the lower leaves and at later stage, drooping down of leaves was observed due to loss in turgidity. With the increase in severity of the disease, the pith of the stem showed brown discolourations with mycelia growth. The inoculated organism was reisolatedin PDA media and confirmed the Koch postulate.

Synthesis of Silver Nanoparticles (AgNPs) from *T. asperellum*

For the synthesis of silver nanoparticles *T. asperellum* (Plate 4 and 5) was purposefully selected based on the earlier work on higher efficacy against soil borne pathogen, faster multiplication rate In the present study silver nanoparticles were synthesized from *T. asperellum* following the modified protocol of Kaman and Dutta (2019) [13]. During the study, it was observed that fungal supernatant of *T. asperellum* without $AgNO_3$ retains its original colour but the $AgNO_3$ treated fungal supernatant turns from green to pale yellow and finally to brown after 192 hrs of reaction (Plate 6). The change in colour from green to brown confirmed the formation of silver nanoparticles. The change in colour from pale green to yellowish brown was due to Surface Plasmon Reasonanace (SPR) phenomenon. Generally the metal nanoparticles have free electrons, which helps in the formation of the Surface Plasmon Resonance absorption band. It happens due to the united vibration of the electrons of metal nanoparticles in resonance with light wave. A possible mechanism for the presence of silver nanoparticles in fungal biomass could be the extracellular reduction of the silver ions in the solution followed by precipitation on to the cells. We observed gradual increase in colour development from yellowish brown to dark brown. This may due to Surface Plasmon Resonance (SPR) phenomenon (Triphati *et al*., 2013). The colour change observed in this study may be due to the deposition of silver nanoparticle as reported by Vahabi *et al.* (2011) [23]. They reported that silver nanoparticles shows yellowish brown to brown colour in water due to surface plasmon vibration in metal nanoparticles. Nanoparticles are biosynthesized when microorganism takes up target ions from their environment and then turns the metal ions into the element metal via action of enzymes produced by it. It can be classified into intracellular and extracellular synthesis depending on the location of biosynthesis. The intracellular method consists of transporting ions into the microbial cell to form nanoparticles in the presence of enzymes. The extracellular synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes (Zhang *et al.*, 2011) [26]. Herein, the synthesis was intracellular or extracellular.

The synthesized Ag NP were characterized by different equipments such as UV-Vis spectrophotometer, DLS and Zeta potential. The UV-Vis spectroscopic study showed the Plasmon resonance property, confirmed the reduction of metal ion and formation of nanoparticles with plasma resonance peak at 408 nm (Fig 1). No peak was observed in supernatant of *T. asperellem* treated without silver nitrate (Fig 2) The result of UV-Vis spectroscopy observed in the present study is in agreement with the earlier report of Basavaraja *et al*. (2007) who reported that SPR band for AgNPs occurs at 300-500 nm. They found SPR band of biosynthesized silver nanoparticles using the fungus *Fusarium semitectum* at 408 nm. Singh and Raja (2011) [21] also found the SPR band of biosynthesized silver nanoparticles from *T. harzianum* at an wavelength of 408 nm. Similarly, San and Don (2013) [19] synthesized AgNPs from *Schizophyllum commune* and observed the characteristic SPR band at a wavelength of 408 nm.

DLS analysis showed that average size of the synthesized silver nanoparticles was 46.94 nm with PDI 0.427 indicated that the biosynthesized silver nanoparticles were polydispersed in nature (Fig 3). Hughes *et al.* (2015) [11] reported that, PDI is dimensionless with value between 0 and 1, which is scaled such that values with 0.10 or less are considered highly monodispersed and above 0.10 are

polydispersed. For determination of average size of nanoparticle, earlier worker (Dhand *et al.*, 2015; Ahmed *et al*., 2016) [8, 3] also used DLS for determination of average size of nanoparticle. Dhand *et al.* (2015) [8], also determined the mean particle size of synthesized silver nanoparticles using *Coffea arrabica* seed extract by DLS as 20-50 nm, respectively. Ahmed *et al.* (2016) [3] synthesized silver nanoparticles using *Azadirachta indica* aqueous leaf extract and average size was determined as 48 nm by using DLS.

Zeta Potential of the biosynthesized AgNPs was found to be negative with a value of -30.9 mV (Fig 4). Negative zeta potential (-30.9 mV) observed in this study indicates that the AgNPs synthesized from *T. asperellum* were highly stable at dispersion. Zhang *et al*. (2009) [25] reported that the large negative zeta potential value (above -0.50 mV) indicates higher electrostatic repulsion among AgNP and stable on their dispersion. They also observed that nanoparticles synthesized from *Penicillum* sp. possessed negative zeta potential and were stable at different pH due to electrostatic repulsion.

Effect of Silver Nanoparticles (AgNPs) on *Fusarium oxysporum* f.sp. *lycopersici*

In the present study green synthesized AgNPs showed inhibitory effect against *F. oxysporum*. The growth inhibition percent was recorded as 41.2% after ten days of inoculation as compared to control (Table 1) which is evident from the image (Plate. 7,8, 9 and 10). The inhibitory effect of biosynthesized AgNPs on *F. oxysporum* may be due to the smaller size of AgNPs which help in rapid accumulation on the fungal mycelium and then penetration inside it as observed by Stroz and Imlay. (1999). Previous studies suggested that nanometer-sized silvers possess different properties, which might come from morphological, structural and physiological changes (Nel *et al*., 2003) [16]. Hwang *et al.* (2008) [12] reported that AgNPs on entering inside the fungal mycelium, causes interruption in the metabolic process and respiration by reaction with cellular molecules. These nanoparticles adhere to the cell walls and membranes of microorganisms and may reach the interior of the cell. It damages the cellular structures, induce the production of reactive oxygen species, and alters the mechanisms of signal transduction (Dakal *et al*., 2016) [7].

Regarding the biomass of *F. oxysporum* it was found to be highest in control fresh weight (7.925 g), air dried weight (1.651 g) and Oven dried weight (0.410 g) as compared to silver nanoparticle treated fresh weight (7.255 g), air dried weight (1.615 g) and Oven dried weight (0.370 g). (Table 2)

In the present study, protein content of the AgNP exposed fungus estimated by Lowry method was found lower as compared to the control. The absorbance of the treated culture was found to be 0.073 and that of control was found to be 0.098 with the help of the equation obtained, the protein concentration was calculated. The protein concentration of treated culture was 0.428 (25 µg/0.1 ml) while that of the control was found to be 0.628 (25 µg/0.1 ml). The lower protein content observed in the treated mycelium may be due to the breakdown of protein by the action of AgNPs. AgNPs are highly active and always have the affinity for agglomeration. Ag nanoparticles used in the present study might react with the oxygen ions and/or hydroxyl ions to stabilize themselves, resulting in the destruction of chemical bond of different elements of protein. This ultimately consequences in the disintegration of protein compound and thus recorded lower concentration of protein as compared to control where protein remained intact. Protein constitutes an important part of fungal cell, especially the cell wall. In *F. oxysporum*, it plays structural and functional role like the chitin synthases (CSs) of class I, class II and class III and the virulence proteins encoded by Fpr 1 gene respectively (Urdiroz *et al*., 2004) [22]. Any inhibition in the formation or function of such proteins will render it vulnerable to external pressures, leading to cell death in absence of proper cell wall. Further, the pathogen growth may also be suppressed due to effects on its proteins, though the nanoparticles may also affect other constituents or metabolic pathway, for this further research is required.

Table 1: Effect of biosynthesized silver nanoparticles on mycelial growth inhibition of *Fusarium oxysporum*

Treatment	Day 5	MGI%	Day 6	MGI%	Day 8	MGI%
T_1: Control (Only *F. oxysporum*)	5.10	-	6.03	-	7.50	-
T_2: *F. oxysporum* + Ag NP	4.70	7.84	5.40	10.40	5.90	16.00

Table 2: Effect of biosynthesized silver nanoparticles on biomass of *Fusarium oxysporum*

Treatment	Fresh weight (g)	Air dried weight (g)	Oven dried weight (g)
T_1 : Control (*F. oxysporum)*	7.925	1.651	0.410
T_2 : Treatment (*F. oxysporum* + Ag NP)	7.255	1.615	0.370

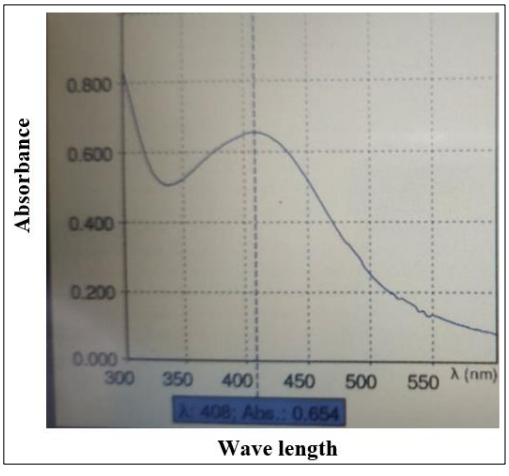

Fig. 1: UV-Vis absorption spectra obtained for silver nanoparticle synthesized by *Trichoderma asperellum*

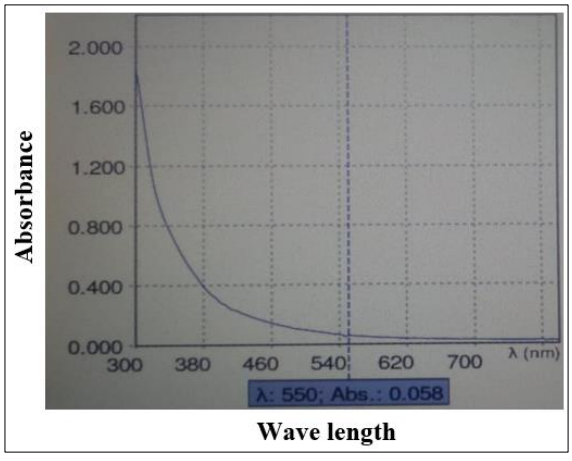

Fig 2: UV-Vis absorption spectra obtained for *Trichoderma asperellum*

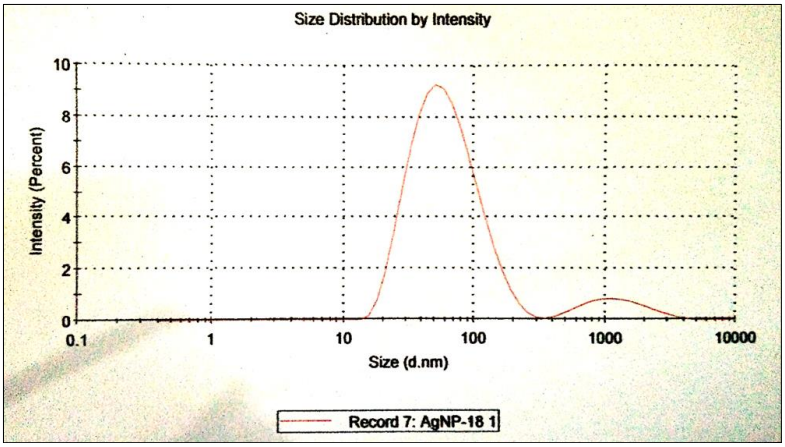

Fig 3: Dynamic light scattering (DLS) pattern of silver nanoparticle synthesized from *Trichoderma asperellum*

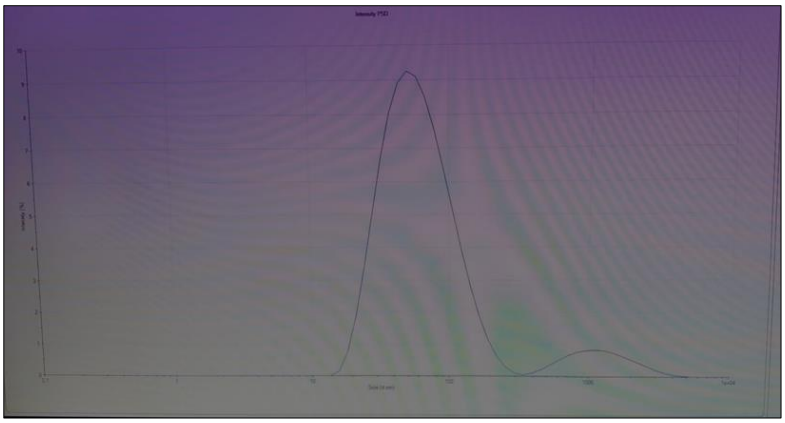

Fig 4: Zeta Potential of silver nanoparticle synthesized from *Trichoderma asperellum*

Plate 1: Pure culture of *Fusarium* spp

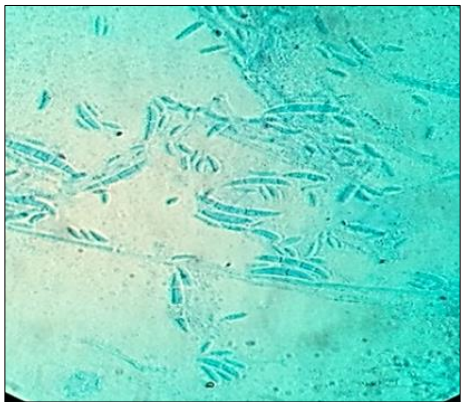

Plate 2: Macro and Micro Conidia of *Fusarium* spp (40X)

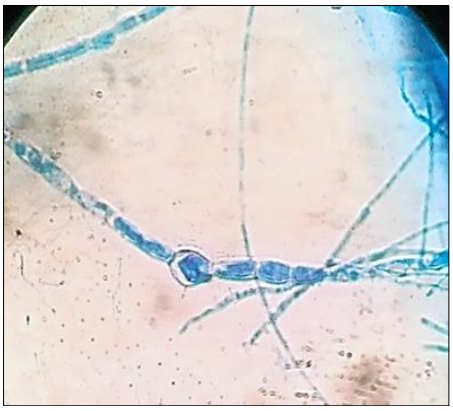

Plate 3: Chlamydospore of *Fusarium* spp (40X)

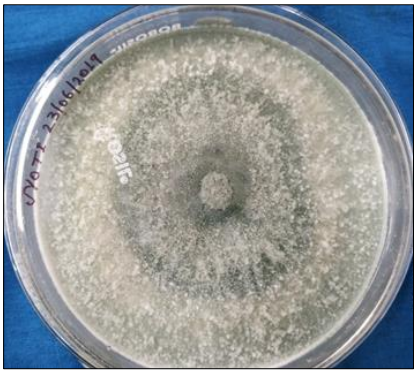

Plate 4: Pure culture of *Trichoderma asperellum*

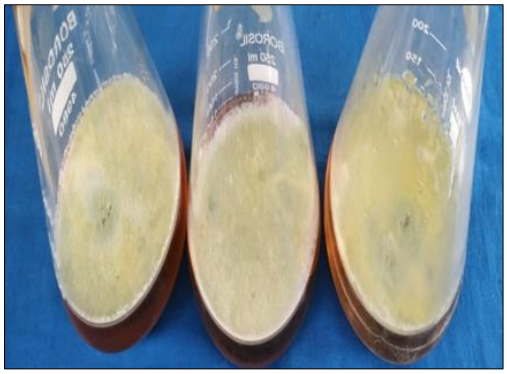

Plate 5: Mass culture of *T. asperellum*

Plate 6: Supernatant of T. asperellum (greenish) and silver nanoparticles (Yellowish brown)

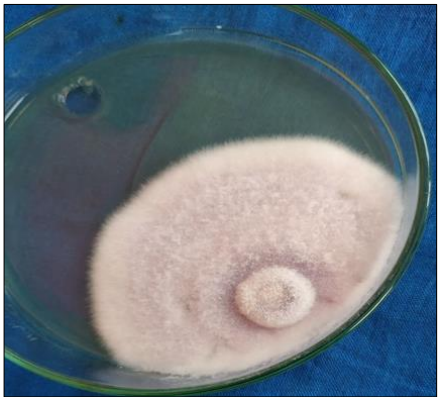

Plate 7: Growth of *Fusarium oxysporum* treated with Ag NPs after Day 8

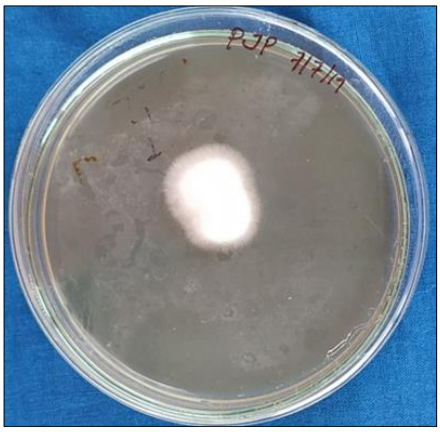

Plate 8: Growth of *Fusarium oxysporum* after Day 3

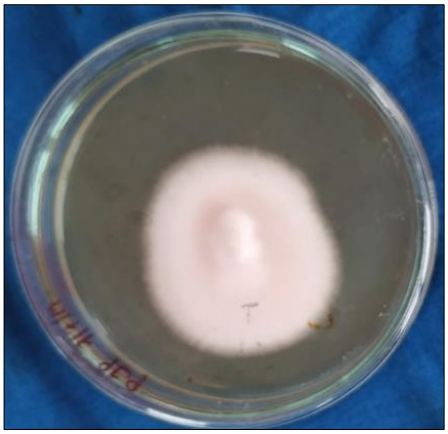

Plate 9: Growth of *Fusarium oxysporum* after Day 5

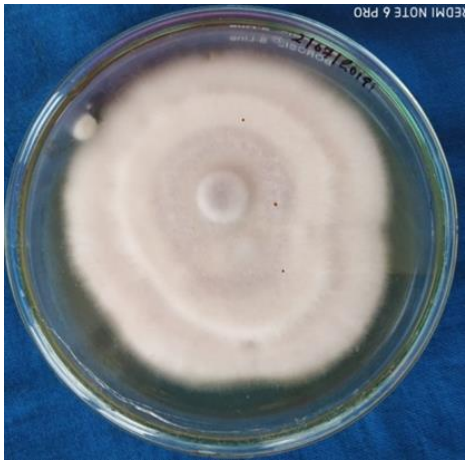

Plate 10: Growth of *Fusarium oxysporum* after Day 8

Conclusion

The present study concludes that AgNP is effective in controlling *F. oxysporum* and thus provides an ecofriendly alternative to chemical fungicides. Reduction in the use of chemical fungicides will ultimately lead to the reduced pesticide pressure on the environment. Our results support the hypothesis that AgNPs are suitable for formulating new types of fungicidal materials. We also obtained some proteins that are involved in virulence, antagonism, defense system and colonization. The present study supports the use of such modern and improvised techniques in building the Agricultural biome organically and naturally. It gives strong and supportive results in the effective control of various diseases that causes severe economic and ecological losses to the farming community.

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