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In vitro Bio-efficacy of *Trichoderma* Based Nano Formulation against *Pyricularia oryzae* and *Bipolaris oryzae*

D. Hazra, A. Debnath, A. Roy and P. Mondal[1]

Department of Plant Pathology, [1]Department of Agricultural Biochemistry,
Uttar Banga Krishi Vishwavidyalaya, Pundibari- 736 165, India
E-mail: dipayanhazra78@gmail.com

***Abstract*:** Biological synthesis of nanoparticle is very popular for its eco-friendly and non-toxic and green nature. Plant, Bacteria, Fungi, Yeast are used in this method. Among them Fungi are now the preferred option for production of nanoparticles due to their many benefits over other microorganisms. *Trichoderma* based biocontrol is a viable option in this situation. The goal of this project was to biologically synthesise silver nanoparticles (AgNP) from *Trichoderma asperellum* and *Trichoderma harzianum*, two possible native biocontrol agents. Silver nitrate (1 mM and 2 mM) served as the precursor for AgNP formation. Fourier Transmission Infrared Scanning and a UV-Vis spectrophotometer were used to characterise the biosynthesized silver nanoparticle. A characteristic UV-Vis absorption peak was observed at 415–420 nm, confirming the presence of AgNPs. In vitro bioefficacy was done by food poison technique. Silver nanoparticles at several concentrations (10 ppm, 5 ppm, and 1 ppm) were tested for their antagonistic activity in vitro against *Pyricularia oryzae* and *Bipolaris oryzae*, two Paddy diseases.

***Keywords*:** *Trichoderma*, Silver nanoparticles, *Pyricularia oryzae*, *Bipolaris oryzae*, Nanotechnology

Global agricultural productivity is severely hampered by plant diseases, managing those diseases one of the most important tasks for scientists. Modern agricultural practices for increasing rice production have recently led to an increase in disease severity. These practices include the use of high yielding crop varieties, excessive nitrogen fertilisation, extensive use of agrochemicals, increased plant population per unit area, and continuous rice cropping, which favours the crop's susceptibility to rice blast (Miah et al., 2017). The current plant disease management strategies have benefits and drawbacks. Although cultural practices are safer, they are not always effective in controlling disease. Chemical management comes with a number of drawbacks. Indiscriminate use of synthetic fungicide result in host toxification, which includes adverse effects on the soil microbiota, residual toxicity, fungicidal resistance, and environmental contamination (Gerhardson 2002). The overuse of pesticides has recently resulted in environmental dangers that have drawn a lot of attention and discussion. Chemical management of plant diseases degrades soil quality, pollutes water supplies, and upsets the ecosystem (Ayla and Rao 2002). In addition to the pathogen's comeback, fungicide resistance is a significant issue brought on by the harmful effects of excessive chemical use. For these reasons researchers are currently trying to develop novel, environmentally sound and more reliable methods of managing diseases. In contrast to traditional physico-chemical methods that have numerous drawbacks, the environmentally friendly method for developing nanoparticles through biological processes has garnered a lot of attention in recent years. The usage of environmentally friendly nanoparticles that don't generate harmful waste is becoming more and more necessary. Since it helps avoid an excessive buildup of chemicals that could cause residual effect in the environment, the use of nanotechnological products for pest management in agriculture is believed to be safer than the use of conventional agrochemicals. Additionally, the biocompatibility of NP usage is enhanced by these novel ecologically friendly synthesis methods (Guilger-Casagrande and Lima 2019). Nanoparticles are required in small amount to check pathogen. Nanoparticle synthesized bioagent become more essential wherever the bioagent is present or absent in a crop habitat. Nanoparticles can be isolated using a variety of techniques, including physical, chemical, and biological ones. The best of these approaches appears to be biological since it is an inexpensive, simple, and environmentally safe way to develop huge quantities of different nanoparticles. The objectives of this research work are synthesization of *Trichoderma* synthesized silver nanoparticle and evaluation of biocontrol efficacy of those nanoparticles against two pathogens infecting paddy plant.

MATERIAL AND METHODS

In this experiment silver nanoparticle was formulated by extracellular biosynthesis procedure. For the production of silver nanoparticles, two strains of *Trichoderma harzianum* (MT876632) and *Trichoderma asperellum* (MT951635) were employed. These two strains' potency against Paddy disease pathogens was evaluated.

Collection and culture of *Trichoderma harzianum* and *T. asperellum*: Two *Trichoderma* species, *Trichoderma harzianum* (MT876632) and *Trichoderma asperellum* (MT951635) were obtained from the Department of Plant Pathology's Biocontrol Lab at U.B.K.V., Pundibari, West Bengal. Throughout the trial time, these two strains' pure cultures are preserved by ongoing subculturing in potato dextrose agar slants.

Trichoderma isolates were identified using morphological analysis using the slide culture method. This procedure involved placing one glass slide within a sterilised Petri plate on top of two glass slides on blotting paper. To keep the humidity level high, a few drops of distilled water that had been sterilised were added to the blotting paper. Different sterilised Petri plate setup was employed for each isolate. Using a sterile cork borer, the mycelial disc of the *Trichoderma* isolate was put onto the glass slide. For 72 hours, plates were stored in BOD at 28±1˚C. Few drops of cotton blue were applied to the slide after the disc was later removed. The slides were examined at 10X and 40X magnifications using a microscope.

Biomass production of *Trichoderma harzianum* and *T. asperellum*: Biomass production is necessary for the manufacture of silver nanoparticles (AgNPs) utilising *Trichoderma asperellum* and *Trichoderma harzianum* culture supernatant. In potato dextrose broth (PDB), biomass was produced. Six mm disc of each bioagents incubated in PDB at 28±1˚C for 7 days. The culture supernatant was collected after seven days of incubation. following the removal of the mycelial mass (the vegetative part of fungi) from the culture broth using a sterile paper filter. The collected culture filtrate of *Trichoderma* isolates was then used to generate silver nanoparticles.

Biogenic synthesis of silver nanoparticle from *Trichoderma harzianum* and *T. asperellum*: Mycelium of

Fig. 1. Change in colour of *Trichoderma* synthesized silver nanoparticles

fungi was subjected to a metal salt for the production of metal nanoparticles by a fungus. Fungi release metabolites and enzymes with the aim to survive. This process transforms hazardous metal ions into non-toxic metallic solid nanoparticles via the catalytic action of the extracellular enzyme and secondary metabolites released by fungi. Fifty millilitres of sterile deionised water were used to create solutions of 1mM and 2 mM of silver nitrate. In a 250 ml Erlenmeyer flask, 50 ml of *Trichoderma* culture supernatant solution and 50 ml of silver nitrate solution were combined or mixed. The entire combination was continuously shaken at 200 rpm in a dark environment. The uninoculated set was used as control. Aluminium foil can be used to keep flasks in a dark condition. AgNP production is indicated by change in colour of supernatant from green to yellowish brown to brown.

Characterization of biogenic synthesized silver nanoparticles

UV-VIS spectrophotometer: Since the optical characteristics of nanoparticles are sensitive to factors like size, shape, concentration, aggregation state, and refractive index close to the nanoparticle surface, UV spectroscopy is an excellent technique for identifying, characterising, and studying nanoparticles. The fundamental requirements for the field of plasmonic are the distinct optical characteristics of materials made of specific metals, such as gold and silver, which interact strongly with particular light wavelengths when synthesised into nanoparticles. Visual observation of the solution and analysis of its UV-visible spectra were employed to regularly track the decline of silver ions by taking aliquots (2 mL) of the aqueous component on a regular basis. To ascertain the production of the biogenic silver nanoparticle resulting from the bio reduction of Ag+, an ultraviolet-visible spectrophotometer was employed. The UV-Vis Spectrophotometer of the UV-1900 series (model number: A12535780139) was utilised in our study to take measurements. The scanning range for the material was 200–800 nm. The baseline correction of the spectrophotometer was carried out using a water blank reference. The 2 ml sample was taken in a cuvette. The UV-Vis absorption spectrum of the sample was recorded, and numerical data was plotted.

Scanning in Fourier Transform Infrared Spectroscopy (FTIR): The infrared absorption spectrum that FTIR

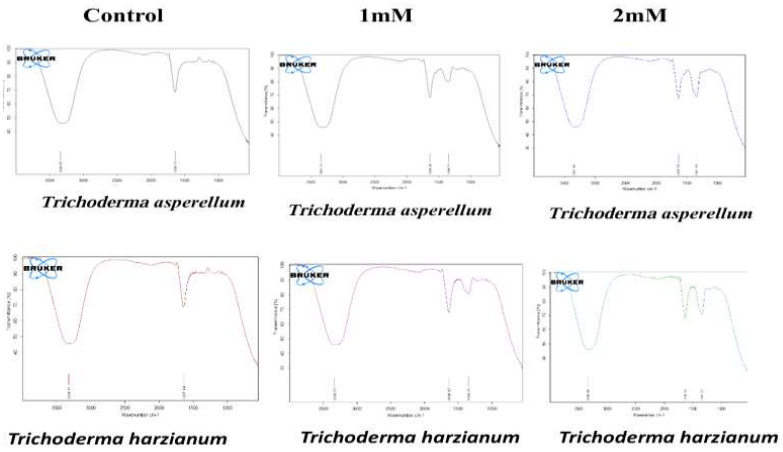


Fig. 2. UV-Vis absorption spectra obtained for silver nanoparticle

Table 1. *In vitro* biocontrol efficacy of *Trichoderma* synthesized nano particles against *Pyricularia oryzae*

Treatments	*Trichoderma harzianum* (MT876632)		*Trichoderma asperellum* (MT951635)	
	Radius of pathogen (cm)	% growth inhibition over control	Radius of pathogen	% growth inhibition over control
1mM (1ppm)	1.93	78.50	3.27	63.67
1mM (5ppm)	1.90	78.90	2.83	68.50
1mM (10ppm)	1.82	79.78	2.34	74.05
2mM (1ppm)	3.22	64.20	2.45	72.79
2mM (5ppm)	2.48	72.45	1.59	82.36
2mM (10ppm)	1.89	79.01	1.09	87.85
CD (p=0.05)	0.328	3.647	2.634	0.237

produces is similar to a molecular "fingerprint" and is a potent instrument for determining the kinds of chemical bonds present in a molecule (Senapati, 2005). This annotated spectrum illustrates how the chemical bond is characterised by the wavelength of light absorbed. FTIR can be utilised for quantitative analysis since the absorption strength is related to the concentration. This spectrometer simultaneously collects high-resolution spectral data throughout a wide spectral range. Compared to a dispersive spectrometer, which measures intensity throughout a small range of wavelengths at once, this offers a significant benefit. Infrared spectroscopy uses a sample to transmit infrared radiation. A portion of the infrared radiation is absorbed by the sample. It is transmitted in part. Since the FTIR spectra in the 1400–1700 cm–1 area reveals information on the existence of "C=O" and "N-H" groups, the measurement can also be used to investigate the presence of a protein molecule in the solution (Senapati et al., 2005). Consequently, the spectrum establishes a molecular fingerprint of the material by representing its molecular structure, transmission, and absorption. This fingerprint aids in the molecule's identification.

Isolation of *Pyricularia oryzae*: Blast affected small leaf and neck samples put in Potato Dextrose Agar (PDA) after surface sterilization with 0.1% Sodium Hypo chloride.

Identification of *Pyricularia oryzae*: Under a light microscope, morphological characteristics are used to identify *Pyricularia oryzae*.

Pathogenicity of *Pyricularia oryzae*: Healthy rice seedlings were used for the pathogenicity test. Rice grains were used to prepare pathogen inoculum. Then the pathogen from the freshly acquired lesion was viewed under microscope, confirmed and was re-isolated on potato dextrose agar media and the colony growth and morphology was evaluated. Rice plants were sprayed with pathogen inoculum at a concentration 10^6 cfu/ml after 21 days growth of pathogen.

Isolation of *Bipolaris oryzae*: *Bipolaris oryzae* was isolated under aseptic conditions in a laminar air flow chamber. Samples selected from the lesion's expanding edge; surface

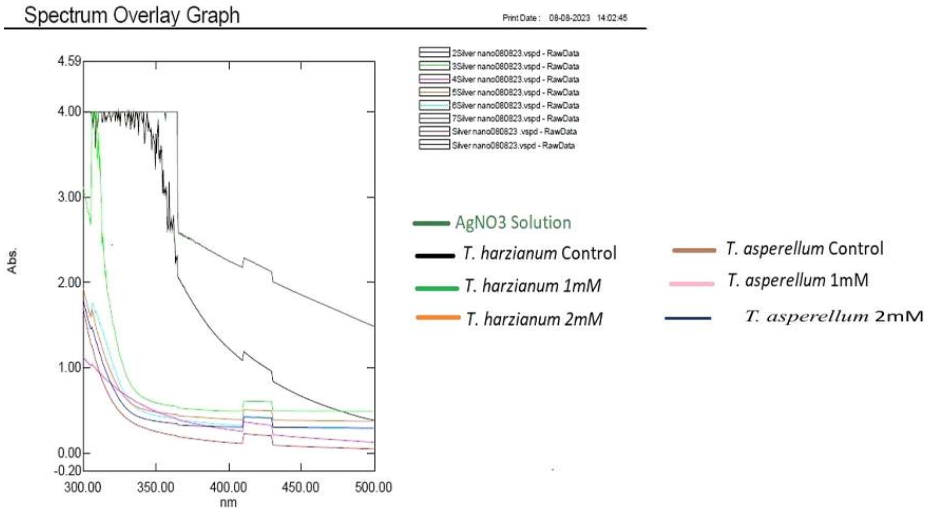


Fig. 3. Scanning in FTIR

Table 2. *In vitro* biocontrol efficacy of *Trichoderma* synthesized nano particles against *Bipolaris oryzae*

Treatments	*Trichoderma harzianum* (MT876632)		*Trichoderma asperellum* (MT951635)	
	Radius of pathogen (cm)	% growth inhibition over control	Radius of pathogen	% growth inhibition over control
1mM (1ppm)	3.93	56.38	3.81	56.05
1mM (5ppm)	3.77	58.30	3.42	62.67
1mM (10ppm)	2.93	67.54	3.01	66.51
2mM (1ppm)	3.70	58.93	3.66	60.56
2mM (5ppm)	3.45	61.74	3.27	63.63
2mM (10ppm)	3.23	64.38	3.21	64.38
CD (p=0.05)	1.945	0.175	0.317	1.455

sterilized by 1% sodium hypochlorite. After that samples put on potato dextrose agar (PDA) plate and were then incubated at 28 ± 1ºC for five to seven days.

Inoculum preparation of *Bipolaris oryzae*: Paddy grain was used to multiply of *Bipolaris oryzae.* Mycelial disc from full grown plate was transferred on autoclaved rice in a conical flask.

Identification of *Bipolaris oryzae*: *Bipolaris oryzae* identification using morphological traits. Conidia pointed at both sides are evidence of *Bipolaris oryzae*.

Pathogenicity test: Inoculum was prepared with the help of rice grain. On rice grains, pathogens doubled in size. Twenty-one days old rice seedlings were sprayed with the conidial suspension (5 x 10^6 spore/ ml) of the isolates of *Bipolaris oryzae*.

Determination of antagonistic potential of *Trichoderma* AgNPs: AgNPs' efficiency against the two infections under investigation (*Pyricularia oryzae* and *Bipolaris oryzae*) was evaluated in an in vitro experiment. Using the Poison Food Technique, the effectiveness of AgNPs was examined at concentrations of 10 ppm, 5 ppm, and 1 ppm (Kim *et al.*, 2012). AgNPs were combined with sterile PDA media for this experiment prior to plating. PDA was put into a sterile Petri plate after being thoroughly mixed. After the AgNPs treated PDA solidified, a 6 mm mycelia disc of the pathogen that was actively growing with an agar plug was cut with a cork borer and kept at the centre of a Petri plate (9 cm in diameter). For ten days, each plate was incubated at 28±1˚C. When the control attained its maximum development, the colony diameter was observed. The growth inhibition rate was computed using the following formula.

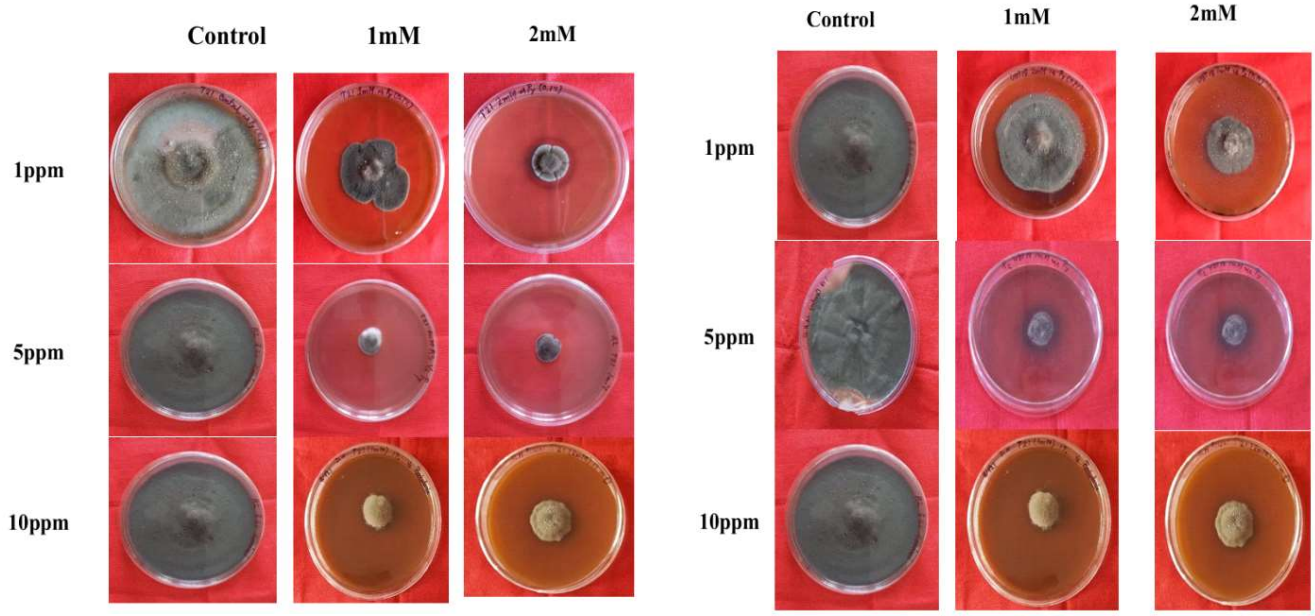
$$\text{Inhibition } \% = [(R - r) / R] * 100$$

where **r** is the radial growth of fungal mycelia on the plate treated with silver nanoparticles and **R** is the radial growth of fungal mycelia on the control plate.

RESULTS AND DISCUSSION

Synthesis of silver nanoparticles: The successful synthesis of silver nanoparticles (AgNP) was achieved using the extracellular synthesis approach. *T. harzianum* and *T. asperellum* culture filtrate was exposed to 1mM and 2 mM solutions of silver nitrate, it was observed that the reduction of silver ions into silver nanoparticles was followed by precipitation onto the cells. The colour shift from pale green to yellowish brown and finally dark brown, which is caused by the Surface Plasmon Resonance phenomenon, served as preliminary confirmation that silver nanoparticles were forming (Tripathi et al., 2013).

Change in the colour: When *Trichoderma* culture filtrate and silver nitrate solution were combined and stored in a shaker incubator under dark conditions, the colour progressively changed from pale yellow to yellowish brown, and after 30 days, it turned dark brown. Various workers also reported similar findings. Devi et al. (2013) observed that the shift in hue from pale yellow to dark brown showed that the *Trichoderma* isolate was clearly creating silver nanoparticles. The primary cause of the hue shift is either the deposited



Trichoderma asperellum *Trichoderma harzianum*

Plate 2A: In vitro efficacy of *Trichoderma harzianum* & *Trichoderma asperellum* synthesized AgNPs against Pyricularia oryzae

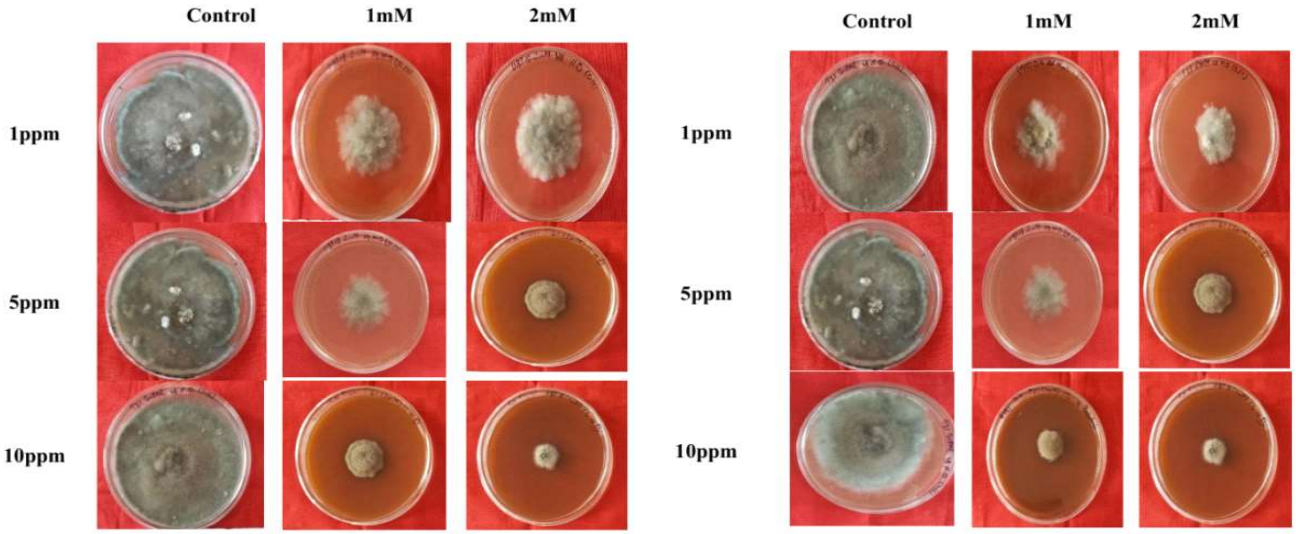


Plate 2B. In vitro efficacy of *Trichoderma asperellum* synthesized AgNPs against *Bipolaris oryzae*:

silver nanoparticles' surface plasmon resonance or the surface electrons' collective and coherent oscillations (Link and El-Sayed 2003).

We found that there is no change in colour in case of control where silver nitrate solution was not mixed. But in case of *Trichoderma* isolate where silver nitrate solution was mixed with culture filtrate, colour had changed into dark brown. The change of colour is the confirmation or evident of producing silver nanoparticle (Devi et al., 2013).

UV-Vis spectrophotometer analysis: It is widely acknowledged that controlled nanoparticles in aqueous solutions can be investigated by applying UV-Vis spectroscopy. The surface plasmon resonance (SPR) absorption band at the crucial wavelength is provided by the free electrons in the metal nanoparticles. The simultaneous vibrating of metal nanoparticle electrons in resonance with light waves is what causes the SPR band. Silver nanoparticles exhibit an SPR peak between 410 nm and 420 nm (Devi et al., 2013). The study revealed that the SPR peak formed between 415 nm and 420 nm. The spectrum unmistakably displays the rise in silver nitrate intensity, which denotes the creation of more silver nanoparticles in the solution.

Scanning in FTIR: There were peaks I, II, and III in the FTIR spectra. Peaks II and III may have been caused by the silver ion. Peak I which was observed in both the control and *Trichoderma* synthesised silver nanoparticle cases, may have been caused by O-H bonding. Peak III originated between 1330 cm^{-1}-1420 cm^{-1}, and might likewise be the result of alcohol's O-H bonding. In contrast, *T. asperellum* synthesised silver nanoparticles produced a III peak, which might be the result of aromatic amine's C-N stretching. These III peaks could be a sign that a silver nanoparticle is forming.

***In vitro* bioefficacy of the *Trichoderma* synthesized silver nanoparticles:** *Trichoderma* synthesized silver nanoparticles in two concentrations (1mM and 2mM) were tested against two paddy pathogens namely *Pyricularia oryzae* and *Bipolaris oryzae in vitro*. Against *Pyricularia oryzae, Trichoderma harzianum* nano formulation used significant variation found in different treatment. Highest percentage inhibition (79.78%) observed in 1mM (10ppm) nano formulation which is closely followed by (79.01%) 2mM (10ppm) nano formulation. When *Trichoderma asperellum*, nano formulation used significant variation found in different treatment. Highest percentage inhibition (87.85%) recorded in 2mM (10ppm) nano formulation which is closely followed by (82.36%) 2mM (5ppm) nano formulation.

Against *Bipolaris oryzae*, *T. harzianum* nano formulation showed variation in growth inhibition (56.38 to 67.54%). Highest percentage inhibition was recorded in1mM 10 ppm nano formulation which is closely followed by 2mM 10 ppm nano. In *T. asperellum* synthesized nano formulation also showed variation in growth inhibition from 56.05%to 64.38%. Highest percentage inhibition was in 1mM 10 ppm nano formulation which is closely followed by in 2mM 10 ppm nano formulation.

CONCLUSION

Silver nanoparticles were developed using *Trichoderma* by green synthesis method without using any harmful chemical. UV-Vis Spectrophotometry, FTIR study are showed formation of silver particle. *T. harzianum* and *T.*

asperellum synthesized AgNPs exhibited antagonistic property to *Pyricularia oryzae* and *Bipolaris oryzae*. With the increase in concentration of *T*. synthesized AgNP increased, antagonism also increased.

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