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Title Key: In-vitro Antifungal Activity…

In- vitro antifungal activity of silver nanoparticles against seed mycoflora of soybean

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ABSTRACT

Fungal diseases cause significant economic agricultural losses and their control have been limited to chemical fungicides in an irrational manner. Silver nanoparticles which have high antimicrobial effects against different fungi .The present investigation on synthesis of silver nanoparticles by using *Trichoderma* spp. and its antifungal activity against different seed mycoflora of soybean i.e. *F.verticillioids*, *M.phaseolina* and *A.alternata*. Characterization of *Trichoderma* silver nanoparticles were carried out by UV-Vis spectroscopy and Transmission electron microscopy (TEM). *Trichoderma asperellum*, *Trichoderma harzianum* and *Trichoderma hamatum* silver nanoparticles at 100 and 150 ppm concentration demonstrated significant antifungal activity against *F.verticillioid*, *M.phaseolina* and *A.alternata* by poisoned food technique. In poison food technique, the suspension of silver nanoparticles of *T.hamatum* at 150 ppm conc. recorded highest 75.22 % inhibition of *A.alternata*. In *M.phaseolina* highest 66.11% inhibition was recorded with silver nanoparticles of *T.harzianum* at 150 ppm conc. and in *F.verticilloids* highest 62.66% inhibition was recorded by *T.asperellum* at 150 ppm conc.

Key Word: Silver nanoparticles (AgNPs), *Trichoderma* spp, UV-Vis spectroscopy, Transmission Electron Microscopy (TEM).

INTRODUCTION

Trichoderma spp. were used as biological control agents against soil borne plant pathogenic fungi. Advantages of using *Trichoderma* in managing seedborne plant pathogens are ecofriendly, effective, ease of mass culturing with less cost of production and growth promoting effect. Biosynthesis of nanoparticles is an attractive possibility of advancement of green nanotechnology, which has potential to find out numerous applications in biology, agriculture in particular. Recently the utilization of biological systems provides a novel idea for the production of nonmaterial.

Silver nanoparticles, have high antimicrobial effects as compared to the bulk silver. In the biosynthesis of nanoparticles by fungus, the fungus mycelium is exposed to the metal salt solution, which prompts the fungus to produce enzymes and metabolize for its own survival. In this process the toxic metal ions are reduced to the non-toxic metal ions through the catalytic effect of extracellular enzymes and metabolites of fungi (Khabat *et al*., 2011). Nanotechnology can offer green and eco-friendly alternatives for plant disease management (Alghuihaymi *et. al*, 2015). Keeping in view importance of *Trichoderma* spp. as biological control agents against soil and seed borne plant pathogenic fungi and green nanotechnology, present study was carried out.

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MATERIALS AND METHODS

Cultures of *Trichoderma asperellum, T. harzianum* and *T. hamatum* which were available at the Department of Plant Pathology, Latur were used for these studies.

Production of biomass *of Trichoderma asperellum, T. harzianum* and *T.hamatum*

A seven days old pure culture of *Trichoderma asperellum, T. harzianum* and *T. Hamatum* was inoculated in 250 ml conical flasks containing 100 ml of Potato Dextrose Broth (PDB) and the culture flasks were incubated at 27±1^{0}C. Then, the mixture was placed in 150 rpm rotating shaker at 28^{0}C for 72 hrs. The biomass was harvested through sterilized Whatman No-1 filter paper. After harvesting of biomass, the culture filtrate was used for the synthesis of silver nanoparticles.

Biosynthesis of silver nanoparticles

Silver nanoparticles were synthesized by treating 50 ml of aqueous solution of 1 mM silver Nitrate with 50 ml of *Trichoderma asperellum, T. harzianum* and *T. hamatum* culture filtrate in a 250 ml conical flask. The colour change of silver nanoparticles from colourless to brown colour indicates formation of silver nanoparticles through reduction of silver ionic forms (Ag+) to (Ag0).

Characterization of silver nanoparticles

UV-Visible spectroscopy

Colour change of cell filtrate after the incubation of silver nitrate solution was observed visually. The reduction of silver ions was monitored by UV-Vis spectrum of the reaction mixture at 24 hrs. The spectra of the surface Plasmon resonance of AgNPs in the reaction mixture were recorded by UV-Vis spectrophotometer at wavelengths between 200 to 800 nm.

Transmission Electron Microscopy (TEM)

The nanoparticles were characterized by transmission electron microscopy (TEM). TEM determine their size and shape from drop-coated films of the silver nanoparticles synthesized by fungal cell filtrate. TEM images of the sample were taken at IIT, Bombay.

In vitro evaluation antifungal activity of biosynthesized silver nanoparticles against mycoflora of soybean seed

Efficacy of silver nanoparticles was evaluated against seed mycoflora *F. verticillioides, M. phaseolina* and *A. alternata* by poisoned food technique and using Potato Dextrose Agar (PDA) as a basal culture medium.

Based on active ingredients, requisite quantity of each test silver nanoparticles was calculated and mixed thoroughly with autoclaving and cooled (40ºC) PDA medium in conical flasks to obtain 100 and 150 ppm concentrations of the test silver nanoparticles. Silver nanoparticles amended PDA medium was then poured aseptically in Petri plates (90 mm dia.). A control was maintained without nanosilver and culture filtrate. 5 mm mycelial disc of seven days old culture of pathogen was inoculated at the center and incubated at 26 ± 2°C until full growth was observed in control.

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RESULTS AND DISCUSSION

Seven days old pure culture of *Trichoderma asperellum*, *T.harzianum* and *T.hamatum* were inoculated in Potato Dextrose Broth (PDB). The culture filtrates were harvested at different time intervals, viz. 4 DAI, 6 DAI, 8 DAI, 12 DAI, 15 DAIand colour of culture filtrate was pale yellow which was clearly observed. To know the effect of incubation on the synthesis of silver nanoparticles, l mM Silver nitrate (AgNO3) solution were added to 10 ml of culture filtrate from each observed DAI. Silver nitrate solution treated with 4 days and 6 days incubated culture filtrate turned into dark brown colour as compare to 8 days, 12 days and 15 days incubated culture filtrate after 24 hrs incubation.

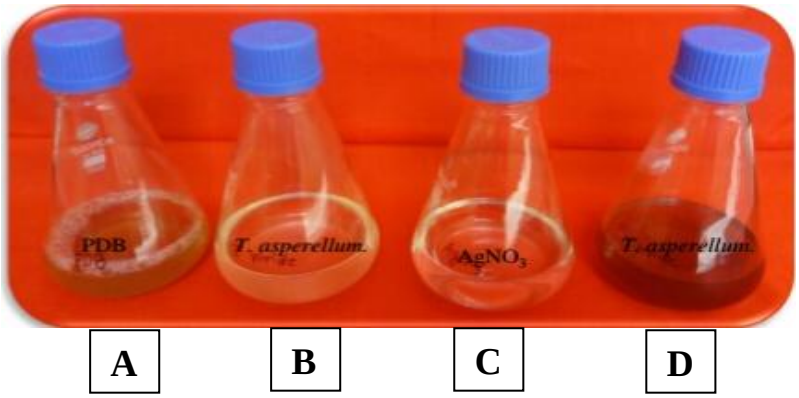

Plate I: Synthesis of silver nanoparticles by using *Trichoderma asperellum*
(A) PDB (Potato Dextrose Broth) alone (B) Trichoderma *asperellum* culture filtrate
(C) 1 mM AgNO3 solution before incubation of Trichoderma *asperellum*culture filterate
(D) 1 mM AgNO3 solution after 24 hrs incubation of Trichoderma *asperellum* culture filterate

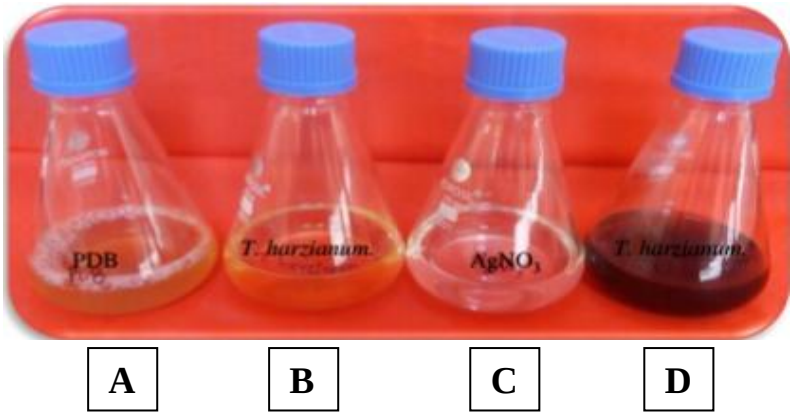

Plate II: Synthesis of silver nanoparticles by using *Trichoderma harzianum*
(A)PDB (Potato Dextrose Broth) alone (B) Trichoderma harzianum culture filtrate (C) 1 mM AgNO3 solution before incubation of Trichoderma harzianum culture filterate (D) 1 mM AgNO3 solution after 24 hrs incubation of Trichoderma harzianum culture filterate

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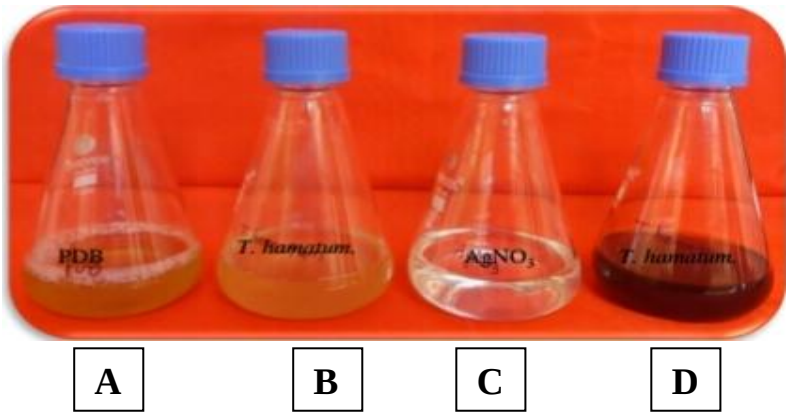

Plate III: Synthesis of silver nanoparticles by using *Trichoderma hamatum*
(A)PDB (Potato Dextrose Broth) alone (B) Trichoderma *hamatum* culture filtrate (C) 1 mM AgNO3 solution before incubation of Trichoderma *hamatum*culture filterate (D) 1 mM AgNO3 solution after 24 hrs incubation of Trichoderma *hamatum* culture filterate

UV -Vis spectroscopy

Silver nanoparticles were synthesized from 1 mM $AgNO_3$ solution treated with four days and six days incubated culture filtrate. A colour change to brown colour with a characteristic surface Plasmon resonance band at 420 nm at 24 hrs after incubation was recorded (Fig. 1). Maximum intensity of synthesized silver nanoparticles were observed for six days incubated culture filtrate treated $AgNO_3$ solution followed by four days incubated culture filtrate treated 1 mM $AgNO_3$ solution. Similar to the present study, UV absorption peak of silver nanoparticles synthesized from *T. asperellum*,*T. koningii* ,*T. harzianum* (Shelar and Chavan 2015), *T. reesei* (Khabat *et al.*,2011) were observed at 400 nm, 413 nm, 440 nm and 420 nm, respectively.

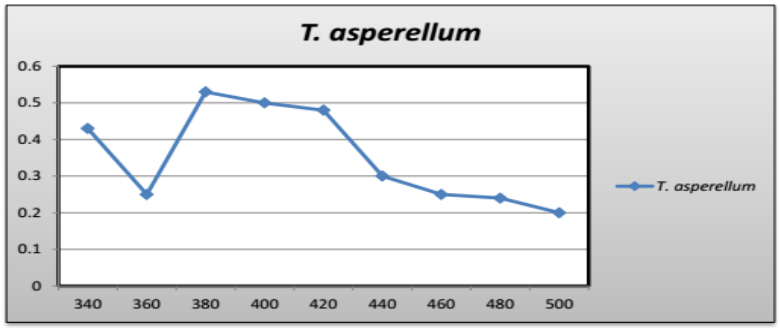

Fig. 1. UV-Vis spectra recorded after the exposure of 1 mM silver nitrate solution in culture filtrate of *T. asperellum*

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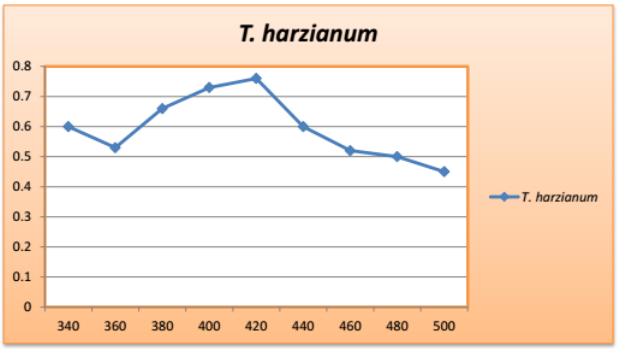
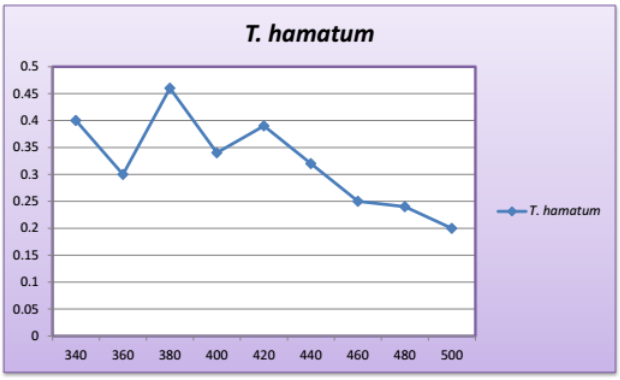


Wavelenghth (nm)

Fig 2. UV-Vis spectra recorded after the exposure of 1 mM silver nitrate solution in culture filtrate of *T. harzianum*



Wavelenghth (nm)

Fig.3. UV-Vis spectra recorded after the exposure of 1 mM silver nitrate solution in culture filtrate of T. hamatum

Transmission Electron Microscopy (TEM) analysis

The Transmission Electron Microscopy studies characterized the shape and size of the synthesized silver nanoparticles (Fig. 4). In general particles were spherical in shape and the sizes of the silver nanoparticles were found in the range of 50 nm. Results of the present study on Transmission Electron Microscopy (TEM) analysis are in consonance with those reported earlier by several workers on size 5-50 nm (Khabat et al. 2011), 10-20 nm (Kaur et al.2012) and 19-63 nm (Shelar and Chavan 2015).

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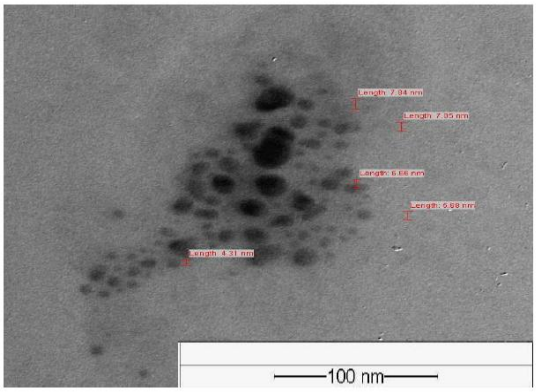
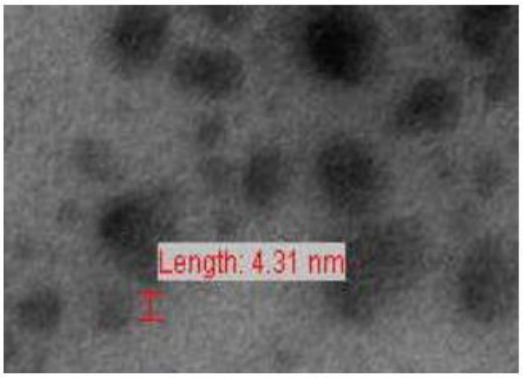
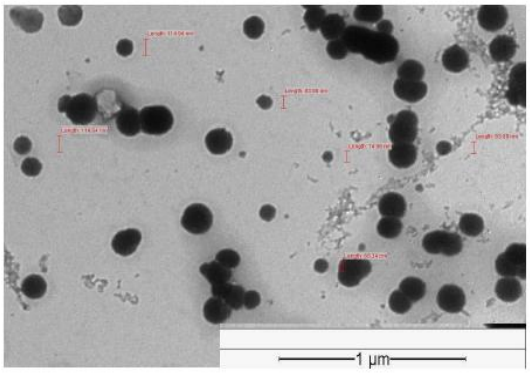
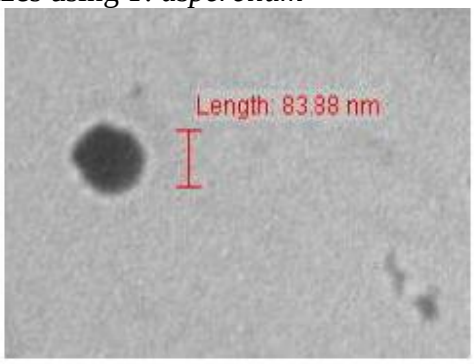




Single particle of silver nanoparticle

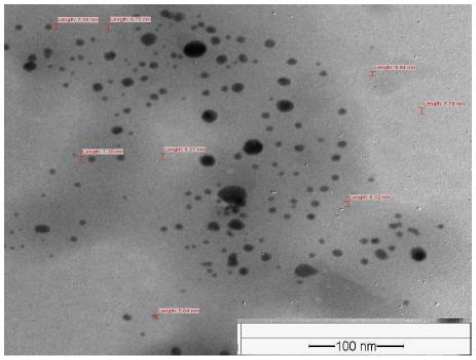
Fig.4. TEM micrographs showing the relatively spherical shape Ag nanoparticles with the range of 4.31-8.00 nm synthesizes using *T. asperellum*

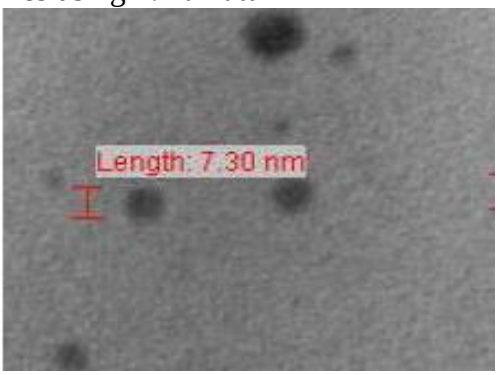




Single particle of silver nanoparticle

Fig.5.TEM micrographs showing the relatively spherical shape Ag nanoparticles with the range of 74.58-114.9 nm synthesizes using *T. hamatum*





Single particle of silver nanoparticle

Fig.6. TEM micrographs showing the relatively spherical shape Ag nanoparticles with the range of 4.31-8.00 nm synthesizes using *T. harzianum*

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Table.1. *In vitro* efficacy of silver nano particles against *F.verticillioids, M.Phaseolina and A.alternata*

Sr. No.	Treatments	Conc. (ppm)	*F.verticillioids*		*M.Phaseolina*		*A.alternata*	
			Colony diameter (mm)	Inhibition * (%)	Colony diameter (mm)	Inhibition * (%)	Colony diameter (mm)	Inhibition* (%)
T1	*T.asperellum* silver nanoparticles	100	40.50	55.00 (47.86)	75.10	16.55 (24.00)	27.80	69.11 (56.23)
T2	*T.harzianum* silver nanoparticles	100	40.60	54.88 (47.80)	73.10	18.77 (25.67)	25.80	71.33 (57.62)
T3	*T.hamatum* silver nanoparticles	100	43.30	51.88 (46.07)	73.50	18.33 (25.34)	25.50	71.66 (57.83)
T4	*T.asperellum* silver nanoparticles	150	33.60	62.66 (52.07)	64.80	28.00 (31.94)	24.50	72.77 (58.54)
T5	*T.harzianum* silver nanoparticles	150	34.00	62.22 (52.07)	30.50	66.11 (54.39)	24.30	73.00 (58.69)
T6	*T.hamatum* silver nanoparticles	150	37.80	58.00 (49.60)	38.50	57.22 (49.15)	22.30	75.22 (60.14)
T7	*T.asperellum* culture filtrate	-	43.80	51.33 (45.76)	81.80	9.11 (17.56)	32.30	64.11 (53.19)
T8	*T.harzianum* culture filtrate	-	43.80	51.33 (45.76)	75.80	15.77 (23.39)	31.80	64.66 (53.52)
T9	*T.hamatum* culture filtrate	-	44.00	51.11 (45.63)	80.10	11.00 (19.36)	29.60	67.11 (55.00)
T10	Control	-	90.00	00 (00.00)	90.00	00 (00.00)	90.00	00 (00.00)
SE±			0.74	0.63	0.67	0.70	0.89	0.63
CD @1%			2.43	2.08	2.20	2.32	2.15	2.07

*: Mean of three replications Figures in parentheses are Arcsine value

***In vitro* evaluation of antifungal activity of silver nanoparticles by Poison food technique method**

Poison food technique method

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Antifungal activity of synthesized silver nanoparticles against mycoflora of soybean seeds *was* evaluated by poison food technique. Effect of Silver nanoparticles was compared with the effect of *Trichoderma asperellum*, *T.harzianum* and *T.hamatum* culture filtrate.

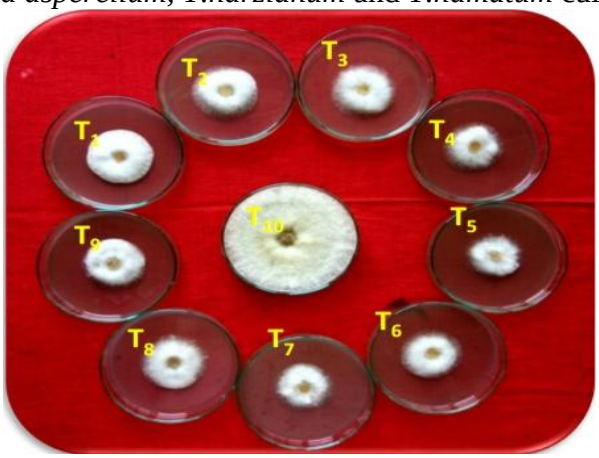
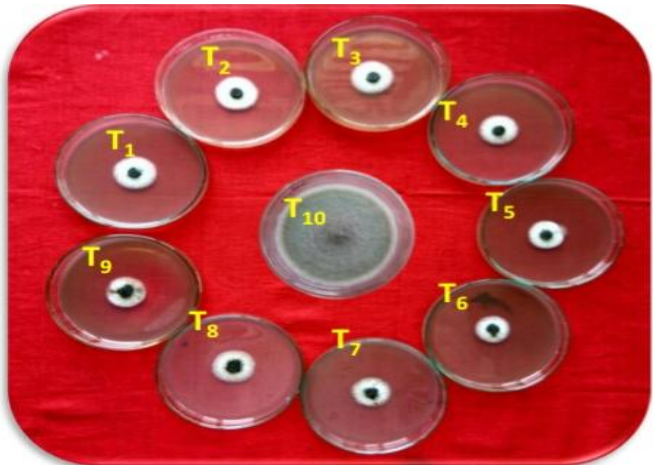


Plate. IV: ***In vitro*** **evaluation of antifungal efficacy of** ***Trichoderma asperellum, T.harzianum*** **and** ***T.hamatum*** **silver nanoparticles by using poison food method against** ***F.verticillioids***

Plate V: ***In vitro*** **evaluation of antifungal efficacy of** ***Trichoderma asperellum, T.harzianum*** **and** ***T.hamatum*** **silver nanoparticles by using poison food method against** ***M.phaseolina***



PlateVI: *In vitro* evaluation of antifungal efficacy of *Trichoderma asperellum, T.harzianum* and *T.hamatum* silver nanoparticles by using poison food method against *A.alternata*

For *F. verticillioides* (Table 1 and plate IV), significantly highest mycelial growth inhibition was with *Trichoderma asperellum* silver nano particles @ 150 ppm (62.66%), followed by, *T. harzianum* silver nano particles @ 150 ppm (62.22%), *T. hamatum* silver nano particles @ 150 ppm (58.00%), *Trichoderma asperellum* silver nano particles 100 ppm (55.00%), *T. harzianum* silver nano particles 100 ppm (54.88%) and *T. hamatum* silver nano particles @ 100 ppm (51.88%). *Trichoderma asperellum* culture filtrate and *T. harzianum* culture filtrate also caused mycelium growth inhibition of 51.33% and *T. hamatum* culture filtrate of 51.11%.

For *M. phaseolina* (Table1 and Plate V), significantly highest mycelial growth inhibition was with *T. harzianum* silver nano particles @ 150 ppm (66.11%), followed by, *T. hamatum* silver nano particles @ 150 ppm (57.22%), *Trichoderma asperellum* silver nano particles @ 150 ppm (28.00%), *T. harzianum* silver nano particles @ 100 ppm (18.77%), *T. hamatum* silver nano particles @ 100 ppm (18.33%), *Trichoderma asperellum* silver nano particles @ 100 ppm (16.55%), *T. harzianum* culture filtrate (15.77%), *T. hamatum* culture filtrate (11.00%) and *Trichoderma asperellum* culture filtrate (9.11%).

For *A. alternata* (Table 1 and Plate VI), significantly highest mycelial growth inhibition was with *T. hamatum* silver nano particles @ 150 ppm (75.22%), followed by, *T. harzianum* silver nano particles @ 150 ppm (73.00%), *Trichoderma asperellum* silver nano particles @ 150 ppm (72.77%), *T. hamatum* silver nano particles @ 100 ppm (71.66%), *T. harzianum* silver nano particles 100 ppm (71.33%), *T. asperellum* silver nano particles @ 100 ppm (69.11%), *T. hamatum* culture filtrate (67.11%), *T. harzianum* culture filtrate (64.66%) and *Trichoderma asperellum* culture filtrate (64.11%).

It has been proved that *Trichoderma* spp. was capable of synthesizing the metal nanoparticles; silver in particular, which is an effective controlling agent of pathogens, *Fusarium verticillioides*, *Macrophomina phaseolina* and *Alternaria alternata*. (Gajbhiye et al. 2009; Savithramma et al. 2011 and Kaur et al. 2012)

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