*In Vitro*Antifungal Efficacy of Silver Nanoparticles against *Fusarium oxysporum* f. sp. *Lycopersici* in Tomato

ABSTRACT

Present investigation on Mycosynthesisof silver nanoparticles and antifungal activity of silver nanoparticles against*Fusarium oxysporum* f. sp. *lycopersici* in tomato was carried out at department of Plant Pathology, College of Agriculture, Latur to find out antifungal activity of silver nanoparticles against *Fusarium oxysporum* f. sp. *lycopersici* in tomato.

Mycosynthesis of silver nanoparticles produced from the fungus *Trichoderma harzianum.* Characterization of silver nanoparticles were carried out by UV-Vis spectroscopy and Transmission Electron Microscopy (TEM) which revealed that synthesized nanoparticles were having the UV absorption peak at 420 nm and nanoprticle size was 50 nm. Silver nanoparticles demonstrated significant antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* in tomato by using Agar well diffusion method and Poisoned food technique.

In Agar well diffusion method, the highest zone of inhibition 18.66 mm was recorded at 100 ppm concentration than other treatments. In poisoned food technique, the suspension of silver nanoparticles at 100 ppm concentration recorded highest (75.19%) inhibition. This was followed by 50 ppm, 30 ppm, 10 ppm conc. and *Trichoderma* culture filtrate which recorded 66.67%, 58.89%, 54.45% and 51.45% inhibition, respectively.

Growth of the *Fusarium oxysporum* f. sp. *lycopersici* in tomato decreased drastically with increase in the concentration of the silver nanoparticles.

Keywords: *[*Antifungal activity, silver nanoparticles *[*AgNPs], *Trichoderma harzianum*,*Fusarium oxysporum* f. sp. *lycopersici*, UV-Vis spectroscopy, Transmission Electron Microscopy [TEM].]

1. INTRODUCTION

‘‘*Trichoderma spp*were used as biological control agents against soil borne plant pathogenic fungi. Advantage of using *Trichoderma* in managing soilborne 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 nanomaterials” (Khabat et al., 2011).

“Silver nanoparticles, which 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). Keeping in view importance of Trichoderma*spp*as biological control agents against soil borne plant pathogenic fungi and green nanotechnology, present study was emphasized on *in vitro* antifungal activity of silver nanoparticles against *Fusarium oxysporum* f. sp. *lycopersici* in tomato was carried out.

2. MATERIAL AND METHODS

2.1 Isolation and identification of *Trichoderma* spp.

For mycosynthesis and characterization of silver nanoparticles, soil required was collected from rhizospheric soil from farm of College of Agriculture, Latur. Serial dilution technique was used for isolation of *Trichoderma* spp. and PDA was used as basal medium for isolation. Identification of *Trichoderma* spp. on the basis of cultural and morphological characters. This identified *Trichoderma* spp. used for synthesis of silver nanoparticles.

2.1.1 Production of biomass *of Trichoderma harzianum*

A seven-day old pure culture of *Trichodermaharzianum*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.

2.2 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 harzianum* culture filtrate in a 250 ml conical

flask. The colour change of silver nanoparticles colourless to brown colour indicates formation of silver nanoparticles through reduction of silver ionic forms (Ag+) to (Ag0).

2.3 Characterization of silver nanoparticles

2.31 UV-Visible spectroscopy

Colour of the cell filtrate changes after the incubation of silver nitrate solution was visually observed. 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 using UV-Vis spectrophotometer at wavelengths between 200 to 800 nm.

2.3.2 Transmission Electron Microscopy (TEM)

The nanoparticles were characterized by transmission electron microscopy (TEM) to determine their size and shape from drop-coated films of the silver nanoparticles synthesized by fungal cell filtrate. TEM micrograph indicates the particles were relatively uniform in nature and also shows that particles were well separated from each other having no accumulation. TEM images of the sample were taken at IIT, Bombay.

2.4 In vitro evaluation antimicrobial activity of biosynthesized silver nanoparticles and *Trichoderma* culture filtrate against *Fusarium oxysporum* f. sp. *Lycopersici* by different methods.

2.4.1 Agar well Diffusion method

The soilborne plant pathogenic fungus (*Fusarium oxysporum f. sp.lycopersici*) was used to determine the antifungal activity of the silver nanoparticles. The experiment was carried out by Agar Well Diffusion Method (Kaur *et al*., 2012).

Fifteen ml of sterilized PDA medium was poured into the 90 mm Petri plate. After solidification, 5 ml of seeded agar containing 0.5×10^6 spores/mycelium of test pathogens per ml was spread uniformly on PDA medium. Appropriate wells were made on agar plate by using sterile cork borer of 9 mm diameter. The required concentrations of nanosilver and culture filtrate(10, 30, 50 and 100 ppm) were prepared using distilled water. The wells on agar plates were filled with each concentration of nanosilver and culture filtrate. In control plates, the wells were filled with distilled water. For each concentration, four plates were maintained. Plates were incubated at 27 ± 1^0C, until the clear zones of inhibitions were observed around the wells. The inhibition zones were measured from the centre of the well. Minimum inhibition concentration of nanosilver and $AgNO_3$ was recorded based on inhibition zones around each concentration.

2.4.2 Poisoned food technique

In vitro evaluation of *Trichoderma harzianum* culture filtrate of non-biosynthesized *Trichodermaharzianum*and synthesized nanoparticles were evaluated against soilborne pathogen (*Fusarium oxysporum*f. sp.*lycopersici*) of tomato crop by poisoned food technique (Nene and Thapliyal, 1993) using PDA as basal medium.

Thirty ml of double strength PDA medium (Potatoes-400 g, Dextrose- 40g, Agar 40g, dist. Water-1000 ml) was mixed with thirty ml of double concentrated nanosilver and culture filtrate solutions to obtain final concentrations of 10, 30, 50 and 100 ppm. Twenty ml of this mixture was poured in 90 cm Petri plate. A control was maintained without nanosilver and culture filtrate.7 mm mycelial disc of seven days old culture of *Fusarium oxysporum*f. sp. *lycopersici*was inoculated at the center and incubated at 27±1^{0}C until full growth was observed in control. Per cent mycelial growth inhibition of*Fusarium oxysporum*f. sp. *lycopersici*over untreated control was calculated by applying the formula (Vincent, 1927).

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where,
C = growth of the test fungus in untreated control plate
I = Per cent inhibition
T = growth of the test fungus in treated plate

Four replications were maintained for each treatment.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of *Trichoderma spp*. from the rhizosphere of tomato

A soil sample was collected from healthy tomato plant present in the research farm, College of Agriculture, Latur. The *Trichoderma* spp. from the rhizospheric soil was isolated by using serial dilution technique. Cultural and morphological identification of the *Trichoderma* isolate was studied, based on the characters of colony, mycelial and spore pattern (Bissett, 1991). Colony characters of *Trichoderma* isolate was studied using 3 days old culture. The *Trichoderma* isolate grew well and formed conidia within 4 days. In the colonies of isolate conidation was effuse, appearing powdery due to dense conidiation. Rapidly turning yellowish to yellowish green with ring like zone. Colorless to dull yellow at the reverse of the Petri plates. The colour of *Trichoderma* isolate was found to be green to light green. The shape of conidia observed irregularly and bottle shaped and size of conidia was 4.5-8.0×3.0-4.5 µm. The chlamydospores of *Trichoderma* sp. was not observed up to 7 days. Based on cultural characteristics colony colour, revers colony colour, growth rate and morphological characters like conidiophores, conidia size and shape, isolate was confirmative with characters of *T. harzianum*. (Plate 1)

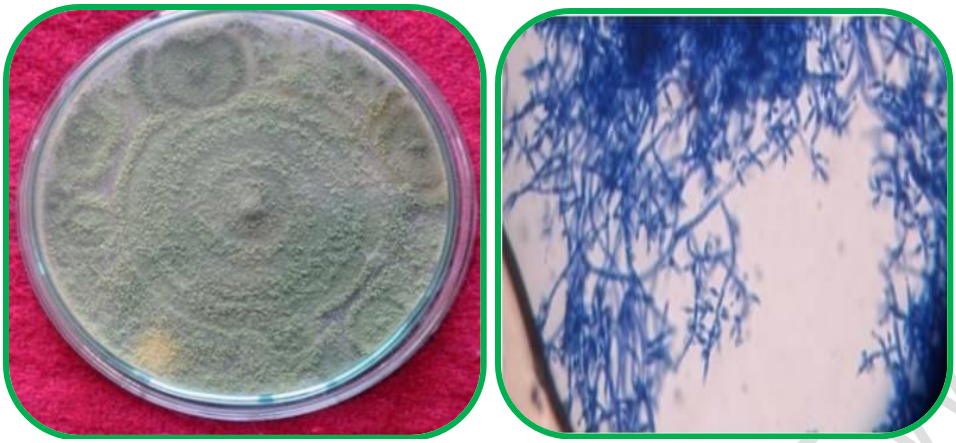

Plate 1: Isolation of *Trichoderma harzianum* and Microphotographs at 100 X in Olympus light microscope of isolated *Trichoderma harzianum* from tomato rhizosphere

3.2 Biosynthesis of silver nanoparticles using *Trichoderma harzianum*

Seven days old pure culture *of Trichoderma harzianum* was inoculated in Potato Dextrose Broth (PDB). The culture filtrate was harvested at different time intervals, *viz.* 4 DAI, 6 DAI, 8 DAI, 12 DAI, 15 DAI (Plate 2). To know the effect of incubation on the synthesis of silver nanoparticles, 10 ml of culture filtrate from each observed DAI was added to the l mM Silver nitrate ($AgNO_3$) solution. 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 3).

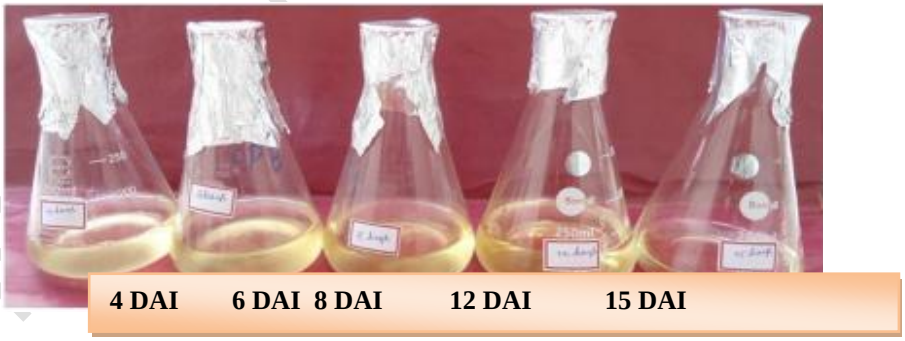

Plate 2: Culture filtrate of *Trichodermaharzianum* at different days of incubation (Left to Right : 4 DAI, 6 DAI, 8 DAI, 12 DAI and 15 DAI)

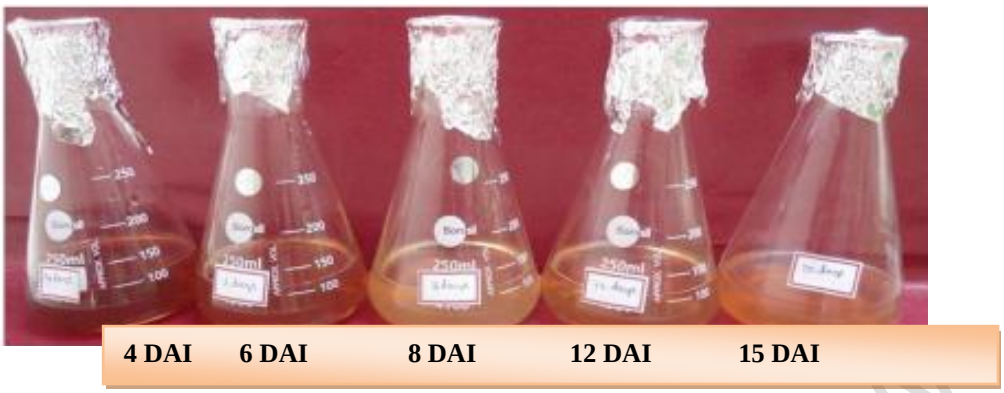

Plate 3: Culture filtrate treated silver nitrate solutions after 24 hrs incubation

3.3Characterization of silver nanoparticles

3.3.1 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. viride*,*T. koningii*(Tripathi *et al*., 2013), *T. harzianum*(Shelar and Chavan 2015), *T.reesei*(Khabat*et al.,*2011) was observed at 400 nm, 413 nm, 440 nm and 420 nm, respectively.

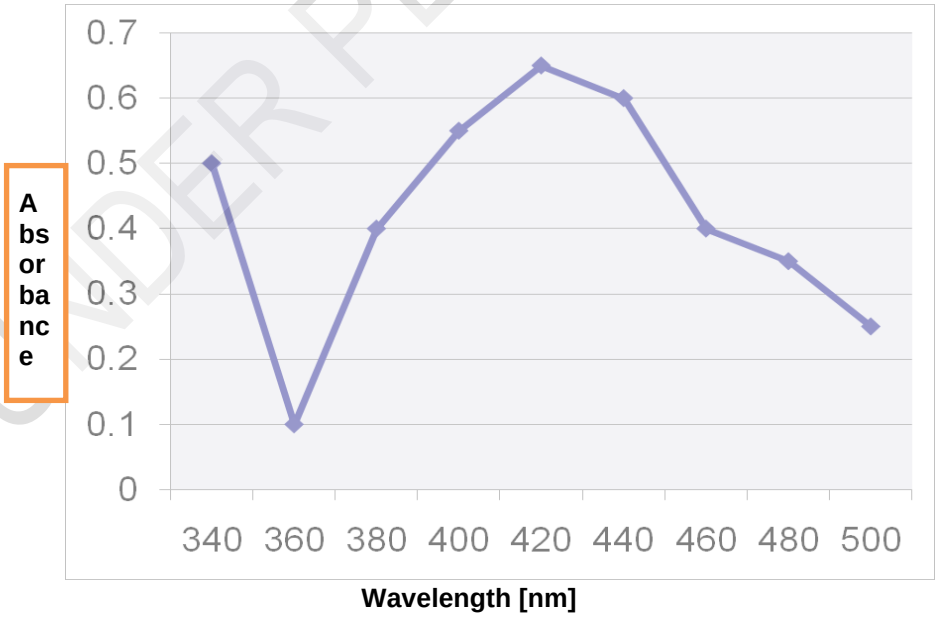

Fig. 1. Spectrum of UV-Vis spectrum absorption of silver nanoparticles of *T. harzianum*

3.3.2 Transmission Electron Microscopy (TEM) analysis

The Transmission Electron Microscopy studies characterized the shape and size of the synthesized silver nanoparticles (Plate 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), 8-24 nm (Tripathi *et al*., 2013) and 19-63 nm (Shelar and Chavan, 2015).

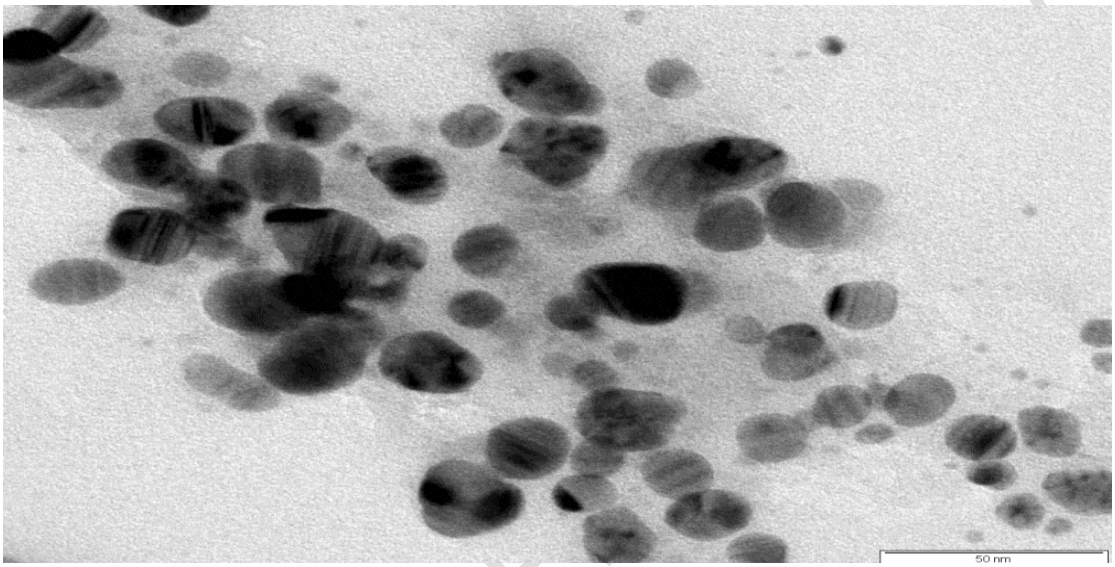

Plate 4: TEM Micrographs showing the relatively spherical shape Ag nanoparticles with the mean size 50 mm synthesized using *Trichoderma harzianum*

3.4*In vitro* evaluation of antimicrobial activity of silver nanoparticles against *Fusarium oxysporum*f. sp. *lycopersici*

3.4.1 Agar well diffusion method

The antimicrobial activity of the synthesized nanoparticles was evaluated with agar well diffusion method. The antimicrobial activity was evaluated against *Fusarium oxysporum*f. sp. *lycopersici*at different concentrations and it was observed that increase in concentration of nanoparticles progressively inhibited the growth.

The required concentrations of nanosilver(10, 30, 50 and 100 ppm) solutions were prepared using distilled water. The wells on agar plates were filled with 120 µl from each concentration of nanosilver and *Trichoderma harzianum* culture filtrate and observed for inhibition zone around the wells. In this method mycelia growth was minimum (71.34 mm) in 100 ppm and higher at *Trichoderma harzianum* culture filtrate (77.00 mm).

The zone of inhibitions was highest at 100 ppm silver nanoparticles (18.66 mm) and lowest against *Trichoderma harzianum* culture filtrate (13 mm). Table 1, Plate 5 and Fig. 2

Plate 5: *In vitro* evaluation of antifungal efficacy of *Trichoderma harzianum* silver nanoparticles by using agar well diffusion against *Fusarium oxysporum*f. sp. *lycopersici*

Table 1. *In vitro* evaluation of antimicrobial activity of silver nanoparticles and *Trichoderma harzianum* culture filtrate by using Agar Well Diffusion method against *Fusarium oxysporum*f. sp. *lycopersici*

Tr. No.	Treatments	Mycelialgrowth* (mm)	Zone of inhibition* (mm)
T_1	TCF	77.00	13.00
T_2	AgNPs (10 ppm)	75.50	14.50
T_3	AgNPs (30 ppm)	74.50	15.50
T_4	AgNPs (50 ppm)	72.34	17.66
T_5	AgNPs(100 ppm)	71.34	18.66
T_6	Control	90.00	00.00
	SE±		**0.245**
	CD at 1%		**0.735**

*-Mean of Four replications, Dia.: Diameter TCF: *Trichoderma* Culture Filtrate

The results were confirmed with the findings of Kaur *et al.,*2012. In agar well diffusion method Ag-Ch exhibited highest inhibition against *Aspergillus flavus*(19.66±0.28) followed by *Alternaria alternata*(16.33±0.29) and *Rhizoctonia solani*(12.66±0.76). In mycelia

growth inhibition method Ag-Ch showed inhibitions 94%, 67% and 78% against *Aspergillus* spp, *Rhizoctonia*spp. and *Altenaria*spp., respectively.

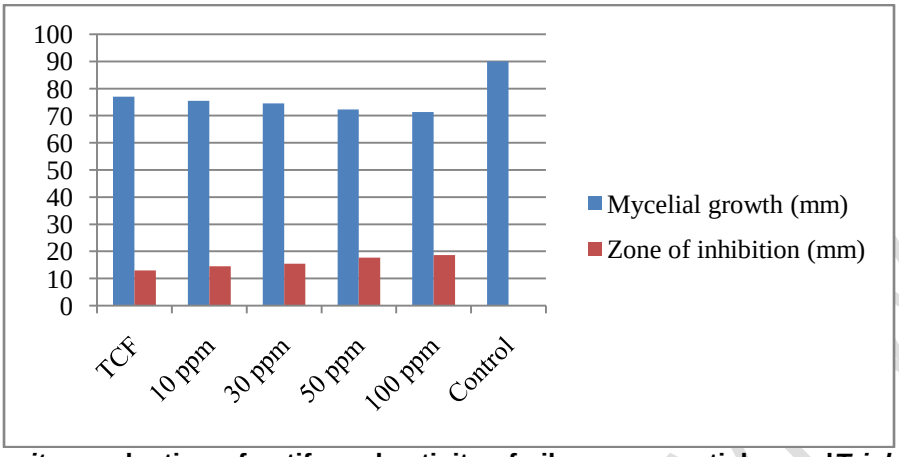

Fig.2: *In vitro* evaluation of antifungal activity of silver nanoparticles and *Trichoderma harzianum*culture filtrate against *Fusarium oxysporum*f. sp. *lycopersici*by using agar well diffusion method

3.4.2 Poisoned food technique method

The suspension of silver nanoparticles was used to study the antifungal activity against *Fusarium oxysporum*f. sp. *lycopersici*by poison food technique. Effect of Silver nanoparticles was compared with the effect of *Trichoderma harzianum* culture filtrate. The per cent inhibitions increased with increase of concentration. In this method minimum colony diameter was observed at 100 ppm (22.33 mm) and maximum mycelial growth was observed in *Trichoderma harzianum* culture filtrate (44.00 mm). At 100 ppm concentration the per cent inhibitions were observed as 75.19% for AgNPs.

Plate 6: *In vitro evaluation* of antifungal efficacy of *Trichoderma harzianum*silver

nanoparticles by using poison food method against *Fusarium oxysporum*f. sp. *lycopersici*

Table 2. *In vitro* evaluation of antimicrobial activity of silver nanoparticles and *Trichoderma* culture filtrate by using poison food technique against *Fusarium oxysporum f.* sp. *lycopersici*

Tr. No.	Treatments	*Fusarium oxysporum*f. sp. *Lycopersici*	
		Colony Dia.* (mm)	% inhibition*
T_1	TCF	44.00	51.45 **(45.64)**
T_2	AgNPs (10 ppm)	41.00	54.45 **(47.55)**
T_3	AgNPs (30 ppm)	37.00	58.89 **(50.12)**
T_4	AgNPs (50 ppm)	30.00	66.67 **(54.73)**
T_5	AgNPs (100 ppm)	22.33	75.19 **(60.12)**
T_6	Control	90.00	00.00 **(00.00)**
	SE±	**0.419**	
	CD at 1%	**1.256**	

*-Mean of Four replications, Dia.: Diameter,
TCF: *Trichoderma* Culture Filtrate

The per cent inhibitions were observed as 66.67%, 58.89%, 54.45% and 51.45% for 50 ppm, 30 ppm, 10 ppm and *Trichoderma harzianum* culture filtrate, respectively. (Table 2, Plate 6 and Fig. 3).

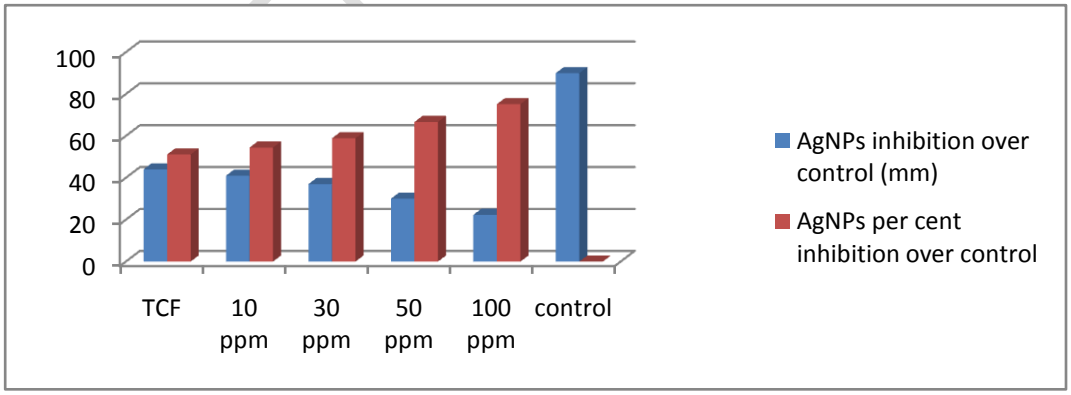

Fig.3: *In vitro* evaluation of antifungal activity of silver nanoparticles and *Trichodermaharzianum*culture filtrate against *Fusarium oxysporum*f. sp. *lycopersici* by using poisoned food technique

Similar result regarding effective ness of silver nanoparticles was reported by several scientist i.e. Lamsal *et al*., 2011., Kaman and Dutta (2018), Pritteshe*t al.* (2018), Hassan *et al.* (2019) The results were confirmed with the similar findings of Lamsal *et al*., 2011. In poison food method AgNPs exhibited highest inhibition against *Colletotrichum spp* at concentration 100 ppm, 50 ppm, 30 ppm and 10 ppm are 90%, 84.56%, 84.50% and 11.33% inhibition, respectively. The results Kaman and Dutta (2018) showed that, "the silver nanoparticle at 100 ppm showed significantly higher efficacy in inhibiting mycelial growth of the against the four pathogens *R. solani, F. oxysporum, S. sclerotiorum*and *S. rolfsii*caused diseases of vegetables and horticultural crops as compared to Carbendazim at 3000 ppm". Also,Pritteshe*t al.* (2018) recorded "the antifungal activity of silver nanoparticles against *Fusarium solani*by inoculating agar medium with different concentrations of AgNPs (25ppm, 50ppm, 100ppm and 250 ppm, respectively). In all cases, silver nanoparticles exhibited higher inhibition of mycelial growth and gave significant results as compared to control. The highest inhibition was observed with 250ppm and the lowest at 25ppm". Tomah *et al.* (2020) studied "the mycosynthesis and antifungal activity of silver nanoparticles and observed that, the highest per cent inhibition of *Sclerotium sclerotiorum*colony at a concentration of 200µg/ml followed by 150, 100, 50µg/ml by poisoned food technique".

4. CONCLUSION

The antifungal activity of silver nanoparticles against *Fusarium oxysporum*f. sp. *lycopersici*was most effective than culture filtrate of *Trichoderma harzianum*. It has been proved that rhizosphere colonies (*Trichoderma harzianum*) were capable of synthesizing the metal nanoparticles: silver in particular, which is an effective controlling agent of pathogens, *Fusarium oxysporum*f. sp. *lycopersici.* Addition of silver metal in relatively smaller quantities at plant rhizosphere leads to the control of soilborne diseases in Tomato crop.

REFERENCES

Bissett, J. (1991). A revision of the genus *Trichoderma*. II. Infrageneric classification. *Can. J. Bot.* 69:2357-2372.

Kaman, P. K. & Dutta, P. (2018). Synthesis, characterization and antifungal activityof biosynthesized silver nanoparticle. Indian Phytopathology. 72(1), 79-88.

Kaur, P., Thakur, R and Choudhary. A. (2012). An *in vitro* study of the antifungal activity of silver/chitosan nanoformulation against important seedborne pathogens. *Inter. J.* of *Sci.* and *Tech. Res.* 1:6

Khabat, V., Mansoori, G. A. and Sedighe Karimi (2011). Biosynthesis of Silver Nanoparticles by Fungus *Trichoderma reesei. Int. sci. J.* 1(1): 65-19.

Hassan, S. A., Hanif, E., Khan, U. H. & Tanoli, A. (2019). Antifungal activity of silvernanoparticles from Aspergillus niger. Pakistan Journal of PharmaceuticalSciences. 32(3), 1163-1166.

Lamsal, K., Sang Woo Kim, Jin Hee Jung, Yun Seok Kim, Kyoung Su Kim, and Youn Su Lee (2011). Application of silver nanoparticles for the control of *Colletotrichum* species *in vitro* and pepper anthracnose disease in field. *Mycobiol.* 39(3): 194-199.

Nene, Y. L. and Thapliyal, P. N. (1993). Evaluations of fungicides in fungicides for plant disease control (3rd ed.) Oxford, IBH Publishing Co. New Delhi. 531-532.

Noshad, A., Iqbal, M., Folkers, L., Hetherington, C., Khan, A., Numan, M. & Ullah, S.(2019). Antibacterial effect of silver nanoparticles (AgNPs) synthesized fromTrichodermaharzianum against Clavibactermichiganensis. Journal of NanoResearch. 58, 10-19.

Shelar, G. B. and Chavan, A. M. (2015). Myco-synthesis of silver nanoparticles from *Trichoderma harzianum*and its impact on germination status of oil seed. *Biolife*3(1): 109-113.

Tripathi, R. M., Gupta, R. K., Shrivastav, A., Singh, M. P., Shrivastav, B. R. and Singh Priti (2013). *Trichoderma koningii*assisted biogenic synthesis of silver nanoparticles and evaluation of their antibacterial activity. *Adv. Nat. Sci.: Nanosci. Nanotechnol.*1-5.

Tomah, A. A., Almer, I. S., Li, B. & Zhang, J. (2020). Mycosynthesis of silver nanoparticlesusing screened Trichoderma isolates and their antifungal activity against Sclerotiniasclerotiorum. Nanomaterials. 10,1955

Vincent, J. M. (1947). Distortion of fungal hypha in the presence of certain inhibitors. *Nature.* 159: 850.