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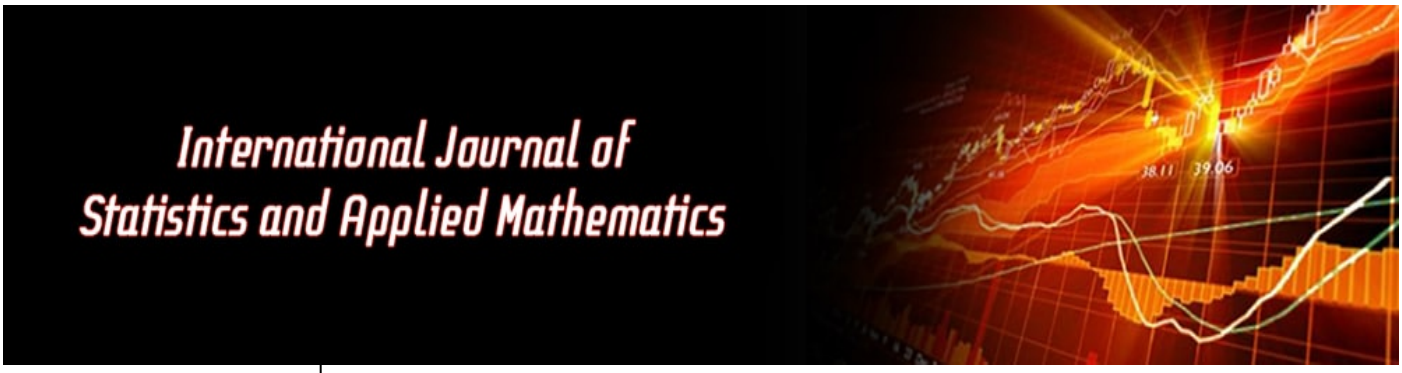

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In vitro evaluation of green synthesized nanoparticles against *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) Snyder & Hansen causal agent of wilt of Tomato

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Abstract
Now-a-days, due to continuous increasing world population, are threatening food security in worldwide. This is also a big challenge for India to provide sufficient amount of food for peoples, it is because India's have highest population in the world. Each year, about 20%-40% of crops are lost due to crop pests and diseases. The conventional methods are not that much effective against the pathogens causes disease in plants due to continuous and blind application of chemical fungicides also causes negative impact on human and environment health. The nanoparticles (nm) synthesized from plant and used as seed or seedling treatment, foliar spray and soil drenching *etc*, it comes in support to the search for alternative, non-hazardous and eco-friendly crop disease management strategies. Three nano-particles with four different concentrations evaluated against *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) Snyder & Hansen causing wilt disease in Tomato crop. All treatments demonstrated inhibitory effect against *F. oxysporum* f.sp. *lycopersici*, the least radial growth colony of pathogen recorded from T_{12} (Silica 100 ppm) treatment used as Food Poison Technique. Generally, vegetative (mycelium) and reproductive (spore production) growth of *Fusarium* significantly affected when treated with nano-particles. So it has direct contribution for the management of wilt disease of Tomato crop.

Keywords: Nanoparticles, fungicides, pathogen, eco-friendly, nanotechnology and mycelium etc

1. Introduction
Among the horticultural crops, Tomato (*Lycopersicon esculentum* Mill.), 2n=24, which is a member of the family Solanaceae, is one of the most remunerable and widely grown vegetables in the world and ranks first among the processed food crops. It is supposed to be originated from Peru, the wild ancestor of the (Tomato) *Solanum pimpinellifolium*, and also native to South America particularly Peru and Ecuador and it was domesticated first in Mexico, (Knapp and Peralta, 2016) [1]. It is called protective food because of having more nutritional values like vitamins, minerals, lycopene, fiber, and a dietary source of antioxidants. The mature fruits of tomato contains vitamin A, B and C, essential amino acids, minerals like Mg, Ca, P, Fe, Na, K, Cu and S *etc* (Lenucci *et al*., 2006) [3].
The total production of tomato in the world is 186.821 million metric tonnes from 5,051,983 hectares of land in 2020. China is the world's largest tomato producer, followed by India, which in 2020 produced 20.573 million mT of tomatoes on 812,000 hectares and had an average yield of 25.3 mT/ha (FAO STAT, 2022) [7]. There are many factors involved in low yield of tomato including the infestations by fungi, bacteria, nematodes or viruses and the competing weeds are predominant. Among them Fusarium wilt disease of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* is one of the most important and widespread disease of the cultivated tomato. This disease was first described by G. E. Massee in England in 1895. It has worldwide importance where at least 32 countries had reported the disease, which is particularly severe in countries with warm climate (Mui-Yun, 2003) [4].
The first symptom of Fusarium wilt is yellowing of the older lower leaves. Browning of the vascular tissue is strong evidence of Fusarium wilt (Snyder and Hans, 2003) [5].

Fusarium wilt of tomato is a soil borne disease caused by the filamentous fungus *F. o.* f. sp. *lycopersici* that leads to significant economic up to 80% losses to tomato production (Harikrushana *et al.*, 2014; Juliano *et al.*, 2005) [6, 13]. Currently, control methods against Fusarium wilt mainly consist of soil fumigation through chemical fungicides, the use of resistant cultivars and biological control *etc*. Fumigation negatively affects the environment and human health. In addition, resistant varieties are losing their effectiveness owing to the continuous emergence of novel races that overcome resistance and biological control strategy well not established due fluctuation of temperature and humidity *etc*.

Therefore, it is highly desirable to establish alternate substitute for Fusarium wilt management. Green synthesized nano-particles used for controlling plant diseases looks to be the most promising approach to managing agricultural phytopathogens. Silver nanoparticles have shown antifungal inhibition of *Alternaria alternata, Sclerotinia sclerotiorum, Macrophomina phaseolina, Rhizoctonia solani, Botrytis cinerea,* and *Curvularia lunata* by well diffusion assay (Awad-Allah *et al.*, 2021) [8]. Natural nano-products have been considered viable alternatives for managing plant diseases. This study investigated *in-vitro* antifungal activity of nanoparticles against *Fusarium oxysporum* f. sp. *lycopersici*.

2. Methods and Materials

2.1 Collection, isolation and purification of *Fusarium oxysporum* f.sp. *lycopersici*

2.1.1 Collection of diseased specimen

The diseased specimens were obtained from the Student Instruction Farm (SIF) of C.S.A. University of Agriculture & Technology Kanpur. The infected tomato plant in the field that are exhibiting wilting like symptoms followed by yellowing were gathered and brought to laboratory for initial analysis.

2.1.2 Isolation and purification of pathogen

The selected stem and root of the wilted diseased plant were first carefully rinsed with running water. Then, a sterilised sharp knife was used to cut the diseased area of the stem into little pieces, each of which contained tiny fragments of both the diseased and healthy tissues. These pieces were carefully dip 0.1% mercuric chloride ($HgCl_2$) solution for 5 seconds then followed by three time washing in plates containing distilled water. These pieces were placed on sterilized blotting paper with the help of sterilized forceps to absorb excess moisture. Therefore, pieces were placed on sterilized poured plates containing (PDA) medium in the inoculation chamber. Daily inspections of the Petri plates were made to spot any mycelial development around the pieces.

Cultural characters were observed by eye and microscopic examination. Colony morphology was observed from PDA plates. The morphological identification of *Fusarium oxysporum* f. sp. *lycopersici* and confirmed by comparing it to an accurate description provided by (Snyder and Hansen, 1940) [9].

Fig 1: Collected specimen, pure culture and conidial structure of *F. o.* f. sp. *lycopersici*

2.2 Collection and green synthesis of nanoparticles

2.2.1 Green synthesis of silver nano-particles

2.2.1.1 Reagent requirements

0.2 M of $AgNO_3$ (212.996 g/mol) solution
0.2 N ammonium hydroxide solution

2.2.1.2 Procedure

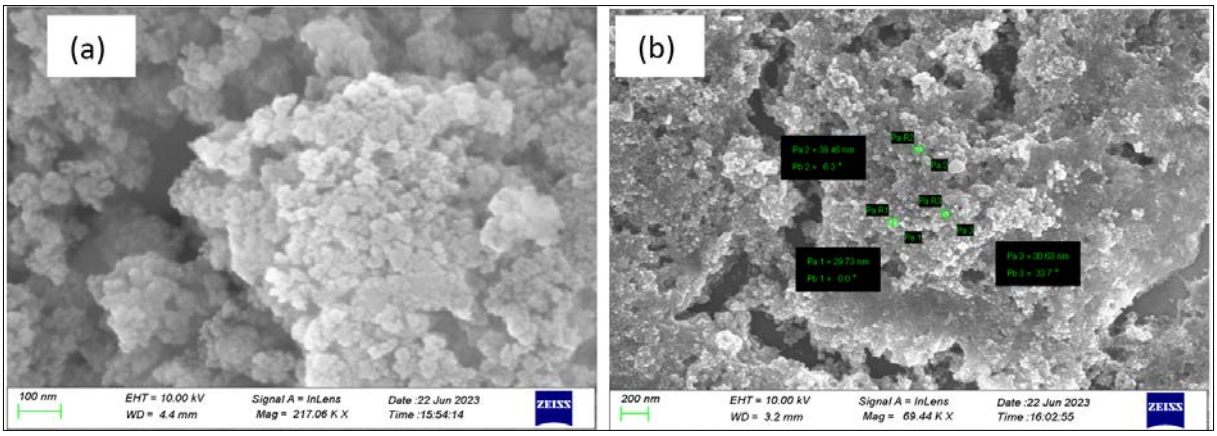
Take 5 g of fresh leaves of *Cannabis sativa* (Hemp), and washes it thoroughly with distilled water. Then dry under shade inside room for 20-30 minutes. Macerate the leaves in mortar and pestle and transfer it into 500 ml beaker. Add 20 ml of water to the leaf extract and heat it over hot plate or sand bath at 80-90 °C for an hour. Filter the extract by Whatman No.1 filter paper. Load the filtrate in burette for using it as capping and reducing agent in synthesis of silver nano-particles. Prepare 0.2 M of $AgNO_3$ (212.996 g/mol) solution of 20 ml in a 250 ml conical flask as precursor. Put that beaker on hot plate with magnetic stirrer at 60 °C and 800 rpm for 1 hour by putting Hemp leaf extract drop wise into the liquid $AgNO_3$ solution. Cover the conical flask by silver foil to prevent photolysis of $AgNO_3$. Add 0.2 N ammonium hydroxide solutions simultaneously from another burette to increase the pH to around 10. Yellowish colour of plant extract changes to black which indicates silver nano particle formation which can be the centrifuged at 5000 rpm for 20 minutes to get the nano silver hydroxide particles at the bottom of test tube which can be then washed thrice by using distilled water. The solid material at the bottom of the test tube is then kept inside distilled water /80% Ethanol in suspended form and stored in refrigerator at 4 °C for characterization and further use.

2.2.1.3 Characterization

The characterization of the green synthesized nano-particles is an important part for proving whether it is a nano-particle or not. Nano properties of synthesized silver product confirmed through Scanning Electron Microscope (SEM) image (Fig.2a) at Centre for Nanoscience, Indian Institute of Technology, Kanpur- India.

2.2.2 Collection of Silica nano-particles

Silica nano-particles collected from Department of Materials Science Programme, Indian Institute of Technology (IIT), Kanpur-India. The characterization of Silica nano-particles was also confirmed through Scanning Electron Microscope (SEM) image (Fig.2b) from the Centre for Nanoscience, IIT, Kanpur.



2.2.3 Agri-tech nano-fungicides

Agri-tech nano- fungicides a product (containing nano-particles of silver + copper + zinc) was obtained from the market prepared by Nano Research Lab Jamshedpur, Jharkhand, India.

After fungicidal activities confirmation of nano-particles further investigation as nano-fungicides against the *Fusarium oxysporum* f. sp. *lycopersici*. Three different nano- particles *viz*., Silver nano- particle, Agri-tech nano- particle and Silica nano- particle with each four different concentrations as 25, 50, 75 and 100 ppm were used.

2.3 Effect of nanoparticles on radial growth *Fusarium oxysporum* f.sp. *lycopersici*

The efficacy of Nano-particles like, silver nano- particles, Agri-tech nano- particles and Silica based nano-particles, were tested in four different concentrations (25, 50, 75 and 100 ppm) of each nano- particles, on radial mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* at different days of interval using through Food Poison Technique. The PDA media amended with nano-particles (Silver, Agri-tech and Silica based nano-particles) at different concentrations (25, 50, 75 and 100 ppm) separately and poured in 90 mm petriplates. The plates were inoculated with mycelial discs of 0.5 cm diameter from the advancing edges of seven day-old pure cultures of *Fusarium oxysporum* f. sp. *lycopersici*. The petriplates were then incubated at a temperature of 25± 1°C.

Inhibition zone of radial colony calculated by given formula:-

$$\text{Inhibition } (\%) = \frac{(C-T)}{T} \times 100$$

Whereas, C = Radial growth of control and T= Radial growth of treatment.

Treatment details

Treatment 1 = Silver nanoparticle 25 (ppm),
Treatment 2 = Silver nanoparticle 50 (ppm)
Treatment 3 = Silver nanoparticle 75 (ppm),
Treatment 4 = Silver nanoparticle 100 (ppm)
Treatment 5 = Agri-tech nanoparticle 25 (ppm),
Treatment 6 = Agri-tech NPs 50 (ppm)
Treatment 7 = Agri-tech nanoparticle 75 (ppm),
Treatment 8 = Agri-tech NPs 100 (ppm)
Treatment 9 = Silica nanoparticle 25 (ppm),
Treatment 10 = Silica nanoparticle 50 (ppm)
Treatment 11 = Silica nanoparticle 75 (ppm),
Treatment 12 = Silica nanoparticle 100 (ppm)
Control = (Un-treated)

2.4 Statistical analysis

The experiment was conducted on the basis of Completely Randomized Design (C.R.D.) with the mean of three (3) replications. Analysis of variance (ANOVA) was used to determine the critical difference (C.D.) between the effect of nano-particles and radial growth of mycelium. All the examination was carried out at 5% (five) level of significance.

3. Results

3.1 Effect of nanoparticles on radial mycelial growth of *F. oxysporum* f. sp *lycopersici*

The result showed that the radial mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* was sharply inhibited by nano-particles over the control. The minimum radial growth was found in T_{12} (Silica 100 ppm) treatment representing the value 8.30, 20.47, 32.10 and 45.33 mm at 2, 4, 6 and 8 days of inoculation, respectively against 24.66, 43.36, 62.23 and 85.90 mm as in case of control. The T_8 (Agri-tech 100 ppm) treatment represented 10.10, 25.33, 36.33 and 47.60 mm radial growth of mycelium at 2, 4, 6 and 8 days of inoculation, respectively which was the second highest radial growth inhibitor nano- particles. Among the treatments, the radial growth of mycelium about 19.00, 32.53, 51.00 and 66.61 mm was recorded in case of T_1 (Silver 25 ppm) treatment which was the inferior than all treatment but superior than control. The highest per cent decreased radial growth against control at 8 days was found in T_{12} (Silica 100ppm) treatment with the value of 47.22%, followed by T_8 (Agri-tech 100 ppm), T_{11} (Silica 75 ppm) and T_4 (Silver 100 ppm) representing as 44.58, 43.86 and 40.98%, respectively. All remaining treated petriplates was also reduces the radial growth of mycelium against non-treated plates. It is evident in Table-1 and Fig.-3, also cleared that all, the tested nano-particles through Poison Food Techniques were statistically significant in reducing the mycelial growth of *F. o.* f. sp. *lycopersici* as compared to control at 2, 4, 6 and 8 days of inoculation.

Fig 3: Effect of nanoparticles on radial mycelial growth after 8 days of *F. oxysporum* f.sp. *lycopersici*.

4. Discussion

In the present study, nano-particles exhibited antifungal activity against *F. o.* f. sp. *lycopersici*. Costa *et al.* (2015) [14] demonstrated that the nano-products of *Ocimum selloi* were efficient inhibitors of mycelium growth and conidium germination of *Moniliophthora perniciosa*. Mycelium growth of *Botrytis fabae* reduced after treated with essential oil of *Ocimum basilicum* reported by Oxenahm *et al.* (2005) [10].

The current research is discussed here with in the light result obtained by previous workers. The pathogen (*F. o.* f. sp. *lycopersici*) tested through Poison Food Technique and found inhibitory effect against nano-particles. Results presented in Table-1 indicated the varying degrees inhibition of *F. o.* f. sp. *lycopersici* by three nano-particles; Silver, Agri-tech and Silica nano-particles with each four different concentrations as 25, 50, 75 and 100 ppm. The potentialities of the used nano-fungicides (nano-particles) could be attributed to their effect on inhibited the mycelial growth, radial growth and spore germination of *F. oxysporum* f. sp. *lycopersici*.

In the present investigation, the radial growth of *Fusarium oxysporum* f. sp. *lycopersici* was sharply inhibited treated with nano-particles. The minimum radial growth was found in T_{12} (Silica 100 ppm) treatment representing the value 8.30, 20.47, 32.10 and 45.33mm at 2, 4, 6 and 8 days of inoculation, respectively. The maximum 47.22% decreased radial growth against control at 8 days was found in T_{12} (Silica 100 ppm) treated nanoparticles. It is evident in Table-1, cleared that all, the tested nano-particles through Poison Food Techniques were statistically significant in reducing the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici*. Similar results were also recorded by El-Argawy *et al.* (2017) [11] the three different concentrations of (25, 50 and 100 ppm) of silver nano-particles inhibited the radial growth of *Sclerotium rolfsii* under *in vitro* condition. Kim *et al.* (2012) [12] also have been found that, the fungicidal properties of silver nano-particles used as an agent for antifungal treatment against plant pathogens at various levels.

Table 1: Effect of Nano-fungicides with different concentrations on radial mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* at different 2, 4, 6, and 8 days intervals.

Treatment details		Radial mycelial growth (mm) at different days				Per cent decreased over control at 8 days
Name of Nano-fungicides	Concentration (ppm)	2nd days	4rth days	6th days	8th days	
Silver Nano-fungicides	T_1 = 25	19.00	32.53	51.00	66.61	22.45
	T_2 = 50	17.66	31.12	50.48	63.00	26.65
	T_3 = 75	16.32	30.75	50.00	62.82	26.86
	T_4 = 100	11.71	26.84	39.56	50.69	40.98
Agri-tech Nano-fungicides	T_5 = 25	14.10	29.86	47.10	61.96	27.86
	T_6 = 50	13.32	29.11	45.85	59.23	31.04
	T_7 = 75	12.66	27.51	42.68	53.10	38.18
	T_8 = 100	10.10	25.33	36.33	47.60	44.58
Silica Nano-fungicides	T_9 = 25	15.74	30.20	49.19	62.31	27.46
	T_{10} = 50	12.93	28.42	44.54	56.44	34.29
	T_{11} = 75	10.42	25.76	38.10	48.22	43.86
	T_{12} = 100	08.30	20.47	32.10	45.33	47.22
Control	(Untreated)	24.66	43.36	62.23	85.90	-
C.D.					4.957	
SE(m)					1.729	
SE(d)					2.269	
C.V.					5.973	

5. Conclusion

The antifungal activity of different nano-fungicides was determined *in-vitro* through Food Poison Technique. The minimum radial growth of *Fusarium oxysporum* f.sp. *lycopersici* was found in plates treated with Silica nanoparticles 100ppm (T_{12}) treatment representing the value 8.30, 20.47, 32.10 and 45.33 mm against 24.66, 43.26, 62.23 and 85.90 cm in control at 2, 4, 6 and 8 days after inoculation, respectively. The maximum decreased radial growth of treated pathogen was also observed in plates treated with Silica 100ppm (T_{12}) as 47.22%, followed by T_8 (Agri-tech 100ppm), T_{11} (Silica 75 ppm) and T_4 (Silver 100ppm) indicating as 44.58, 43.86 and 40.98% against control, respectively.

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