BIOSYNTHESIS OF SILVER NANOPARTICLES (AgNPs) BY USING *Trichoderma harzianum* AND ITS EFFICACY AGAINST SOILBORNE PLANT PATHOGENS OF TOMATO

Submitted by

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B.Sc. (Agri.)

MASTER OF SCIENCE

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IN

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DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE, LATUR

413 512 (M.S.), INDIA

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DISSERTATION

Submitted to
The Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani
In partial fulfillment of the requirements
For the Degree of

MASTER OF SCIENCE
(Agriculture)
IN
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DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF AGRICULTURE, LATUR
413 512 (M.S.), INDIA

2017

CANDIDATE'S DECLARATION

I hereby declare that the dissertation

or part thereof has not been

previously submitted by

me for a degree of any

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CERTIFICATE - I

This is to certify that the dissertation entitled "**BIOSYNTHESIS OF SILVER NANOPARTICLES (AgNPs) BY USING *Trichoderma harzianum* AND ITS EFFICACY AGAINST SOILBORNE PLANT PATHOGENS OF TOMATO"** submitted by **Mr**. **SHENDGE VIJAY SUBHASH** to the Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **PLANT PATHOLOGY** is record of original and bonafide research work carried out by him under my guidance and supervision. It is of sufficiently high standard to warrant its presentation for the award of the said degree.

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This is to certify that the dissertation entitled "**BIOSYNTHESIS OF SILVER NANOPARTICLES (AgNPs) BY USING *Trichoderma harzianum* AND ITS EFFICACY AGAINST SOILBORNE PLANT PATHOGENS OF TOMATO"** submitted by **Mr**. **SHENDGE VIJAY SUBHASH** Reg. No. 2015A/101ML to the Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **PLANT PATHOLOGY** has been approved by the student's advisory committee after viva-voce examination in collaboration with the external examiner.

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ABBREVIATIONS

%	-	Per cent
&	-	And
<	-	Less than
>	-	Greater than
µl	-	Microliter
µm	-	Micrometer
^0C	-	Degree Celsius
Ag^+	-	Silver ions
$AgNO_3$	-	Silver Nitrate
AgNPs	-	Silver Nanoparticles
CD	-	Critical Difference
cm	-	Centimeter
Conc.	-	Concentrations
CRD	-	Completely Randomized Design
CV	-	Coefficient of Variation
DAS	-	Days After Sowing
Dia.	-	Diameter
DLS	-	Dynamic Light Scattering
et al	-	*et alia*, and other people
etc.	-	Etcetera
Fig.	-	Figure
gm	-	Gram

ha	-	Hectare
i.e	-	That is
Kg h^{-1}	-	Kilogram per hectare
Kg	-	Kilogram
m	-	Meter
m^2	-	Square meter
min	-	Minutes
ml	-	Milliliter
mm	-	Millimeter
nm	-	Nanometer
p.s.i	-	Pounds per square inch
ppm	-	Parts per million
Sr. No.	-	Serial Number
Sp & Spp	-	Species (Singular and Plural)
TEM	-	Transmission Electron Microscopy
TSM	-	*Trichoderma* Selective Media
UV-Vis	-	Ultra Violet Visible
viz.	-	Namely

INTRODUCTION

Chapter I

INTRODUCTION

Fungal species belonging to the genus *Trichoderma* are worldwide in occurrence and easily isolated from soil, decaying wood and other forms of plant organic matter. Rapid growth rate in culture and the production of numerous spores (conidia) that are varying shades of green colour which characterize fungi in this genus. The reverse sides of colonies are often uncolored, buff, yellow, amber or yellow-green and many species produced prodigious quantities of thick-walled spores (chlamydospores) in submerged mycelium. The potential of *Trichoderma* species as biocontrol agents of plant diseases was first recognized in the early 1930s and in subsequent years, for the control of many diseases (Howell, 2003).

The mechanism of action of *Trichoderma* consists of hyper-parasitism, antibiosis, production of antibiotics which restricts the growth of pathogenic fungal organisms and also enhances plant growth when applied as amendment in soil.

The genus *Trichoderma* belongs to Phyllum- *Ascomycetes,* Class- *Sordriomycetes,* Order- *Hypocreales* and Family- *Hypocreaceae.* The genus *Trichoderma* was first described more than two hundred years ago by Persoon (1794) in Germany. The systematics and taxonomy of these fungi have been studied since 1794, when Persoon introduced the name *Trichoderma*.

Species of *Trichoderma* were used as biological control agents against soil borne plant pathogenic fungi (Kucuk and Kivank, 2003). The 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.

Plant disease plays a direct role in the destruction of natural resources in agriculture. In particular, soilborne pathogens cause important losses, as the fungi being most aggressive. The distribution of several phytopathogenic fungi such as *Fusarium, Pythium, Phytophthora, Botrytis* and *Rhizoctonia* has spread during the last few years due to changes introduced in fanning with detrimental effects on crops of economic importance.

Nanotechnology ("nanotech") is the manipulation of matter in an atomic, molecular and supramolecular scale. The earliest, widespread description of nanotechnology referred to the particular technological goal of precisely manipulating atoms and molecules for fabrication of macroscale products, also now referred to as molecular nanotechnology. A more generalized description of nanotechnology was subsequently established by the National Nanotechnology Initiative, which defines nanotechnology as the manipulation of matter with at least one dimension sized from 1 to 100 nanometers. This definition reflects the fact that, quantum mechanical effects are important at this quantum-realm scale and so the definition shifted from a particular technological goal to a research category inclusive of all types of research and technologies that deal with the special properties of matter that occur below the given size threshold. It is therefore common to see the plural form "nanotechnologies" as well as "nanoscale technologies" to refer to the broad range of research and applications whose common trait is size. Because of the variety of potential applications (including industrial and military), governments have invested billions of dollars in nanotechnology research.

Nano Silver is the most studied and utilized nano particle for bio-system. It has long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities. Silver nanoparticles, which have high surface area and high fraction of surface atoms, have high antimicrobial effects as compared to the bulk silver. Double capsulized nano silver was prepared by chemical reaction of silver ion with aid of physical methods, reducing agents and stabilizers. They were highly stable and very well dispersive in aqueous solution. It eliminates unwanted microorganisms in soils and hydroponics systems. It is being used as foliar spray to stop fungi, moulds, rot and several other plant diseases. Moreover, silver is an excellent plant-growth stimulator (Singh *et al*., 2014).

Nanotechnology is an emerging field in the area of interdisciplinary research especially in biology. The advancement of nanotechnology mainly requires the development of reliable and ecofriendly protocols for the synthesis of nanomaterial over a range of biological composition, sizes, shapes and high monodispersity. Nanoparticles possess exceptional physical and chemical properties which lead to rapid commercialization. Nanoparticles are considered as fundamental molecular building blocks for nanotechnology. They are pre-requisites for preparing many nanostructure materials and devices. 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. Bansal *et al*., (2011) reported the biosynthesis of nanoparticles is a kind of bottom-up approach which involves reduction or oxidation process. Recently the utilization of biological systems provides a novel idea for the production of nanomaterials.

Silver is a prehistoric element. The use of silver for medicine as local antibacterial agent was recognized in 19th Century, since then the antimicrobial property of silver has been investigated thoroughly than any other inorganic antimicrobial agent. Silver is blown to attack a broad range of biological processes in microorganisms including alteration of cell membrane structure and functions (Mc Doimell and Russel, 1999). The prepared Silver nanoparticles can be characterized using UV spectrophotometer, X-ray diffractometer, Fourier Transform Infrared spectrophotometer, Scanning Electron microscope (SEM) and Transmission Electron Microscope (TEM).

It is shown that, certain fungi have the ability to produce extracellular metabolites that serves as an agent for their own survival when exposed to environmental stresses like toxic material, predators and temperature variations. 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). Since *Trichoderma* is an environmental friendly fungus and is well known for its formation of extracellular enzymes and metabolites in very large amounts, much higher than other fungi. The present study is aimed at biosynthesis of silver nanoparticles (AgNPs) using *Trichoderma harzianum* and its efficacy against soilborne pathogens of tomato with the following objectives.

OBJECTIVES

1. To isolate and identify the *Trichoderma harzianum* from rhizosphere soil of tomato.
2. Biosynthesis of silver nanoparticles by using selected *Trichoderma harzianum*
3. Characterization of silver nanoparticles (UV-Vis and TEM)
4. *In vitro* evaluation of isolates of *Trichoderma harzianum* (non-biosynthesized) and biosynthesized silver nanoparticles against soilborne pathogens of tomato.

REVIEW OF LITERATURE

Chapter II

REVIEW OF LITERATURE

The literature available on soilborne diseases of solanacious crops caused by *Fusarium oxysporum f. sp. lycopersici and Sclerocium rolfsii,* antagonistic effect of *Trichoderma* spp. synthesis of silver nanoparticles and their antipathogenic efficacy has been reviewed and presented under the following subheads.

2.1 History of *Trichoderma spp.*

The genus *Trichoderma* was described in 1794 in Germany. Most of the species were identified as *T. lignorum* because of its globose conidia or as *T. koningii* because of its oblong conidia. The potential for use of *Trichoderma* as biocontrol agents was suggested more than 75 years ago by Weindling (1932), who was the first to demonstrate the parasitic activity of members of this genus against pathogens such as *R. solani.*

The genus *Trichoderma* belongs to the Phylum- *Ascomycetes***,** Class- *Sordriomycetes***,** Order- *Hypocreales* and Family- *Hypocreaceae***.** The genus *Trichoderma* was first described more than two hundred years ago by Persoon (1794) in Germany. The systematic and taxonomy of these fungi have been studied since 1794, when Persoon introduced the name *Trichoderma***.**

Hermosa *et al*., (2000) reported the most common biological control agents (BCAs) of the genus *Trichoderma* have been reported to be strains of *Trichoderma virens, T. harzianum* and *T. viride.* Since, *Trichoderma* BCAs use different mechanisms of biocontrol, it is very

important to explore the synergistic effects expressed by different genotypes for their practical use in agriculture.

Cigdem and Merih (2003) studied *Trichoderma spp.* as biological control agents against soil-borne plant pathogenic fungi (5-8). Results from different studies showed that, several strains of *Trichoderma* had a significant reducing effect on plant diseases caused by pathogens such as *Rhizoctonia solani, Sclerotium rolfsii, Pythium aphanidermatum, Fusarium oxysporum, Fusarium culmorum* and *Gaeumannomyces graminis* var. *tritici* under greenhouse and field conditions.

2.2 Isolation and Identification of *Trichoderma* spp.

Chet and Baker (1981) studied the isolation and biocontrol potential of *Trichoderma hamatum* from soils naturally suppressive of *Rhizoctonia solani.*

Elad and Chet (1983) developed a specific medium named *Trichoderma* selective medium - TSM for quantitative isolation from soil.

Samuels *et al.*, (1998) studied morphological characters of different isolates of *Trichoderma* spp., using Special Nutrient Agar, Corn meal - Dextrose Agar and Potato Dextrose Agar media.

Hermosa (2000) studied the molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. and revealed that, characterization of 16 biocontrol strains, previously identified as *Trichoderma harzianum* Rifai and one biocontrol strain recognized as *T. viride*, was carried out using several molecular techniques. A certain degree of polymorphism was detected in hybridizations using a probe of mitochondrial DNA. Sequencing of internal transcribed spacers 1 and 2 (ITS 1 and ITS 2) revealed three different ITS lengths and four different

sequence types. Phylogenetic analysis based on ITS l sequences, including type strains of different species, clustered the 17 biocontrol strains into four groups: *T. harzianum* - *T. hamatum* complex, *T. longibrachiatum, T. asperellum* and *T. atroviride* - *T. koningii* complex. ITS 2 sequences were also useful for locating the biocontrol strains in *T. atroviride* within the complex *T. atroviride* - *T. koningii.* None of the biocontrol strains studied corresponded to biotypes Th2 or Th4 of *T. harzianum,* which cause mushroom green mold.

Cigdem and Merih (2003) studied that, *Trichoderma* isolates were obtained from 31 different Eskisehir soil samples. All isolates showed different behaviors depending on the physiological tests carried out such as growth in the presence inhibitory substrates, P^H limits of growth and hydrolysis of gelatin. *T. harzianum* isolates were grown on the chitin, which is the sole carbon source. The chitinase activity determined from *T. harzianum* T_{15} by SDS-PAGE was nearly 73 kDa.

Sitansu *et al*., (2009) isolated *Trichoderma* spp. from rhizosphere soils of different ecological habitat of West Bengal by dilution plate technique using *Trichoderma* Selective Medium.

Otadoh *et al*., (2011) observed that, *Trichoderma* spp. were isolated from soils collected from farmers field in Embu and Taita Districts in Kenya with the plants showing *Fusarium* wilt symptoms.

Kamala and Indira (2012) studied the *Trichoderma* colonies were initially observed as white specks on the TSM which then enlarged of 5 to 6 mm within 5-6 days. By this time, the white colony turned into green/yellow/off-white on the medium. The colonies rapidly and readily developed with typical yellow-green colour, which make their identification easy from other soil-borne fungi. After 7 to 8 days, 50 to

60% of the colonies appeared as dark green with compact conidiophores throughout the 90mm plate.

Kumar *et al*.**,** (2012) found *Trichoderma***,** a soilborne filamentous fungus, which was capable of parasitizing several plant pathogenic fungi. Twelve isolates of *Trichoderma* spp**.** isolated from different locations of South Andaman were characterized for their cultural, morphological and antagonistic activity against soilborne and foliar borne pathogens. The sequencing of these isolates showed seven different species. The isolates revealed differential reaction patterns against the test pathogens *viz*., *Sclerotium rolfsii, Colletotrichum gloesporioides* and *C. capsici.*

Trichoderma is widely distributed all over the world and is known to occur in almost all soils and in other natural habitats containing organic matter. The genus *Trichoderma* is a soil dweller that grows rapidly producing a scintillating array of secondary metabolites and sporulates profusely and has been promoted as biocontrol agent and plant growth stimulator. The potential of *Trichoderma* spp. as biological control agents of plant diseases was first recognized by Weindling (1932). *Trichoderma* is observed to be an early colonizer of substrates and known to reduce the activity of other fungi simply by substrate occupation and depletion. Mechanisms employed by *Trichoderma* to affect the disease control are competition mycoparasitism and antibiosis (Khan and Sinha, 2007). The following scientists isolated *Trichoderma* spp. from rhizosphere of different crops from different regions mentioned in the Table 1.

Table 1. Isolation of *Trichoderma* spp. isolates from the rhizosphere region of different crops at different places

Sr. No.	Antagonistic *Trichoderma* spp.	Rhizosphere	Reference
1.	Six strains of *Trichoderma* spp.	Carnation plants in the Bogota plateau (Colombia)	Marquez *et al.,* (2002)
2.	*T. viride, T. harzianum* and *T. hamatum*	Rhizosphere of elephant foot yam.	Sreenivasulu *et al.,* (2005)
3.	*T. harzianum, T. koningii and* T. *viride*	Forest sites in higher altitudes of Indian Himalayan region.	Ghildiyal and Pandey (2008)
4.	12 isolates of Three *Trichoderma strains (T. virens, T. viride, T. harzianum)*	Groundnut crop rhizosphere	Gajera *et al*., (2011)
5.	Fourteen isolates of *Trichoderma* spp.	Groundnut crop rhizosphere	Nandeesh *et al.,* (2013)

Khan and Sinha (2007) reported that, twenty one isolates of *Trichoderma* spp. were isolated from 24 soil samples collected from different agricultural fields by serial dilution technique using potato dextrose agar medium.

Dehariya *et al*., (2015) mentioned that, *Trichoderma* easily isolated from soil by using *Trichoderma* selective medium by dilution plate method.

2.3 Concept of Nanotechnology

The idea of Nanotechnology was first conceived by Nobel laureate Richard Feynman in his famous lecture at the California Institute of Technology, on 29th December, 1959. In one of his articles published in 1960 entitled, "There is plenty of room at the bottom" discussed about the properties of nanomaterials. He pointed out that, if a bit of information required only 100 atoms, then all the books ever written could be stored in a cube with sides 0.02 inch long. Taniguchi (1974) first defined the term Nanotechnology, in 1970.

A nanoparticle is defined as a small object or particle that, behaves as a whole unit in terms of its transport and properties. Nanoparticles are particles that have at least one dimension in the range of 1 to 100 nm. Nanotechnology takes advantage of the fact that, when a solid material becomes very small, its specific surface area increases, which leads to an increase in the surface reactivity and quantum-related effects. Nanomaterials often show unique and considerably changed physical, chemical and biological properties compared with their macroscaled counterparts (Sharma *et al*., 2009).

Nanotechnology is defined as the science and technology of small things i.e. ‘nano’. This term originated from Greek word meaning ‘dwarf’. This term was firstly used by 'Richard Feynman' in 1959.

“Nano-technology” mainly consists of the processing of separation, consolidation and deformation of materials by one atom or by one molecule. Nanotechnology and Nanoscience got started in the early 1980s with two major developments; the birth of cluster science and the invention of the Scanning Tunneling Microscope (STM). In another development, the synthesis and properties of semiconductor nanocrystals

was studied; this led to a fast increasing number of metal and metal oxide nanoparticles and quantum dots.

Singh *et al*., (2014) defined nanotechnology as relating to materials, systems and processes which operate at a scale of 100 nm or less. Nanotechnology has many applications in all stages of production, processing, packaging and transport of agricultural products. Nanotechnology will revolutionize agriculture and food industry by innovation of new technique such as: precision farming techniques, enhancing the ability of plants to absorb nutrients, more efficient and targeted use of inputs, disease detection and control of diseases, withstand environmental pressures and effective systems for processing, storage and packaging.

Alghuihaymi *et al*., (2015) found that, nanotechnology can offer green and eco-friendly alternatives for plant disease management. Apart from being eco-friendly, fungi are used as bio-manufacturing units, which will provide an added benefit in being easy to use, as compared with other microbes. The non-pathogenic nature of some fungal species in combination with the simplicity of production and handling will improve the mass production of silver nanoparticles. Recently a diverse range of fungi has been screened for their ability to create silver nanoparticles.

Yadav *et al*., (2015) observed that, nanotechnology is an emerging cutting edge technology, which involves inter disciplinary subjects such as physics, chemistry, biology, material science and medicine.

An attractive possibility of green nano-technology is to use micro-organisms in synthesis of nanoparticles.

2.4 Importance of Silver and Nano silver

Silver has been valued throughout the history for many of its properties that are useful to humans. It is used as a precious commodity in currencies, ornaments, jewelry, electrical contacts and photography and others. One of the most beneficial uses of silver has been as a potent antibacterial agent that is toxic to fungi, viruses and bacteria. Silver has long been used as a disinfectant: for example, the metal has been used in treating wounds and burns because of its broad-spectrum toxicity to bacteria as well as because of its reputation of limited toxicity to humans. Silver is incorporated in textiles to inhibit the growth of bacteria and to keep odor at minimum (Clement *et al*., 1994). In 1954, silver was registered in the US as pesticide for to use as disinfectant, sanitizer and fungicide.

Silver nanoparticles are fine particles of metallic silver that have at least one dimension less than 100 nm. Nanosilver is not a new discovery, it had been known for over 100 years. Previously, nanosilver or suspensions of nanosilver were referred to as colloidal silver. To produce colloidal silver, a positive electrical current was applied through pure silver bars suspended in water resulting in colloidal silver particles with a size range of 15-500 nm (Lindemann, 1997). By converting bulk silver into nanosized silver, its effectiveness for controlling bacteria and viruses was increased multifold, primarily because of the nanomaterials extremely large surface area when compared to bulk silver, lints resulting in increased contact with bacteria and fungi. Nanosilver, when in contact with bacteria and fungus adversely affected the cellular metabolism of the electron transfer systems and the transport of substrate in the microbial cell membrane.

2.5 Principles of Biosynthesis of Nanoparticles

Nanoparticles were viewed as the fundamental building blocks of nanotechnology (Mansoori *et al*., 2015). This was the starting point for preparing many nanostructure materials and devices. Their synthesis ***is*** an important component of the rapidly growing research efforts in nano-science and nano-engineering. The nanoparticles of a wide range of materials can be prepared by a number of methods. In synthesis and assembly, strategies of nanoparticles or nanomaterials, precursors from liquids, solid or gas phase are used.

Currently, there is a growing need to using environment friendly nanoparticles that do not produce toxic wastes in their process synthesis protocol**.** Advantage of nano biotechnology is the development of reliable processes for the synthesis of nano materials over a range of sizes (with good monodispersity) and chemical composition. The utilization of such micro-organisms like bacteria, fungi, herbal extracts and yeasts in the synthesis of nanoparticles is a relatively recent activity. It is known that, certain bacteria, yeasts and now fungi play an important role in remediation of toxic metals through reduction of the metal ions so long they are not toxic in other ways. For example, environment friendly microorganism could minimize the toxicity in the process of metallic nanoparticle production by reduction of the metal ions or by formation of insoluble complexes with metal ions (e.g. metal sulfides) in the form of colloidal particles, cell mass or extracellular components from microorganisms, such as *Klebsiella pneumoniae*, *Bacillus licheniformis*, *Fusarium oxysporum*, *Aspergillus flavus*, *Cladosporium cladosporoides*, *Aspergillus clavatus* and *Penicillium brevicompactum* (Ahmad *et al*.,

2003; Shaligram *et al*., 2009) have been utilized for the reduction of silver ions to AgNPs, The principle of preparation of silver nanoparticles by using microorganism is a bio-reduction process; the silver ions arc reduced by the extracellular reductase enzymes produced by the microorganisms to silver metal in nanometer range.

Mohamed *et al*., (2015) studied nanotechnology as one of the most emerging fields in the recent years. In the current investigation, we reported the biosynthesis of iron nanoparticles (Fe-NPs) employing *Alternaria alternata* fungus, which is an eco-friendly process for the synthesis of metallic nanoparticles,

It is demonstrated that, using the dissimilatory properties of an eukaryotic organism such as fungi can be used to biosynthesize and grow nanoparticles. It is shown that, certain fungi have the ability of producing extracellular metabolites that, serve as agent for their own survival when exposed to such environmental stresses like toxic materials (such as metallic ions), predators and temperature variations. In the biosynthesis of metal nanoparticles by a fungus, the fungus mycelium is exposed to the metal salt solution that prompts the fungus to produce enzymes and metabolites for its own survival. In this process, the toxic metal ions are reduced to the non-toxic metallic solid nanoparticles through the catalytic effect of the extracellular enzyme and metabolites of the fungus.

Mohammed *et al*., (2015) studied that, in recent years the nanoparticles of II-VI group are very much important due to its emission properties. These nanoparticles (CdS, ZnS, CdSe etc.) are used for a wide range of application in the field of cathode ray tube, flat-panel display,

sensor, laser devices, etc. But for biological application, these particles are not very successfully applicable. On the other hand, the intense light emission properties of noble metals (gold, silver, etc.) nanoparticles have caught a lot of attention. These nanoparticles are extensively used for biological labeling, easy to prepare and have a good chemical and thermal stability.

Ingale and Chaudhari (2013) reported that, the conception or synthesis of material with nanometer-scale precision (nanoparticles), by means of material science is nanotechnology. Here, we discussed different synthesis methods i.e. chemical, physical and biogenic synthesis of nanoparticles. The potential come together between nanotechnology and biological science is enormous. Realistic biologics depends on units that have nanoscale dimensions (proteins, viruses, molecular motors, extra cellular matrix).

2.6 Methods of Synthesis of Nanosilver

Generally, metal nanoparticles can be prepared and stabilized by chemical, physical and biological methods: In chemical approach, techniques such as chemical reduction, electrochemical techniques, photochemical reduction and pyrolysis are used. *et al*.,

Turkevich *et al*., (1951) reported a wet chemistry technique to synthesize nanosilver using silver nitrate as a silver ion source and sodium citrate as the reducing agent for the first time.

Janardhanan *et al*., (2009) synthesized silver nanoparticles by an aqueous chemical method with an organic base and with no external capping agents. Silver nanoparticles of 40-80 nm size are formed

in the process of oxidation of glucose to gluconic acid by amine in the presence of silver nitrate and the gluconic acid caps the nanosilver.

In physical methods, nanoparticles were produced from larger structures (top down) by use of ultrafine grinders, lasers and vaporization followed by cooling.

Biological synthesis method has been developed to obtain biocompatible, inexpensive, eco-friendly and size-controlled nanoparticles, which were considered as main drawbacks in physical and chemical methods (Sadasivum *et al*., 2010). In biological synthesis, living organisms such as bacteria, fungi and plants were used for the production of metal nanoparticles. Among all the living organisms, fungi has gained much importance for synthesis of silver nanoparticles because they are easy to culture and they produce higher amount of proteins than bacteria (Sastry *et al*., 2003). Fungal-mediated synthesized nanoparticles have good monodispersity and good dimensions (Rai *et al*., 2009). The intracellular and extracellular methods were used for the synthesis of nanoparticles from fungi. The extracellular method is more advantageous than intracellular method because the intracellular method needs an additional step to obtain the purified nanoparlicles (Kuber *et al*., 2006).

2.7 Biosynthesis of silver nanoparticles

Mansoori (2008) used *Trichoderma reesei* for the extracellular synthesis of silver nanoparticles. In the biosynthesis of metal nanoparticle by a fungus, one or more enzymes or metabolites are produced that reduce a salt to its metallic solid nanoparticles through a catalytic process.

Mukherjee *et al*., (2008) studied the green synthesis of highly stabilized nanocrystalline silver particles by a non pathogenic and

agriculturally important fungus *T. asperallum* for the first time. In this study, culture filtrate of *T. asperellum* was used for the synthesis of silver nanoparticles.

Gajbhiye *et al*., (2009) reported the extracellular biosynthesis of Ag-NPs using a common fungus, *Alternaria alternata*. Also in this study, these nanoparticles were evaluated for their part in increasing the antifungal activity of fluconazole against *Phoma glomerata*, *Phoma herbarum*, *Fusarium semitectum*, *Trichoderma* spp. and *Candida albicans.*

Fayaz *et al*., (2010) biologically synthesized the silver nanoparticles at the size of 2-10 nm by exposing the silver (Ag^{+}) ions to the culture filtrate of *Trichoderma viride.*

Shaligram *et al*., (2009) observed biosynthesis of silver nanoparticles using aqueous extract from the compactin producing fungal strain. An eco-friendly process for the synthesis of nanomaterials using a fungus, *Penicillium brevicompactum* WA 2315 has been attempted. The fungus has been previously utilized for compactin production. Supernatant of seed culture was used for the biosynthesis of silver nanoparticles. The aqueous silver ions were reduced to silver metal nanoparticles when treated with the fungal supernatant. After 72 hrs of treatment, silver nanoparticles obtained were in the range of 23-105 nm as obtained from TEM. The nanoparticles were characterized by UV, FTIR, SEM, TEM and XRD. The use of supernatant of the seed media of the said fungus opens up the exciting possibility of rational strategy of biosynthesis of nanomaterials.

Khabat and Sedighe (2011) reported that, extracellular production of metal nanoparticles by several strains of the fungus *Trichoderma viride* was carried out. It was found that, aqueous silver ions when exposed to several *Trichoderma viride* strains are reduced in solution, thereby leading to the formation of silver nanoparticles.

Khabat *et al*., (2011) reported that, biosynthesis of AgNPs by using *Trichoderma reesei.* The fungal mycelium was exposed to the silver nitrate solution, that prompts the fungus to produce enzymes and metabolites for its own survival. In this process, the toxic Ag^{+} ions were reduced to the non-toxic metallic AgNPs through the catalytic effect of the extracellular enzymes and metabolites of the fungus.

Singh and Raja (2011) screened four different fungal species *(Alternaria alternata*, *T. harzianum*, *Fusarium oxysporum* and *Phoma glomerata)* for biological synthesis of silver nanoparticles and among them *T. harzianum* was found to be capable of synthesizing silver nanoparticles with high stability.

Devi *et al*., (2013) screened different isolates of *Trichoderma* spp. *(T. asperellum, T. harzianum, T. longibrachiatum*, *T. pseudokoningii* and *T. virens)* for biosynthesis of stable silver nanoparticles. Among the all isolates, *T. virens* VN-11 produced maximum nanoparticles as evident from UV-Vis spectroscopy.

Ingale and Chaudhari (2013) studied the conception or synthesis of material with nanometer-scale precision (nanoparticles), by means of material science, is nanotechnology. Nanoparticles are defined as particulate dispersal or solid particles with a size in the range of 1-100 nm.

Shelar and Chavan (2014) used the fungus *Aspergillus terreus* for the biosynthesis of silver and gold nanoparticles. The cell free filtrate of *Aspergillus terreus* reacted with $AgNO_3$ and $HAuCl_4$ ions separately, resulting formation o f silver and gold nanoparticles.

Ahluwalia *et al*., (2014) reported that, silver nanoparticles were successfully synthesized from agriculturally beneficial fungus *Trichoderma harzianum* through a simple green and eco-friendly route using cell filtrate. The silver nanoparticles formation was evaluated at different temperatures and concentrations of $AgNO_3$.

Mohamed *et al*., (2015) reported that, biosynthesis of iron nanoparticles (FeNPs) employing *Alternaria alternata* fungus, which is an eco-friendly process for the synthesis of metallic nanoparticles. FeNPs were synthesized through the reduction of aqueous Fe^{3+} ion in the dark conditions.

2.8 Characterization of Silver Nanopaticlcs

Characterization refers to the study of materials features such as composition, colour, size, structure and various properties like physical chemical and magnetic properties. Nanoparticles characterization is necessity to establish understanding and control of nanoparticle synthesis and applications. Characterization was done by using a variety of different techniques.

Mukherjee *et al*., (2001) reported the biological synthesis of silver nanoparticles using the fungus *Verticillium* and found the size of silver nanoparticles in the range of 25 ± 2 nm.

Shaligram *et al*., (2009) observed that, the aqueous silver ions were reduced to silver metal nanoparticles, when treated with the fungal supernatant. After 72 hrs of treatment, silver nanoparticles obtained were in the range of 23-105 nm as obtained from TEM. The nanoparticles were characterized by UV, FTIR, SEM, TEM and XRD. The use of supernatant of the seed media of the said fungus opens up the exciting possibility of rational strategy of biosynthesis of nano materials.

Mohammed *et al*., (2010) synthesized silver nanoparticles at the size of 2-4 nm using biological route and studied fluorescence property of these nanoparticles and observed that, these silver (Ag^{+}) ions when extremely stable silver hydrosol. These silver nanoparticles were characterized by means of UV-Vis spectrophotometer, FTIR, HrTEM, EDX, XRD and fluorescence spectroscopy. The nanoparticles exhibited maximum absorbance at 405nm in UV-Vis spectrum. The presence of proteins was identified by FTIR. The HrTEM micrograph revealed the formation of mono-dispersed spherical nanoparticles and the presence of elemental silver was confirmed by EDX analysis and XRD. These mono-dispersed silver nanoparticles showed emission in the range of 320-520 nm wavelength.

Khabat *et al*., (2011) recorded the FTIR spectrum from a drop-coated film of an aqueous solution incubated with *Trichoderma reesei* and reacted wilh Ag^{+} ions for 72 hrs. The amide bands are identified at 1650 cm^{-1} and 1430 cm^{-1} which are due to *C=0* and *N-H* stretch vibrations present in the amide linkages of the proteins, respectively.

Singh and Raja (2011) studied the size and shape of the silver nanoparticles synthesized from *T. harzianum* and found that, they were in the range of 30-50 nm in size and spherical in shape.

Devi *et al*., (2013) screened 75 isolates belonged to five *Trichoderma* species for AgNPs, Their results indicated that the high Plasmon band was observed at 420 nm at every 24 hrs.

Ahluwalia *et al*., (2014) evaluated the silver nanoparticles formation at different temperatures and concentrations of $AgNO_3$. The synthesized nanoscale particles were analyzed by UV-Vis spectrum, TEM, XRD, SEM and EDS, which revealed that, the synthesized nanoparticles had face centered cubic symmetry. Size range (51.10 nm) and stability of the synthesized silver nanoparticles were evaluated by DLS and found stable for three months.

Gopinath and Arumugam (2014) observed that, the UV-Vis spectra of the fungal culture filtrate medium containing gold ion showed peak at 527 nm corresponding to the Plasmon absorbance of gold nanoparticles. FTIR spectra provide an evidence for the presence of heterocyclic compound in the culture filtrate, which increases the stability of the synthesized gold nanoparticles. The X-ray analysis respects the Bragg's law and confirmed the crystalline nature of the gold nanoparticles. AFM analysis showed the results of particle sizes (41 nm). Transmission electron microscopy (TEM) showed that, the gold nanoparticles are spherical in shape with the size ranged from 20 to 50 nm.

Mendes *et al*., (2014) synthesized AgNPs and further been characterized by UV-Visible spectroscopy, biophysical techniques like

Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM). The average diameter of the prepared silver colloidal nanoparticles was about 52 nm. Absolute inhibitions (100%) were observed on treatment with a 270 and 540 µg ml^{-1} concentration of AgNPs.

Shelar and Chavan (2014) characterized the silver and gold nanoparticles by Visual analysis, UV-Vis absorption spectroscopy and transmission electron microscopy (TEM). The silver and gold nanoparticles exhibited maximum absorbance at 430 and 540 nm in UV-Vis spectroscopy. TEM micrograph showed polydisperse, spherical and ellipsoid nanoparticles in the size ranged from 1-50 nm.

Shelar and Chavan (2015) studied the biosynthesis of silver nanoparticles by using the fungus *Trichoderma harzianum*. The cell filtrate of *Trichoderma harzianum* reacted with 1 mM silver nitrate solution, resulting the formation of silver nanoparticles within 3 hours. The silver nanoparticles were characterized by Visual analysis, UV-Vis absorption spectroscopy and Transmission electron microscopy (TEM). Biosynthesized silver nanoparticles exhibited maximum absorbance at 440 nm in UV-Vis spectroscopy. TEM showed polydisperse spherical and occasionally ellipsoid nanoparticles in the size ranged from 19-63 nm and average size 34.77 nm.

Mansoori *et al*., (2015) reported that, biological synthesis of AgNPs by using white radish *(Raphanus sativus var. aegyptiacus)*. The biosynthesis was regularly monitored by UV-Vis spectroscopy, X-ray diffraction spectra revealed peaks of crystalline nature of AgNPs and the

transmission electron micrographs further confirmed the size of the synthesized nanoparticles ranging from 6 to 38 nm.

Mohammed (2015) reported that, AgNPs were characterized by colour changes and UV spectroscopy a peak absorption value between 400-450 nm for the extract and the colour changed to dark brown were corresponding to the Plasmon absorbance of AgNPs.

Mohamed *et al*., (2015) synthesized Fe-NPs through the reduction of aqueous Fe^{3+} in the dark conditions. Ultraviolet-Visible spectrum of the aqueous medium containing iron ion showed a peak at 238 nm and another peak at 265 nm. The forming of nanoparticles was confirmed by transmission electron microscope, scanning electron microscope and energy-dispersive X-ray. The morphology of nanoparticles is found to be cubic shapes mostly and the average particle diameter as determined by scanning electron microscope was found to be 9±3 nm.

2.9 Evaluation of biosynthesized silver nanoparticles against plant pathogenic fungi

Jo *et al*., *(*2009) carried out studies on the applicability of silver to control plant diseases. Various forms of silver ions and nanoparticles were tested in the current study to examine the antifungal activity on two plant pathogenic fungi, *Bipolaris sorokiniana* and *Magnaporthe grisea. In vitro* Petri dish assays indicated that, silver ions and nanoparticles had a significant effect on the colony formation of these two pathogens.

Gajbhiye *et al*., (2009) studied on the extracellular biosynthesis of Ag-NPs using a common fungus, *Alternaria alternata.* Also in this study, these nanoparticles were evaluated for their part in increasing the antifungal activity of fluconazole against *Phoma glomerata, Phoma herbarum, Fusarium semitectum, Trichoderma* spp. and *Candida albicans.* The antifungal activity of fluconazole was enhanced against the test fungi in the presence of Ag-NPs. Fluconazole in combination with AgNPs showed the maximum inhibition against *C. albicans* which was confirmed from the increase in fold area of inhibition, followed by *P. glomerata* and *Trichoderma* spp., which showed less increase in the fold area, whereas no significant enhancement of activity was found against *P. herbarum* and *F. semitectum.*

Lamsal *et al*., (2011a) evaluated the inhibition effect of silver nanoparticles (WA-CV-WA13B) against powdery mildews on cucumber and pumpkin. The disease incidence was observed as 57.8, 48.8, 40.2 and 20% in 10, 30, 50 and 100 ppm concentrations of silver nanoparticles treated after disease outbreak on plants. In similar way, the disease incidence was observed as 15, 40, 27 and 18% in 10, 30, 50 and 100 ppm concentrations of silver nanoparticles treated before the disease outbreaks on plants, respectively.

Lamsal *et al*., (2011b) applied the silver nanoparticles (WA-PR-WB 13R) for the control of *Colletotrichum* species *in vitro* and pepper anthracnose in the field. Complete inhibition was observed on PDA treated with 100 ppm silver nanoparticles against isolates C-3 and C-5. The C-7 and C-8 isolates also showed more than 90% inhibition on PDA treated with 100 ppm silver nanoparticles. The inhibition of fungi was significantly high, when silver nanoparticles were applied before disease

outbreak on the plants when compared with the application after the disease outbreak. The lowest disease incidence was observed on plants treated with 50 ppm silver nanoparticles before the disease outbreak (9.7%).

Savithramma *et al*., (2011) tested phyto synthesized silver nanoparticles for antibacterial and antifungal activities using disc diffusion method. The test cultures are *Proteus*, *Pseudomonas*, *Klebsiella*, *Bacillus* and *E. coli* species of bacteria and *Aspergillus*, *Fusarium*, *Curvularia* and *Rhizopus* species of fungi were used. The microbial property of silver nanoparticles was analyzed by measuring the inhibition zone. In bacterial pathogens, the high inhibition zone was found in *Pseudomonas* (15 mm) and in that of less inhibition zone was observed in *Proteus* (7 mm). In fungal pathogens, the high inhibition zone was observed in *Rhizopus* (15 mm) and least inhibition zone was found in *Fusarium* spp. (8 mm).

Kaur *et al*., (2012) used silver nanoparticles, chitosan nanoparticles and silver-chitosan nanocomposite as an antifungal agent against seedborne pathogens. In this study, seedborne pathogens *viz. Rhizoctonia solani, Aspergillus flavus and Alternaria alternata* were isolated from chickpea seeds. The zone of inhibitions of Ag-Ch nanoformulations was much higher than silver or chitosan nanoparticles used independently. In agar well diffusion method, Ag-Ch exhibited highest inhibition against *Aspergillus flavus* (19.66±0.25) followed by *Alternaria alternata* (16.33±0.29) and *Rhizoctonia solani* (12.66±0.76). In mycelial growth inhibition method, Ag-Ch showed inhibitions 94%, 67% and 78%, respectively against *Aspergillus* spp., *Rhizoctonia* spp. and *Alternaria* spp., respectively.

Kim *et al*., (2012) studied the effect of silver nanoparticles (AgNPs, WA-CV-WA13B (CV), WA-AT-WB13R (AT) and WA-PR-WB13R (PR)) provided by Bio plus Co. (Pohang, Korea) against eighteen plant pathogenic fungi on different media (PDA, MEA and CMA). Higher inhibition of fungal growth was recorded at a concentration of 100 ppm. Most of the fungi showed growth inhibition with the increment of incubation tune and the inhibition showed similar patterns for each type of media. Absolute inhibition (100%) was observed on PDA medium against A-3, C-10, F-5, P-S and P-9. and greater than 90% inhibition on PDA against C-l, D-L, G-L, M-l and S-3. The lowest level of inhibition was observed against fi-1 on PDA treated with 10 ppm concentration of AgNPs.

Tripathi *et al*., (2013) studied on supernatant of seed culture was used for the biosynthesis of silver nanoparticles. The biosynthesis of silver nanoparticles using *Trichoderma koningii* and evaluation of their antibacterial activity. *Trichoderma koningii* secretes proteins and enzymes that act as a reducing and capping agent. The antibacterial activity of biosynthesized AgNPs was evaluated by growth curve and inhibition zone and it was found that the AgNPs showed potential effective antibacterial activity.

Ahluwalia *et al*., *(*2014*)* evaluated the antibacterial activities of synthesized nano-silver against two bacteria; *Staphylococcus aureus* and *Klebsiella aeruginosa* and it was found that, bacterial growth was significantly reduced in a dose dependent manner.

Shelar and Chavan (2014) used *Aspergillus terreus* for biosynthesis of silver and gold nanoparticles. *Aspergillus terreus* synthesized silver nanoparticles found having strong antibacterial activity

against *Staphylococcus aureus* and *Shigella* spp. However, gold nanoparticles do not showed any antibacterial activity. Biological approach using the fungi is a novel way towards the safe, cost effective and eco-friendly method for the synthesis of gold nanoparticles, which was gaining importance in the field of nanotechnology.

Mansoori *et al*., (2015) evaluated silver nanoparticles using white radish to control land snails and found that, results indicated that, the exposure of the snails and soil matrix to AgNPs in a laboratory experiment reduced the activity and the viability of the land snail (20% of AgNPs treated snails died) as well as the frequency of fungal population in the surrounding soil. Moreover, histology and ultra structure alterations have been found in both kidney and the digestive gland of AgNPs treated land snails. The synergistic effect of synthesized AgNPs as antifungal was evaluated and clearly revealed that, AgNPs could be effectively used against various plant pathogenic fungi. The present study results may open a new avenue to use the snails as bio-indicator organism of environmental pollution.

Shelar and Chavan (2015) studied the effect of mycosynthesis of silver nanoparticles from *Trichoderma harzianum* on seed germination of oilseed crops. It was observed that, percentage of seed germination was enhanced irrespective of the myco-synthesized silver nanoparticles solution. *T. harzianum* synthesized silver nanoparticles showed increase in the percentage of seed germination with increase in the soaking time of silver nanoparticles solution. *T. harzianum* synthesized silver nanoparticles observed optimistic effect on seed germination. Therefore, biosynthesized silver nanoparticles have

biological assay used in agricultural purposes to increase the viability of seeds.

MATERIAL AND METHODS

Chapter III

MATERIALS AND METHODS

This chapter includes all the materials used and methods adopted in the investigations and the techniques were detailed wider in respective headings.

3.1 Experimental site

The present experiments were carried out in the Department of Plant Pathology, College of Agriculture Latur, Vilasrao Deshmukh College of Agricultural Biotechnology Latur and SAIF, IIT, Bombay.

3.2 MATERIAL USED

3.2.1 Glassware

Glasswares with standard measurements were used throughout the present investigation. The glasswares used were Petri plates (90 mm diameter), conical flasks (100, 250, 500 and 1000 ml), measuring cylinders (10, 50 and 100 ml), test tubes and pipettes (0.1, 1.0, 2.0, 5.0 and 10 ml) etc.

3.2.1.1 Cleaning of Glassware

The glassware were first washed with a detergent followed by thorough cleaning with tap water before placing them in cleaning solution (Potassium dichromate 60 gm, Concentrated sulphuric acid 60 ml and Distilled water 1000 ml) for 24 hours, 3-4 times rinsed with distilled water and air dried.

3.2.1.2 Sterilization of Glassware

All the glasswares were sterilized in Hot air oven at 180^0C for 2 hrs and the culture media were sterilized at 15 lbs pressure for 15-20 min in Autoclave.

3.2.2 Pouring of Petri plates

The media in solid state were melted and allowed to cool down to 45^0C before pouring. Approximately, 15-20 ml of liquid media was poured in previously sterilized Petri plates and allowed them to solidify. Pouring of Petri plates was always done on Laminar Air flow cabinet under aseptic conditions.

3.2.3 Chemicals

Chemicals of Analytical Reagent (AR) and Guaranteed Reagent (GR) grades of standard make were used. The pH of the media was adjusted using either 0.1 N HCl or 0.1 N NaOH.

3.2.4 Equipments

Compound microscope (10x, 40x and 100x magnifications) were used for observing the fungal structures. Hot air oven and autoclave were used for sterilization of glassware and media respectively. BOD Incubators were used for incubating test materials at different temperatures. Rotary shaker were used for continues shaking of fungal broth. The cultures were stored at 4^0C in a refrigerator. Weighments were done on a single pan electronic balance with a sensitivity of 0.001 g. Other tools which were used in the present investigation for various purposes included camel brush, inoculation needle, pots etc. UV-Visible Spectrophotometer was used for the estimation of wavelength of silver nanoparticles and Transmission Electron Microscope (TEM) was used for the characterization of silver nanoparticles.

3.3 LABORATORY TECHNIQUES

3.3.1 Collection and isolation of the pathogens

Tomato crop showing typical wilt symptoms were collected from the field for the isolation of *Fusarium oxysporum* f. sp. *lycopersici*. The roots and stem parts of infected plant were washed in running tap water to remove the soil particles before isolation to avoid contamination. The roots were cut into small bits of the size 2.5 mm with sterilized blade. These bits then surface sterilized with 2-3 per cent sodium hypochlorite solution for two minutes and washed with three changes of sterilized water to remove the traces of sodium hypochlorite. Each bit was blot dried and four bits each were placed on the solidified potato dextrose agar (PDA) plates. These plates were then incubated at 27 ± 1^{0}C for seven days. The fungal growth was transferred to the PDA plates.

Sclerotium rolfsii was isolated from infected tomato crop showing typical stem rot symptoms. Stems with infected collar regions were washed in running tap water and cut into pieces of 3 mm each. These pieces were surface sterilized with sodium hypochlorite (2-3%) solution for 60 sec. and then washed thoroughly in sterile distilled water three times to remove traces of sodium hypochlorite and transferred aseptically to sterilized potato dextrose agar plates and were incubated at 27 ± 1^{0}C for 4-5 days for growth of the fungus.

3.3.2 Identification of the pathogens

The pathogens associated with the diseases in the pure form was obtained on Potato Dextrose Agar medium and was identified based on mycelial growth and spore production characters.

3.4 Collection and isolation of *Trichoderma* sp. from rhizospheric soil

3.4.1 Collection of rhizosparic Soil sample

Collection of soil sample was done from field of Biotech College Latur. Tomato plants were uprooted and soil was collected from rhizospheric area. Nearly ½ Kg soil was collected in plastic bag. Collected soil was air dried and used for further study.

3.4.2 Isolation of *Trichoderma* sp. from rhizosphere soil sample

Serial dilution technique was used to isolate the *Trichoderma* from the samples collected. The collected samples were air dried in shade and finely ground before serial dilutions. PDA media was used for Isolation.

Test tubes labeled with glass marking pencil as 10^{-1}, 10^{-2} up to 10^{-5}. In each test tube, 9 ml of water was poured. Test tubes were plugged with non absorbent cotton and sterilized in an autoclave as mentioned earlier. After cooling, initial dilution was prepared in test tube labelled as 10^{-1} by addition of 1 g representative soil sample into the first test tube containing 9 ml of sterilized water. Contents were mixed by rolling the test tubes to and between palms of hands for 5 minutes to obtain uniform distribution of the soil sample. From the first dilution, 1 ml of suspension while in motion was transferred to the tube labeled as 10^{-2} having 9 ml sterilized water with fresh sterilized pipette. Again same procedure was repeated till the original sample was diluted to 10^{-5}. Each time fresh sterilized pipettes was used. With the help of sterilized pipette, 1 ml of suspension from each dilution (while rotating the test tubes in palms of hands) was placed in centre of the sterilized Petri plate. An antimicrobial agent was added to it in required quantity. The medium (PDA) was poured over soil water suspension in Petri plate with rotary

motion of the plate to mix it thoroughly. PDA was poured at the rate of 20 ml/plate. Plates were labeled with glass marking pencil and inverted on solidification of the medium. These plates were incubated in BOD incubator at 28± 1^0 C up to a week's period. Incubated plates were watched every day for growth of *Trichoderma* spp. Preliminary screening for *Trichoderma* species was carried out by observing both macroscopic and microscopic features of the fungal colonies.

3.5 Maintenance of the culture

The culture of the *Trichoderma* was sub-cultured on potato dextrose agar slants and kept in laboratory at 28°C for 15 days. Such mother culture slants were preserved at 5°C in refrigerator. Further, this culture was sub-cultured once in a month and used for future studies.

3.6 Identification of *Trichoderma* sp.

The cultural and morphological characteristics of the test *Trichoderma* sp. strain was studied using PDA. Cultural characteristics such as growth rate, colony colour, reverse colony colour and colony edge etc. were studied.

For morphological characterization of the test *Trichoderma* sp. strains, temporary mount of pure culture of the test strain was prepared on clean glass slide and observed under research microscope to record morphological characters like conidiophores, their branching pattern, phialide numbers and their arrangement, conidial shape and size etc.

Colony appearance and pigmentation of *Trichoderma* isolate was studied on PDA plates. By using a cork borer, 5 mm mycelial plugs were cut and transferred to the center of the agar plates. The inoculated

agar plates were then incubated at 27±1^{0}C for 7 days. The culture was observed daily and colonies that produced sweet coconut odor were noted.

The growth rate of each *Trichoderma* isolate was studied on PDA plates. Mycelial plugs (5 mm) from the margin of growing fungal colonies were cut and placed at the center of the 9 cm vented agar plates. The culture plates were then incubated at 27±1^{0}C. A duplicate plate for each isolate was prepared. For culture plate incubated at 27±1^{0}C, the diameter of each colony was measured after every 24 hours interval until the agar plate was fully colonized. The growth trial was repeated once independently and the average diameter of the colonies was taken from the two independent growth trials.

Trichoderma isolates were inoculated on PDA and incubated at 27±1^{0}C prior to microscopic identification. Culture plates that incubated for 3-5 days were used to observe branching patterns of conidiophores, whereas culture plates that incubated for 7-10 days were used to observe the chlamydospores. A slide of each isolates was prepared by placing a small piece of mycelia onto a drop of distilled water on a slide and the mycelium was gently dispersed using an inoculating needle. The slide was then observed under a light microscope with 100x and 400x magnification. Observations focused on the sizes, shapes and colours of conidia, the branching patterns of conidiophores and the appearance of chlamydospores. The sizes of conidia were measured using an ocular micrometer. The macroscopic and microscopic features were compared to the characteristics described by Samuels *et al*., (1998).

3.7 Synthesis of silver nanoparticles from culture filtrate of *Trichoderma harzianum*

3.7.1 Production of biomass *of Trichoderma harzianum*

A seven day old pure culture of *Trichoderma harzianum* was inoculated in 250 ml conical flasks containing 100 ml of Potato Dextrose Broth (PDB) and the culture flasks were incubated at 27 ± 1^0C. Then, the mixture was placed in 150 rpm rotating shaker at 28^0C 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.

3.7.2 Synthesis of silver nanoparticles

In typical biosynthesis of silver nanoparticles, 50 ml of aqueous solution of 1 mM Silver nitrate ($AgNO_3$) was treated with 50 ml of *Trichoderma* culture filtrate for the extracellular synthesis of silver nanoparticles in a 250 ml conical flask. The whole mixture was incubated at room temperature for 24 hrs. The colour change of silver nitrate from colourless to brown colour indicates the formation of silver nanoparticles through reduction of silver ionic forms (Ag^+) to Ag^0.

3.8 Characterization of silver nanoparticles

3.8.1 UV-Visible spectroscopy

The reduction of silver ions was monitored by UV-Vis spectrum of the reaction mixture at 24 hrs. and their absorbance was recorded at 380, 400, 420 nm. The spectra of the surface Plasmon resonance of AgNPs in the reaction mixture were recorded using UV-Vis spectrophotometer (Shimodzu, UV-2150) at wavelengths between 200 to 800 nm.

3.8.2 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 are relatively uniform in nature and also shows that particles are well separated from each other having no accumulation. TEM were performed at accelerating voltage of 200.0 kV with 20000X magnification.

3.9 *In vitro* evaluation antimicrobial activity of biosynthesized silver nanoparticles and *Trichoderma* culture filtrate against soilborne pathogens of Tomato by different methods.

3.9.1 Agar well Diffusion method

The soilborne plant pathogenic fungi (*Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii*) were 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 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.

Experiment details :-

Design - CRD

Replications - Four

Treatments - six (6)

Test pathogens - 1. *Fusarium oxysporum* f. sp. *lycopersici*
2. *Sclerotium rolfsii*

Treatment details :-

T_1 : *Trichoderma* culture filtrate

T_2 : AgNPs 10 ppm

T_3 : AgNPs 30 ppm

T_4 : AgNPs 50 ppm

T_5 : AgNPs 100 ppm

T_6 : control

3.9.2 Poisoned food technique

In vitro evaluation of *Trichoderma* sp. culture filtrate of non biosynthesized *Trichoderma* and synthesized nanoparticles were evaluated against soilborne pathogens (*Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii*) of tomato crop by poisoned food technique 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 9 cm petriplate. A control was maintained without nanosilver and culture filtrate. 7 mm mycelial disc of seven days old culture of *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* was inoculated at the center and incubated at 27 ± 1^0C until full growth was observed in control. Four replications were maintained for each treatment.

Experiment details :-

Design - CRD

Replications - Four

Treatments - six (6)

Test pathogens - 1. *Fusarium oxysporum* f. sp. *lycopersici*
2. *Sclerotium rolfsii*

Treatment details :-

T_1 : *Trichoderma* culture filtrate

T_2 : AgNPs 10 ppm

T_3 : AgNPs 30 ppm

T_4 : AgNPs 50 ppm

T_5 : AgNPs 100 ppm

T_6 : control

Observations on radial mycelial growth/colony diameter of the test isolates were recorded at an interval of 24 hours and continued till untreated control plates were fully covered with mycelial growth of the test pathogen. Per cent mycelial growth inhibition of the test pathogens

over untreated control was calculated by following formula (Vincent, 1927).

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

Where,

I = Per cent inhibition

C= Radial growth (mm) of test pathogen in control

T= Radial growth (mm) of test pathogen in treatments

3.10 Statistical analysis

The data obtained in all the experiments (*in vitro*) were statistically analyzed. The standard error (S.E) and critical difference (C.D) at 1 % level significance were worked out. The per cent data were transformed into Arc sine values before statistical analysis.

RESULTS & DISCUSSION

Chapter IV

RESULTS AND DISCUSSION

4.1 Collection and Isolation of the pathogens

Tomato plants showing typical wilt and stem rot symptoms were collected from the field. The roots and stem parts of infected plant were washed in running tap water to remove the soil particles before isolation to avoid contamination. The roots were cut into small bits of the size 2.5 mm for isolation of *Fusarium* sp. and collar rot infected stems were cut into 3 mm size with sterilized blade. These bits then surface sterilized with 2-3 per cent sodium hypochlorite solution and washed with three changes of sterilized water to remove the traces of sodium hypochlorite. Then these bits placed on the solidified potato dextrose agar (PDA) plates. These plates were then incubated at 27 ± 1^{0}C for seven days. Colonies of *Fusarium* sp. and *Sclerocium* sp. was obtained on PDA medium on 7^{th} days and 4-5 days after inoculation, respectively.

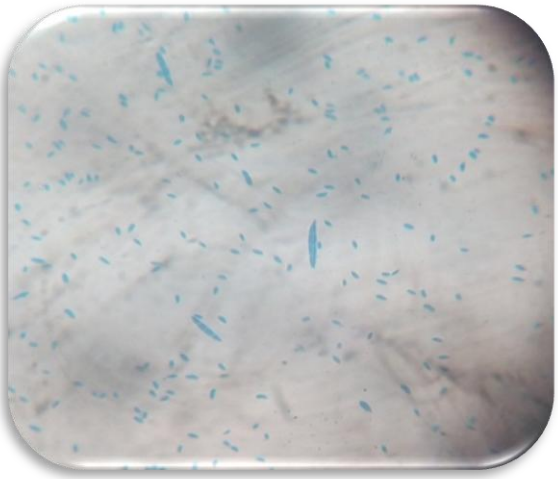
4.2 Identification of the pathogens

The pathogens in the pure form associated with the disease were obtained on Potato Dextrose Agar medium and were identified based on mycelial growth and microscopic observations the pathogens was identified as *Fusarium oxysporium* f. sp. *lycopersici* and *Sclerocium rolfsii.* (Plate I a&b)

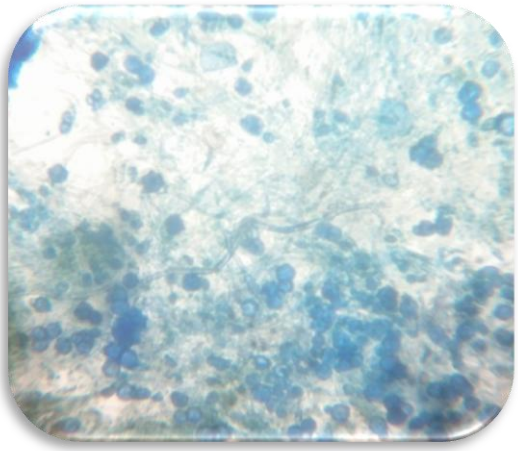
4.3 Isolation of *Trichoderma* sp. from the rhizophere of Tomato

A soil sample was collected from healthy tomato plant present in the field of Biotech college Latur. The *Trichoderma* sp. from the rhizospheric soil was isolated by using 10^{-4} to 10^{-5} dilutions by

Pure culture



Micro and Macroconidia



Chlamydospore

Plate I a. Microphotographs at 100 X in Olympus light microscope of *Fusarium oxysporum* f. sp. *lycopersici*

Pure Culture

Microphotograph of *S. rolfsii*

Plate I b. Microphotographs at 100 X in Olympus light microscope of *Sclerotium rolfsii*

dilution plate technique. Colonies of *Trichoderma* sp. was obtained on Potato Dextrose Agar medium on 7th day after inoculation.

4.4 Identification of *Trichoderma* sp.

Cultural and morphological identification of the *Trichoderma* isolate was studied, based on the characters of colony, mycelial and spore pattern (Bissett, 1991).

For the identification of *Trichoderma* sp., 3-7 old days culture grown on PDA was used. The *Trichoderma* strain morphologically identified by using characters such as colony growth rate, reverse colony colour, colony edge, alongwith morphology of conidia, shape and size, phialids size and shape, conidial shapes, size, branching of conidiophores etc.

4.4.1 Colony characters

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.

4.4.2 Growth rate

These isolate was also identified on the basis of growth rate when cultured on PDA and incubated at 27±1^{0}C. The colony diameters of *Trichoderma* isolate was observed. The isolate of *Trichoderma* sp. showed 8-9 cm growth rate in 4 days.

4.4.3 Microscopic features

Further above observations was confirmed by microscopy. The microscopic features of isolate were identified based on the shape of conidiophores, conidia and phialides. 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 conidiophores were highly branched and difficult to differentiate and measure under light microscope. The colour of conidia of *Trichoderma* isolate was found to be green. The different intensities of green colour (light green, yellowish green and dark green) of mature conidia, observed on PDA plates can hardly be observed under light microscope. The chlymadospores of *Trichoderma* sp. were 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 II).

Results of the present studies on identification and morphological characterization of *Trichoderma* isolate are in consonance with those reported earlier by several workers (Rifai, 1969; Bissett, 1991; Samuels *et al*., 1998; Prameela *et. al.*, 2012; Kamala and Indira, 2012; and Shrivastava *et al*., 2015).

4.5 Maintenance of Pure culture of *Trichoderma harzianum*.

The antagonistic *Trichoderma harzianum* purified from mother culture was grown separately on then respective medium and also maintained in PDA slants for further experimental studies.

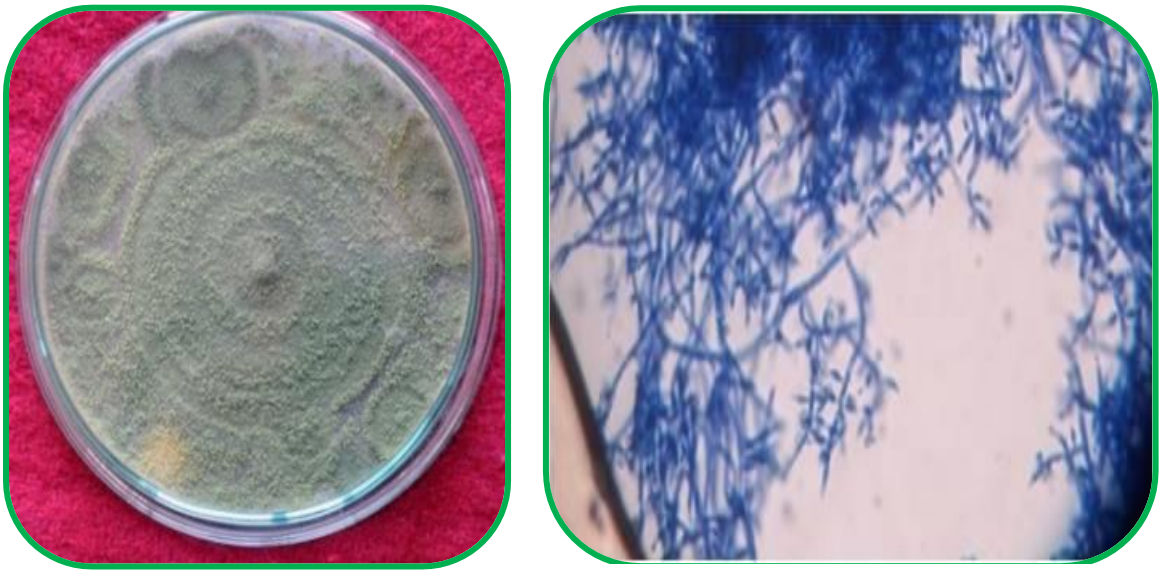


Plate II: Isolation of *Trichoderma harzianum* and Microphotographs at 100 X in Olympus light microscope of isolated *Trichoderma harzianum* from tomato rhizospare

4.6 Biosynthesis of silver nanoparticles using *Trichoderma harzianum*

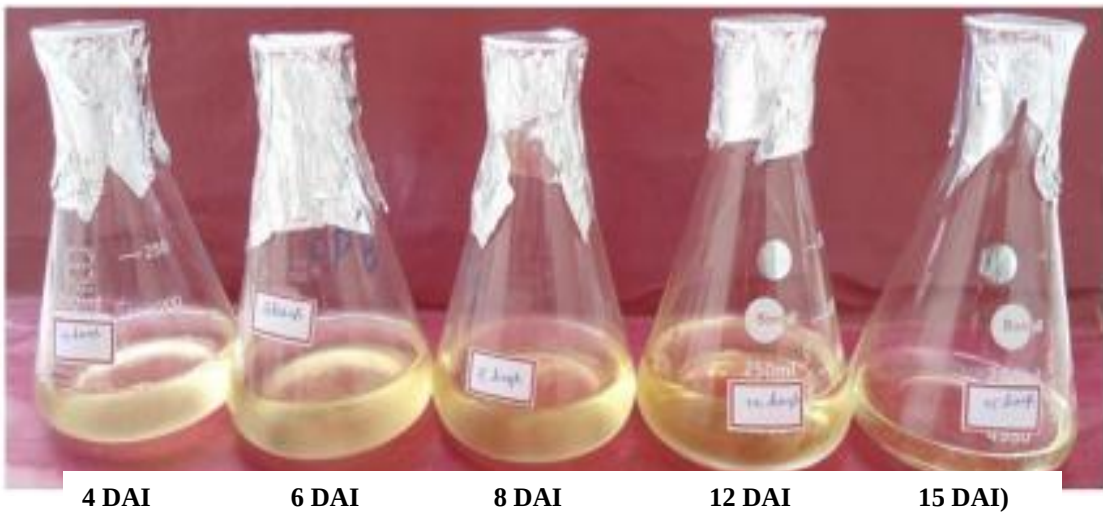
Seven day old pure culture *of Trichoderma harzianum* was inoculated in Potato Dextrose Broth (PDB). The culture filtrate was harvested at different time intervals, *viz.* 4 DAI, 6 DAI, 8 DAI, 12 DAI, 15 DAI (Plate III). To know the effect of incubation on the synthesis of silver nanoparticles, 10 ml 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 IV). The colour changes in the solutions, which were used in silver nanoparticles synthesis were clearly observed in Plate V.

4.7 Characterization of silver nanoparticles

4.7.1 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 440 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. The silver nanoparticles synthesized from six days incubated culture filtrate treated 1 mM $AgNO_3$ solution were used for further characterization and evaluation of its antifungal activity against pathogen.

Formation and stability of Ag nanoparticles in aqueous solution are continued by UV-Vis spectral analysis. It is well known that



4 DAI 6 DAI 8 DAI 12 DAI 15 DAI)

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

4 DAI 6 DAI 8 DAI 12 DAI 15 DAI)

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

Plate V: Synthesis of silver nanoparticles:

(A) PDB alone

(B) ***Trichoderma harzianum*** **culture filtrate**

(C) 1 mM $AgNO_3$ solution before incubation of ***Trichoderma*** **culture filtrate and**

(D) 1 mM $AgNO_3$ solution after 24 hrs incubation of ***Trichoderma*** **culture filtrate**

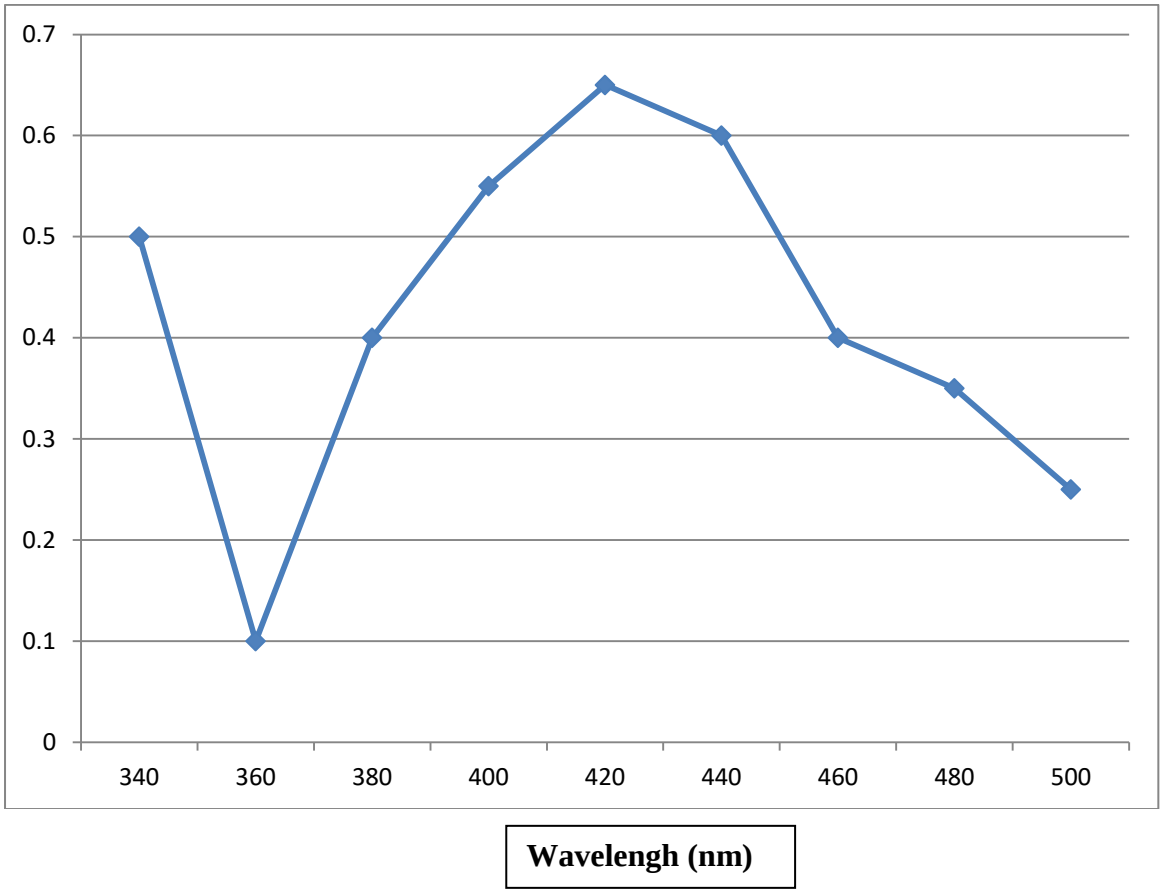


Fig. 1. UV-Vis spectra recorded after the exposure of 1 mM silver nitrate solution in culture filtrate of *T. harzianum*.

silver nanoparticles exhibit brown color in aqueous solution due to excitation of surface Plasmon vibrations hence, it confirmed the reduction of silver ions extracellular (Krishnaraj *et al*., 2010). 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.

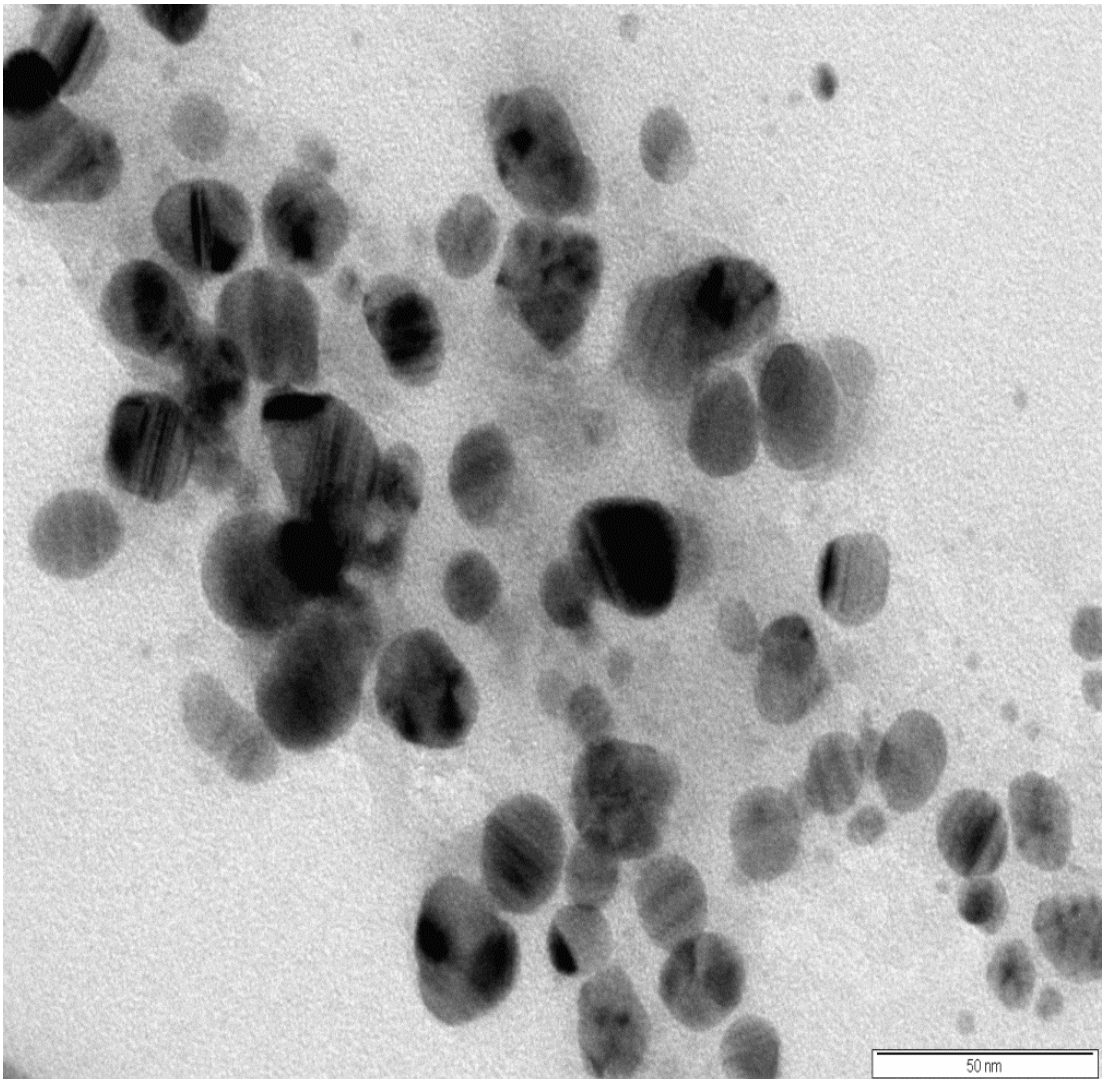
Among the all treatments, six days aged *Trichoderma* culture filtrate produced high amount of silver nanoparticles after 24 hrs incubation and this was found to be similar reported by Devi *et al*., (2013).

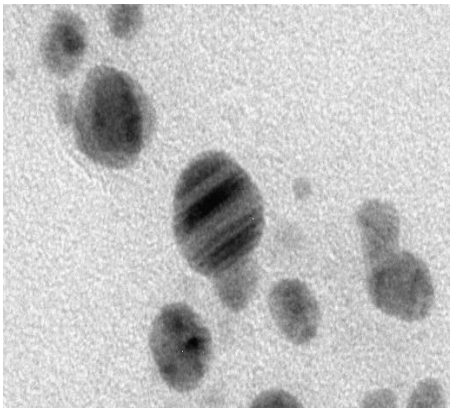
4.7.2 Transmission Electron Microscopy (TEM) analysis

The Transmission Electron Microscopy studies characterized the shape and size of the synthesized silver nanoparticles (Plate VI a&b).This picture shows individual silver nanoparticles as well as a number of aggregates. In general particles were spherical in shape and the sizes of the silver nanoparticles were found in the range of 50 nm.

The TEM micrograph shows the particle size ranges from 50 nm. When compared with $AgNO_3$ nanosilver having the increased contact with fungi.

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), 8-24 nm (Tripathi *et al*., 2013) and 19-63 nm (Shelar and Chavan 2015).





Single particle of silver nanoparticle

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

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

4.8.1 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* 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* culture filtrate (77.00 mm). The zone of inhibitions was highest at 100 ppm silver nanoparticles (18.66 mm) and lowest against *Trichoderma* culture filtrate (13 mm). Table 2, Plate VII and Fig. 2.

Table 2. *In vitro* evaluation of antimicrobial activity of silver nanoparticles and *Trichoderma* 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, 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 *Rhizocionia 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.

In the present investigation, Ag nanoparticles are used as fungicidal solution for the control of *Fusarium oxysporum* f. sp. *lycopersici* which causes soilborne disease in solanacious crops.

4.8.2 Poison 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* culture filtrate. The per cent inhibitions

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

T_1 : *Trichoderma* culture filtrate

T_2 : AgNPs (10 ppm)

T_3 : AgNPs (30 ppm)

T_4 : AgNPs (50 ppm)

T_5 :AgNPs (100ppm)

T_6 : Control (untreated)

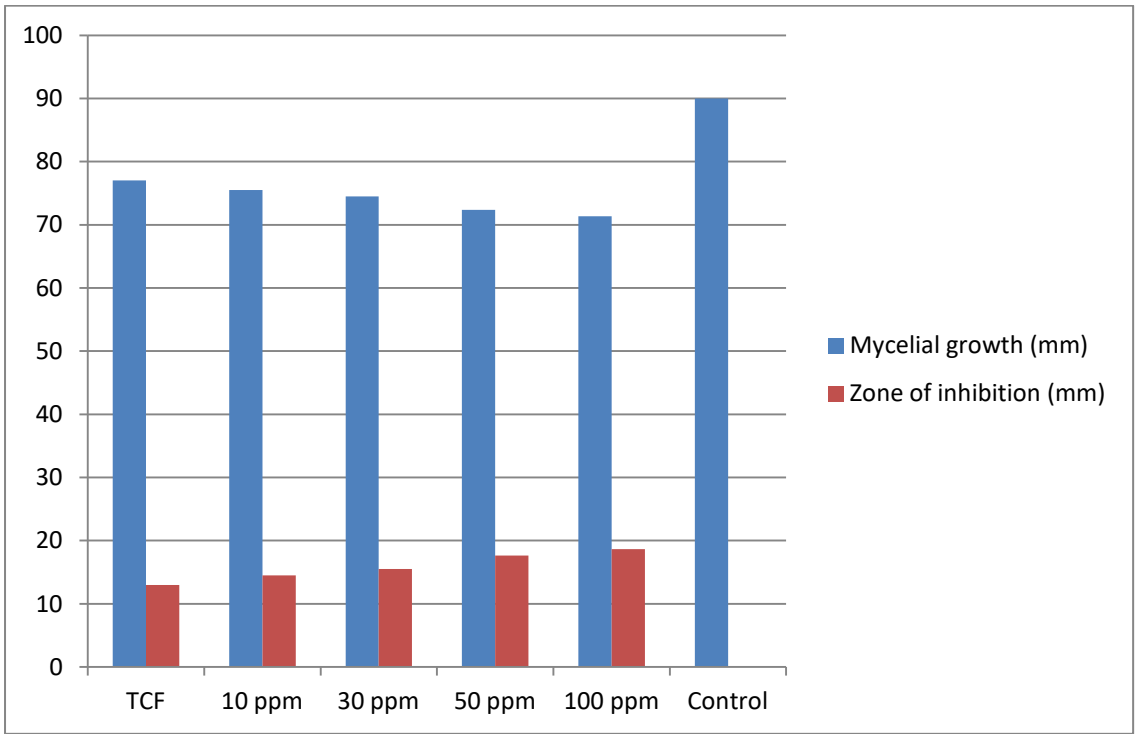


Fig. 2. *In vitro* evaluation of antifungal activity of silver nanoparticles and *Trichoderma* culture filtrate against *Fusarium oxysporum* f. sp. *lycopersici* by using agar well diffusion method

T_1 : *Trichoderma* culture filtrate **T_4 : AgNPs (50 ppm)**

T_2 : AgNPs (10 ppm) **T_5 : AgNPs (100ppm)**

T_3 : AgNPs (30 ppm) **T_6 : Control (untreated)**

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* culture filtrate (44.00 mm). At 100 ppm concentration the per cent inhibitions were observed as 75.19% for AgNPs. 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* culture filtrate, respectively. (Table 3, Plate VIII and Fig. 3).

Table 3. *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**
Figure in Parentheses are transformed in arc sine values.

The results were confirmed with the similar findings of Lamsal *et al*., 2011. In poison food method AgNPs exhibited highest inhibition against *Colletotrichum species* at concntration 100 ppm, 50 ppm,

Plate VIII: ***In vitro*** **evaluation of antifungal efficacy of silver nanoparticles against** ***Fusarium oxysporum*** **f. sp.** ***lycopersici***

T_1 : *Trichoderma* culture filtrate

T_2 : AgNPs (10 ppm)

T_3 : AgNPs (30 ppm)

T_4 : AgNPs (50 ppm)

T_5 : AgNPs (100ppm)

T_6 : Control (untreated)

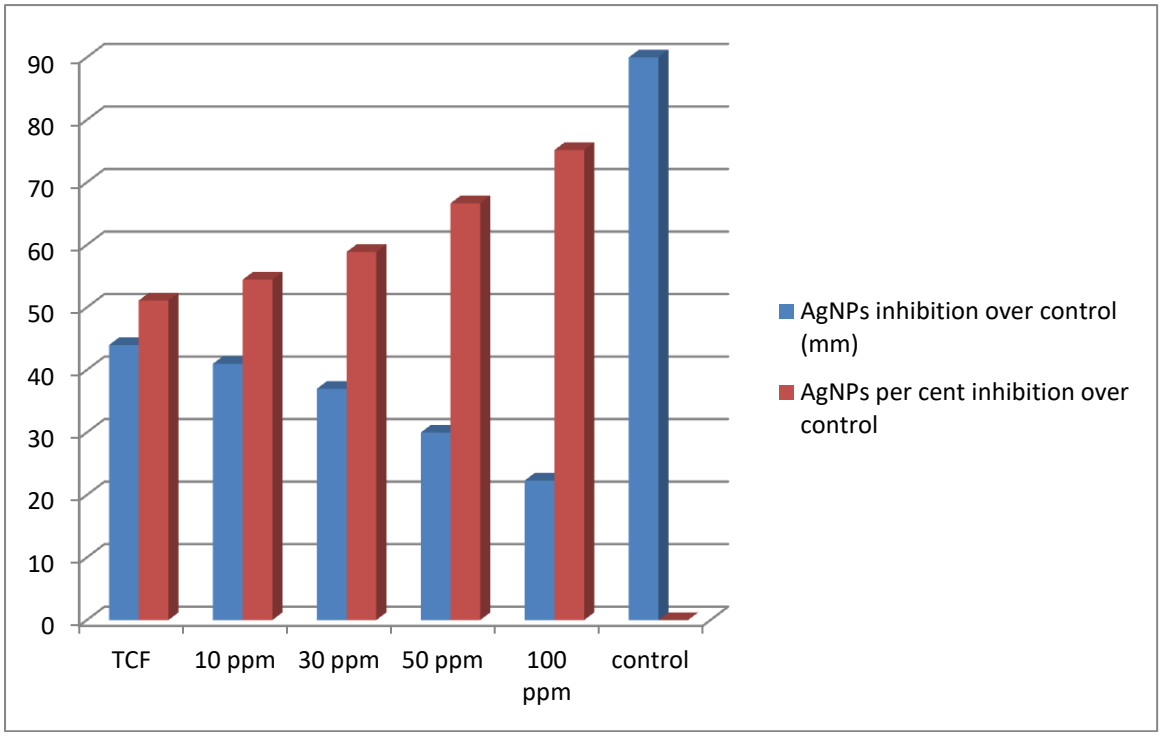


Fig. 3. *In vitro* evaluation of antifungal activity of silver nanoparticles and *Trichoderma* culture filtrate against *Fusarium oxysporum* f. sp. *lycopersici* by using poison food technique

T_1 : *Trichoderma* culture filtrate **T_4 : AgNPs (50 ppm)**

T_2 : AgNPs (10 ppm) **T_5 : AgNPs (100ppm)**

T_3 : AgNPs (30 ppm) **T_6 : Control (untreated)**

30 ppm and 10 ppm are 90%, 84.56%, 84.50% and 11.33% inhibition, respectively.

4.9 *In vitro* evaluation of antimicrobial activity of silver nanoparticles *Trichoderma* culture filtrate against *Sclerotium rolfsii*

4.9.1 Agar well diffusion method

The antimicrobial activity of the synthesized nanoparticles was evaluated with agar well diffusion method. The antimicrobial activity was evaluated against *Sclerotium rolfsii* at different concentrations and it was observed that increase in concentration of nanoparticles progressively inhibit 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* culture filtrate and observed for inhibition zone around the wells. In this method the zone of inhibitions were highest at 100 ppm silver nanoparticles (17 mm) and lowest against *Trichoderma* culture filtrate (11 mm). (Table 4, Plate IX and Fig. 4).

Table 4. *In vitro* evaluation of antimicrobial activity of silver nanoparticles and *Trichoderma* culture filtrate by using Agar Well Diffusion method against *Sclerotium rolfsii*

Tr.No.	Treatments	Mycelial growth* (mm)	Zone of inhibition* (mm)
T_1	TCF	79.00	11.00
T_2	AgNPs (10 ppm)	77.67	12.33
T_3	AgNPs (30 ppm)	75.67	14.33
T_4	AgNPs (50 ppm)	74.34	15.66
T_5	AgNPs (100 ppm)	73.00	17.00
T_6	Control	90.00	00.00
	SE±		**0.289**
	CD at 1%		**0.864**

***-Mean of four replications, TCF: *Trichoderma* Culture Filtrate**

The results were confirmed with the similar 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 *Rhizocionia 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.

4.9.2 Poison food technique method

The suspension of silver nanoparticles was used to study the antifungal activity against *Sclerotium rolfsii* by poison food technique, Effect of Silver nanoparticles were compared with the effect of *Trichoderma* culture filtrate. The per cent inhibitions were increased with increase of concentration. At 100 ppm concentration the per cent inhibitions were observed as 68.53% for AgNPs and the per cent

Plate IX: *In vitro* evaluation of antifungal efficacy of silver nanoparticles by using agar well diffusion method against *Sclerotium rolfsii*

T_1 : *Trichoderma* culture filtrate

T_2 : AgNPs (10 ppm)

T_3 : AgNPs (30 ppm)

T_4 : AgNPs (50 ppm)

T_5 : AgNPs (100ppm)

T_6 : Control (untreated)

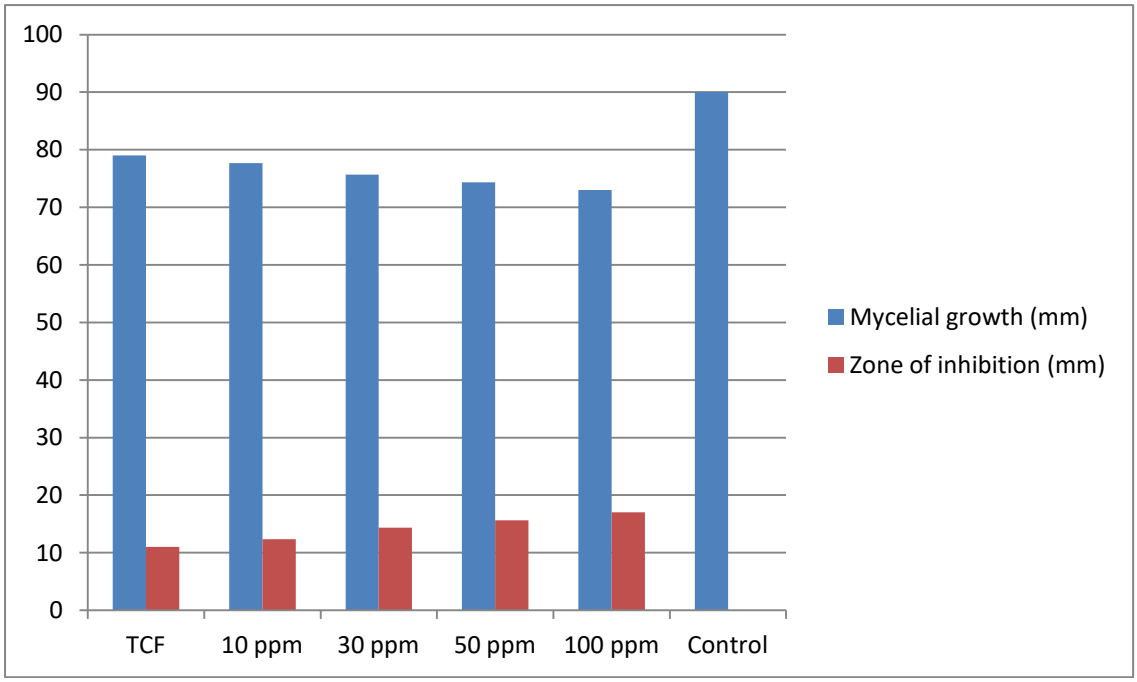


Fig. 4. *In vitro* evaluation of antifungal activity of silver nanoparticles and *Trichoderma* culture filtrate against *Sclerotium rolfsii* by using agar well diffusion method

T_1 : *Trichoderma* culture filtrate **T_4 : AgNPs (50 ppm)**

T_2 : AgNPs (10 ppm) **T_5 : AgNPs (100ppm)**

T_3 : AgNPs (30 ppm) **T_6 : Control (untreated)**

inhibitions were observed as 64.45%, 55.56%, 49.64%, and 44.45% for 50 ppm, 30 ppm, 10 ppm and *Trichoderma* culture filtrate, respectively. (Table 5, Plate X and Fig. 5).

Table 5. *In vitro* evaluation of antimicrobial activity of silver nanoparticles and *Trichoderma* culture filtrate against *Sclerotium rolfsii* by using Poison food technique

Tr. no.	Treatments	*Sclerotium rolfsii*	
		Colony Dia.* (mm)	**% inhibition***
T_1	TCF	50.00	44.45 **(41.80)**
T_2	AgNPs (10 ppm)	45.33	49.64 **(44.79)**
T_3	AgNPs (30 ppm)	40.00	55.56 **(48.19)**
T_4	AgNPs (50 ppm)	32.00	64.45 **(54.39)**
T_5	AgNPs (100 ppm)	28.33	68.53 **(55.87)**
T_6	Control	90.00	00.00 **(00.00)**
	SE±	**0.319**	
	CD at 1%	**0.956**	

***-Mean of four replications, Dia.: Diameter**
Figure in Parentheses are transformed in arc sine values.

The results were confirmed with the similar findings of Lamsal *et al.,* 2011. In poison food method AgNPs exhibited highest inhibition against *Colletotrichum species* at concntration 100 ppm, 50 ppm, 30 ppm, and 10 ppm are 90%, 84.56%, 84.50% and 11.33% inhibition, respectively.

Plate X: *In vitro* evaluation of antifungal efficacy of silver nanoparticles against *Sclerotium rolfsii*

T_1 : *Trichoderma* culture filtrate

T_2 : AgNPs (10 ppm)

T_3 : AgNPs (30 ppm)

T_4 : AgNPs (50 ppm)

T_5 : AgNPs (100ppm)

T_6 : Control (untreated)

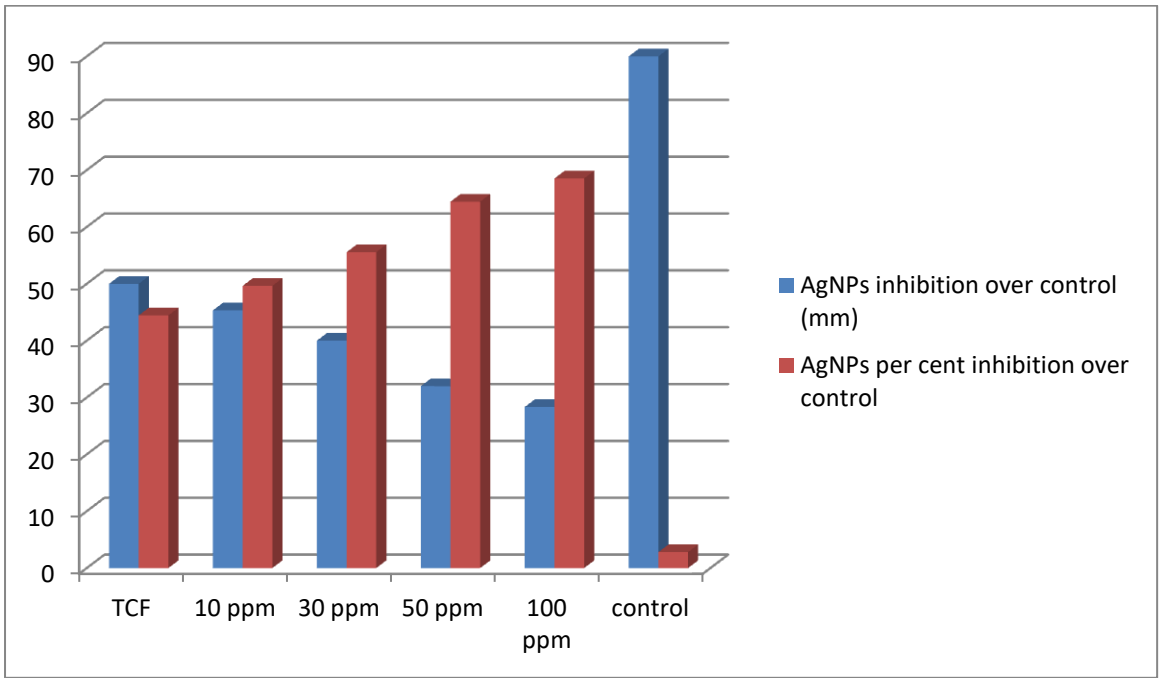


Fig. 5. *In vitro* evaluation of antifungal activity of silver nanoparticles and *Trichoderma* culture filtrate against *Sclerotium rolfsii* by using poison food technique

T_1 : *Trichoderma* culture filtrate **T_4 : AgNPs (50 ppm)**

T_2 : AgNPs (10 ppm) **T_5 : AgNPs (100ppm)**

T_3 : AgNPs (30 ppm) **T_6 : Control (untreated)**

It has been proved that rhizosphere colonies *(Trichoderma harzianum*) was capable of synthesizing the metal nanoparticles: silver in particular, which is an effective controlling agent of pathogens, *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* points to the addition of silver metal in relatively smaller quantities at plant rhizosphere leads to the control of soilborne diseases in Tomato crop.

SUMMARY AND CONCLUSION

Chapter V

SUMMARY AND CONCLUSION

The Present investigation was earned out to biosynthesis of silver nanoparticles (AgNPs) by using *Trichoderma* spp. and its efficacy against soilborne pathogens of tomato. The effective *Trichoderma* sp. was isolated and used for synthesis of silver nanoparticles which were tested against *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii.* The results were summarized and presented below.

Fusarium oxysporum f. sp. *lycopersici* and *Sclerotium rolfsii* isolates were isolated from tomato crop. A soil sample was collected from tomato field of Biotech College Latur. Isolation of *Trichoderma* sp. was undertaken on Potato Dextrose Agar by using serial dilution method.

Identification and characterization of *Trichoderma harzianum* was done by observing characters include various parameters such as colony growth rate, colony colour, reverse colony colour, colour edge and mycelial form speed along with morphology of conidia and phialids, conidia colour, shapes, size, branching of conidiophores and the presence or absence of chlamydospore etc. The chlamydospores of *Trichoderma harzianum* were not observed up to 7 days.

The isolate of *Trichoderma harzianum* showed 80-90 mm growth rate in 4 days. The shapes of conidia observed irregularly and bottle shape, green coloured and size of conidia was 4.5-8.0×3.0-4.5 µm. The conidiophores were highly branched and difficult to define. Characters are confirmative with the characters of *Trichoderma harzianum.*

Biological synthesis of silver nanoparticles using silver nitrate solution treated with 4 days and 6 days incubated culture filtrate of *Trichoderma harzianum* turned into dark brown color after 24 hrs of incubation.

A characteristic surface Plasmon resonance band was observed at 420 nm after 24 hrs of incubation. Maximum intensity of synthesized silver nanoparticles were observed with six day incubated culture filtrate treated $AgNO_3$ solution followed by four day incubated culture filtrate treated $AgNO_3$ solution.

Transmission Electron Microscopy studies showed the synthesized silver nanoparticles were spherical in shape with the average size of 50 nm.

Synthesis and confirmation of silver nanoparticles were evaluated for its antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* by Agar well diffusion method and Poison food technique. In agar well diffusion method, the zone of inhibition of silver nanoparticles at 100 ppm concentration, recorded 18.66 mm and 17.00 mm inhibition against *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* isolates, respectively.

In Poison food technique, the suspension of silver nanoparticles at 100 ppm concentration the per cent inhibitions were observed as 75.19% for AgNPs. The per cent inhibitions were observed as 66.67%, 58.89%, 54.45% and 51.45% for 50 ppm, 30 ppm, 10 ppm and *T. harzianum* culture filtrate, respectively against *F.oxysporum* f. sp. *lycopersici* and at 100 ppm concentration the per cent inhibitions were observed as 68.53% for AgNPs and the per cent inhibitions were observed as 64.45%, 55.56%, 49.64%, and 44.45% for 50 ppm, 30 ppm, 10 ppm and *Trichoderma* culture filtrate, respectively.

It has been proved that *T. harzianum* is capable of synthesizing the metal nanoparticles: silver in particular, which is an effective inhibition agent of *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii*. Points to the addition of silver metal in relatively smaller quantities at plant rhizosphere leads to the control of soilborne diseases in Tomato crop.

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THESIS ABSTRACT

ABSTRACT

"Biosynthesis of silver nanoparticles (AgNPs) by using *Trichoderma harzianum* and its efficacy against soilborne plant pathogens of Tomato"

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The genus *Trichoderma* was first proposed as a genus more than two hundred years ago by Persoon in 1794 at Germany. *Trichoderma*, a fungi, which grow saprophytically in soils have proved as an effective biocontrol agent of soilborne plant diseases specifically wilt caused by different pathogens like; *Pythium* spp., *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Phytophthora* spp.

A soil sample was collected from rhizosphere of tomato crop. The *T. harzianum* from this sample was isolated on PDA by using serial dilution method. It was morphologically identified by using characters such as colony growth rate, reverse colony colour, colony colour, colony edge, alongwith morphology of conidia, shape and size, phialids numbers, conidial shapes, size, branching of conidiophores etc.

The culture filtrate of *Trichoderma harzianum* was used for the reduction of silver ions (Ag^{+}) in $AgNO_3$ solution to the silver (Ag^{0}) nanoparticles. The different ages (4 days, 6 days, 8 days, 12 days and 15 days) of culture filtrates were screened for the synthesis of silver nanoparticles. Synthesized silver nanoparticles were characterized using

UV-Vis spectrophotometer and Transmission Electron Microscopy (TEM). Among the all treatments the silver nitrate solution treated with six days aged culture filtrate of *Trichoderma harzianum* showed the UV absorption peak at 440 nm with maximum intensity after 24 hrs of incubation. The TEM micrographs showed the spherical shape silver nanoparticles with an average size of 50 nm.

The antifungal activity of silver nanoparticles evaluated against *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* pathogens by Agar well diffusion method and Poison food technique. In Agar well diffusion method, the zone of inhibition of silver nanoparticles at 100 ppm concentration observed as 18.66 mm and 17.00 mm against *F. oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* isolates, respectively.

In Poisoned food technique, the suspension of silver nanoparticles at 100 ppm concentration, the per cent inhibition were observed as 75.19% and 68.53% against *F.oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* isolates, respectively. The results points to the usage of these mycogenic AgNPs in agriculture to control plant diseases.

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2) It would give me a good career as well as professional satisfaction.

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Quick and Enthusiastic learner, including the ability to learn from the experiences of experts with having a good ability of leadership.

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Course	University/Board	Year of passing	Subject of Specialization	percentage	Class
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B.Sc. (Agri.)	M.P.K.V., Rahuri.	June, 2015	Agriculture	73.70	Second Class
H.S.C.	Pune	Feb., 2011	Science	62.33	First Class
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