Mycosynthesis of Silver Nanoparticles (AgNPs) using *Tricoderma* species and its efficacy against plant pathogenic fungi

DETAILED OUTLINE OF THE RESEARCH WORK SUBMITTED FOR THE AWARD OF THE DEGREE OF

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INTRODUCTION

Systematics of *Trichoderma*

Division - Ascomycota
Sub division - Pezizomycotina
Class - Sordariomycetes
Order - Hypocreales
Family - Hypocreaceae
Genus - *Trichoderma*

Trichoderma species are economically important for their production of industrial enzymes (cellulases and hemicellulases) antibiotics and their action as biocontrol agents against plant pathogen fungi based on various mechanisms such as the production of antifungal metabolites, competition for space and nutrients and mycoparasitism **(Howell 2003)**. The ecological importance of this genus, particularly of its mycelium, is to take part in the decomposition of plant residues in soil. Mycoparasitic *Trichoderma* strains are able to recognize the host hyphae, to coil around them, develop haustoria and penetrate the cell wall of the host **(Abdullah 2007)**.

Characterization of the antagonistic effect of *Trichoderma* species is the first step in utilizing the full potential of *Trichoderma* species for specific applications. *In vitro* screening with different bioassays is an effective and rapid method for identifying strains with antagonistic potential. For evaluating the potential of different *Trichoderma* species arrange of mechanisms have to be considered production of antibiotic, volatile and non-volatile chemicals. These substances influence the permeability of cell membranes and result in an efflux of the cytoplasm **(Howell 1998)**.

• Mycoparasitism and excretion of lytic enzymes. The antifungal enzyme system of *Trichoderma* species played an important role for detection and destroying the host cell wall **(Schirmbock *et al.*, 1994)**.

• Competitiveness is based on rapid growth and the production of various asexual generated conidia and chlamydospores **(Chet *et al.*, 1998)**.

• The ability to promote growth and induce resistance in plants is a mechanism which has also been described for members of this genus **(Harman, 2006)**.

History of *Trichoderma*

The genus *Trichoderma* was described in 1791 in Germany. Most species were identified as *T. lignorum* because of its globose conidia or as *T. koningii* because of its oblong condia. 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 to pathogens such as *R. solani*.

Nanotechnology

Micro and Nano word comes from Greek. Micro means very small, usually smaller then 1mm, denotes 10^{-6} so a micrometer is 1 millionth of a meter but nano means dwarf goes from small to tiny: the word nano is 10^{-9} so a nano mater is 1 billionth of a meter. Nanotechnologies mainly consist of the processing of separation, consolidation and deformation of materials by one atom or one molecule. Nanotechnology is the application of science and technology to control matter at the molecular level. At the nano scale level, the properties of matter are significantly different from their macroscopic bulk properties. Nanotechnology is also referred to the ability for designing, characterization, production and application of structures, devices and systems by controlling shape and size at the nanometer scale **(Mansoori, 2005)**.

A nanoparticle of particular shape and size depends on specific requirements **(Godsell, 2004)**. In the 1980s the basic idea of this definition was explored in much more depth by Drexler, who promoted the technological significance of nano-scale phenomena and devices through speeches and the books Engines of Creation: The Era of Nanotechnology (1986) and Nanosystems: Molecular Machinery, Manufacturing, and Computation **(Drexler, 1991)**, and so the term acquired its current sense. The reduction of the metal ions occurs by a nitrate-dependent reductase and a shuttle quinone extracellular process. The potentialities of this Nanotechnological design based in fugal biosynthesis of nanoparticles for several technical applications are important, including their high potential as antibacterial material.

History of Nanotechnology

The term "Nanotechnology" was defined by Tokyo Science University in a paper **(Taniguchi 1974)** as follows: "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 Sanning 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.

In 2000, the United States National Nanotechnology Initiative was founded to coordinate Federal nanotechnology research and development and is evaluated by the President's Council of Advisors on Science and Technology.

Principle of Biosynthesis of Nanoparticles

Nanoparticles were viewed as the fundamental building blocks of nanotechnology **(Mansoori *et al.*, 2007)**. This was the starting point for preparing many nanostructured materials and devices. Their synthesis is an important component of the rapidly growing research efforts in nanoscience 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 environmentally friendly nanoparticles that do not produce toxic wastes in their process synthesis protocol. Advantage of nanobiotechnology 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 as they are not toxic in other ways. For example, environmentally-friendly microorganisms 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 **(Mehra and Winge 1991)**. Cell mass or extracellular components from microorganisms, such as *Klebsiella pneumoniae*, *Bacillus licheniformis*, *Fusarium oxysporum*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Aspergillus clavatus*, and *Penicillium brevicompactum* **(Ahmad *et al.*, 2003; Shahverdi *et al.*, 2007; Kalishwaralal *et al.*, 2008; Balaji *et al.*, 2009; Shaligram *et al.*, 2009; Verma *et al.*, 2010)** have been utilized for the reduction of silver ions to AgNPs. The principle of preparation of silver nanoparticles by using microorganism is a bioreduction process; the silver ions are reduced by the extracellular reductase enzymes produced by the microorganisms to silver metal in nanometer range. The reaction involved in this process is shown in figure 1:-

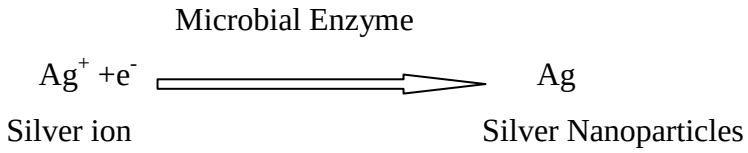

Fig-I: Reaction showing preparation of silver nanoparticles.

Mechanism of Nanoparticles production through fungi

It is demonstrated that using the dissimilatory properties of an eukaryotic organism such as fungi may 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. The presence of hydrogenase in fungi, such as *Fusarium oxysporum* **(Gilbert *et al*., 2003),** *Trichoderma reesei* **(Rautio *et al*., 2006)** and *Trichoderma viride***,** was demonstrated with washed cell suspensions that had been grown aerobically or anaerobically in a medium with glucose and salts amended with nitrate **(Chovanec *et al*., 2005)**. The nitrate reductase was apparently essential for ferric iron reduction **(Ottow *et al*., 1969)**. Many fungi that exhibit these characteristic properties, in general, are capable of reducing *Au* (III) or *Ag* (I) **(Lloyd *et al*., 2003)**. Besides these extracellular enzymes, several naphthoquinones **(Medentsev *et al*., 1998; Duran *et al*., 2002; Bell *et al*., 2003)** and anthraquinones **(Baker *et al*., 1998)** with excellent redox properties, were reported in *Fusarium oxysporum* that could act as electron shuttle in metal reductions **(Misko *et al*., 1993; Klittich *et al*., 1988; Kumar *et al*., 1997)**.

Target plant pathogenic fungi:-

(i) *Sclerotium rolfsii*

Sclerotium rolfsii, an omnivorous, soil-borne fungal pathogen, causes disease on a wide range of agricultural and horticultural crops. Colonies of *S. rolfsii* are readily distinguished on plant material or artificial media by gross morphological characteristics. Rapidly growing, silky-white hyphae tend to aggregate into rhizomorphic cords **(Aycock 1966)**. Both in culture and in plant tissue,

a fan-shaped mycelial expanse observed growing outward and branching acutely **(Takahashi 1927)**. Sclerotia forming on a host tend to have a smooth texture, whereas those produced in culture may be pitted or folded **(Paolo 1933)**. Sclerotia (0.5-2.0 mm diameter) begin to develop after 4-7 days of mycelial growth **(Backman and Brenneman 1984; Weber 1931)**. Sclerotia serve as the principle overwintering structures and primary inoculum for disease.

(ii) ***Fusarium oxysporum***

The coloration of *F. oxysporum* mycelium is initially white but later becomes purple. Its conidiophores are asexually reproduced, short, single, lateral monophialides (flask-shaped projections) in the aerial mycelium, later arranged to densely branched clusters.

These fungi have been found in soils. *F. oxysporum* strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes, and degrade lignin **(Rodriguez 1996; Sutherland 1983)** and complex carbohydrates **(Christakopoulos *et al.*, 1995)** associated with soil debris. Pathogenic strains of *F. oxysporum* have been studied for more than 100 years. The host range of these fungi is extremely broad, and includes animals, ranging from arthropods **(Teetor and Roberts 1983)** to humans **(Nelson *et al.*, 1994)** as well as plants, including a range of both gymnosperms and angiosperms. While collectively, plant pathogenic *F. oxysporum* strains have a broad host range, individual isolates usually cause disease only on a narrow range of plant species.

(iii) ***Cercospora canescens***

This fungus survives in crop debris, and on the seed coat. However, lesions on primary leaves may serve as a source of infection for trifoliolate leaves after they have matured. Vigorously growing leaves rarely become infected. Leaf spots are sub circular to broadly irregular, sometimes confluent, generally brown with a pale tan to gray center surrounded by a dark brown or reddish margin. Conidiophores occur in fascicles, are sometimes dense, divergent, mostly straight, rarely branched, uniform in color, pale to medium brown, multi septate, medium to large size, and have a conidial scar present on the rounded apex with uniform width of 20 to 175 x 3 to 6.5 μm.

(iv) ***Rhizoctonia bataticola***

Rhizoctonia is genus of anamorphic fungi in the order Cantharellales species do not produce spores, but are composed of hyphae and sclerotia (hyphal propagules) and are asexual states of fungi in the genus *Thanatephorus*. *Rhizoctonia* species are saprotrophic, but are also facultative plant pathogens, causing commercially important crop diseases. "Rhizoctonia" means

"root killer" and de Candolle's original species. *Rhizoctonia crocorum* is the causal agent of violet root rot of carrots and other root vegetables **(Andersen and Stalpers 1994)**. Subsequent authors added over 100 additional names to the genus, most of them plant pathogens bearing only a superficial resemblance to the type species. *Rhizoctonia* thus became an artificial form genus comprising a diverse range of unrelated species.

REVIEW OF LITERATURE

Biocontrol agent should be in active state in a right place and at a right time for successful disease control. The strategies used for the biocontrol of soil borne pathogens include protection of the infection sites, impeding the progress of pathogen in soil and inactivation of surviving structures of the pathogen. Bio-agents that work through high competitive ability or antibiosis are employed for protecting the infection court and restrict the growth of pathogen in the soil where as the mycoparasites are often used for the destruction of surviving structures **(Lumsden *et al.*, 1995).** Mycoparasitism is a phenomenon of one fungus parasitizing another fungus and is well known to occur in *Trichoderma* spp. Four stages can be distinguished in Mycoparasitism. The first stage is chemotropic growth in which chemical stimuli from the pathogenic fungus attracts the mycoparasite **Chet *et al.*, (1981)**.

Reddy *et al.*, (1990), Amin (1993), Gholve and Kurundkar (2003) evaluated Being primarily a soil borne disease various practices, cultural operations and all those means that reduce *F. udum* population in the soil help to reduce wilt incidence. Various measures suggested for the management of pigeon pea wilt are cultural practices, cropping systems, resistant varieties, chemicals, amendments and biocontrol agents.

Trichoderma spp., and other beneficial root-colonizing microorganisms, also enhances plant growth and productivity. Intuitively, this might seem counterproductive, as most of these species also induce resistance in plants, and switching on resistance pathways must be energetically expensive to the plant. However, many resistance-inducing fungi and bacteria do increase both shoot and root growth. The specific examples that follow are from research on *Trichoderma*, **Kloepper (1993)** but many other organisms also have similar effects; in fact, resistance-inducing rhizobacteria are widely known as plant-growth-promoting rhizobacteria.

In vitro purified endochitinase, chitobiosidase, N. acetyle B glucosidase and glucan 1, 3-6 glucosidase are produced by *Trichoderma* spp., in combination there by greatly suppressing the spore germination and germ tube elongation of plant pathogenic fungi **Loritio *et al.*, 1993; Di Pietro *et al.*, 1993; Loritio *et al.*, (1994). Hammond-Kosack *et al.*, (1995)** observed the fungi themselves (both *Trichoderma* spp. and other beneficial fungi) have many proven abilities to affect plant productivity and health positively; these can be exploited much more efficiently with a better understanding of the

mechanisms and systems that operate in interactions between *Trichoderma* spp. and plant pathogens. Moreover, genes such as those encoding the *Trichoderma* Avr proteins might be more useful for engineering plants for disease resistance than their homologues from pathogenic fungi 52, 85, because they can be active in many plant species and cultivars.

Elad and Kapat (1999) found *Trichoderma* strains can colonize leaf surfaces under some conditions. However, biocontrol might not be dependent on the growth of *Trichoderma* on leaf surfaces, as the presence of the organism can induce systemic resistance or negatively affect growth or penetration of the pathogen into plants.

Bari *et al.*, (2000) studied the black scurf disease on potato caused by *Rhizoctonia solani* using fungal antagonists *Trichoderma harzianum, T. koningii, T. viride* and *Gliocladium virens.* Both volatile and non-volatile compounds of culture filterates of the antagonists *T. harzianum* and *T. koningii* significantly inhibited the radial growth of *R. solani, T. harzianum* was Comparative radial growth of *R. solani* was inhibited by volatile and non-volatile compounds produced in the culture medium of *Trichoderma* spp. and controlled potato black scurf disease under field conditions. All bioagents significantly reduced sclerotium index.

Saikia *et al.*, (2000) evaluated three antagonistic fungi viz. *Trichoderma viride, Trichoderma harzianum* and *Gliocladium virens in-vitro* for their efficacy in suppressing growth and sclerotia formation of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum.* **Ashwani *et al.*, (2004)** explored three isolates of *Trichoderma viride* (Tv1, Tv2, Tv3), *T. harzianum* and *G. virens* inhibiting the growth of *Dematophora necatrix* (*Rosallinia necatrix*). The volatile compounds released by antagonists of different age showed varying degrees of inhibition on growth of *D. necatrix*.

Steyaert *et al.*, (2004) found *Trichoderma hamatum* the expression of Mycoparasitism genes, namely chitinase*chit42* and proteinase *prb1* were analyzed. The expressions of these genes were analyzed by confrontation assay against the plant pathogen *Sclerotinia sclerotiorum*. During sequence analysis the presence of motifs was discovered and that helps in the regular expression of the genes that enhances the parasitic activity against pathogens. **Podder *et al.*, (2004)** evaluated four systematic fungicides in which carbendazim showed maximum growth inhibition of pathogen.

Kapil and Kapoor (2005) evaluated eight organic substrates for mass multiplication of bioagents. Maximum multiplication was found in FYM followed by in *Lantana camara* and wheat bran. **Chaudhary (2005)** occurred the ratio of component crops were again worked out and Root exudates of some crops like groundnut have been found inhibitory to *F. udum* and encourage growth of antagonist *Trichoderma* spp.

Oksanenb *et al.*, (2000) observed in the biosynthesis of metal nanoparticle by a fungus, enzymes are produced which reduce a salt to its metallic solid nanoparticles through the catalytic effect. Compared to other filamentous fungus, the *Trichoderma reesei* is considered to be the most efficient extracellular enzyme producer, and has a long history in the production of industrial enzymes.

Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*. Development of reliable and eco-friendly process for synthesis of metallic nanoparticles is an important step in the field of application of nanotechnology. One of the options to achieve this objective is to use natural processes such as use of biological systems. In this work we have investigated extracellular biosynthesis of silver nanoparticles using *A. fumigatus*. The synthesis process was quite fast and silver nanoparticles were formed within minutes of silver ion coming in contact with the cell filtrate. UV–visible spectrum of the aqueous medium containing silver ion showed a peak at 420 nm corresponding to the plasmon absorbance of silver nanoparticles. TEM micrograph showed formation of well-dispersed silver nanoparticles in the range of 5–25 nm. XRD-spectrum of the silver nanoparticles exhibited 2θ values corresponding to the silver nanocrystal. The process of reduction being extracellular and fast may lead to the development of an easy bioprocess for synthesis of silver nanoparticles **Bhainsa and Souza (2006).**

Pandey and Khuller (2007) studied nanoparticle for the development of oral drug delivery system and suggested that nano-encapsulation might be useful for developing a suitable oral dosage form for streptomycin and for other antibiotics that are otherwise injectable. **Sadowski *et al.*, (2008)** reported biosynthesis of silver nanoparticles using *Penicillium* fungi has been reported. The extracellular mechanism of silver nanoparticles creation was investigated by UV-Vis spectroscopy, electron microscopy and laser diffraction. The zeta potential of silver nanoparticles has also been determined.

Purshottam *et al.*, (2008) conducted large scale *demonstration* with *Trichoderma* farmer's field in Bundelknand region of Uttar Pradesh. They found that higher biomass, better root growth, dark leaf

colour, higher nodulation and more plant vigor in chickpea. Six organic substrates Wheat bran alone or in combination with F.Y.M in the ratio 1:1 and 1:2 supported maximum multiplication of *T. harzianum* followed shelled maize cob powder, maize flour and FYM **Kapoor (2008).**

Mishra *et al*., (2008) observed t is now widely recognized that use of eco-friendly biopesticide is a distinct possibility for the future and can be successfully exploited in modern agriculture especially within the framework of integrated pest management system without affecting our precious ecosystem. **Kathiresan *et al.*, (2009)** reported **s**tudies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment. In this work, *in vitro* biosynthesis of silver nanoparticles was achieved using $AgNO_3$ as a substrate by *Penicillium fellutanum* isolated from coastal mangrove sediment. The biosynthesis was faster within minutes of silver ion coming in contact with the cell filtrate. Presence of silver nanoparticles in the culture filtrate was confirmed by absorption peak at 430 nm, as well under TEM.

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 h 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.

Antifungal activity of silver ions and nanoparticles on phyto-pathogenic fungi. Silver in ionic or nanoparticle forms has a high antimicrobial activity and is therefore widely used for various sterilization purposes including materials of medical devices and water sanitization. There have been relatively few studies on the applicability of silver to control plant diseases **Jo *et al.*, (2009). Ruocco *et al*., (2009)** occurred the antagonist activity against pathogens such as *R. solani*, *B. cinerea*, and *P. ultimum* was done by dual culture plate assay with *T. atroviride* wild and mutant type strains.

Gade *et al.*, (2010) reported Mycogenic metal nanoparticles: progress and applications. Nanotechnology is relevant to diverse fields of science and technology. Due to the many advantages over non-biological systems, several research groups have exploited the use of biological systems for the synthesis of nanoparticles. Among the different microbes used for the synthesis of nanoparticles, fungi are efficient candidates for fabrication of metal nanoparticles both intra- and extracellulary. The nanoparticles synthesized using fungi present good polydispersity, dimensions and stability.

Naveen *et al.*, (2010) reported extracellular biosynthesis of silver nanoparticles using the filamentous fungus *Penicillium* sp. The aim of the study was to synthesize silver nanoparticles by using filamentous fungus *Penicillium* sp. The fungal culture was isolated from the soil samples collected from agriculture fields in Vellore district, Tamil Nadu, India. The purified fungal isolates were inoculated in minimal medium and incubated at room temperature for three days. For the synthesis of silver nanoparticles, 50 ml of cell filtrate was mixed with equal volume of 1mM silver nitrate (1 mM) and agitated at room temperature in dark. The synthesis of silver nanoparticles was investigated by UV-Vis spectroscopy, AFM and FTIR analysis. Results indicate the synthesis of silver nanoparticles in the reaction mixture. Mechanism of silver nanoparticles synthesis was determined by nitrate reduction test.

Green synthesis of silver nanoparticles using *Agrimone Mexicana* leaf extract and evaluation of their antimicrobial activities. Nanoparticles were characterized using UV-Vis absorption spectroscopy, FTIR, XRD and SEM. further these biologically synthesize nanoparticles were found to be highly toxic against different bacterial spp. (*Aspergillus flavus, E.coli, Psuedomonas* sp.). The most important outcome of this work will be development of value added products from *Agrimone mexicana* (a potential weed of India) for biomedical and nanotechnology based industries **Khandelwal *et al.*, (2010)**.

Production of antimicrobial silver nanoparticles in water extracts of the fungus *Amylomyces rouxii* strain KSU-09. A fungal strain, KSU-09, isolated from the roots of date palm (*Phoenix dactylifera*), was identified as *Amylomyces rouxii* based on sequence analysis of the internal transcribed spacer (ITS) region of its rRNA genes. Mycelia-free water extracts obtained from mycelium suspended in water for 72 h facilitated the production of stable, predominantly mono dispersed and spherical silver nanoparticles (AgNPs) in the size range of 5–27 nm upon addition of 1 mM silver nitrate, as determined by the XRD, AFM and TEM. The AgNPs exhibited antimicrobial activity against *Shigella dysenteriae* type I, *Staphylococcus aureus*, *Citrobacter* sp., *Escherichia coli*, *Pseudomonas*

aeruginosa, *Bacillus subtilis*, *Candida albicans* and *Fusarium oxysporum* reported by **Musarrat *et al.*, (2010)**.

Marcello *et al*., (2010) reported *T. asperellum* (Enzymology Group collection, UFG-ICB) (*tag* 3 gene) Production of cell wall degrading enzyme glucanase and showed significant biocontrol activity. **Tijerino *et al*., (2011)** observed *T. brevicompactum* IBT40841 (*tri*5 gene) Production of *Trichoderma* in and antifungal activity against *C. albicans*, *C. glabrata* and *A. fumigatus* shows enhanced biocontrol.

Biosynthesis, characterization and antimicrobial studies of AgNPs extract from *Bacopamonniera* whole plant. UV.VIS spectrum, XRD, EDX evidences the presence of silver nanoparticles in the aquatic solution of leaf extract reported by **Mahitha *et al*., (2011)**.

OBJECTIVES

1. Collection of soil samples for Isolation and Identification of *Trichoderma* species.
2. Biosynthesis of silver nanoparticles by isolates of *Trichoderma* species.
3. Screening of isolates of *Trichoderma* for the production of silver nanoparticles.
4. Characterization of silver nanoparticles (UV-Vis, XRD, SEM, FTIR).
5. Effect of isolates of *Trichoderma* (non-biosynthesized) and biosynthesized Silver Nanoparticles against plant pathogenic fungi.

METHODOLOGY

(1) Collection of soil samples for Isolation and Identification of *Trichoderma* species:-

Collection of soil sample, Isolation and Identification will be done in following steps:

(i) Collection of soil samples

Soil samples will be collected up to a depth of 15 cm from the upper surface including rhizoplane randomly from Agra and adjacent areas for Isolation and Identification of *Trichoderma* species.

(ii) Isolation of *Trichoderma* Strains (Singh and Singh 1970; Thakur *et al.*, 1928; Johson and Curl 1972)

For the isolation of bio agents soil samples will be ground to very fine powder. 10 gm fine soil powder will be added in 100 ml sterilized water and shacked properly to prepare a stock solution. One ml diluted solution will be poured in PDA plates containing **Potato Dextrose Agar** (PDA) medium and spread properly as film over the PDA surface and incubated at $28\pm1^{o}C$ for 3-7 days. After 2-3 days incubation many soil mycoflora grow in the Petri plate.

(iii) Identification of *Trichoderma* strains (Bisset 1991)

Those resembling *Trichoderma* species will be transferred to another PDA plate and incubated at $28\pm1^{o}C$ in BOD incubator. Isolates of *Trichoderma* will be grown on PDA and incubated at $28\pm1^{o}C$ for 3-5 days. Identification of isolates of *Trichoderma* will be done on the basis of colony type, growth, its colour, phialdes and conidia. The identified *Trichoderma* isolates will be sent for identification of *Trichoderma* species. The chemical constituent of PDA medium: Peeled potato 250 g, Glucose 20.0 g, Agar 15.0 g, in 1 litter distilled water.

(2) Biosynthesis of silver nanoparticles by Isolates of *Trichoderma*:-

In a typical biosynthesis production scheme of silver nanoparticles, 10*g* of *Trichoderma* species fungus wet biomass will be mixed with a 100 ml aqueous solution of 1 mM silver nitrate ($AgNO_3$). Then the mixture will be placed in a 100 rpm rotating shaker at 28 °C for 120 hours duration. In this process silver nanoparticles will produce through reduction of the silver ions to metallic silver.

(3) Screening of Isolates of *Trichoderma* for the production of silver nanoparticles:-

Screening procedure will be done on the basis of antagonist effect. in this, we will measure the activity of biologically synthesis silver nanoparticles of best two isolates of *Trichoderma* which show maximum inhibition against *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium oxysporum* and *Cercospora canescens*.

(4) Characterization of silver nanoparticles:-

(i) UV-Vis spectroscopic studies

The bioreduction of Ag+ in aqueous solution will be monitored by periodic sampling of aliquots (0.2 ml) of the suspension, then diluting the samples with 2 ml deionized water and subsequently measuring UV–vis spectra of the resulting diluents.

(ii) Scanning electron microscopy (SEM)

This will be performed by fabricating a drop of suspension onto a clean electric stubs and allowing water too completely evaporate. JEOL-5800-LV SEM will be used with an accelerating voltage of 15 KW and a sample current of 41 µA. the samples will sputter coated with gold.

(iii) X-ray diffraction measurements

X-Ray diffraction (XRD) measurements of the bioreduced silver nitrate solution drop-coated onto glass substrates will be done, operating at a voltage of 45 kV and a current of 40 mA with Cu Kα radiation.

In order to determine the functional groups on the *Trichoderma* species and their possible involvement in the synthesis of silver nanoparticles, **Fourier Transform Infra Red (FTIR)** analysis will be carried out.

(5) Effect of isolates of *Trichoderma* and biosynthesized silver nanoparticles against plant pathogenic fungi:-

(i) Effect of isolates of *Trichoderma*

The effect of Isolates of *Trichoderma* will be evaluated against plant pathogenic fungi *Sclerotium rolfsii, Rhizoctonia bataticola, Fusarium oxysporum* and *Cercospora canescens* by **Dual culture technique** (Johnson and Curl 1972). Percent inhibition over control will be calculated by applying the following formula:-

$$I = \frac{C-T}{C} \times 100$$

Where,

I = Percent inhibition

C = Colony diameter in control (mm)

T = Colony diameter in treatment (mm)

(ii) Effect of biosynthesized silver nanoparticles

The plant pathogenic strains of *Sclerotium rolfsii*, *Rhizoctonia bataticola*, *Fusarium oxysporum* and *Cercospora canescens* will be used to determine the antifungal activity of the silver nanoparticles. The experiments will be carried out by **Agar Well Diffusion Method** (Shanmuga *et al*., 2002), **Paper Disk method** (Okigbo *et al*., 2005), **Minimum Inhibitory Concentration** (Shanmuga *et al*., 2002).

SIGNIFICANCE

Trichoderma is a potent biocontrol agent and used extensively for post-harvest disease control. The application of *Trichoderma* strains with plants such as grasses increases the number of deep roots, thereby increasing the plant's ability to resist drought.

Trichoderma strains play an important role in the bioremediation of soil that are contaminated with pesticides and herbicides.

Nanoparticle synthesis is an important component of rapidly growing research efforts in nano scale science and engineering. So the outcome of this study may s focus on the exploitation of *Trichoderma* species for the synthesis of Silver Nanoparticles to industrial applications and extracellular production of AgNPs. Biotechnology approach towards the synthesis of nanoparticles has many advantages, such as ease with which the process can be scaled up, economic viability, possibility of easily covering large surface areas by suitable growth of the mycelia, and its green chemistry nature provided the microorganism medium is safe.

Silver nanoparticles have several characteristics that make it currently among the most widely used nanoparticles in science. One highly useful characteristic is its antimicrobial property. Silver has long been recognized as having an inhibitory effect towards fungus strains and micro-organisms commonly present in industrial process

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