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**“EVALUATION & ELUCIDATION OF EFFECT OF SILVER
NANOPARTICLES ON DIFFERENT PLANT PATHOGENIC FUNGI”**

**SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF**

**MASTER OF TECHNOLOGY
IN
BIOTECHNOLOGY**

SUBMITTED BY:

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UNDER THE SUPERVISION OF

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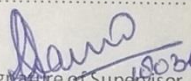
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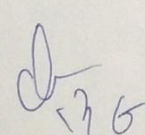
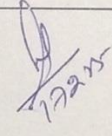
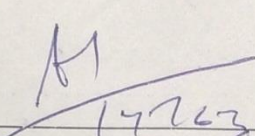
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1. Evaluation & elucidation of effect of silver nano particles on different plant pathogenic fungi
2.
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ABSTRACT

Plant pathogens causing harm to various plants and traditional use of fungicide became failure, for this nanotechnology come forward and by the help of nanotechnology science silver nanoparticles were synthesized. Fungus mediated or green synthesis of silver nanoparticles was obtained from *Alternaria solani*, *Cladosporium colocassiae*, *Ascochyta phleina* *Aspergillus flavus* and *Aspergillus niger*. The U.V spectral analysis of of *A. phleina*, *A. solani*, *A. flavus*, *C. colocassiae* and *A. niger* with absorption peaks at 420nm, 400nm, 420nm, 425nm and 420nm were observed respectively. XRD analysis showed an average size of nanoparticles ranging from 20nm to 75nm. Fungus mediated silver nanoparticles exhibited good antifungal activity alone and in combination with fluconazole. *A. flavus* mediated silver nanoparticle 18nm and a zone of inhibition of 23mm in combination with fluconazole followed by *C. colocassiae* mediated nanoparticles (22mm) and 21mm in combination with fluconazole.

Keywords: *Alternaria solani*, *Cladosporium colocassiae*, *Ascochyta phleina*, *Aspergillus flavus*, Silver Nanoparticles

CERTIFICATE

This is to certify that **Mohit Shekhar (11307405)** has completed Dissertation project report (BTY 731), entitled “**Evaluation & Elucidation of effect of silver nanoparticles on different plant pathogenic fungi**” under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the report has ever been submitted for any other degree at any university.

This report is fit for submission and the partial fulfilment of the conditions for the award of M. Tech. in Biotechnology.

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DECLARATION

I hereby declare that this thesis entitled “**Evaluation & Elucidation of effect of silver nanoparticles on different plant pathogenic fungi**” is an authentic record of my own work carried out at School of Biotechnology and Biosciences, **Lovely Professional University, Phagwara**, for the partial fulfillment of the award of Master of Technology in Biotechnology under the guidance of Er. Robinka Khajuria, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara.

This work is my original and has not been submitted for any degree/diploma in this or any other University. The information furnished in this dissertation is genuine to the best of my knowledge and belief.

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Date:

(Mohit Shekhar)

Place:

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CHAPTER 1

INTRODUCTION

Fungal infections destroy approximately 125 million tonnes of the top food crops- rice, maize, soybeans and potatoes. The destruction caused by fungi to wheat, rice and maize solely costs global agriculture \$60 billion per year. More than 600million people can be fed each year by avoiding fungal diseases in world's five most important crops. The data reviewed by scientist suggests that in 70% of cases where infectious disease causes extinction of a type of plant, an emerging species of fungus is behind the problem. Tress damaged by fungi are unable to absorb 230-570 mega tonnes of atmospheric carbon dioxide, equivalent to 0.07% of global atmospheric CO₂ (Fisher et al., 2012). On other hand, micro-organisms fungi cause most of the disease in plants like potato late and early blight, sudden oak death, black rot disease, great Irish famine disease etc. The only way of controlling the growth of these pathogens and subsequent destruction of crops is to spray fungicides on standing crops. Many antifungal chemicals like Azoles, Carbendazim , Diethofencarb, Qol and Dicarboximide, Fluconazole are used to prevent the fungal diseases in plant (Sinclair, 2005). Fluconazole consists of imidazole ring with a spectrum that includes mostly *Candida* and *Aspergillus* species. Like other azole based antifungal it acts as alpha-demethylase inhibitor in affecting membrane fluidity (Ghannoum et al., 1999).

Excessive use of these fungicides has led to emergence of resistant to azole .For instance *Botrytis cinerea* has been reported to shown resistance to both azoles and dicarboximide fungicides, Black sigatoka is important pathogen of banana, has developed resistant to Qol fungicides (Prabhushreenivasan et al., 2006). Brown rot caused by fungus *Monilinia fructicola* causes disease in peach fruit develops resistance to fungicides and causes disaster of peaches in Nov, 2010 in Southeast of Southscape Fall. In Oct ,2013 in North Carolina, frogeye leaf spot fungus (*Cercospora sojina*) causes disease in soybean and become resistant to strobilurin fungicides (FRAC code 11; Headline, Quadris, Evito and Approach) and was reason of disaster of soybean crop. Due to this problem, there is need of developing new

types of fungicides which can stop these types of disaster and this is where nanotechnology comes into play (Wood et al., 2013).

Nanotechnology is the branch of science, engineering and technology of functional system which deals with the dimensions and tolerances of less than 100 nanometres i.e.1 to 100nm, mainly with the manipulation of individual molecules and atoms. The word “Nanotechnology” was coined by Professor Taniguchi, University of Tokyo of science in the year 1975 to define an accurate production of things at the nanometre measure (Drexler et al., 1992). Nanotechnology now is employed in various techniques used for diagnose and treatment of diseases, improvement uptake of nutrients by plants, micro fabricated xylem vessels used for water holding from soil, nano oligocellulosoic materials type polymeric matrix used in food packaging etc.(Ditta, 2012). One of the applications of nanotechnology is in control of plant diseases. Control of plant diseases by site-targeted delivery of nanoformulated agrochemicals, development of disease resistant plant varieties by nanomaterial-mediated genetic transformation and early diagnosis of plant and pathogens are some of the possible key applications plant pathology

There are several methods used to synthesize silver nanoparticles by laser ablation of metallic bulk materials (Malfune, 2000), by chemical reduction of silver ions using reductants like borohydride, citrate (Levinis, 2010) and biological synthesis by microbes like fungi, bacteria, algae and plant extract. In comparison to physical and chemical methods, biological methods are gaining rapid acceptance because they don't form chemically toxic compounds and are easy to synthesize and less expensive (Kim et al., 2007). The effectiveness of less sized antimicrobials like silver nanoparticles because of those smaller particles can pass through the cell membrane and cell wall and that relative to larger sized nanoparticles; smaller particles have a greater surface area to volume ratio (Yamamoto, 2001). The greater surface area to volume ratio means higher per unit mass of silver, thus more availability of the antifungal agents at lower concentrations (Kim et al., 2007; Moronoes et al., 2005).

The aim of this study was to synthesise silver nanoparticles using *Alternaria solani*, *Aspergills niger*, *Aspergillus flavus*, *Ascochyta phleina*, *Cladoporium colocassiae* and to evaluate their antifungal properties against these plant pathogens

CHAPTER 2

TERMINOLOGY

Nanotechnology: is the branch of science, engineering and technology of functional system which deals with the dimensions and tolerances of less than 100 nanometres i.e.1 to 100nm, mainly with the operation of individual molecules and atoms.

Agrochemicals: is a chemical based broad range of pesticides, including insecticides, herbicides, fungicides and nematicides. It may also include synthetic fertilizers, hormones and other chemical growth agents, and concentrated stores of raw animal manure.

Antimicrobial: is a chemical agent that kills micro-organisms or inhibits their growth. Antimicrobial medicines can be assembled according to the micro-organisms they act principally against.

Pathogens: Pathogens are the infectious agents are biological agents which kills or ill to its host. They causes diseases in plants and human

Fungicide: Fungicides are the chemical liquid or in powder form which are used to control or kill the plant pathogenic fungi.

XRD: X-ray diffraction is an analytical techniques stands on the dual property of x-rays to produce information about the structure or phase of crystalline materials

SEM: Stands for scanning electron microscope used to obtain image of sample by scanning it with focused beam of electrons of high energy.

Green Synthesis: Green synthesis means that by development of material using green chemistry whose material are less hazardous and more environment friendly.

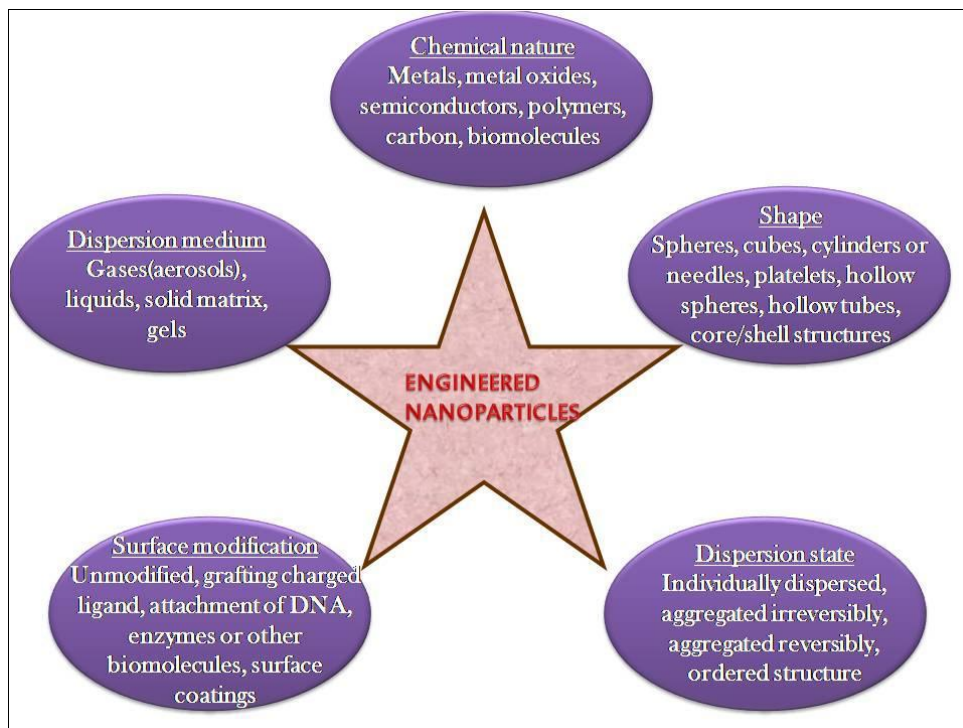
CHAPTER 3

REVIEW OF LITERATURE

Nanotechnology is the branch of science, engineering and technology of functional system which deals with the dimensions and tolerances of less than 100 nanometres i.e.1 to 100nm, mainly with the operation of individual molecules and atoms. The word “Nanotechnology” was given by Professor Taniguchi, University of Tokyo of science in the year 1975 to define accurate production of things at the nanometre measure (Drexler et al., 1992). Nanotechnology now is engaged in various techniques used for diagnosis and treatment of diseases, improvement uptake of nutrients by plants, micro fabricated xylem vessels used for water holding from soil, nano-oligocellulosoic polymeric matrix used in food packaging to name a few (Ditta, 2012). The undergoing research activities in nanotechnology in the health sector research focus in the areas of nanometer systems and tissue engineering, drug delivery tissue, regenerative, nanomaterials for therapeutic applications and other biomaterials, nanomaterial based diagnostics, nanodevices and nanosensors, interaction of nanomaterials with human systems and toxicological studies. (Joshi et al., 2009)

Bio-nanotechnology has developed up as combination between biotechnology and nanotechnology for developing biosynthetic and eco-friendly technology for production of nanomaterials. Amongst the latest line of technological novelties, nanotechnology occupies a noticeable position in renovating agriculture and food production. The growth of nano-devices and nanomaterials possibly will open up innovative applications in plant biotechnology and agriculture. (Scrinis et al., 2007; Min et al., 2009) Another application of nanotechnology deals with the crop management techniques such as use of nanoparticle based antimicrobial agents against common plant pathogens. Traditionally used agrochemicals suffer from drawbacks such as leaching of chemicals, degradation by photolysis, hydrolysis and in some cases microbes. Hereafter frequent application is essential to have an effective control which might cause some contrary effects such as soil and water pollution. Nano-encapsulated agrochemicals have been reported to have improved targeted activity and minimum eco-toxicity with eco- friendly delivery thus escaping frequent application (Green et al., 2007; Boehm et al., 2003; Tsuji, 2001).

Figure 1 shows the vast diversity of the nanoparticles arising from their wide chemical nature, shape and morphologies, the medium in which the particles are present, the state of dispersion of the particles and most importantly, the many possible surface modifications the nanoparticles can be subjected to make this an significant active field of science now-a-days.



3.1 Approaches to synthesis of Nanoparticles

The synthesis of nanoparticles can be divided into three main categories, viz., Chemical, Physical and Biological depending upon the type of method chosen. Biological synthesis also known as green synthesis is gaining more acceptance these days due to simpler synthesis methods, non-production of toxic by-products and high yields. (Jain et al., 2009). The following section discusses these methods in detail:

3.1.1 Physical Approach

The particle generation by physical method is very stable because of furnace temperature. Physical method can be useful as it generates long-term nanoparticle for long term.

experiments for inhalation toxicity. In physical approach of synthesis of nanoparticles is usually done by:

Condensation & Evaporation method: It is done in tube furnace at atmospheric pressure. The source material within a boat centred at the furnace is vaporized into a carrier gas. Nanoparticles of various materials, such as Ag, Au, PbS and fullerene, have earlier been synthesized using the evaporation-condensation technique. Small ceramic heater is used as a evaporate source material. It was demonstrated that silver nanoparticles can be formed by small ceramic with local field area. The evaporated vapours cool very rapidly because the temperature at surface of furnace is very steep (Kruis et al., 2000; Magnusson et al., 1999; Schmidt-Ott, 1988).

Physical ablation: It is another technique in which laser is incident on bulk metal from which nanoparticles formed. Synthesis of silver nanoparticles has some drawbacks like furnace occupies large space and releases lots of heat. It has advantage there is no solvent contamination in thin films and uniformity of nanoparticle distribution (Dutta et al., 1994)

Arc Discharge Method: This method used to fabricate silver nanoparticles suspension in deionised water without adding any surfactants. In this silver wires dipped in deionised water and used as electrodes. By this method uniform distribution of silver nanoparticles were obtained (Tien et al., 2008)

3.1.2 Chemical Approach

Chemical Reduction of Metal Salts: Silver nanoparticles are synthesized by chemical reduction of silver nitrate by reducing agent like sodium borohydride, hydrazine and ascorbic acid. Some capping agents are used and these reactions occurred at room temperature Dihydroxy benzene reagent can be used to reduce silver ions to form stable silver nanoparticles (with an average diameter of 30 nm) in air-saturated aqueous suspension solution (Ghorbani, 2014).

Photochemical Method (Irradiation): In this technique, system is excited by radiation and therefore it is forms active reducing agents such as radicals, electrons and other excited components. It has advantage over chemical method as it obtains fewer impurities at low temperature (Ghorbani, 2014).

Electrochemical Method :In this method, electrolysis phenomenon is used to reduce the metal ions. Yang et al., 2012, reported synthesis of silver nanorods by electrochemical method. Physical structure and sizes of nanoparticles can be controlled by changing some experimental parameters such as the concentration of metallic precursors, surfactant polymers, temperature and solvents. (Iravani et al., 2014)

Thermal Decomposition: Thermal decomposition of metals is chemical based decomposition by heat. Temperature at which substance chemically decomposes is known as decomposition temperature. (Ghorbani, 2014).

Tollen's Method: has been used for the synthesis of silver nanoparticles with a controlled size. In the modified Tollens procedure, silver ions are reduced by saccharides in the presence of ammonia, yielding silver nanoparticle films (50-200 nm), silver hydrosols (20-50 nm) and silver nanoparticles of different shapes (Yin et al., 2002).

Microwave-assisted synthesis: Microwave-assisted synthesis is a hopeful method for synthesis of silver nanoparticles. Microwave heating is well than a conventional oil bath when it comes to constantly yielding nanostructures with smaller sizes, narrower size distributions, and a higher degree of crystallization. Microwave heating has shorter reaction time, reduced energy consumption, and improved product produces which prevent the accumulation of the particles formed. Starch has been engaged as a template and reducing agent for synthesis of silver nanoparticles with an average size of 12 nm, using microwave-assisted synthetic method. Starch functions as a template, preventing the aggregation of produced silver nanoparticles (Nadagouda et al., 2011).

3.1.3 Biological Approach

Biological approach for synthesis of metal nanoparticles is important feature of nanotechnology research and biological approach also called eco- friendly approach. The biological approaches escapes the ruthless process used in physical and chemical methods.

Plant Extract

In this, Plants are used to synthesize the nanoparticles by using plant extract in certain solutions or chemical reducing agents like eugenol and carbazoles etc. Plant extract like Latex of *Jatropha curcas*, Callus extract of *Carica papaya* etc. (Karnani et al., 2013). The major advantage of using plant extracts for silver nanoparticle synthesis is that they are easily available, safe, and nontoxic in most cases, have a broad variety of metabolites that can aid in the reduction of silver ions, and are quicker than microbes in the synthesis. The main mechanism considered for the process is plant-assisted reduction due to phytochemicals. The main phytochemicals involved are terpenoids, flavones, ketones, aldehydes, amides, and carboxylic acids. Flavones, organic acids, and quinones are water-soluble phytochemicals that are responsible for the immediate reduction of the ions. Studies have revealed that xerophytes contain emodin, an anthraquinone that undergoes tautomerization, leading to the formation of the silver nanoparticles. In the case of mesophytes, it was found that they contain three types of benzoquinones: cyperoquinone, dietchequinone, and remirin. It was suggested that the phytochemicals are involved directly in the reduction of the ions and formation of silver nanoparticles (Jha et al., 2009).

Bacteria

Bacteria have lead over plant extract as it can easily produce in less time. Bacteria forms variety of silver nanoparticles like hexagonal or in pyramid shapes (Karnani et al., 2013). Bacteria have been explored in the synthesis of silver NPs. It was reported that highly stable silver nanoparticles (40 nm) formed by bio reduction of aqueous silver ions with a culture supernatant of non-pathogenic bacterium, *Bacillus licheniformis* .Furthermore, well-dispersed silver nano-crystals (50 nm) were formed during the bacterium *B. licheniformis* . Saifuddin and co-workers (2008) had defined a new combinational synthesis approach for the synthesis of silver nanoparticles by using a combination of culture supernatant of *B. subtilis* and microwave

irradiation in water. They stated the extracellular biosynthesis of mono-dispersed silver nanoparticles (5-50 nm) using supernatants of *B. subtilis* (Iravani et al., 2014).

3.2 Fungus

Fungi are used mainly for synthesis of nanoparticles as it produces great amount of nanoparticles. Silver nanoparticles are synthesized by biomass production of fungi and treated in deionised water kept for overnight and then it water is filtered out through wattman filter paper no. 1 and silver nitrate is treated with it which further shows dark red to brown colour solution which indication of development of silver nanoparticles (Karnani et al., 2013). The mechanism of silver nanoparticle manufacture by fungi is said to follow the subsequent steps: trapping of silver ions at the surface of the fungal cells and the following reduction of the silver ions by the enzymes present in the fungal system (Mukherjee et al., 2001). The extracellular enzymes like naphthoquinones and anthraquinones are said to ease the reduction. Considering the example of *Fusarium oxysporum*, it is supposed that the NADPH-dependent nitrate reductase and a transport quinone extracellular process are responsible for nanoparticle formation (Ahmad et al., 2003). However the exact mechanism involved in silver nanoparticle production by fungi is not fully deciphered, it is believed that the above stated phenomenon is responsible for the process. A main disadvantage of using microbes to produce silver nanoparticles is that it is a very slow process when in contrast with plant extracts. Henceforth, the use of plant extracts to produce silver nanoparticles becomes an option that is possible (Karnani et al., 2013).

3.3 Nanotechnology and Nanoparticles in agriculture

Agriculture field facing various problems like climate changes, urbanization, global challenges sustainable use of resources, environmental issues like deposition of fertilizers and pesticides etc. These problems are further worsened by the growing food demand that will be needed to tolerate an estimated population growth from the present level of about 6 billion to 9 billion by 2050. Nanoparticles play vital role in delivery of agrochemicals like pesticides, fertilizers, plant growth regulators, herbicides. To monitor the environmental stresses like temperature, climate and crop management like crop nutrient status, insect, plant diseases etc. field sensing can be applied. With help of nanoparticles plant traits can be treated well to oppose the environmental stresses like drought, flood and salinity (Chen et al., 2011). Nanoparticles include nano-fertilizers, nano-pesticides, nanosensors. Nano-fertilizers

generally growth promoters encapsulated in nano chelates, polymers or emulsions. Nano-sensors are used detect pathogens or detect monitor local, nano-conditions in the field like temperature, water availability, humidity, nutrient status, pesticide levels. They increases stability or solubility, increase uptake and in some cases targeted delivery.

3.4 Fungus Pathogens

Every year, major part of crop destroyed by the plant pathogens like fungi. It is estimated that by solely fungal diseases, plant yields remains to 20%. Plant diseases cause major economic losses among farmers. The FDA estimated that due to fungal diseases and pests are of 25% of total loss by other diseases and environmental stresses. More than 600million people can be fed each year by avoiding fungal diseases in world's five most important crops. The data reviewed by scientist suggests that in 70% of cases where infectious disease causes extinction of a type of plant, an emerging species of fungus is behind the problem. Fungal infections presently destroy at least 125 million tonnes of the top food crops- rice, maize, soybeans and potatoes. The destruction caused by fungi to wheat, rice and maize solely costs global agriculture \$60 billion per year. Trees damaged by fungi unable to absorb 230-570 mega tonnes of atmospheric carbon di oxide, equivalent to 0.07% of global atmospheric CO₂ (Fisher et al., 2012).

Mechanism of plant fungal pathogens

Fungi rarely cause disease in healthy plant but when adjacent plants get affected by fungus then near healthy plant gets affected. Fungus accidently penetrates host barriers or other conditions exist that favour fungal growth and entry. Fungi generally form both virulence mechanisms (e.g., yeasts, hyphae, sclerotic bodies) that facilitate their proliferation within the host. Dissemination of fungi in plant indicates a breach or deficiency of host defences. Severity of disease depends on factors such as inoculum, magnitude of tissue destruction, ability of fungus to proliferate in the tissue. Keratinase like enzyme, the presence of capsule in *Cryptococcus neoformans*, the ability to proliferate at 37°C, dimorphism, and other as yet indeterminate factors contribute to fungal pathogenesis which involves a complex relationship of many fungal and host factors (Kobayashi et al., 1993).

Alternaria solani

It is a fungal plant pathogen that produces disease in tomato and potato called as Early and Late blight of tomato and potato respectively. It produces distinctively “bullseye” patterned leaf spots. The name “Early” foliar symptoms usually occur on older leaves, uncontrolled, early blight could cause measurable yield reductions. This disease can be prevented by long wetness of leaf surfaces and fungicides (Olanya et al., 2009).

Aspergillus niger

It is the most common plant disease causing plant pathogen. It causes “black mould” on certain vegetables and fruits such as apricots, onions, grapes and peanuts and causes contamination in foods. Many species produces mycotoxins called ochratoxins. They also produces isoflavone orobol. It is cultured for the industrial production of many substances like preparation of citric acid and gluconic acid (Samson et al., 2001)

Asprgillus flavus

A. flavus is plant pathogen and saprophytic too. It is well known for causing diseases in cereals grains, legumes and tree nuts. It produces mycotoxins which consumed by mammals causes toxicity. Management can be done by decreasing the moisture up to 15 percent and temperature below 5°C. Infection could be present in the field, preharvest, post harvest, during storage and transit. It has potential to infect seedlings by sporulation on injured seeds (Masayuki et al., 2010).

Ascochyta phleina

It is also a plant pathogenic fungus. It causes patch disease in turfgrass. It is dark green in colour. Its taxonomy is still incomplete. Symptoms are generally elliptical roots that are initially chlorotic and after that become a necrotic brown. Management of this fungi includes fungicide applications like propiconazole 41.8% and pyraclostrobin 3.6% and cleanliness of diseases plat tissue residue (Johnson, 1950).

Cladosporium colocassiae

These are common occurring indoor and outdoor moulds. Brown leaf spot or ghost spot is a fungal disease of older leaves caused by this fungus. Disease caused called as ghost spot as lesions caused by this fungus is less evident on other surface of leaf. This fungus also causes leaf blotch. They also found on dead plant material. They also cause allergens in human like

asthma (Rivas et al., 2005). Some of the common plant fungus pathogens and disease symptoms are enlisted in Table 1.

Table –1 Some common occurring fungal diseases and their symptoms

Fungal disease	Factors conducive to spread	Crops affected	Symptoms
White blister/White rust (<i>Albugo candida</i>)	Optimum conditions for disease development are 3-4 hours in mild temperatures (6- 24°C).	<i>Brassic</i> as (including Asian leafy brassicas).	White blisters and swellings on leaves and heads of affected plants; blisters consist of masses of white dust-like spores; up to 100% losses have been reported.
Downy mildews	High humidity, leaf wetness and cool to mild temperatures (10-16 °C).	Wide host range including onions; peas; lettuce; celery; spinach; kale; herbs; cucurbits; brassicas; Asian leafy brassicas.	Yellowish leaf spots which then turns brown; downy growth appears on underside of leaves.
Powdery mildews (some species are restricted to particular crops or crop families)	Moderate temperatures (20-25°C); relatively dry conditions (unlike downy mildews).	Wide host range and very common, especially in greenhouse crops: cucumber; melons; pumpkin; zucchini; parsnip; beetroot; potato; herbs; peas; bitter melon;	Small, white, powdery patches on most above-ground surfaces; usually observed first on undersides of leaves but eventually cover both surfaces; affected leaves become yellow then brown and papery
Clubroot (<i>Plasmodiophora brassicae</i>)	Warm weather; acidic soil (pH less than 7); high soil moisture.	<i>Brassic</i> as (including Asian leafy <i>brassic</i> as).	Plants are yellow and stunted and may wilt in hotter parts of the day; large malformed ‘clubbed’ roots which prevent the uptake of water.
<i>Fusarium</i> wilts and rots (Various <i>Fusarium</i> species including <i>F. solani</i> And <i>F. oxysporum</i>)	Warm and hot weather	Wide hot range including <i>brassic</i> as, carrots, potato, tomato etc.	Causes severer root or crown rot and wilt diseases by attacking roots and basal stems.

3.5 Currently available Antifungal agents

Fungicides are biocidal chemical formulas or inactivated micro-organism used to inhibit the activity of fungus or or to kill them. Many fungicides are in liquid form having common substance sulphur ranging from 0.8% as weaker concentration to 0.5% as potent fungicides (Table 2). Fungicides are in powder form contains 90% of sulphur which very toxic to the plant too. Traditionally many fungicides are available in market and used by farmers which are as following Tea tree oil, cinnamaldehyde, citronella oil, monocerin, Ampelomyces quisqualis azoles etc. (Jayakumar et al., 2006). Fungal pathogens develop resistance to fungicides by mutation. In the field many types of fungal mutations have been identified like sugarbeet leaf blotch remains resistant to azaoles fungicides, Botrytis species is resistant to both carbendazium and diethofencarb, Black sigotoka is remains resistant to Qol fungicides due single nucleotide change resulting replacement of one amino acid to another. Therefore it is better to use another approach to control diseases rather than relying on fungicides.

Fluconazole ($C_{13}H_{12}F_2N_6O$) is antifungal agent used as medication against much fungus and it is a triazole antifungal drug (Fig 2). Its structure consists of imidazole ring. Fluconazole's spectrum includes most candida and *Aspergillus* species. Like other azole based antifungal it has also mode of action that are 14 alpha-demethylase inhibitors in which it acts as bio-inhibitor of membrane fluidity (Ghannoum et al., 1999).

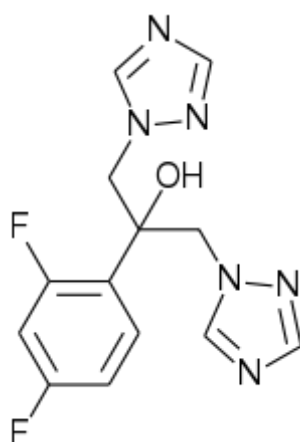


Fig 2: Fluconazole structure (Ghannoum et al., 1999).

Table 2: Some conventional fungicides and their doses (Yani et al., 2005)

Fungicides	Doses	Target fungus
Butylamine	150mg/m ³	<i>Candida</i> and <i>Botrytis</i> species
Fluconazole	150mg/m ³	<i>Candida</i> and <i>Aspergillus</i> species
Ketonazole	200mg/m ³	<i>Aspergillus</i> and <i>Alternaria</i> species
Carboxin	350mg/m ³	Wild <i>Armiliaria</i> species
Isotianil	160mg/m ³	<i>Cyathus</i> species
Quinazamide	250mg/m ³	<i>Fusarium</i> , <i>Aspergillus</i> and <i>Candida</i> species
Salicylanilide	300mg/m ³	<i>Pichia</i> and <i>Candida</i> species

3.6 Nanoparticles as Antimicrobial agents:

Activity of silver nanoparticle as an antimicrobial is essential traditional antifungal used till date. Silver nanoparticle is used as nanoparticle for various eco systems therefore many studies on this is going on. It has known as strong antimicrobial for inhibition of fungal activities, silver nanoparticle of average diameter of 1.5nm has effective colloidal solution against rose powdery mildew caused by fungi *Sphaerotheca panmosa*. Rose powdery mildew disease causes leaf distortion, curling of leaves and less flowering in no. Nano silver colloidal solution is a stable and dispersed silver nanoparticle colloidal solution is more interrelated towards bacteria and fungus, therefore it is a good fungicide and pesticide. Anderson described it as pesticide. Silver is now conventionally growing as agrochemical substitute. It avoids organism in hydroponics. Now scientist describing silver as a plant growth enhancer (Sharma et al., 2012)

Sulphonamides were the first effective antimicrobial used against many diseases. In earlier years, among all inorganic antimicrobial agents like zeolite, zinc oxide etc., silver used to oppose and suppress infections and diseases because once the antimicrobial was used widely, resistant strains capable of inactivating the drug became widespread, and synthetic studies were undertaken to modify (Silver, 1996). The inorganic antimicrobial properties of silver have been known to scientist all around the world as they are working on it. The Phoenician's stored water and other liquids in silver coated bottles to depress contamination by microbes. An inorganic antimicrobial silver ions cause's protein inactivation, associate with DNA to cause damage. Fox and Modak (1974) explored the mechanism of prevention of burn wound infections by silver sulfadiazine. There are various forms of silver which are used to prevent infectious diseases like silver salts like AgNO_3 , silver zeolites, silver nanoparticles etc. Silver nanoparticles studied as antimicrobial materials. Their synthesis and having highly efficiency experimental antimicrobial activity make them a very good form of silver.

The effectiveness of less sized antimicrobials like silver nanoparticles because of those smaller particles can pass through the cell membrane and cell wall and that comparative to larger sized nanoparticles; smaller particles have a greater surface area to volume ratio. The greater surface area to volume ratio of smaller nanoparticles means that per unit mass of silver, because in solution there are more silver ions when they are in small in size. For smaller nanoparticles, it means that more of the silver atoms confined in the nanoparticles are able to cause more cell damage. If only the outer layer of silver atoms of a silver nanoparticles are capable of ionized to silver ions, then a large nanoparticles harvest less silver ions than a lot of small nanoparticles. Because silver ions are having antimicrobial properties to a given silver-containing material, it makes generous reason that smaller silver nanoparticles have more antimicrobial effectiveness than bigger silver nanoparticles.

3.7 Different Types of silver nanoparticles

Carbon nanotubes

These are the dissimilar forms of carbon division whose nano-structure and shape is cylindrical in shape and structure. Recently scientists have debated that when they planted seeds of tomato in a soil that contained carbon nanotubes; these carbon nanotubes can not only enter into the hard surface of germinating seeds but also employed growth enhancing

effect. Scientists explored that the enhancing growth was due to better water consumer caused by permeation of carbon nanotubes. This may be advantage for using carbon nanotubes as vehicle to bring desired molecules into the seeds during sprouting that can guard them from the diseases and other effects. Since it is plant growth enhancer and stimulator, it won't have any harmful or inhibiting effect on the plant (Sharma et al., 2012).

Nanosilver

Activity of silver nanoparticle as an antimicrobial is essential traditional antifungal used till date. Silver nanoparticle is used as nanoparticle for various eco-systems therefore many studies on this is going on. It has known as strong antimicrobial for inhibition of fungal activities, silver nanoparticle of average diameter of 1.5nm has effective colloidal solution against rose powdery mildew caused by fungi *Sphaerotheca panmosa*. Rose powdery mildew disease causes leaf distortion, curling of leaves and less flowering in no. (Sharma et al., 2009)

Nano-silica

Silicon is important constituent in our earth soil, it is best known for its stress freeness and resistance to disease. Many physiological activities and plant growth is improved by it. It generates resistance towards pathogens and enhances plant growth (Gupta et al., 2012)

Nano-silica & Nano-silver Composite

A novel structure of having nano size is used for management of plant diseases which have been developed by Paek et al., 2008, which constitute the silver-silica molecules combination in nano scales. This combination signals antimicrobial activity. The plant pathogens vanished from the infected leaf within 3 days after spraying and plant remain as in good condition as its shape does not have changed thereafter (Gupta et al., 2012).

3.8 Silver Nanoparticles as antifungal agents

It is well known that silver ions and their salts are highly toxic to micro-organisms. Silver is generally used in silver nitrate form to induce its antimicrobial activity, when silver is used it known that it provides greater surface area to microbes (Prabhu, 2012). Y.K Jo et al., reported, "Silver inhibits and hinders the expression of proteins associated with production of ATP, but its specific antimicrobial mechanisms are still not known. Micro molar doses of

silver ions are sufficient to fungi. Silver can be harmful or toxic at high doses to mammals and marine and freshwater organisms, definitely compromising the shape and growth of animal cells by interfering different of biological functions”. Kim et al. 2012 reported the mode of action of nano-silver nanoparticles on fungi. Results showed that silver nanoparticles affected yeast cells by attacking yeast cell membranes, thus disrupting membrane potential. It was also observed by transmission electron microscopy (TEM) examination that the interface between nano silver particle and the membrane structure. Silver nanoparticles have the ability to anchor to the fungal cell wall and subsequently penetrate it, therefore causing physical changes in the cell membrane and the permeability of the cell membrane and cell dies. There is development of ‘pits’ on the cell surface, and there is accumulation of the nanoparticles on the cell surface. The formation of free radicals caused by the silver nanoparticles could be measured to be different mechanism by which the cells die. There have been an electron spin resonance spectroscopy study that says that there is formation of free radicals caused by the silver nanoparticles when in contact with the fungi, and these free radicals have the tendency to damage the cell membrane and make it porous which can ultimately lead to death of cell”.

CHAPTER 4

RATIONALE AND SCOPE OF THE STUDY

It has been reported that fungal infection annually destroyed approx. 125 million tonnes of top food crops such as rice, maize, soybean and potatoes. The destruction caused by fungi to wheat, rice and maize solely costs global agriculture \$60 billion per year. More than 600 billion people can be fed each year by avoiding fungal diseases in the world's five most important crops. Emergence of fungicide resistant pathogenic strain has made the management of fungal diseases even more difficult, thereby forcing the scientific community to search for alternative methods to manage fungal infection. The aim of this project was to synthesize silver nanoparticles using *Alternaria solani*, *Ascochyta phleina*, *Aspergillus niger*, *Cladosporium colocassiae*, *Aspergillus flavus* and to evaluate their antifungal properties against these plant pathogens.

CHAPTER 5

OBJECTIVES OF THE STUDY

The aim of this study was to synthesise silver nanoparticles using *Alternaria solani*, *Aspergillus niger*, *Aspergillus flavus*, *Ascochyta phleina* and *Cladoporium colocassiae* and to evaluate their antifungal properties against these plant pathogens. Work was carried out to meet the following objectives:

1. To maintain fungal biomass
2. To synthesise of silver nanoparticles using *A. solani*, *A.niger*, *A. flavus*, *A. phleina* and *C. colocassiae* respectively and see the effect of antifungal activity of silver nanoparticles on same fungi
3. Silver nanoparticles was characterised by U.V visible spectra analysis and by X-ray Diffraction
4. To evaluate the anti-fungal property of silver nanoparticles.

CHAPTER 6

RESEARCH METHODOLOGY

6.1 Organism

The organism used in this study *Alternaria solani* (NCIM-887), *Cladosporium colocassiae* (MTCC-10796), *Ascochyta phleina* (MTCC-2279) and *Aspergillus flavus* (NCIM-519), *Aspergillus niger* (NCIM-501) were obtained from Institute of Microbial Technology, Chandigarh and CSIR- National Chemical Laboratory, Pune. The culture was maintained in Potato dextrose agar and incubated at 25°C until confluent growth was achieved. These plates were stored and 4°C maintained in the active stage by transferring Mycelia plugs aseptically on fresh plates of PDA from time to time.

6.2 Biomass production of organism

Alternaria solani, *Cladosporium colocassiae*, *Ascochyta phleina* and *Aspergillus flavus*, *Aspergillus niger* were inoculated in the potato dextrose broth for and incubation at 25°C under stationary conditions or till confluent growth was achieved.

6.3 Synthesis of nanoparticles

Fungal mycelia was filtered, washed thrice with sterile deionised water to remove media and inoculated in sterile deionised. This was followed by incubation at 25°C for 3 days under stationary conditions. The biomasses were filtered by whattman filter paper no.1. In the filtered biomass was treated with 4mM silver nitrate and incubated in dark conditions under shaking conditions at 30°C for 24 hours (Kim et al., 2009).

6.4 Characterization of the nanoparticles

6.4.1 UV-visible spectra studies

Conformation of bio reduction of silver ions by fungus in aqueous solution to silver nanoparticles was carried out UV-Vis Spectroscopy at room temperature. The samples were subjected to a wavelength scan ranging between 300-500nm (Sun et al., 2001).

6.4.2 XRD Studies

The phase variety and grain size of formation of Ag-nanoparticles was determined by X-Ray Diffractometer. The vacuum-dried nanoparticles were used for powder X-ray diffraction (XRD) analysis. The spectra recorded in a X'Pert Pro PAnalytical X-ray Diffractometer (Cu K α radiation, λ 1.54060) running at 45 kV and 30 mA. The diffracted intensities were recorded from 20 degrees to 80 degrees 2θ angles. The scan axis used was Gonio and type was continuous. The particle size of the prepared samples were determined by using Scherrer's equation as follows

$$D \approx 0.9\lambda / \beta \cos\theta$$

Where D is the crystal size, λ is the wavelength of X-ray, Θ is the Braggs angle in radians and B is the full width at half maximum of the peak in radians (Klug et al., 1974).

6.5 Evaluation of Antifungal activity

Antifungal activity of the silver nanoparticles was evaluated using disk diffusion assay (Rai et al., 2009) . Potato dextrose agar plates were inoculated with 20 μ L spore suspension having spore count of 108/mL, 95/mL, 102/mL, 115/mL, 85/mL of test fungi viz., *Alternaria solani*, *Cladosporium coloccassiae*, *Ascochyta phleina* and *Aspergillus flavus*, *Aspergillus niger* respectively. Disk coated with freshly prepared Ag-NPs suspension. Fluconazole and combination of silver nanoparticles and fluconazole were placed onto the petri-plates respectively. Antifungal activity of nanoparticles of nanoparticles synthesized by each fungus was evaluated against the same fungi. Fungal cell filtrate used for the synthesis of silver nanoparticles was used as negative control. These plates were then incubated at 25°C for 48 hours. Similar experiments were carried out with only Ag-NPs. After incubation of 48hrs, the zones of inhibition were measured. The set ups were carried out in duplicates.

CHAPTER 7

RESULT AND DISCUSSION

7.1 Organism

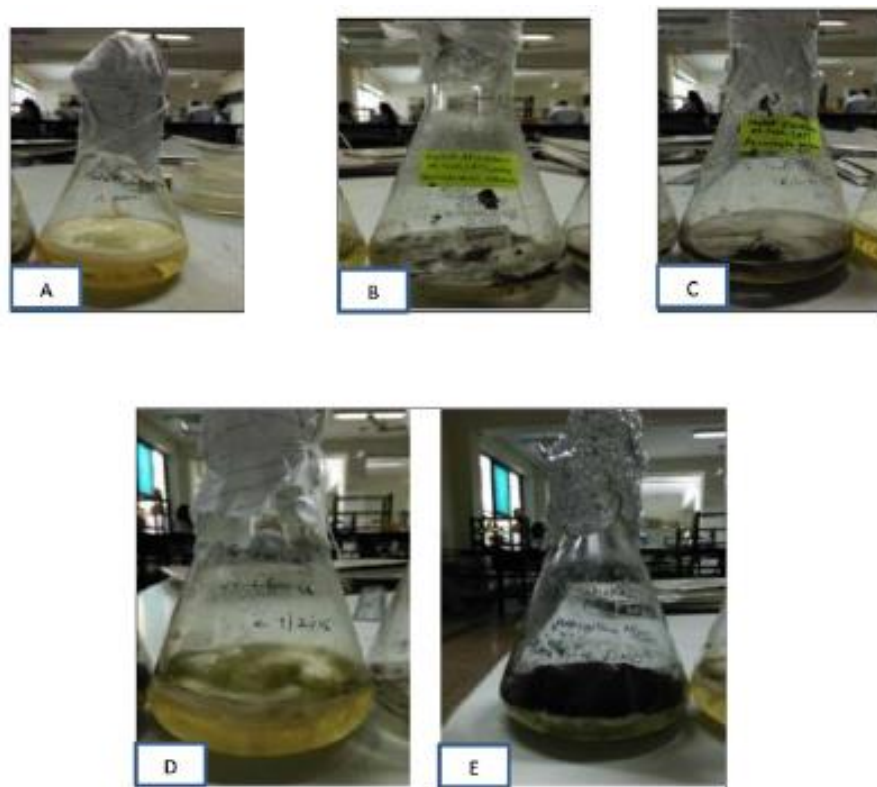
Fungal strains *Alternaria solani* (NCIM-887), *Cladosporium colocassiae* (MTCC-10796), *Ascochyta phleina* (MTCC-2279) and *Aspergillus flavus* (NCIM-519), *Aspergillus niger* (NCIM-501) were maintained in Potato dextrose agar and incubated at 25°C until confluent growth was achieved. *A. solani* forms spherical colonies with cottony growth, while in case of *A. niger*, light black colour spherical growth was seen Fig 3 (A and B). *A. flavus* shows a dark brown colour growth (Fig 3C) while green-black colour colony and dark-brownish colour fluffy growth were seen in case of *A. phleina* and *C. colocassiae* respectively (Fig 3D and Fig 3E). Similar morphological growth characteristics for these fungi have been reported in several works. (Olanya et al., 2009; Samson et al., 2001, Johnson, 1950 Rivas et al., 2005, Masayuki et al., 2010)



Fig no. 3 showing colony morphology of A) *A.solani* B) *A. phleina* C) *A. flavus*
D) *A. niger* and E) *C. colocassiae*

7.2 Biomass production of Organism

A. solani, *C. colocassiae*, *A. phleina* and *A. flavus* and *A. niger* were inoculated in the potato dextrose broth for 5-7 days and incubation at 28⁰C under stationary conditions or till confluent growth was achieved. Fig No. 4A-E shows biomass production of *A. solani*, *C. colocassiae*, *A. phleina* and *A. flavus* and *A. niger* respectively mycelia matte form.



**Fig 4: Biomass production of A) *A.solani* B) *A. phleina* C) *A. flavus*
D) *A. niger* and E) *C. colocassiae***

7.3 Synthesis of Silver nanoparticles

7.1 From Fungal Mycelia

Fungal mycelia were filtered, washed thrice with sterile deionised water to remove media and inoculated in sterile deionised water and treating with 4mM of silver nitrate and kept for 72 hrs. at room temperature. Initial indication of silver nanoparticles is given by the change in the colour of the suspension from transparent to dark brown-red colour (Fig.5). Similar colour changes have been reported by Rai et al., 2009 for silver nanoparticles synthesized from *Alternata alternata*. Birla et al., 2013, silver nanoparticle synthesized from Fungi *Fusarium oxysporum*. Change in colour to dark brown was also reported for nanoparticles synthesized from *Gloriosa superba* has been reported by Sharma et al., 2009.



Fig 5: Change in the colour of the suspension after 72 hrs. of incubation indicating synthesis of silver nanoparticles from mycelium

7.2 From Water Extract

Biomass of *A. flavus*, *C. colocassiae*, *A.phleina*, *A. niger* and *A. solani* was crushed mixed with deionised water and kept in water bath for 15 minutes at 50°C. Then this water extract of mycelia was used for synthesis of nanoparticles. Initial indication of silver nanoparticles is given by the change in the colour of the suspension from transparent to dark brown-red colour (Fig.6). Similar colour changes have been reported by Rai et al., 2009, synthesized the nanoparticles from *Alternata alternata*, Birla et al., 2013, silver nanoparticles synthesized from Fungi *Fusarium oxyporum*. Change in colour to dark brown was also reported for silver nanoparticles synthesized from *Gloriosa superba* have reported by Sharma et al., 2009.

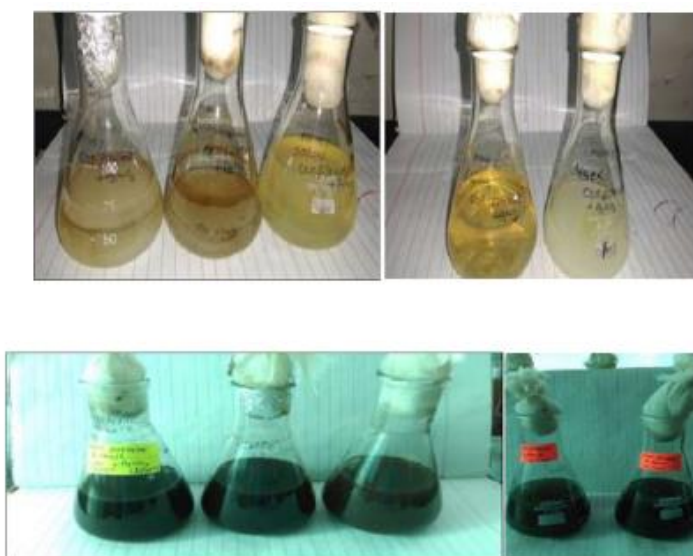
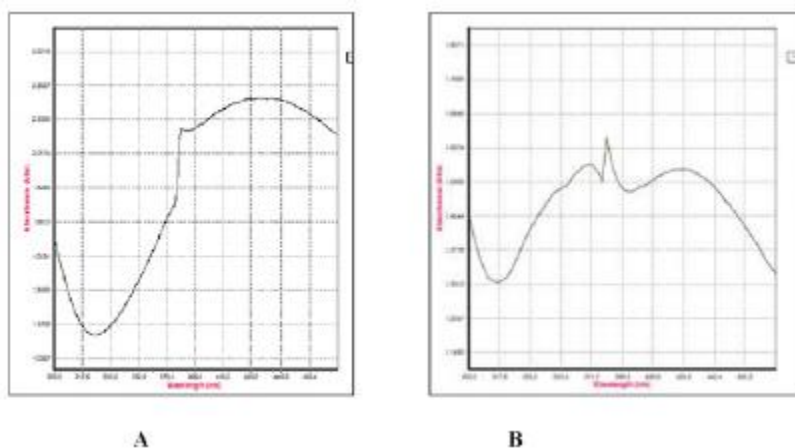


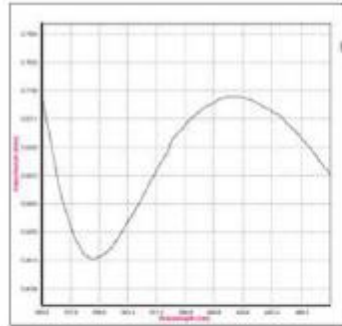
Fig no 6: Change in the colour of the suspension after 72 hrs. of incubation indicating synthesis of silver nanoparticles from fungal water extract

7.4 Characterization of silver nanoparticles

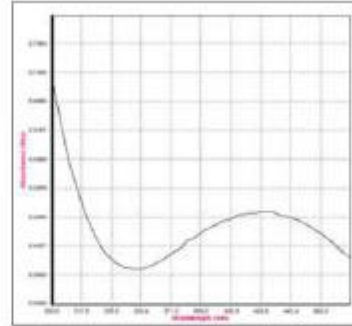
U.V visible spectral analysis

Conformation of fungus dependent bio reduction of silver ions in aqueous solution to silver nanoparticles was carried out UV-Vis Spectroscopy at room temperature. The samples were subjected to a wavelength scan ranging between 300-500nm (Sun et al., 2001). Figure no. 12 shows spectral analysis of *A. phleina*, *A. solani*, *A. flavus*, *C. colocassiae* and *A. niger* with absorption peaks at 420nm, 400nm, 420nm, 425nm and 420nm respectively. Figure no. 13 of shows spectral analysis of *A. phleina*, *A. solani*, *A. flavus*, *C.colocassiae* and *A. niger* with absorption peaks at 440nm, 440nm, 440nm, 440nm and 400nm respectively. Several workers have reported absorption peaks at 440nm for silver nanoparticles synthesised from *A. phleina* 420nm for *A. solani* mediated silver nanoparticles (Bhosle et al., 2012; Gaikwad et al., 2012) Kim et al., 2009 reported absorbance peak at 400nm for silver nanoparticles synthesised from *A. flavus* while 420nm and 425nm was reported in case of *C. colocassiae* and *A. niger* mediated nanoparticles respectively (Yargoli et al., 2012).

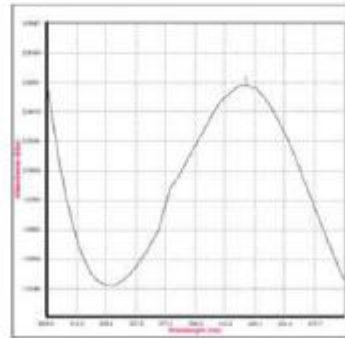




C



D



E

Fig 7: U.V visible spectral analysis of silver nanoparticles synthesized from deionised water filterate of A) *A. phleina* B) *A. solani* C) *A. flavus* D) *C. colocassiae* E) *A. niger*

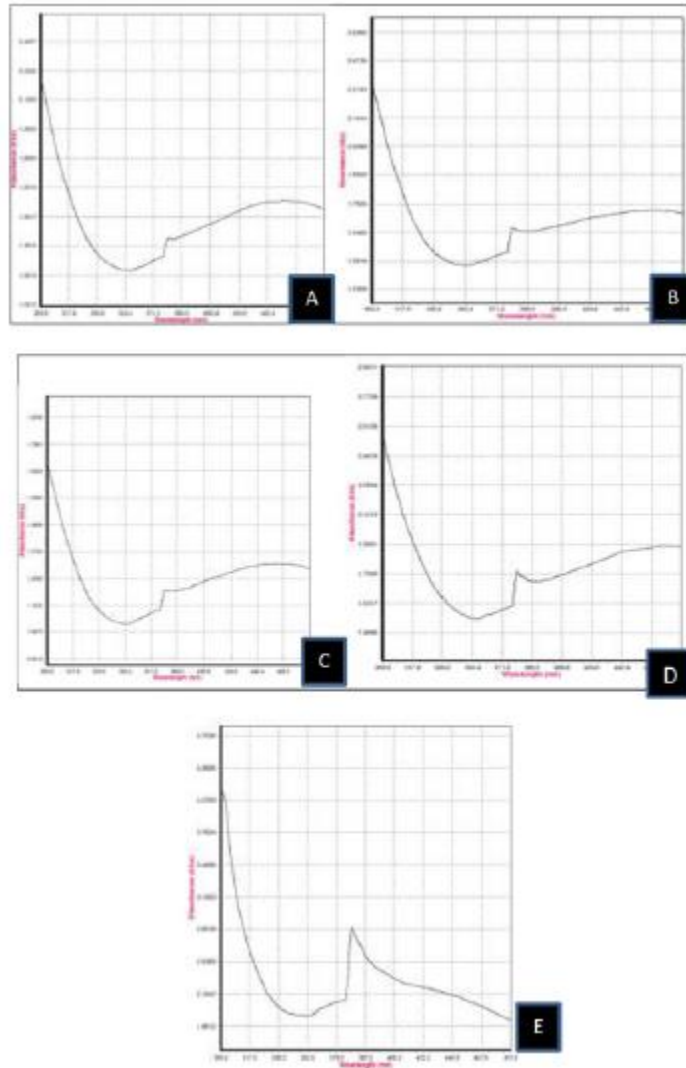
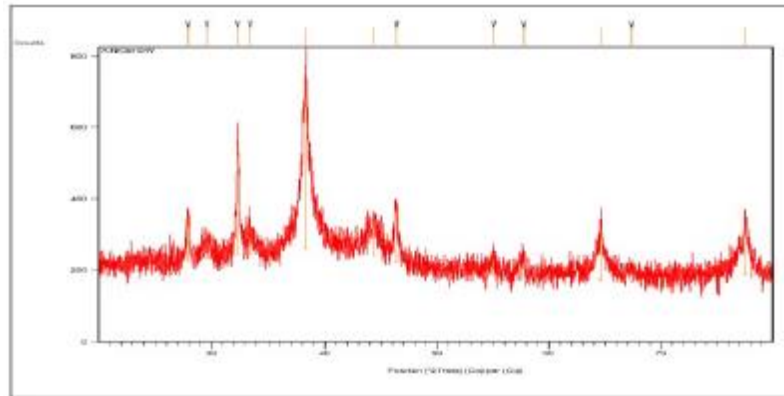


Fig 8: U.V visible spectral analysis of silver nanoparticles synthesized from water extract of
 A) *A. phleina* B) *A. solani* C) *A. flavus* D) *C. colocassiae* E) *A. niger*

XRD Analysis:

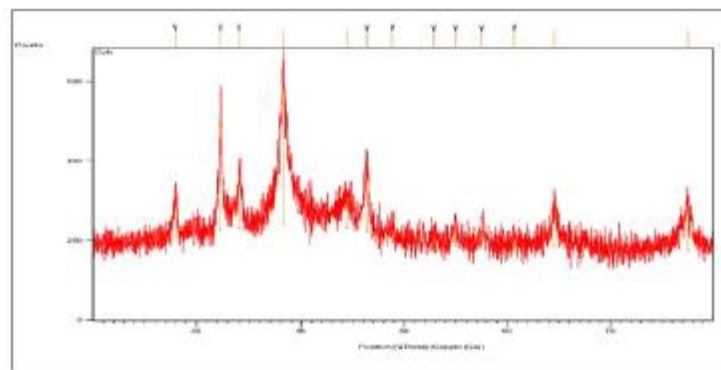
The phase variety and grain size of formation of Ag-nanoparticles was determined by X-Ray Diffractometer. The vacuum-dried nanoparticles were used for powder X-ray diffraction (XRD) analysis. The spectra recorded in a X'Pert Pro PAnalytical X-ray Diffractometer (Cu K α radiation, λ 1.54060) running at 45 kV and 30 mA. The diffracted intensities were recorded from 20 degrees to 80 degrees 2θ angles. Figure 9 depicts of XRD analysis of silver nanoparticles. Nanoparticles synthesized using *A. niger* gave peak at 27°, 77°, 64°, 46°, 38° with an average particle size of 5nm. In case of nanoparticles synthesized from *A. phleina* mycelium peaks at 28°, 32°, 38°, 46° with an average size of 54nm. The nanoparticles synthesized using *A. solani* was observed at 28°, 32°, 38°, 46° with an average size of 70nm.

The nanoparticles synthesized using *A. flavus* and *C. colocassiae* mycelium was seen at 28°, 32°, 38°, 46°, 27°, and 32°, 38°, 46° and 33° with an average size of 30nm and 29 nm respectively. Similar work of XRD patterns for silver nanoparticles have been reported by Gaikad et al., 2012; Sharma et al., 2012, Yen et al., 2009.

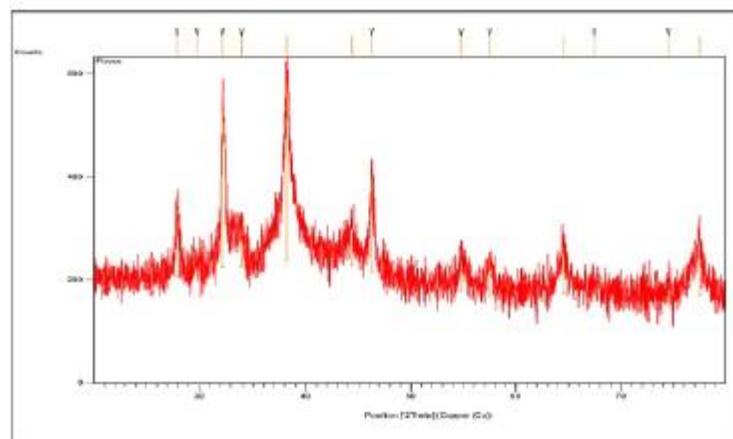


A

Fig 9: XRD Analysis for silver nanoparticles synthesized by A) *A. niger*



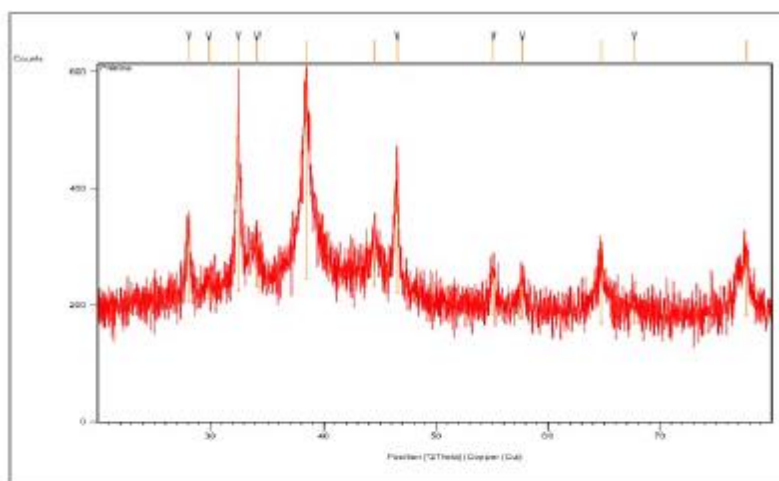
B



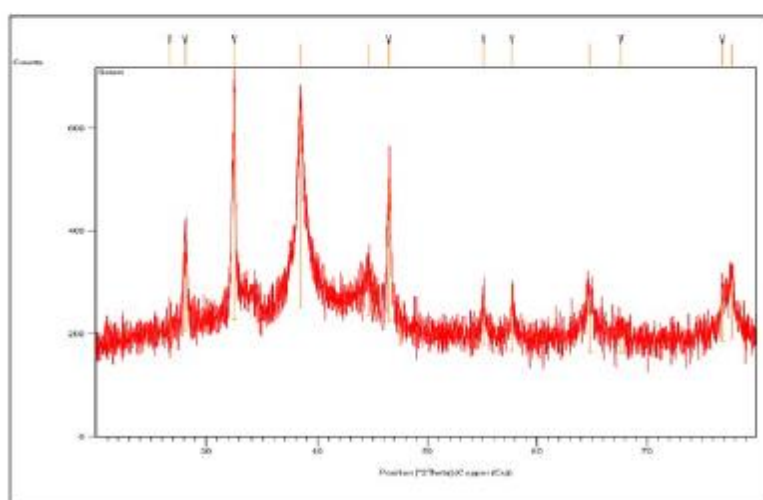
C

Fig 9: XRD Analysis for silver nanoparticles synthesized by B) *C. colocassiae*

C) *A. flavus*



D



E

Fig 9: XRD Analysis for silver nanoparticles synthesized by D) *A. phleina* E) *A. solani*

7.5 Evaluation of Antifungal activity

Disk diffusion assay was used to evaluate combined effects disk diffusion method, used to evaluate in vitro antifungal activity of silver nanoparticles and fluconazole. Figure 10 and Table 3 exhibit the antifungal activities of silver nanoparticles, fluconazole and nanoparticles in combination with fluconazole. Combination of silver nanoparticles and fluconazole exhibited higher activities than nanoparticles alone. The best results were observed from nanoparticles synthesised by water extract of *A. flavus* with zone of inhibition(ZOI) 22 mm against *A. flavus* and least ZOI was observed in *A. niger* with ZOI of 12mm (Figure 10). Kesharwani et al., 2009 reported similar results against *Candida albicans* and *Trichoderma sp.*

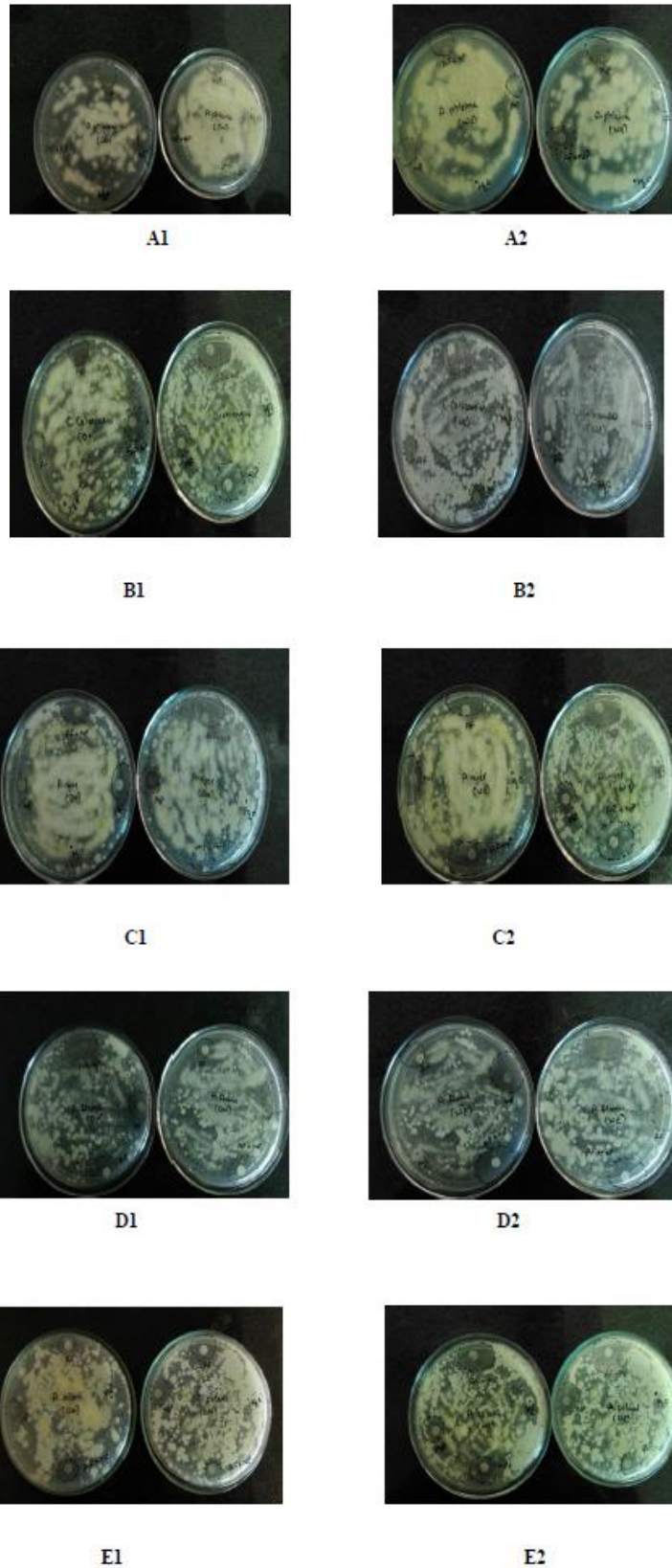


Fig no. 15: Zone of inhibition of NPs (DW) and NPs (WE), Fluconazole+NPs, Fluconazole and water in A1 & A2) *A. phleina*, (C1 & C2) *C. colocassiae*, (B1 & B2) *A. solani*, (D1&D2) *A. flavus* , E1 & E2) *A. niger*

Table no. 3: Zone of Inhibition of NPs (Deionised water), Fluconazole + NPs, Fluconazole and water (all values are in mm)

Test Fungi	ZONE OF INHIBITION (in mm)			
	Ag-NP(S)	F	Ag-NP(S)+F	DW
<i>A. solani</i>	15.5 ±0.707	13.5	19.5±3.535	0
Test Fungi	Ag-NP(P)	F	Ag-NP(P)+F	DW
<i>A. phleina</i>	15±1.414	12	18.5±0.707	0
Test Fungi	Ag-NP(F)	F	Ag-NP(F)+F	DW
<i>A. flavus</i>	17.5±0.707	16	18±3.535	0
Test Fungi	Ag-NP(N)	F	Ag-NP(N)+F	DW
<i>A. niger</i>	13.5±2.121	18	19±4.242	0
Test Fungi	Ag-NP(C)	F	Ag-NP(C)+F	DW
<i>C. colocassiae</i>	17.5±2.121	20	22±2.121	0

Where Ag-NP (S)= Silver nanoparticle synthesised by *A.solani* ,

Ag-NP (S)= Silver nanoparticle synthesised by *A.phleina*

Ag-NP (S)= Silver nanoparticle synthesised by *A.flavus*

Ag-NP (S)= Silver nanoparticle synthesised by *A. niger*

Ag-NP (S)= Silver nanoparticle synthesised by *C. colocassiae*

F= Fluconazole, DW= Distil. Water,

Ag-NPs= Combination of respective silver nanoparticle and fluconazole.

Table no. 3: Showing Zone of Inhibition of NPs (WE), Fluconazole + NPs, Fluconazole and water (all values are in mm)

Test Fungi	ZONE OF INHIBITION (in mm)			
	Ag-NP(S)	F	Ag-NP(S)+F	DW
<i>B. solani</i>	16.5±.707	14	19.5±.707	0
Test Fungi	Ag-NP(P)	F	Ag-NP(P)+F	DW
<i>B. phleina</i>	13±0	12	20±1.414	0
Test Fungi	Ag-NP(F)	F	Ag-NP(F)+F	DW
<i>B. flavus</i>	15.5±.707	17	22.5±.707	0
Test Fungi	Ag-NP(N)	F	Ag-NP(N)+F	DW
<i>B. niger</i>	15±4.242	14	17±2.828	0
Test Fungi	Ag-NP(C)	F	Ag-NP(C)+F	DW
<i>C. colocassiae</i>	17±0	16	19±1.414	0

Where Ag-NP (S)= Silver nanoparticle synthesised by *A.solani* ,

Ag-NP (S)= Silver nanoparticle synthesised by *A.phleina*

Ag-NP (S)= Silver nanoparticle synthesised by *A.flavus*

Ag-NP (S)= Silver nanoparticle synthesised by *A. niger*

Ag-NP (S)= Silver nanoparticle synthesised by *C. colocassiae*

F= Fluconazole, WE= Water Extract,

Ag-NPs= Combination of respective silver nanoparticle and fluconazole.

CHAPTER 8

CONCLUSION AND FUTURE SCOPE

The green synthesis of silver nanoparticles was carried out using *A. solani*, *A. phleina*, *A. flavus*, *A. niger*, *C. Colocassiae*. XRD analysis revealed the size of silver nanoparticle ranging from 20nm to 80nm in size. The antifungal activity was done by using disk diffusion method which shows the zone of inhibition of nanoparticle and combination of silver nanoparticle and fluconazole. The best results came from nanoparticles synthesized water extract of *A. flavus* which was having 23mm and 22mm of zone of inhibition in combination of silver nanoparticle with fluconazole against *A. flavus*. These results reveal that use of silver nanoparticles alone and in combination with fungicide can serve as an alternative to use of excessive fungicides and further studies need to be carried out to evaluate the toxicity of silver nanoparticles towards plants cell and optimize concentrations so as to achieve practical outcome.

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