Exploring potential microbial agent against *Curvularia lunata* and its utilization for nanoparticle Bio-synthesis

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN MICROBIOLOGY (HONOURS)

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DECLARATION

I, Monika, student of M.sc. (Honours) Microbiology hereby declare that the presented in Dissertation-1 entitled "**Exploring Potential Microbial Agent against *Curvularia lunata* and its utilization for Nanoparticle Biosynthesis"** is an authentic record of my own work that has been carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, for the partial fulfilment of the award of Masters of Science in Microbiology (Honours) under the guidance of **Dr. Gaurav Kumar**.

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

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CERTIFICATE

This is to certify that the work embodied in the Dissertation- II report entitled **"Exploring potential microbial agent against *Curvularia lunata* and its utilization for nanoparticle biosynthesis"** has been carried out by **Monika** (11506874) under my guidance and supervision to the best of my knowledge. The present work is the result of her original research and study. No part of this dissertation has ever been submitted for any other degree or diploma. The work has been carried out by her at the School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab. She has fulfilled the requirement for the award of the degree M.Sc. (Hons.) Microbiology.

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Chapter 1

Introduction

Rice (*Oryza sativa*) has a major role in the nutrition of the people all over the world and after wheat it is the most cultivated agricultural product (Yamaguchi et al., 2008). It is taken as staple food by more than half of the population all over the world. It is considered as the third highest agricultural product but the attack of various microorganisms such as fungi, bacteria, pests etc. Causes major economic loss of rice by decreasing its productivity (IRRI Rice facts, 2012). Every year there are reports of diseases which are destroying rice crop and causes a major loss of about $5 billion (Asgher et al., 2007).

Curvularia lunata is considered as plant pathogen which is mainly originated from the soil and causes the Black Kernel Disease which is found all over the world. Its infection causes molds in its grains and spot diseases in its leaves, which results in major loss for the dry land crops (Mian and Ahmed, 1978). Although there is availability of chemical fungicides for the black kernel disease, but they have very bad impact on both soil as well as on plant. They are toxic for the crop and may cause soil pollution (Meister and Sine, 2010). Therefore biological method can be used as an alternative method because it is more cheaper as well as ecofriendly. The use of soil borne microorganisms in order to control the disease is known as biological control. Biological fungicides are used to control the growth of plant pathogen. They allow the plants to quickly escape the effects of the pathogen, or to damage them by the production of toxins (Cook, 2000; Gilreath, 2002).

Biocontrol agents are mainly obtained from natural environments such as animals, plants, bacteria, fungi etc. So therefore the use of a biological agent may help to control the disease without harming the environment. The most important condition for introducing a microorganism as a biocontrol agent is by ensuring that the microorganism would not damage the main crop (Watson, 1985). Therefore, it should be proved that these fungal isolates should not damage rice and it is considered to be of great significant importance.

Today Nanoscience is considered one of the most advanced and fast developing field that is covering a wide range of application in a variety of areas of science and technology where new materials are synthesized at nano scale (Banerjee et al., 2014). The word 'nano' is originated from the Greek Word "nanos" which means "dwarf". And from the Greek Prefix, this means "extremely small" and can be translated to "1 billionth" as in nanosecond. Size ranges from 1-100 nm in at least one dimension. The dimension factor is significant for the reason that it allows materials such as carbon nanotubes, which are several micrometre long by few nanometer wide. These particles until now have actually attracted a great number of attentions because of their unusual and interesting properties, and their various uses over their mass and size. These extremely small particles with ever increasing applications have the potential to shape our Future. Nanotechnology is a technique which involves designing, characterization, production & application of the structures, by taking into consideration their shape and size at nanometer scale. Nanotechnology investigates electrical, optical and magnetic activity as well as structural behavior at the sub-atomic and sub-molecular level (Hasan, 2015).

Nanoparticles consist of two types- Metal and Non-metal nanoparticles. Metal nanoparticles include Au, Ag, Ti, Cu etc. There are a variety of methods which can synthesize these nanoparticles; it can be formed through physical method, chemical method and biological method (Mohanpuria et al., 2008). Even though these techniques are better in forming these particles, but because of the harmful chemicals created in the course of synthesizing, it greatly limits and affects various areas in the field of medicines. For that reason, forming environmental friendly means while synthesizing these particles is very important. An alternative to attain this aim is by using biological method where microorganisms are used to produce these particles. Different types of nanoparticles can be synthesized by methods such as chemical, physical and biological. Biological method or Biogenic synthesis of nanoparticles has been widely accepted and followed as it can be achieved by several organisms including bacteria, yeast, fungi, lichens, algae, plant and plant extracts (Sharma et al., 2009). And these organisms are easily available and more affordable. An advantage of biogenic synthesis, over conventional chemical synthesis, is that they are more safe and accompanied by easier handling of microbial cultures. The nanoparticles synthesized by biological means are considered much better option compared to the chemical method. There are a variety of methods to synthesize these nanoparticles; it can be

synthesized through physical method, chemical method and biological method (Liu *et al.*, 2011). Metal Nanoparticles exhibits unique physical, chemical and biological properties in contrast to maximum chemical composition materials because they have high surface to volume ratio (Iravani, 2014).

Nanoparticles are biologically synthesized when the microorganisms comes in contact with the target ions from their environment and then they convert the metal ions into the element state through enzymes which are generated mainly by the cell activities involved. It can be categorized into intracellular and extracellular synthesis according to the location of their formation (Simkiss and Wilbur, 1989; Mann, 2001). The intracellular method involves transportation of ions into the microbial cell so that they can form nanoparticles in the presence of enzymes. The extracellular synthesis of nanoparticles consists of metal ions which are trapped on the surface of the cells and then ions got reduced in the presence of several enzymes (Zhang et al., 2011).

Silver Nanoparticles (AgNPs) are considered one of the most important fraction of nano materials which is used in consumer products, as this particle is said to have the strong antimicrobial property (Sharma et al., 2009), electronic properties, optical properties, magnetic properties, resistance to oxidation and high thermal conductivity. In medicines Silver and Silver nanoparticles have enormous applications including topical creams containing silver to avert infection of open injuries (Duran et al.,2005) medical devices and inserts prepared with silver impregnated polymers (Becker, 1999; Lok et al., 2007; Marambio-Jones and Koek, 2010).

Biologically synthesized Silver nanoparticles have various applications which may involve biosensors, biolabelling, in cancer therapeutics and in coating of medical equipments (Duran et al., 2005). Hence, biologically synthesized NP represents a very interesting greener and more environmental friendly manufacturing alternative (Mukherjee et al., 2001). The need for eco-friendly, non-harmful engineered protocols for nanoparticle synthesis promotes the growing importance in biological methods which are free from the use of toxic chemicals as byproducts (Singhal et al., 2011).

Silver Nanparticles can have both positive as well as negative impact on the growth of rice crop. Plant cells can interact with the synthesized nanoparticles which can lead to the modification of plant gene expression and can also connect various biochemical pathways, which can affect the plant growth and development. Biologically synthesized Silver nanoparticles are also extensively known for their antimicrobial and antioxidant activities. These properties are known to greatly work against several diseases and cancer. Synthetic antioxidant is reported to have various properties such as anti-allergen, anti-carcinogenicity, anti-ageing activity and anti-mutagenicity.

So therefore, in present investigation biologically synthesized nanoparticles by using fungus were isolated from soil and used for controlling *Curvularia lunata*. Silver Nanoparticles were synthesized so that it can be detected whether they are used as bio-control agents or not and if they can produce better results than microorganisms which can directly act as biocontrol. Further studies were conducted to determine the effect of these nanoparticles on growth of rice by Seed germination tests and Pot Analysis. The Antimicrobial and antioxidant activities were also determined. These biogenic nanoparticles were characterized by several Microscopic and Spectroscopic methods such as UV-Visible Spectroscopy (UV-VIS), Fourier Transform Infrared Spectroscopy (FT-IR), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), X-Ray Diffraction (XRD) etc.

Chapter 2

Terminology

Nanoparticle (Np)	Particles of size between 1-100 nm are referred as nanoparticles.
AgNPs	Silver Nanoparticles.
UV-Vis Spectroscopy	Ultra-Violet Visible Spectroscopy.
FT-IR Spectroscopy	Fourier Transform Infra Red Spectroscopy.
XRD	X-Ray Diffraction
DLS	Dynamic Light Scattering
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

Chapter 3

Review of Literature

Black kernel disease of rice is caused by *Curvularia lunata* which is a plant pathogen distributed all over the world. Its infection causes molds in the grains and leaf spot diseases, which results in major economic loss for the dry land crops. Infection of *C.lunata* in human beings has resulted into severe problems such as allergic fungal sinusis and bronchial pulmonary fungal diseases (Travis *et al*., 1991; Halwig *et al*., 1985).

3.1 Plant: *Oryza sativa*

Rice has the third-highest worldwide production, after maize (corn) and wheat, according to data for 2009. It is the most useful cereal crop which is grown all over the world. In a report about 350 species of 150 genus and 60 families have been considered as rice weeds throughout the world (Hill *et al*., 1990).

3.2 Taxonomy

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Order	Poales
Family	Poaceae
Genus	Oryza
Species	*Oryza sativa*

3.3 Description and Morphology of Rice

Rice is an annual plant, growing to 1–1.8 m tall, occasionally more, with long slender leaves 50–100 cm long and 2–2.5 cm broad. The small wind-pollinated flowers are produced in a branched

arching to pendulous inflorescence 30–50 cm long. The seed is a grain (caryopsis) 5–12 mm long and 2–3 mm thick.

Figure: 3.1 Morphology of Rice

(Source: Growing upland rice: a production handbook – Africa Rice Center, WARDA)

3.4 Cultivation of Rice

Rice is a major dietary staple crop for maximum part of the world's human population, which makes it the most consumed cereal crop. It is the world's third largest crop, after maize and wheat. Its cultivation is mainly in the countries and areas with low labour costs and having high range of rainfall, as it's cultivation is very labour-intensive and requires enormous amount of waterfor irrigation. But, it can also be grown practically anywhere, even on steep hillsides. The cultivation of its species are native to South Asia and certain parts of Africa, Because of several centuries of trade and its export, Rice has acquired common place in many countries of the world.

3.5 *Curvularia lunata*

The morphology of *C.lunata* is shiny and velvety mainly black in colour, fluffy growth appears on the surface of the colony formed. It is distinguished by septate dematiaceous hyphae which produces brown, geniculate conidiophores. Its colonies show rapid growth on Potato Dextrose Agar (PDA) medium. Conidiophores can appear as isolated in single or in groups, they can be straight or bent in shape which may show simple or geniculate growth pattern & its colour ranges from pale to dark brown (Webster and Gunnell, 1992).

3.6 **Symptoms of Black Kernel Disease caused by *C.lunata***

Curvularia species cause discoloration of the aleurone and starch layer, and the hulls become brown. A Close association exists between discoloration of glumes, empty glumes and discoloration of kernels. When an empty glume dies, severe smudge of the kernels is observed. They are found on rice grains which mainly causes discoloration and they may also cause spots on the surface of the leaf under certain conditions (Ranganathaiah, 1985).

3.7 Chemical Control of Black Kernel Disease

C.lunata can be fully inhibited by the usage of 50 µg/ml of chemical fungicide Carbomar. Shikha et al., (2013) used Carbendazim (10 mg/ml) to control the growth of *C.lunat*a. Topsin and mencozeb suppresses the Curvularia spp. to grow by 50% (Butt *et al*., 2011). Even though there is presence of effective and efficient control of pathogenic fungi by the usage of synthetic fungicides, but they cannot be applied to grains because of their toxic nature (Anon, 2005; Harris

et al., 2001). Thus, there should be a safe, nontoxic, eco-friendly and cheap alternative approach. Therefore, Biological controls are required so that they may inhibit the pathogenic effects (Munusamy et al., 2003).

3.8. Biological Control

Biological fungicides may act as inhibitor to the growth of pathogen by competing with the pathogenic organism. But there may be a chance that they may also act as stimulator for the plant growth which allow the plant to inhibit any pathogenic effects or damage the pathogen by producing toxins (Cook, 2000; Gilreath, 2002). Both *C.lunata* and *C.aeroa* have been observed as biological control agents in *Echinochloa* spp. (Tsukamoto *et al*., 1998). *C.lunata* isolated from barnyard grass was evaluated for controlling weeds in bean fields (Bisen, 1983). It was found that this fungus was not effective in rice cultivars, but caused disease in bean varieties (Bisen, 1983). Also, *C.lunata* act as biocontrol for the *Echhornia crassipes* by 15-20% (Praveena and Nasecma, 2004). In the Philippines, isolates from *C.tuberculata* and *C.oryzae* were evaluated as probable biocontrol agent of *Cyperus difformis* and *Fimbristylis miliacea* (de Luna *et al*., 2002). Therefore, it should be proved that these fungal isolates would not damage rice crop and they can act as better biocontrol rather than using Chemical agents.

3.9 Nanoparticles

Nanotechnology is the technique for designing, characterizing, producing & studying the application of structures, by observing their shape & size at nanometer scale.

3.9.1 Size of Nanoparticles

The word “Nano” is derived from the greek word “nanos” which means dwarf or extremely small. Nanoparticles have a size of 1 Billionth of a meter (i.e. 10-9 m). Particles of size between 1-100 nm are said to be Nanoparticles. These nanoparticles can be made of materials of varied chemical nature, they can be metal oxides, silicates, non-oxide ceramics, polymers, organics, carbon nanotubes, liposomes and biomolecules. Nanoparticles appear in several shapes such as spheres, cylinders, tubes etc.

3.9.2 Types of Nanoparticles

Nanoparticles are mainly divided into two groups- Inorganic Nanoparticles which include magnetic nanoparticles, metallic nanoparticles (gold and Silver), nanoshells and ceramics and organic nanoparticles which include carbon nanotubes, quantum dots, polymers, liposomes and dendrimers.

3.10 Methods of Nanoparticle Synthesis

Nanoparticles are synthesized by using Physical, Chemical and Biological Methods. But both Physical and Chemical methods are non- ecofriendly as well as these methods have several side effects so therefore biological methods are mostly preferred. Chemical approach includes Chemical reduction, Electrochemical techniques, Photochemical Reduction and Pyrolysis. Physical Methods includes Arc-discharge and Physical Vapor Condensation. But biological method using plants and plant extracts are of huge potential for the synthesis of nanoparticles because this method is ecofriendly as well as it does not leads to any kind of toxicity.

3.10.1 Chemical Method

Chemical Method includes reduction of various chemicals, electrochemical techniques etc. for the synthesis of nanoparticles. In Chemical reduction method, common reducing agents such as polyols, $NaBH_4$, N_2H_4, Sodium citrate and N,N- dimethylformamide are used (Jiang et al., 2006). To avoid agglomeration of NPs, certain capping agents are required to stabilize it such as Sodium dodecyl sulphate (SDS), Polyvinyl pyrrolidone (PVP), Tri-Sodium Citrate (Ghorbani et al., 2011). Some other chemical methods includes electrochemical method, pyrolysis, photochemical that is by irradiation method. In this method, No reducing agent is required (Sharma et al., 2009).

3.10.2 Physical method

Physical methods include physical Vapor Condensation and Arc-Discharge. In Chemical methods, toxic chemicals are used which can be harmful to our environment and also these methods successfully produce pure nanoparticles but they depend upon stabilizers to prevent aggregation of nanoparticles. Apart from this, there are several disadvantages as these methods

are generally costly and have potential to create threat to the environment (Tien at al., 2008). While In comparison to this Physical methods do not use any kind of toxic Chemicals and are less time consuming.

In Physical Vapor condensation, the materials are vaporized by heat source and then immediately condensed to synthesize nanoparticles while nanoparticles having a different composition with the target are usually obtained by Chemical vapor Condensation (CVC), because the chemical reaction occurs between the vapor and other system components during the vaporization and condensation (Tavakoli et al., 2007).

3.10.3 Biological Method

Green synthesis of Silver nanoparticles with desired morphology and size using natural reducing, capping and stabilizing agents with desired morphology have become a main focus of researchers. In such biological methods, AgNPs are synthesized without using any harmful, toxic or costly chemicals (Ahmad et al., 2003 and Ankamwar et al., 2005). Certain biomolecules present in plant extracts such as enzymes, proteins, amino acids etc. causes bio-reduction of metal ions.

3.11 **Need for Green Synthesis**

There is a need of green synthesis of Nanoparticles because the conventional methods i.e. Physical and Chemical methods are costly as well as time consuming. There are various other disadvantages of using these methods as chemical synthesis involves some toxic chemicals which are harmful to the environment and cannot be used for medical purpose. In green synthesis, plant extract is used and there is no such issue of toxic chemicals. Plants contain various Phytochemicals which are responsible for the reduction of metal ions into the NPs. Thus green synthesis is preferred as it is less costly, eco-friendly and there is no use of toxic chemicals in green synthesis.

3.12 Synthesis of AgNPs using different Biological Organisms

Microbial source to produce the silver nanoparticles has been shown by the precipitation of nanoparticles due to its metabolic activity. Few reports have been published in literature on the

synthesis of silver nanoparticles biologically by using fungi as source (Husseiny *et al*., 2007). Various chemical & biological methods have been used for silver nanoparticles production, micro-organisms shows very good results in this process (Vigneshwaran, 2007). Some microorganism have inorganic material either intra or extracellularly (Mann, 1996).

3.12.1 Bacteria as a source of Synthesis

Silver Nanoparticles were first synthesized by using a bacterial strain of *Pseudomonas stutzeri* AG259 that was screened from silver mine (Haefeli et al., 1984). Some microorganisms have the ability to resist metals and can survive on high metal ion concentration and they can grow very well under such conditions. The various mechanisms by which these microbes can resist metals are some efflux systems, conversion of solubility and toxicity through reduction or oxidation, biosorption, bioaccumulation, extracellular complex formation or precipitation of metals and due to the absence of particular metal transport systems (Husseiny et al., 2007).

3.12.2 Fungi as a source of synthesis

Fungi can synthesize enormous amount of NPs in comparison to bacteria because fungus can secrete larger amount of proteins which facilitates the higher yield of NPs (Mohanpuria et al., 2008). The mechanism which can be followed to synthesize AgNPs from fungus involves: Entrapping of silver ions at the fungal cell surface followed by immediate reduction of the silver ions with the help of enzymes that are present in the fungal system (Mukherjee et al., 2001). But this is quite slow process in comparison to the plant extracts.

3.12.3 Algae as a source of synthesis

Among all other biological sources, algae is called as “bionanofactories” because in case of algae both live and dead dried biomass was utilized for fabrication of metallic nanoparticles. Other advantage of using algae for green synthesis is that it is less costly, eco-friendly, large-sized material and has the capacity of high metal uptake (Davis et al., 2003). Several reports demonstrated the successful use of algae for the synthesis of silver nanoparticles such as using marine alga *Caulerpa racemosa*, *Sargassum longifolium* etc.

3.12.4 Plant as a source of synthesis

Various plants are the major source of nanoparticles synthesis ant the main advantage behind using these plants is that they are readily available, non-toxic and safe. Plants contain huge variety of metabolites which can reduce silver ions quickly than microbes. The mechanism involved in this Plants based reduction due to phytochemicals such as terpenoids, flavones, ketones, aldehydes, amides and carboxylic acids. Some of the reports shows that Xerophytes contain emodin, an anthraquinone that undergoes tautomerization which further leads to the fabrication of the silver nanoparticles while mesophytes contain three types of benzoquinones i.e. cyperoquinone, dietchequinone and remirin. Jha et al., 2009 reported that these phytochemicals are directly involved in the reduction of silver ions and leads to the formation of Silver Nanoparticles.

3.13 Silver Nanoparticles as Biocontrol Agents

Some of the reports on nanoparticles have been used for biocontrol and some of them are showing promising results. They have antimicrobial as well as antioxidant activity. Ag nanoparticles have been studied in the previous years due to their antibacterial and therapeutic activities. Ag shows a similar way reaction as that of the gold (Au) nanoparticles where an antibody photosensitizer- nanoparticle complex was formed and it has been targeted to cancer cells so that free radicals can be generated which would then kill the affected cells (Stuchinskaya et al., 2011).

3.14 Applications of Silver Nanoparticles

Among all other nanoparticles, Silver Nanoparticles are most commonly used because of their unique properties such as size and shape depending optical, electrical and magnetic properties. Because of presence of such properties AgNPs can be used as antimicrobial agents, biosensor component, composite fibers, cryogenic superconducting materials, textile industries, for water treatment, cosmetic products and electronic components etc. They are mainly used in Pharmaceuticals, Drug delivery, Food Packaging, Cosmetics, Cell Labelling, Agriculture. Among various metal nanoparticles, Silver nanoparticles shows important applications in the field of bio-labeling & sensors (Dickson,1999).

Silver is commonly utilized in the nitrate form to promote antimicrobial activity, but when silver nanoparticles are synthesized using this silver nitrate; there is quite enormous increase in the surface area. It shows its effects on the cellular metabolic activity and the membrane striking damage to the cells and shows its secondary effects such as formation of reactive oxygen species and DNA damage. Its ability to cause cell damage depends on the size and type of cell. Due to the antimicrobial activity of AgNPs they are used in various fields of medicine, agriculture, packaging, cosmetics etc. Biologically synthesized nanoparticles have various applications which may include biosensors bio-labeling, in cancer therapeutics and in coating of medical equipments (Duran et al., 2005).

3.15 Toxicity of Silver Nanoparticles

Besides having so many advantages; AgNPs can cause several harmful effects on humans as well as on environment. The toxic effect of Silver NPs is due to the release of free silver ions into the environment. The exposure of these free silver ions have various harmful effects on humans such as Formation of Black spots on the skin which is known as Agyria or in the eyes which is known as Argyrosis. Excessive exposure to silver compounds can produce toxic effects in liver and kidney which further leads to organ damage. AgNPs has toxic effect on aquatic animals also as sliver ions can connect with the fish gills and suppress the sodium- potassium ATPase activity which in turn inhibits osmoregulation in fish (Wood et al., 1999). AgNPs also shows adverse effect on seed germination and plant growth when applied in high concentrations.

3.16 Effect of Nanoparticles on Plant Growth

Several reports have been published on both beneficial as well as harmful effect of AgNPs on Plant growth and development. The interaction of NPs with plants can cause several morphological as well as physiological changes and it depends upon the size, shape of NPs. The effect of NPs on plants generally depends on their concentration i.e. smaller concentration induces stimulating effect whereas large concentration induces an inhibitory effect. AgNPs can cause several side effects in root and shoot length as well as it can either promote or suppress germination rate of seedlings. Its effect can also be observed by certain Biochemical Estimations such as Chlorophyll Content, Carbohydrate Content, Protein Content, Total Phenolics and Flavonoids etc.

3.17 Characterization of Nanoparticles

The Characterization of Nanoparticles depends upon their size, shape, surface area and dispersity. The techniques involved for the Characterization of Nanoparticles are as follows: UV-Visible Spectroscopy (UV-VIS), Dynamic light Scattering (DLS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FTIR) and X-Ray Diffraction (XRD).

3.17.1 UV-Visible Spectroscopy (UV-Vis)

In this technique light wavelength generally lies in the range of 300-600 nm are used for characterizing different metallic nanoparticles. The spectrophotometric absorption reading in the range of wavelength 400-450 nm is used for Silver NPs Characterization.

3.17.2 Dynamic Light Scattering (DLS)

The Dynamic Light Scattering is used for the characterization purpose and is based on the surface charge and size distribution of the nanoparticles suspended in a liquid.

3.17.3 Scanning Electron Microscopy and Transmission Electron Microscopy

SEM and TEM are used for morphological characterization at the nanometer to micrometer scale. The Transmission Electron Microscopy has a 1000 fold higher resolution compared with the Scanning Electron Microscopy.

3.17.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR Spectroscopy is used for characterizing the surface chemistry of Nanoparticles. This method is used to determine the organic functional groups such as carbonyls, hydroxyls, amines etc. that are attached to the surface of nanoparticles.

3.17.5 X-Ray Diffraction (XRD)

X-Ray Diffraction Method is utilized for the phase identification and for the characterization of the crystal structure of the various nanoparticles. X-Rays easily penetrate into the nanoparticles and the results of diffraction pattern are further compared with given standards to determine structural information.

Chapter 4

Rationale and Scope of the Study

Rice is an important staple crop of maximum population of the world. But the most effective pathogen against rice plant is *Curvularia lunata*; which damages the crop and causes Black kernel disease to the crop. So instead of using Chemical control agents; Bio-control agents are used which are not only ecofriendly but cost effective as well. Various fungal isolates were used as antagonist against it. Their zone of inhibition were checked by Well diffusion method and Dual plate culture method in order to assess their antagonistic activity. Silver Nanoparticles were synthesized from fungal isolates and used for controlling *Curvularia lunata* by Seed germination as well as Pot studies. Therefore, the scope of the study is to check whether the biologically synthesized Silver Nanoparticles or the biocontrol fungal isolate itself can act as better biocontrol against *Curvularia lunata* and helps in controlling Black Kernel Disease.

Chapter 5

Objectives of the Study

The broad objective involved in this study is to isolate the potential microbial biocontrol agent against *C.lunata* and its utilization for nanoparticle biosynthesis; so that it can be used as commercial product in long run. The specific objective of the study includes:

- Isolation of antagonistic fungi from various soil samples
- Assessment and Characterization of antagonistic potential of various isolates.
- Synthesis of nanoparticles using selected antagonistic isolate.
- Assessment of nanoparticles to act as biocontrol agent against *Curvularia lunata*.
- Pot studies to assess the antagonistic activity of fungal isolate and nanoparticle
- To determine the effect of nanoparticle on the growth of rice plant (seed germination and pot studies)
- Characterization of nanoparticles by microscopic and spectroscopic methods.
- Characterization of potential fungal isolate.

Chapter 6

Research Methodology

6.1 Collection of Samples

6.1.1 Obtaining Plant pathogen Sample (*Curvularia lunata*)

The sample for isolation of pathogen was collected from the Microbiology Laboratory of the LPU. It was originally obtained from The Plant Pathology Laboratory, Punjab Agriculture University (PAU), Ludhiana.

6.1.2 Collection of Soil Sample

Soil from various areas of Phagwara was collected for the isolation of Antagonist. The details of the sample collection are as follows:

Table 6.1 Sources of different antagonists from soil sample

	Sample1	**Sample 2**	**Sample3**	**Sample4**
Collection date	02-09-2016	09-09-2016	09-09-2016	10-09-2016
Place of Collection	Near Dera Sant Gobind Dass, Gobind pura, Phagwara	Near Pyramid College NH-1 , Phagwara	Fields of Chak Hakim, Phagwara	Fields of LPU near GH-6.
Latitude	31.2049784	31.247041	31.2426024	31.25318851
Longitude	75.7639070	75.738772	75.7472304	75.70306319

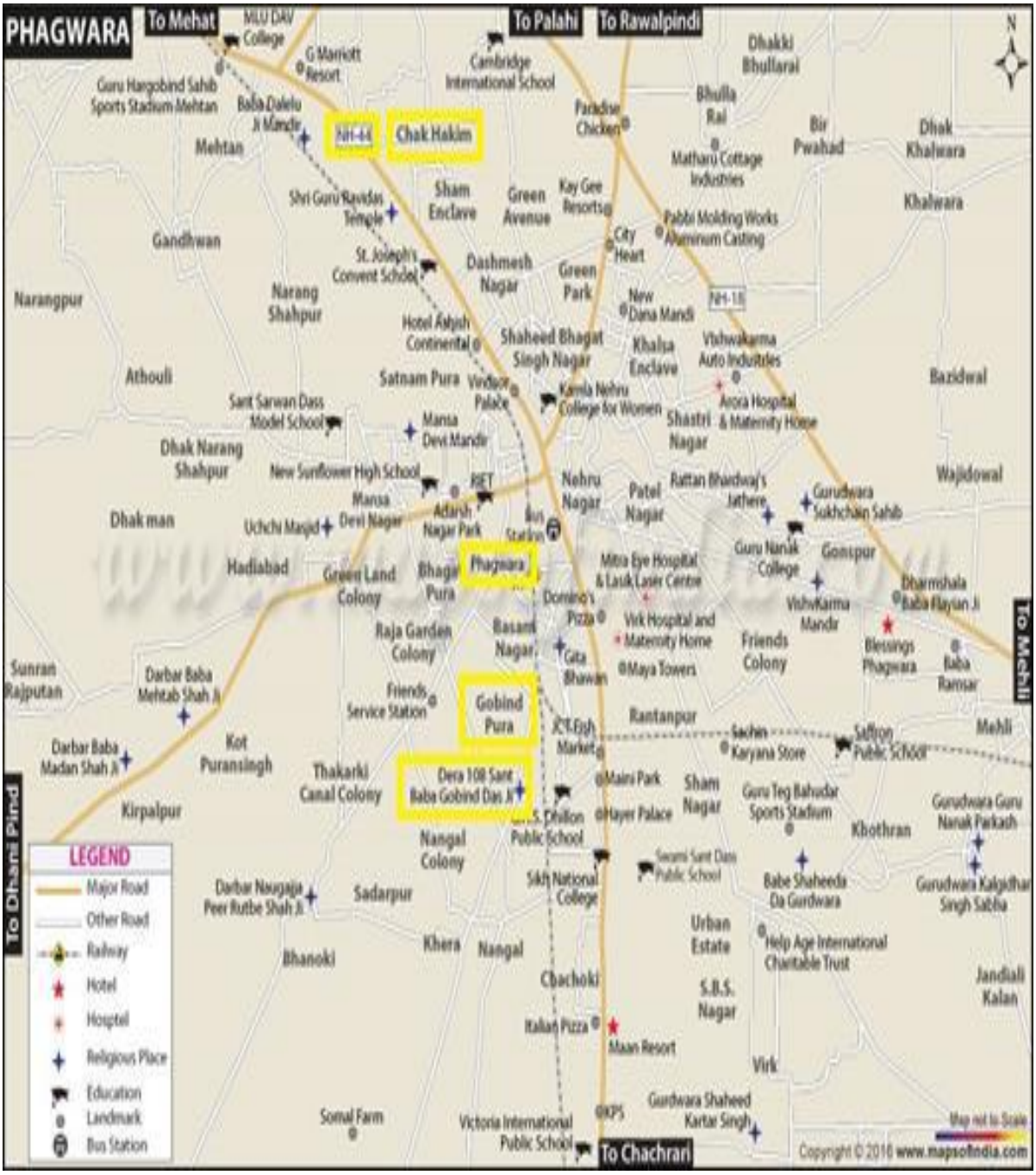

Figure 6.1: Areas of Sample Collection in Phagwara.

Figure 6.2: While collecting soil sample from agriculture fields.

6.2 Plant Pathogenic Sample

6.2.1 Purification of Pathogen (*Curvularia lunata)*

The pathogen sample was collected from the Microbiology Laboratory, LPU and it was obtained originally from the Plant Pathology Laboratory, Punjab Agriculture University (PAU), Ludhiana. It was sub-cultured onto Potato Dextrose Agar (pH4) plates and then incubated for 5 days under room temperature. It was preserved in PDA slants.

6.2.2 Characterization

Identification and characterization of the pathogen was done according to their morphological and microscopic examination.

6.2.2.1 Morphological characteristics

The appearance of *C.lunata* is shiny and velvety. It is black in colour. There is fluffy growth on the surface of the colony. It is characterized by the presence of septate dematiaceous hyphae which produces brown, geniculate conidiophores. Colonies rapidly grow on PDA (Potato

Dextrose Agar) medium. Conidiophores can be isolated singly or they may appear in groups, straight or bent. They show simple or geniculate growth pattern and its colour ranges from pale to dark brown (Webster and Gunnell, 1992).

6.2.2.2 Lactophenol Cotton Blue Mounting

This is a mounting technique where a drop of Lactophenol cotton blue was taken on a clean glass slide. With the help of a sterile forcep, a small portion of the fungal colony was taken and teased into small pieces with the help of the teasing needle. A cover slip was then placed on top of the fungal colony. Observation was done at 10x and 40x using light microscope (Aneja, 2003).

6.3 Isolation and Characterization of Fungi

6.3.1 Isolation of fungi from soil (Aneja, 2003)

Soil samples were collected from different areas of Phagwara. The isolated fungus were used as a potential source of antagonist against *Curvularia lunata*. The collected samples were subjected to serial dilution 10^{-1}, 10^{-2} and 10^{-3} in autoclaved distilled water for isolation of fungus by using pour plating technique into petriplates containing Potato Dextrose Agar. 100µl of the dilution was added to the petri plates and then PDA was poured onto it. PDA plates were allowed to solidify and then further incubated at 27^{o} C for 5 days. Different colonies that were visible on the plates were purified by inoculating on PDA plates and then sub-cultured onto plates and slants (Aneja, 2003). From the different sources of soil samples, total 20 fungal isolates of different morphological characteristics were isolated.

6.3.2 Characterization

The identification and characterization were done on the basis of the morphology and microscopic examination.

6.3.2.1 Morphological Characterization

Identification of fungus on the basis of morphology was done according to Gilman (1957); Aggarwal and Hasija, (1980).

6.3.2.2 Lactophenol Cotton Blue Mounting

This is a mounting technique where a drop of Lactophenol cotton blue stain was taken on a clean glass slide. With the help of a sterile forcep, a small portion from fungal colony was taken and tease into pieces with the help of the teasing needle. A cover slip was then placed on top of the fungal colony. Observation was done at 10x and 40x using light microscope (Aneja, 2003)

6.4 Production of Intracellular and Extracellular material

20ml of Potato Dextrose Broth (PDB) was inoculated with a loopful of fungal culture isolate into an Erlenmeyer flask and kept in incubator at 27^{o}C for 5 days. A layer or mat of fungal culture was formed on the surface of PDB. This is referred to as Extracellular material. The supernatant of the extracellular material was collected in a separate flask and 20 ml of deionized water was added to the carpet culture of fungus. It was kept in an incubator at 27^{o}C for 5 days. This is referred to as Intracellular material because the deionized water will extract out the intracellular material of the fungus by the process of osmosis.

6.5 Synthesis of Silver Nanoparticles

6.5.1 Preparation of fungal biomass

The fungal isolate was cultured, to produce broth inoculum into Potato Dextrose broth medium for 5-7 days at 27^{0}C. After the mat culture is obtained, the broth is separated and same amount of Deionized water is added to it and kept at orbital shaker for 27^{0}C at 120 rpm. The biomass was taken out after 24 hours of growth and processed for silver nanoparticle synthesis (Natarajan *et al*., 2010).

6.5.2 Synthesis of silver nanoparticles

The sample were added separately to the reaction mixture containing 1mM silver nitrate ($AgNO_3$) in 20 ml of deionized water and control (without the silver nitrate, only biomass) were also placed along with the experimental condition. The reaction between this supernatant and Ag+ ions were carried out in dark conditions for 24 hours and more if required (Natarajan et

al.,2010).The batch flask were inoculated with 2 ml of fungal culture, flasks were incubated at 27^0C on orbital shaker (120 rpm). Prepared sample were removed periodically & subjected to UV-visible analysis by taking their dilution with deionized water as 2:1 (Deionized water: Silver Nitrate solution) and concentrated as well. Simultaneously, a negative control was maintained under identical conditions (Patil et al., 2011). The Change in Colour from Yellow to Dark Brown shows the formation of Silver Nanoparticles and it can further be confirmed by obtaining peak at 420 nm on UV-Visible Spectroscopy.

6.6 Determination of Antagonistic Activity

Well Diffusion Method

PDA media was poured into the petri plates and *C.lunata* (spores were suspended in autoclaved distilled water, they were found to be hydrophilic in nature) was swabbed onto the surface with the help of sterilised swab sticks at 3 different angles so as to cover the whole surface area and make it into carpet culture. After this, wells were punctured into the plate with the help of borer. 50µl of extracellular and intracellular material were added to the wells and the plates were incubated at 27^oC for 5 days. Zone of inhibition was measured with the help of scale.

6.7Antagonistic Activity of Silver Nanoparticles against *Curvularia lunata*

Silver nanoparticles formed by using the fungal extract were further used for its antagonistic activity against *C.lunata*. Different concentrations such as 1, 5 mg and Control were used to check the activity by Well Diffusion Method. In this method, *C.lunata* spores were swabbed onto the PDA plates and wells were made with the help of sterilized borer. Then Silver nanoparticle solution was poured into it and kept for incubation at 27^OC. Zone of Inhibition was observed which shows its antimicrobial activity.

6.8 Other Activities of Silver Nanoparticles

6.8.1 Antioxidant Activity

6.8.1.1 DPPH Method

2,2- Diphenyl-1-Picrylhydrazyl is an important method used to study natural antioxidant products. It measures compound that are radical scavengers. Tris HCl buffer is also used in this technique to maintain the pH. From the stock solution, 8mg of DPPH was dissolved in 100 ml of methanol to make 0.22 mM DPPH solution. Different concentrations of AgNO3, Intracellular and Extracellular were taken (250, 500, 1000, 2000). In each 2 ml sample, 1 ml of DPPH solution was added. After which the solution was incubated in dark condition at 20 degree Celsius for 20 minutes. The absorbance of the DPPH solution was recorded at 517 nm in a UV-visible spectrophotometer. Control used was 1ml DPPH solution and 2ml of distilled water. Blank used was 2ml methanol with 4 ml distilled water (Kumar; et al 2003).

6.8.1.2 Iron chelating activity

In iron chelating activity, 0.05% of 1,10- Phenanthroline monohydrate and 200mM of Ferric chloride solution was used. From the stock solution, 25 mg of 1,10- Phenanthroline monohydrate was dissolved in 50 ml of methanol. And 0.4 mg of Ferric chloride was dissolved in 100 ml of distilled water. Three concentrations of AgNO3, Intracellular and Extracellular were taken respectively (250µl, 500µl, 1000µl). In each 2 ml sample, 1 ml of 1,10- Phenanthroline monohydrate and 2 ml of Ferric chloride solutions were added. Incubation was done at room temperature for 10 minutes and absorbance was recorded at 510 nm in UV-Visible spectroscopy. Blank used was 2ml distilled water, 1 ml of 1,10- Phenanthroline monohydrate and 2 ml of Ferric chloride solution (Kumar; et al 2003).

6.8.2 Antibacterial Activity

Well Diffusion Method

Nutrient Agar was poured into the Petri plates and different bacterial cultures were swabbed onto the surface with the help of sterilised swab sticks at 3 different angles so as to cover the whole surface area and make it into carpet culture. After this, wells were punctured into the plate with

the help of borer. 100µl of AgNPs solution were added to the wells and the plates were incubated at 37^{o}C for 24 hrs. Zone of inhibition was measured with the help of scale

6.9Effect of Stability

UV-Visible Spectroscopy was taken at different time intervals such as After 24 hrs, 48 hrs, 72 hrs, 96 hrs to check its effect on Stability of Silver Nanoparticles formed. It can be optimized by UV-Visible Spectroscopy.

6.10 Effect of substrate concentration and physical factors on nanoparticle synthesis

6.10.1 Effect of Concentration of Silver nitrate

Different concentrations of Silver Nitrate ($AgNO_3$) such as 1mM, 2mM, 3mM, 4mM, 5mM and Control were added to 20 ml of deionized water and 2 ml of Fungal extract under Dark Conditions and kept at 27^{o}C on orbital shaker till the synthesis of Silver Nanoparticles were observed. The optimum concentration for the synthesis of silver nanoparticles can be observed by UV-Visible Spectroscopy.

6.10.2 Effect of incubation temperature

Different temperature ranges such as 27°C, 37^{o}C and 47^{o}C were used for the optimization of AgNPs production. Its effect can be observed by UV-Visible Spectroscopy.

6.10.3 Effect of pH

Different pH values such as 4, 6, 7 and 8 were used to check the effect on AgNP production. Different pH can be adjusted by adding 1 N Sodium hydroxide and 1 N Hydrochloric Acid Solutions. Optimum pH can be obtained by UV-Vis Spectroscopy.

6.11 Characterization of Silver Nanoparticles

Nanoparticles are known to have unique shapes and sizes. These size and shapes can be measured by certain techniques such as microscopy or spectroscopy method. Microscopic method such as Scanning electron microscopy (SEM), Transmission electron microscopy (TEM)

and Atomic force microscopy (AFM) are used. Spectroscopic methods involve DLS, UV-VIS etc.

6.11.1 UV-Visible (UV-Vis)

In case of spectroscopic technique, Ultra violet-visible spectroscopy is mostly used. In Silver nanoparticles when aggregation is formed the particles become coupled electronically, the coupled system have different Surface Plasmon resonance (SPR) than the individual particles. Aggregation of multi-nanoparticles take place. This aggregation can be observed as the intensity increases in the red or infrared region of the spectrum. UV-visible spectrum should be carefully monitored to see if any aggregation of nanoparticles have occurred. A wavelength of 420 nm should be observed under UV-visible spectrum for proper conformation of silver nanoparticles.

6.11.2 Fourier Transform Infrared Spectroscopy (FT-IR)

For FTIR analysis, powdered sample of Silver Nitrate, Intracellular material of fungus and Silver Nanoparticle were used. These powdered samples were mixed with KBr and pressed to form a pellet. This pellet was used to determine functional group present in nanoparticle as well as intracellular material. Silver nitrate was taken as control. FTIR spectra were taken in the range of 4000-500 cm^{-1}.

6.11.3 Dynamic Light Scattering (DLS)

The aggregation state of the nanoparticle can also be measured by Dynamic light scattering (DLS) to know their effective size particles in solution.

6.11.4 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is able to scan the surface of the nanoparticles and thus revealing information about the external morphology. The scanning electron microscopy use focused beam of high energy electron to generate the surface information of the nanoparticles. And thus the external morphology can be obtained. In this, the beam scans the surface of the nanoparticles and reflects back to the observer instead of transmitting into the particles.

6.11.5 Transmission electron microscopy (TEM)

TEM on the other hand is used to determine the size of the nanoparticle and its structural morphology. It uses high energy electron beam, but instead of scanning it transmits or pass through the nanoparticles and thus the physical size and morphology of the nanoparticles can be obtained.

6.12 Seed Germination Test

In this test, Rice (*Oryza sativa*) seeds were used to check the effect of Silver Nanoparticles on their germination. Firstly, seeds were sterilized using 1% sodium hypochlorite solution for 3 minutes. After sterilization, seeds were rinsed thrice with deionized water to remove everything. Experiment was carried out in 3 sets of each Control where only deionized water was used for soaking seeds and other two was containing 5 mg/ml and 10 mg/ml concentration of AgNPs. In these solutions seeds were soaked for 3 hours. For further experiments seeds which were settled to the bottom were taken.1% agar solution plates were made and 6 seeds were placed in a petri dish. Experiments were done in triplicates for each set. Then after this, these petri plates were sealed and incubated for 7 days. The control which contains only deionized water was taken for comparing the toxicity of different concentration of AgNPs onto the treated seeds. After 7 days, seeds were used to check the effect of silver nanoparticles on their germination by studying several parameters such as Root and Shoot length (measured using vernier caliper).Fresh and Dry Weight of Seeds (measured using Weighing balance) and Germination % by the formula as follows:

Germination % = (No. of Seeds germinated/ No. of total seeds) * 100

6.11 Pot Studies

Here, the test species was Rice (*Oryza sativa*) on which *Curvularia lunata* attacks and causes Black Kernel Disease. Seeds of Rice were obtained from Agriculture Research Center, Phagwara. The variety used is Pussa-1121. These seeds were sterilized in 5% Sodium hypochlorite solution for 3 minutes and then rinsed thoroughly with deionized water several times. Soil was collected from nearby area of LPU campus and autoclaved. Urea (19.53g/ 1000

ml of distilled water) and Diammonium Phosphate (DAP) (21.46g/1000 ml) was added 2.85 ml/Kg of soil and 2.84 ml/Kg of Soil. Experiment was done in 6 setups as described in Table no 6.2.

Table 6.2: Different Setups Used for Pot Study

Groups	**Treatment given to Rice Seeds**
1.	Control
2.	Treated with spores of *Curvularia lunata*
3.	Treated with spores of Biocontrol Fungus
4.	Treated with spores of *Curvularia lunata* + Spores of Biocontrol Fungus
5.	Treated with Nanoparticle suspensions
6.	Treated with spores of *Curvularia lunata* + nanoparticle suspension

For these setups, 30 pots were taken and filled with soil. After this, two seeds were sown into each pot. Five replicates of each treatment were prepared for a total of 30 pots. After 25 days of growth, the shoot and root lengths were measured using a précised ruler scale. Fresh and Dry weights were measured by Weighing balance.

6.11.1 Preparation of Stock Solution

0.5 gm of leaves from different treatments were crushed in 20 ml of 0.1 mM Phosphate Buffer (NaH_2PO_4 ; 2.34 gm + Na_2HPO_4 ; 2.12 gm) of pH 7 using mortar and pestle, maintaining cold temperature. After crushing extract was centrifuged at 10,000 rpm for 10 minutes.

6.11.2 Phytochemical Analysis

It includes some of the following tests:

6.11.2.1 Phenolic Compounds Test

To the 100 µl of Stock Solution of different samples; 2.5 ml of FC Reagent (1/10 diluted in water) and 2ml of 7.5% Na_2CO_3 were added and kept at 45^oC for 15 minutes. Note down the Absorbance at 765 nm. 100 µl of Phospahte buffer, 2.5 ml of Folin-Ciocalteu's (FC) Reagent and 2ml of Na_2CO_3 is taken as Blank. Gallic Acid was used as Standard.

6.11.2.2 Flavonoids Test

Prepare 2% of $AlCl_3$ solution in Ethanol. Add 100 µl of stock solution to 900 µl of Distilled water. Now put 1 ml of $AlCl_3$ solution to every sample and keep it in dark condition for 1 hr. Take Absorbance at 420 nm.1 ml of $AlCl_3$and 2 ml of Distilled Water is taken as Blank.

6.11.2.3 Chlorophyll Estimation (Arnon, 1949)

Chlorophyll is a green pigment present in plant leaves which contains tetrapyrole ring with a magnesium ion in the center. Here, chlorophyll a, chlorophyll b and Carotenoid pigment was measured. 1gm of leaves from each treatment along with control was taken and crushed in 20 ml of 80% chilled acetone using mortar and pestle. It was then filtered using Whatman filter paper. The filtrate was then used for taking spectrophotometer reading at wavelength 645, 470 and 663 nm. Acetone was taken as blank. It can be measured as:

Total Chlorophyll content: 20.2 (A645) + 8.02 (A663)
Chlorophyll a: 12.7(A663) – 2.69 (A645)
Chlorophyll b: 22.9(A645) – 4.68 (A663)
Carotenoids: 4.69 (A470) – 0.268 (20.2(A645) + 8.02 (A663))

6.11.2.4 Protein Estimation Test (Bradford, 1976)

Preparation of Bradford Reagent : Dissolve 5 mg of Coomassie Brilliant Blue G-250 in 5 ml of Methanol and add 10 ml of 85% Phosphoric Acid. Add the acid solution mixture slowly into 85 ml of distilled water and let the dye dissolve completely. Filter it with Whatman paper and store in dark conditions. To 100 µl of stock solution, add 5 ml of Bradford Reagent. After mixing each sample, the absorbance reading was taken at 595 nm. Distilled water is taken as Blank. For the Standard Curve, Bovine Serum Albumin (BSA) (1mg/ml) for 10, 20, 40, 60, 80 µl of stock solution and then volume is made upto 100ul by adding distilled water and then 5 ml of Bradford reagent is added to each.

6.11.2.5 Carbohydrate Estimation Test (Dubois et al., 1956)

Here supernatant was taken from the stock solution prepared from the fresh plant and mixed gently with 1 ml of 5% phenol and 5 ml of Conc. Sulphuric acid and then incubated for 10

minutes at room temperature. The Absorbance was taken at 490 nm using spectrophotometer. The reading was then compared with the standard prepared with the glucose. 1ml of 5% phenol and 5 ml of Conc. Sulphuric Acid is taken as blank. Glucose is taken as Standard.

6.12 Characterization of Potential Fungal Isolate

6.12.1 Morphological Characteristics

The appearance of fungus on the petri plate i.e according to its colony morphology.

6.12.2 Lactophenol Cotton Blue mounting

This is a mounting technique where a drop of Lactophenol cotton blue was taken on a clean glass slide. With the help of a sterile forcep, a small portion of the fungal colony was taken and teased into small pieces with the help of the teasing needle. A cover slip was then placed on top of the fungal colony. Observation was done at 10x and 40x using light microscope (Aneja, 2003).

6.12.3 Sequencing

Molecular Characterization was performed by 18s rRNA sequencing method. The data of the gene sequencing was further submitted to NCBI (National Centre for Biotechnology Information) and a phylogenetic tree was constructed to study the phylogenetic relationship of the organism.

Chapter 7

Results and Discussion

7.1 Procurement of Pathogen

7.1.1Morphology of *Curvularia lunata*

The appearance of *C.lunata* is shiny and velvety, black in colour, fluffy growth on the surface of the colony. It was characterized by septate dematiaceous hyphae producing brown colour. Colonies rapidly grow on Potato Dextrose Agar (PDA) medium. This is represented in Figure 7.1 (A and B).

7.1.2LPCB mounting of *Curvularia lunata*

Conidiophores were observed which can be present in isolated form or in groups. They appeared as straight or bent and they may show simple or geniculate growth a pattern & colour range from pale to dark brown (Webster and Gunnell, 1992). This is represented in Figure 7.2 (C and D).

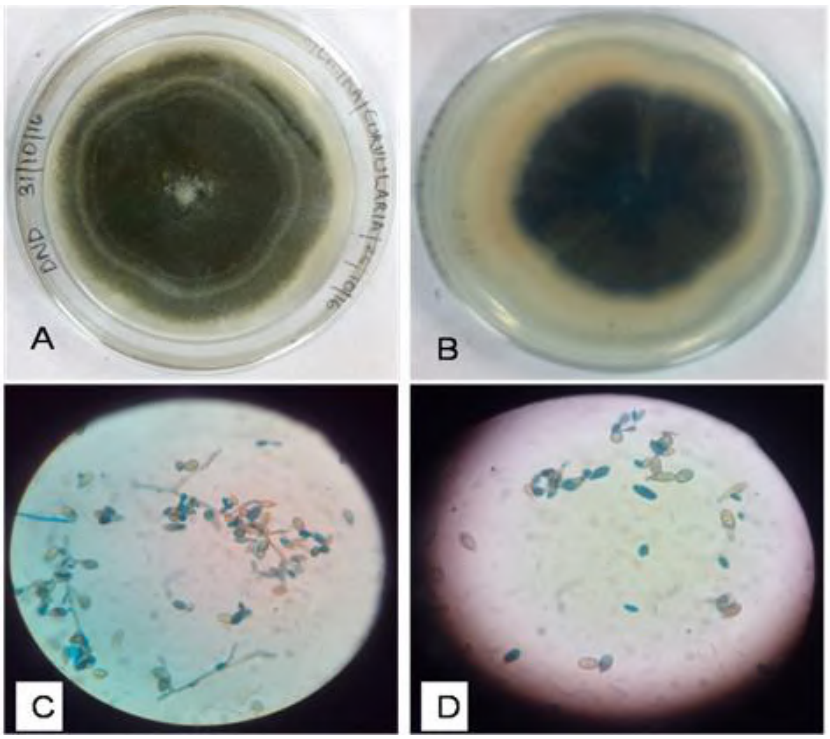

Figure 7.1: Morphology of *C.lunata*. Here,Front view of petri plate B) Back view of petri plate, C and D) LPCB mount of *C.lunata* (at 40x)

7.2 Isolation of antagonists

From the different sources, total 20 fungal isolates of different morphological characteristics were isolated. The details of which are presented in Table 7.1

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Table no.7.1 Number of fungal isolates obtained from the different soil samples.

Soil sample No.	**No. of Isolates**
Sample1	4 Isolates
Sample2	7 Isolates
Sample3	2 Isolates
Sample4	7 Isolates

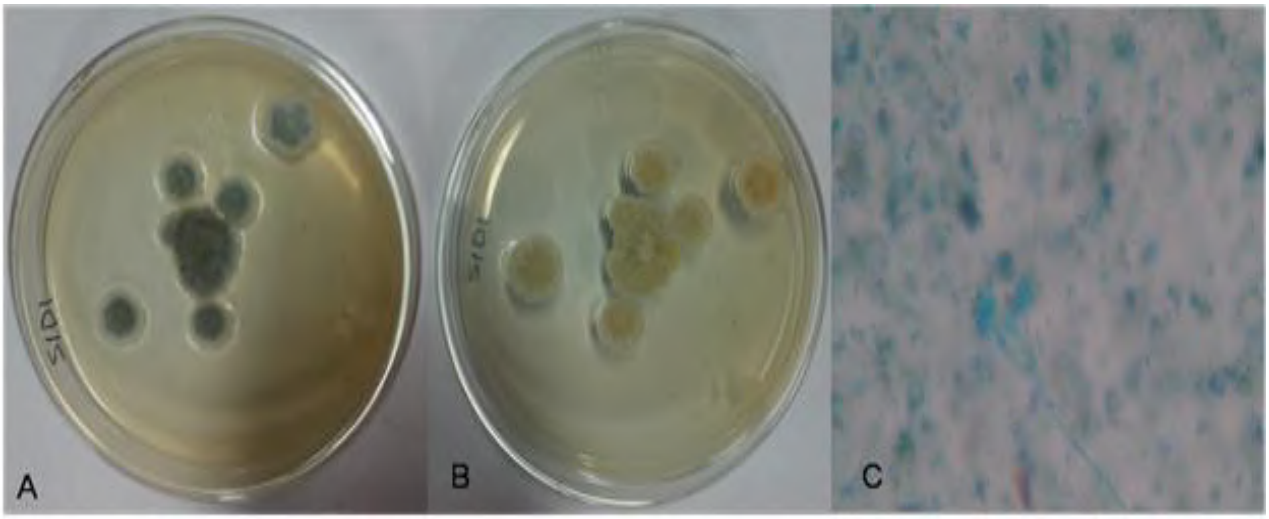

Figure 7.2 : Isolate S1D1 A) Front view of Petri Plate, B) Back View of Petri Plate, C) LPCB Mount (40 x)

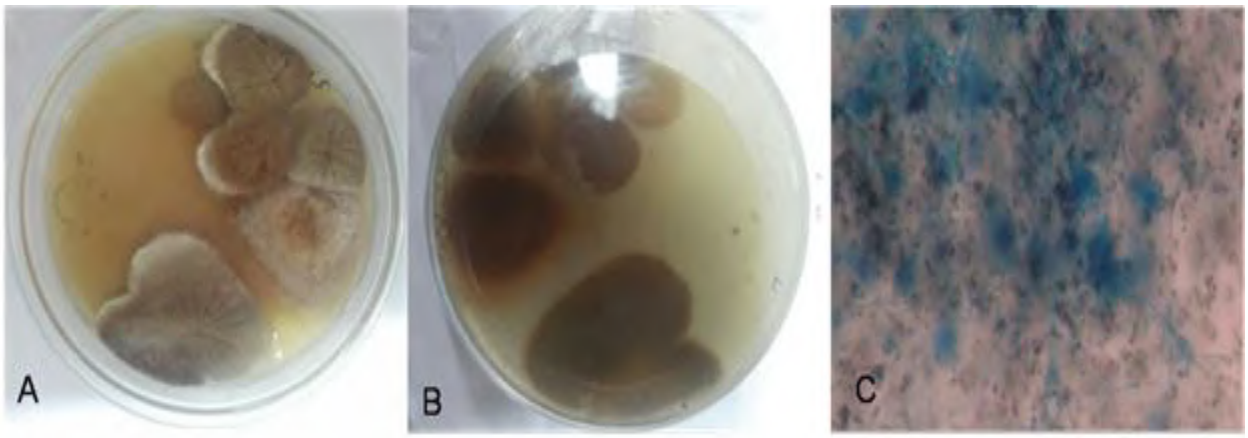

Figure 7.3: Isolate S3D2 A) Front view of Petri Plate, B) Back View of Petri Plate, C) LPCB Mount (40 x)

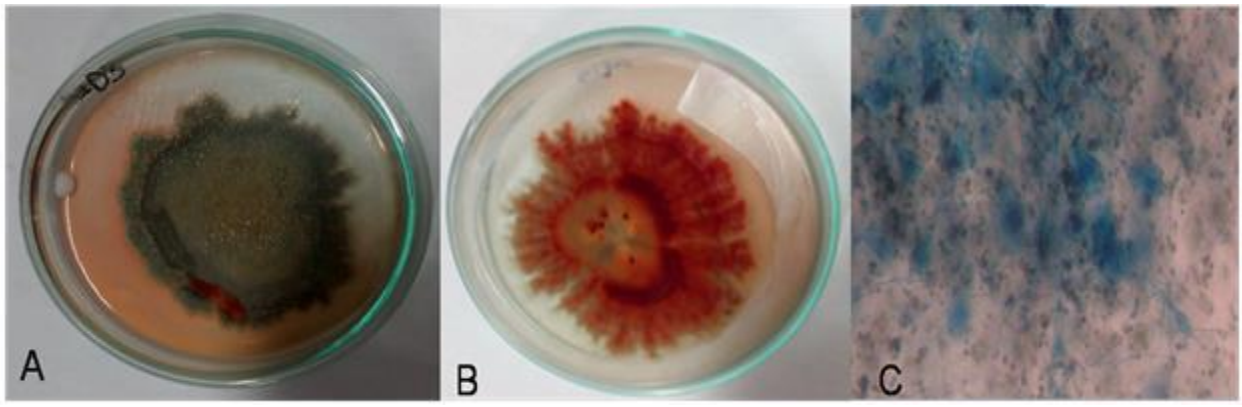

Figure 7.4: Isolate S2D3 A) Front view of Petri Plate, B) Back View of Petri Plate, C) LPCB Mount (40 x)

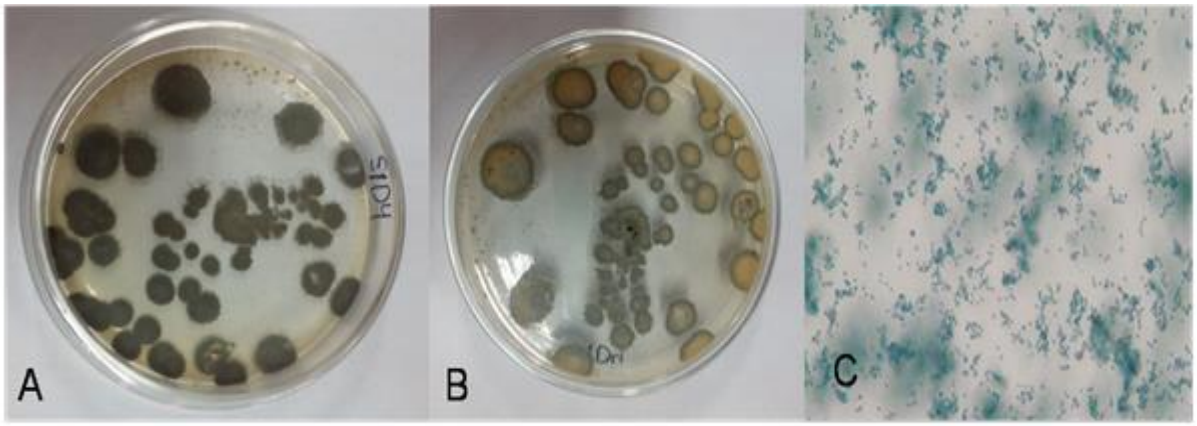

Figure 7.5: Isolate S1D4 A) Front view of Petri Plate, B) Back View of Petri Plate, C) LPCB Mount (40 x)

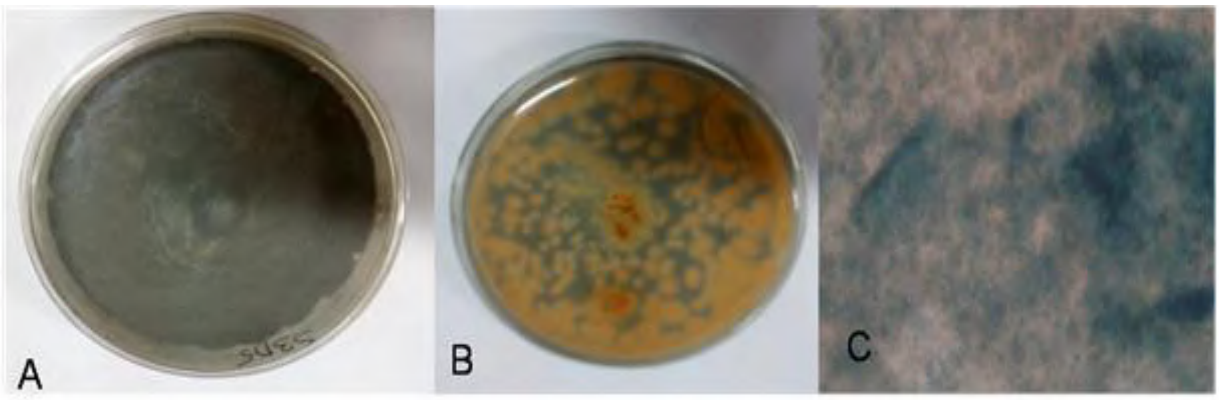

Figure 7.6: Isolate S3D5, A) Front view of Petri Plate B) Back View of Petri Plate, C) LPCB Mount (40 x)

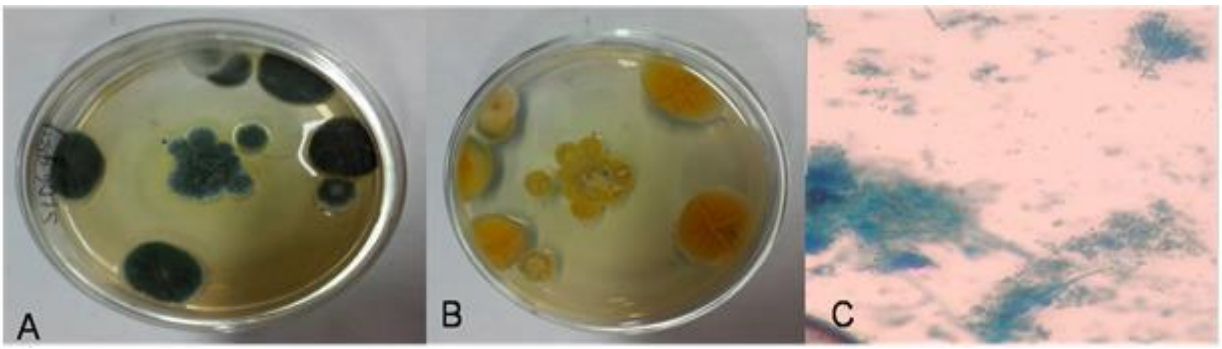

Figure 7.7: Isolate S1D6 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

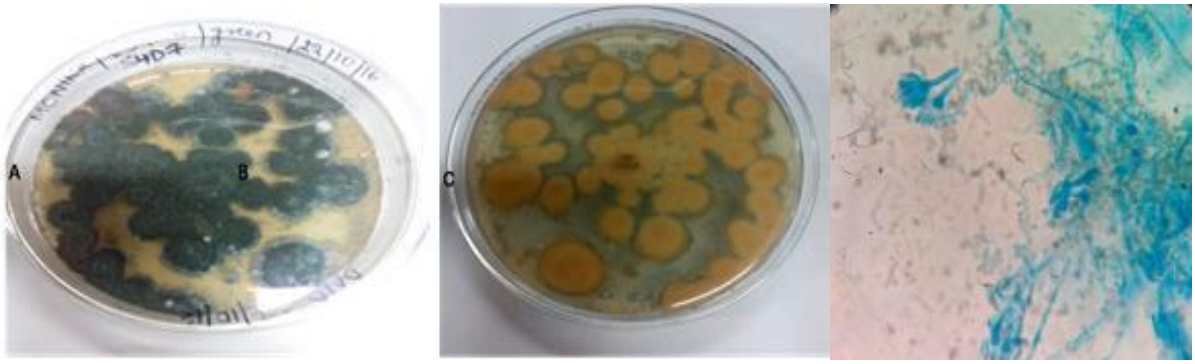

Figure 7.8: Isolate S4D7 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

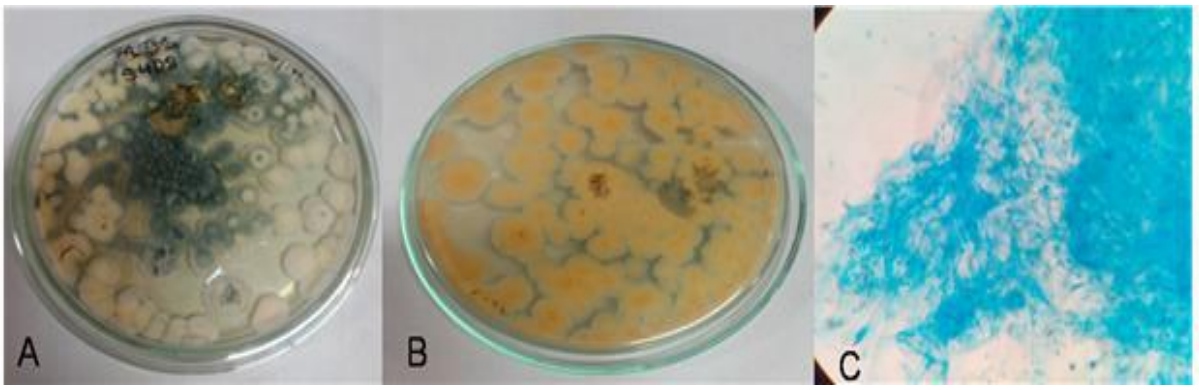

Figure 7.9: Isolate S4D8 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

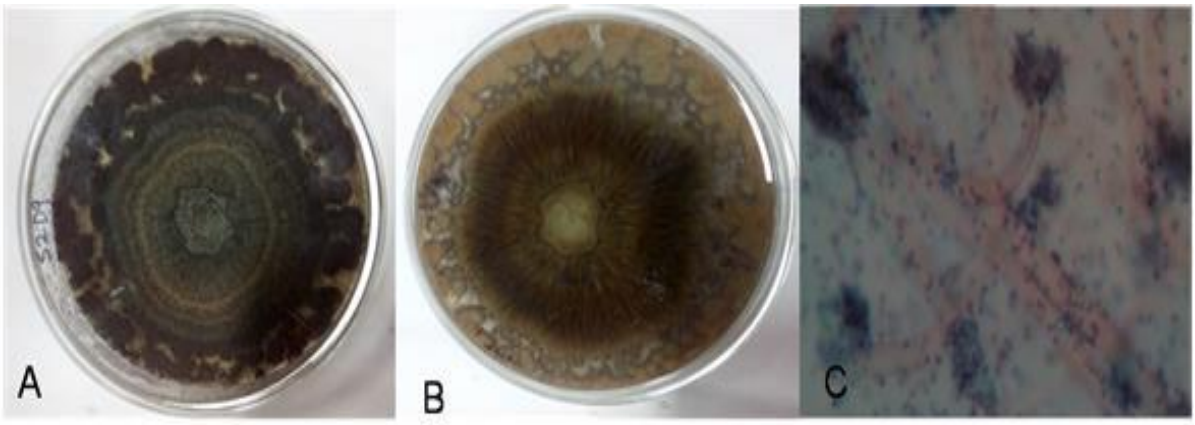

Figure 7.10: Isolate S2D9 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

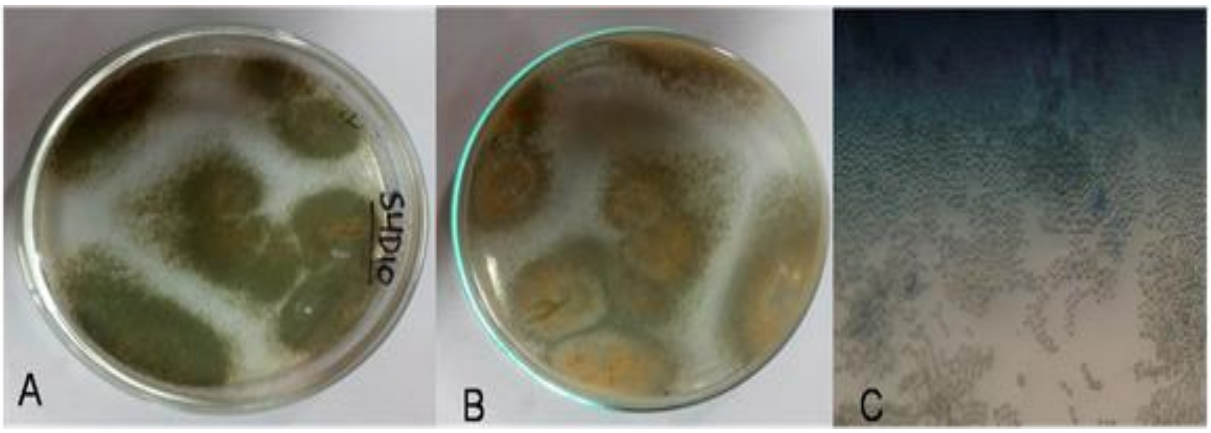

Figure 7.11: Isolate S4D10 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

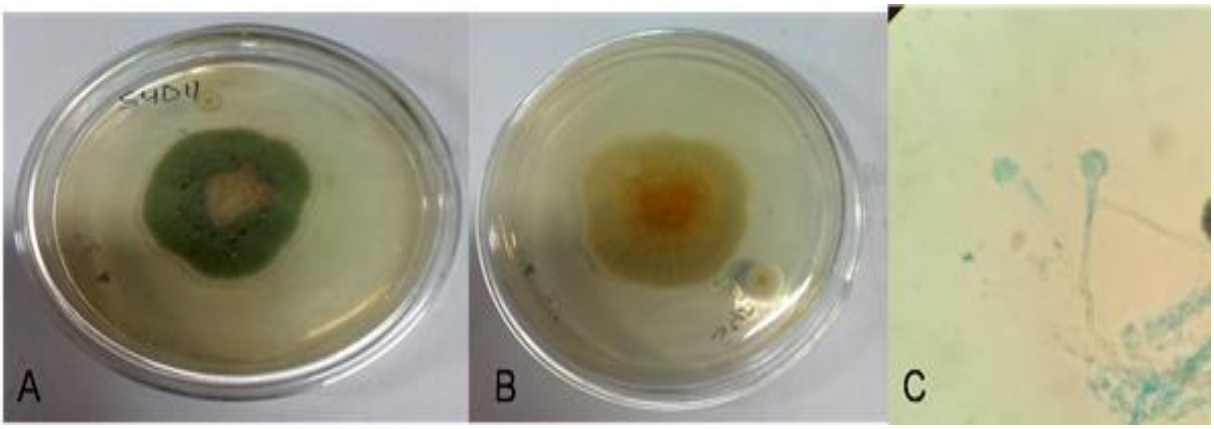

Figure 7.12: Isolate S4D11 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

Figure 7.13: Isolate S3D12 A) Front view of Petri Plate B) Back view of Petri Plate C)LPCB Mount (40x)

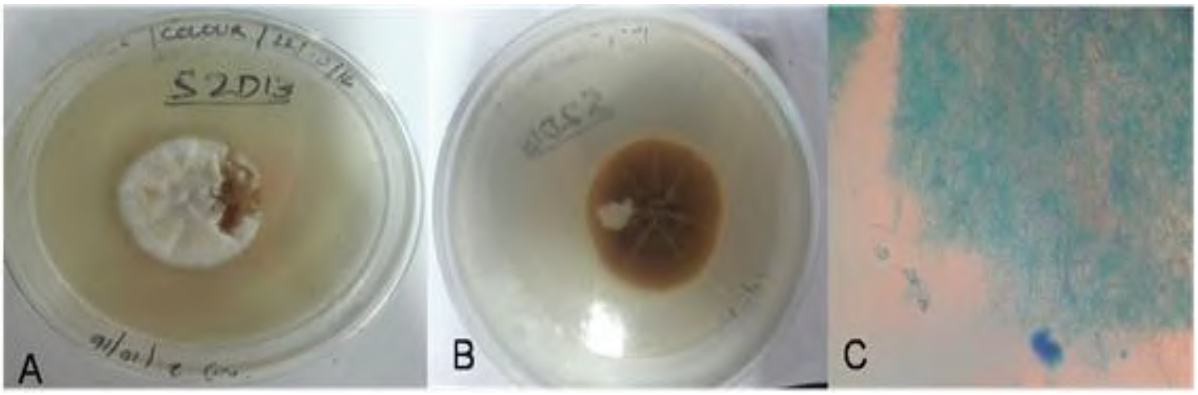

Figure 7.14: Isolate S2D13 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

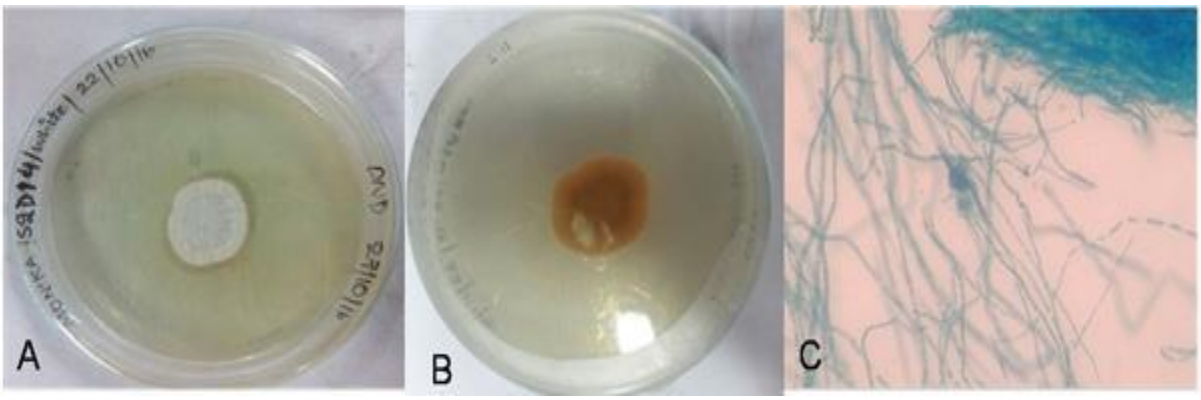

Figure 7.15: Isolate S2D14 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

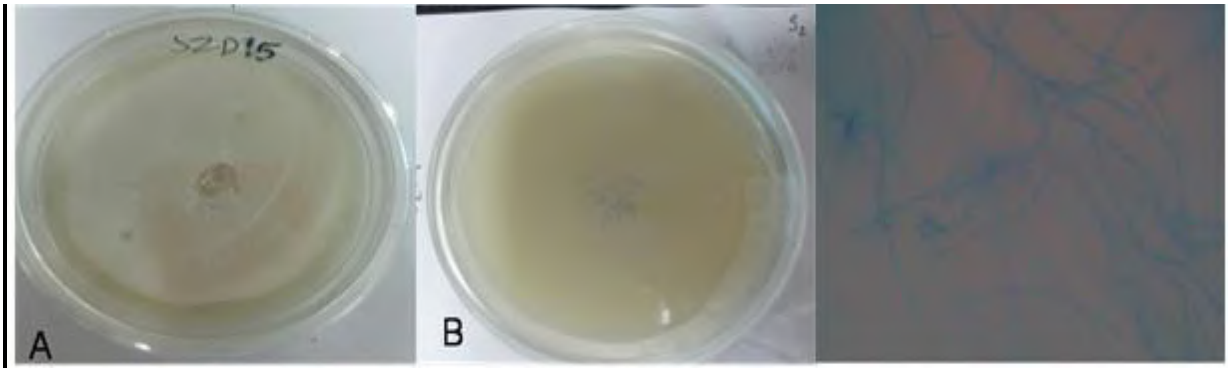

Figure 7.16: Isolate S2D15 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

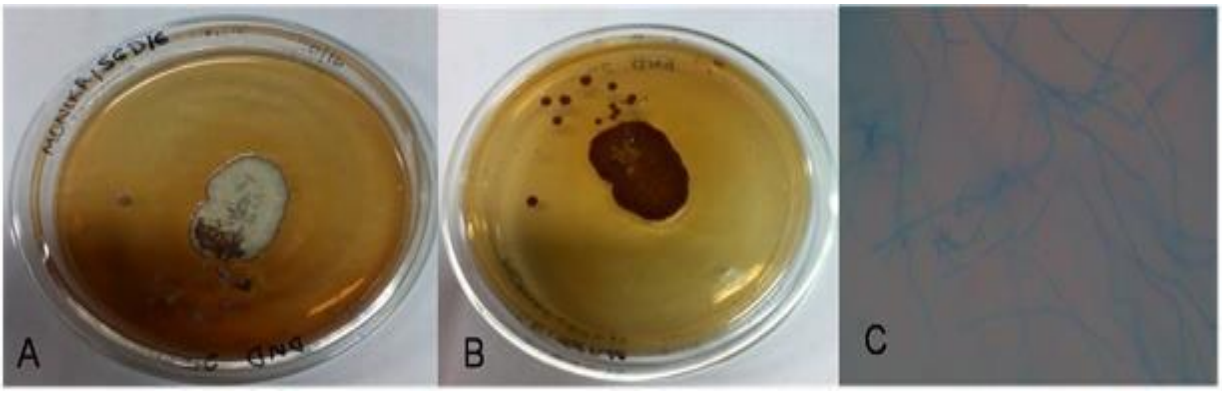

Figure 7.17: Isolate S4D16 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

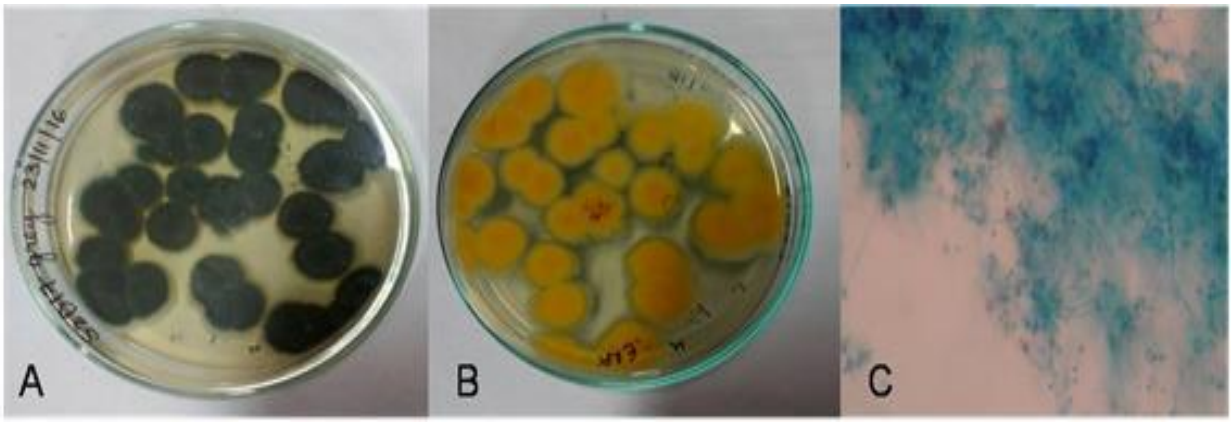

Figure 7.18: Isolate S2D17 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

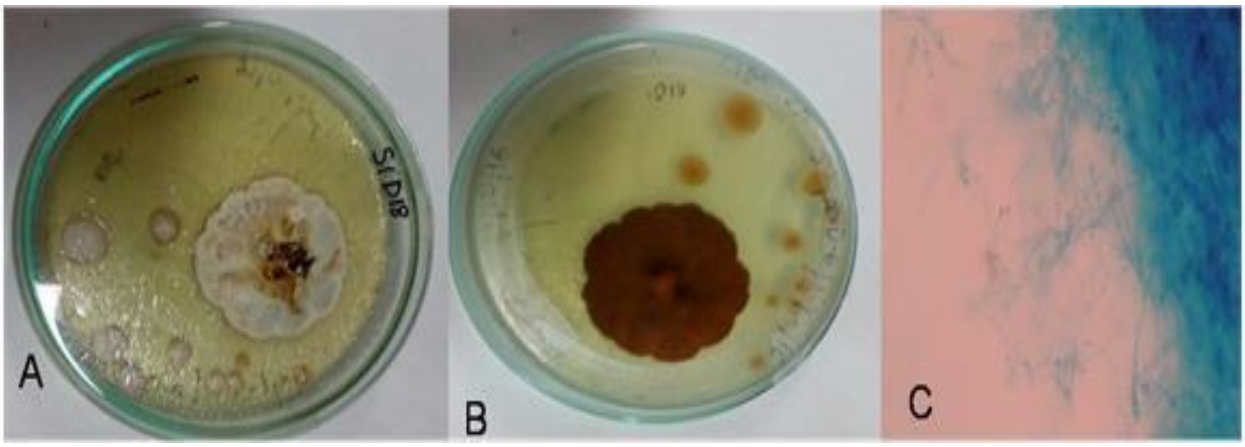

Figure 7.19: Isolate S1D18 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

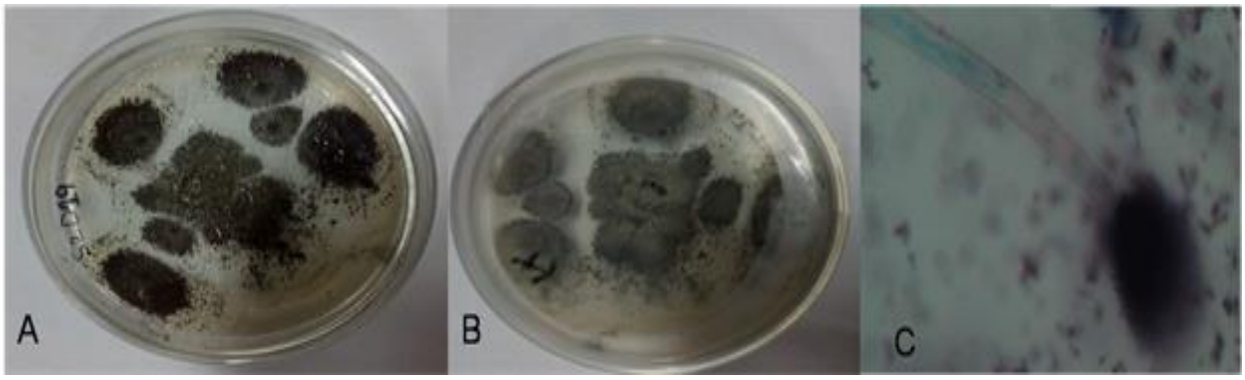

Figure 7.20: Isolate S2D19 A) Front view of Petri Plate B) Back View of Petri Plate C) LPCB Mount (40 x)

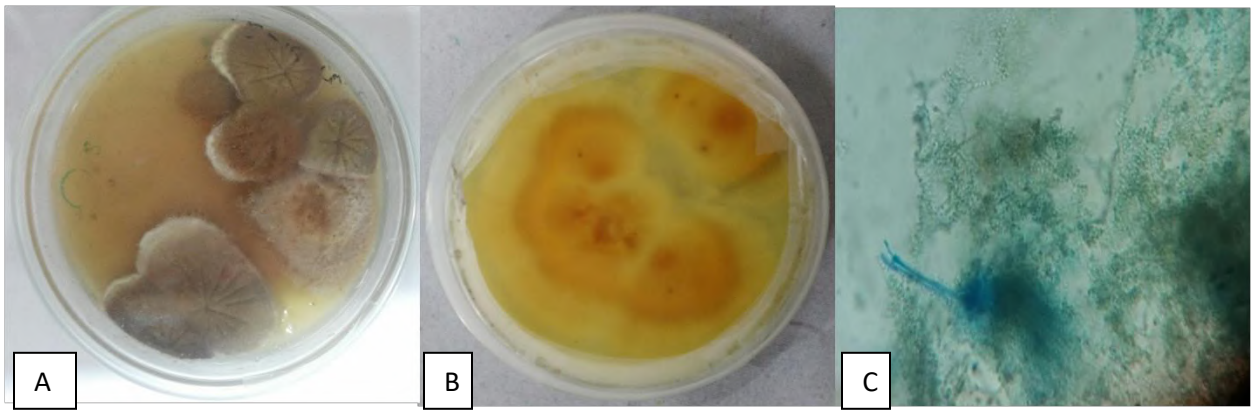

Figure 7.21: Isolate S4D20 A) Front view of Petri Plate B) Back View of Petri Plate C)LPCB Mount (40 x)

7.2.1 Morphology of the antagonistic isolates

The details of the morphological samples are presented in Table no.7.2.

Table no.7.2 Characteristics of fungal isolates

Sample No.	Isolates	Morphological features
Sample no.1	S1D1	Grey coloured round colony with creamish yellow colour at the base.
	S1D4	Green coloured spores with yellow-greenish base.
	S1D6	Grey coloured round colony with greenish tinge and yellow colour at the base.
	S1D18	Shiny White coloured colony with dark brown colour at the base.
Sample no.2	S2D3	Greenish coloured dry colony with red pigment at the base.
	S2D9	Sporulated black colony with black veined round pattern at base.
	S2D13	White coloured velvety round colony with dark brown veined base.
	S2D14	White coloured dry round colony with brownish base.
	S2d15	White coloured round colony.
	S2D17	Grey coloured round colony with dark center, base is yellow coloured.
	S2D19	Black Coloured sporulating colony, Creamish mix blackish base.
Sample	S3D2	Brown coloured velvety colony with brownish colour at the base.

no.3	S3D5	Green coloured spores with yellow-orangish colour at the base.
Sample no.4	S4D7	Green coloured round colony with yellow colour at the base.
	S4D8	White and greenish velvety colonies with yellow base
	S4D10	Light green sporulating colonies with creamish yellow and green base.
	S4D11	Light greenish sporulating colony and pink colour velvety part at the center.
	S4D12	White colored dry colony.
	S4D16	Creamish white coloured dry colony with dark brown base.
	S4D20	Cottony white coloured colony with creamish yellow base.

7.3 Production of Extracellular and Intracellular material

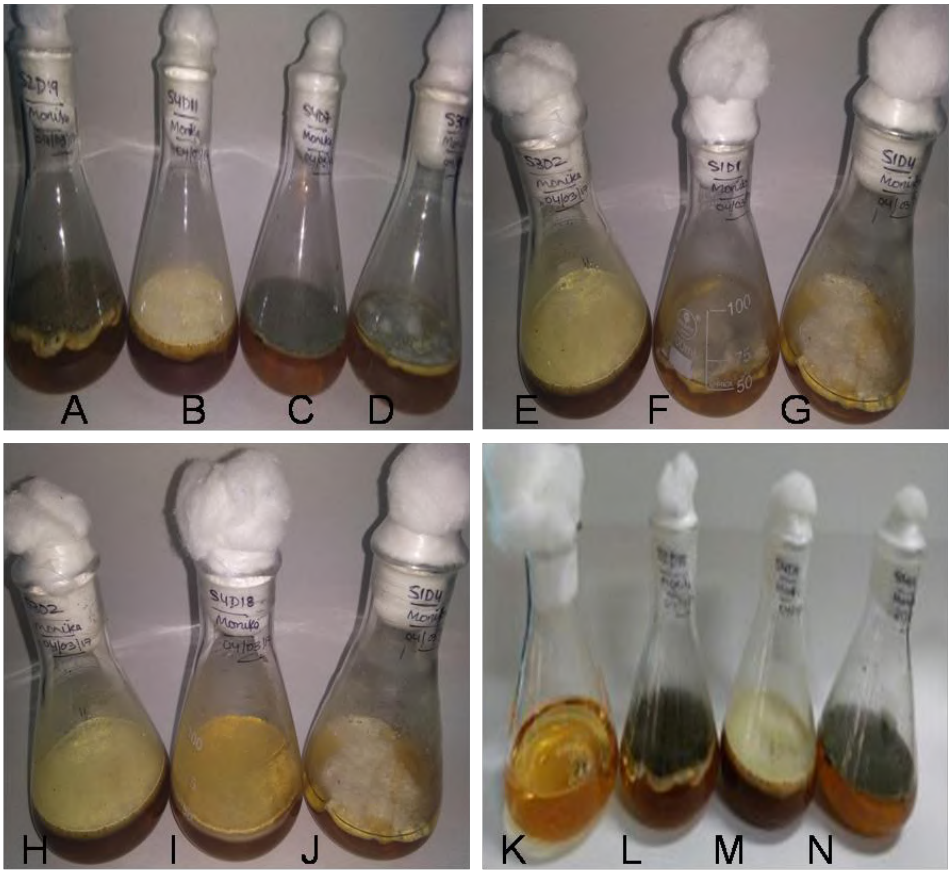

Figure 7.22: Here, It represents Fungal Isolates grown in Potato Dextrose Broth (PDB), Here A)S2D19 B)S4D11 C)S4D7 D)S3D5 E)S3D2 F)S1D1 G)S1D4 H)S2D13 I)S4D18 J)S2D14 K)Control L)S2D9 M)S2D15 N)S4D12

7.4 Silver Nanoparticle synthesis

Among all the fungal isolates which are isolated from the soil; only S2D3, S1D4 and S4D7 are showing colour change due to Silver Nanoparticle production. Change in Colour and UV peak is represented in figure 7.23, 7.24 and 7.25.

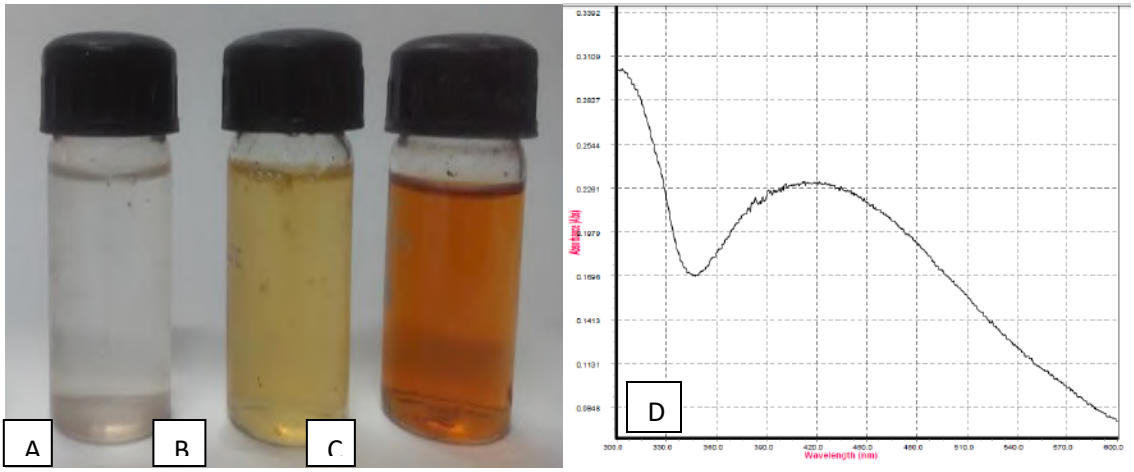

Figure7.23: Isolate S1D4 shows colour changes as: A) Solution of $AgNO_3$B) Intracellular material of isolate C) Silver Np solution formed by the isolate D) UV-Vis Peak at 420nm

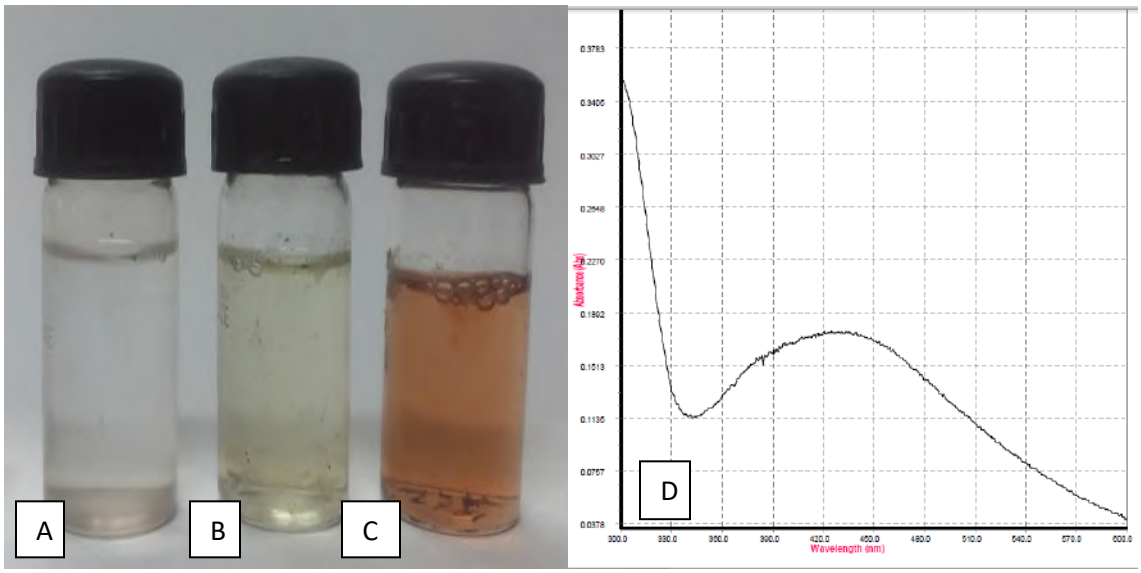

Figure 7.24: Isolate S4D7 shows colour changes as: A) Solution of $AgNO_3$B) Intracellular material of isolate C) Silver Np solution formed by the isolate D)UV-Vis Peak at 420nm

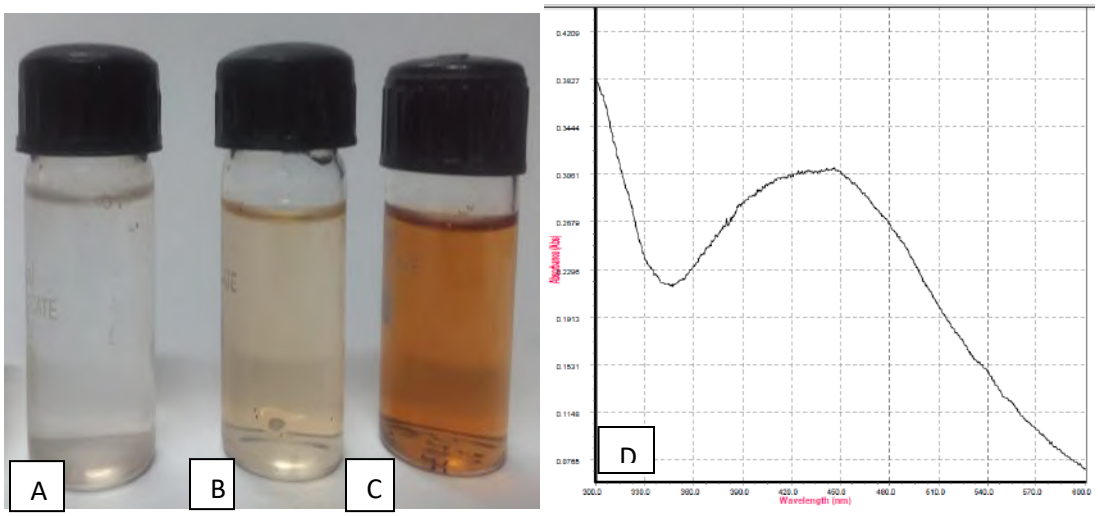

Figure7.25: Isolate S2D3 shows colour changes as: A) Solution of $AgNO_3$B) Intracellular material of isolate C) Silver Np solution formed by the isolate D)UV-Vis Peak at 420nm

7.5 Screening of antagonistic activity

Well Diffusion method

Zone of Inhibition is formed which shows the antimicrobial activity of the fungal isolate. Among all the fungal isolates; Extracellular Fungal Extract of Isolate coded as S4D7 and S2D3 have shown antagonistic activity towards *C.lunata* as shown in figure 7.26 and Table no.7.5 Zone can be measured by using scale.

Table 7.3 Antagonistic activity by the Well Diffusion Method.

	Zone of Inhibition (mm)		
	S4D7	S2D3	S1D4
Extracellular	25±1.0	16.5±1.52	0.0±0.0
Intracellular	0.0±0.0	0.0±0.0	0.0±0.0

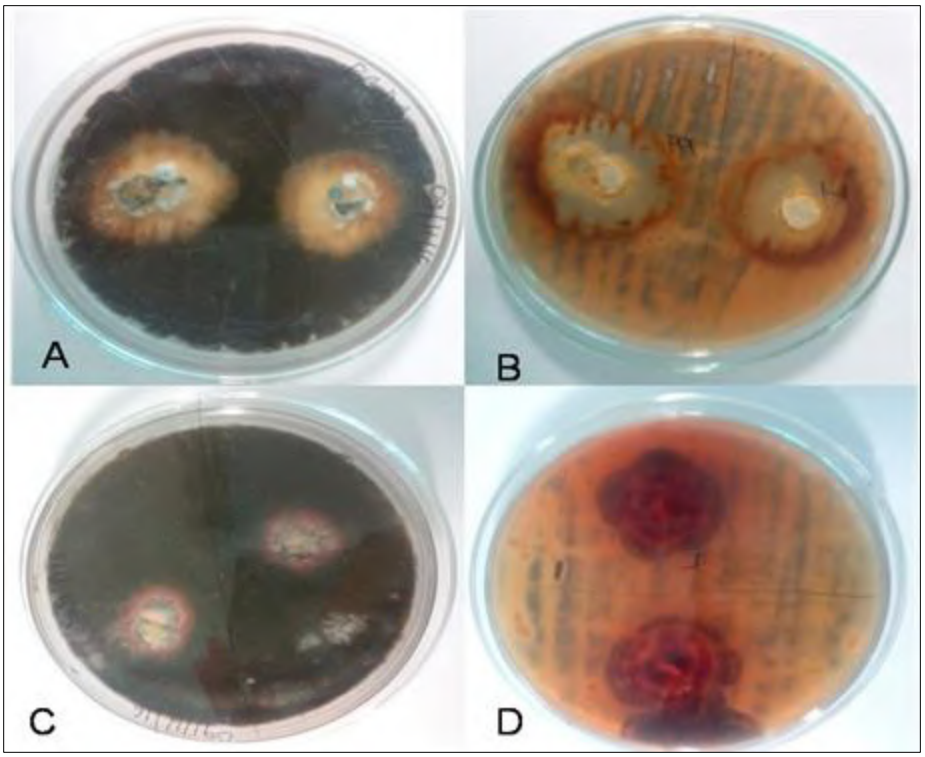

Figure 7.26: Antimicrobial Activity in S4D7 sample A) Front side of petriplate B) Back side of petriplate. Antimicrobial Activity in S4D18 sample C) Front side of petri plate D) Backside of petriplate

7.6 Antifungal Activity of Silver Nanoparticles

Among the isolates S2D3 and S4D7; only the Silver Nanoparticles formed from S4D7 shows Antifungal Activity against *Curvularia lunata.*

Table 7.4 Zone of Inhibition formed by Silver Nanoparticles against *Curvularia lunata*

Isolate	**Zone of Inhibition (mm) Mean± SD**
S2D3	07.66±1.52
S4D7	21.33±3.05
Control (Fluconazole)	35.00±1.00

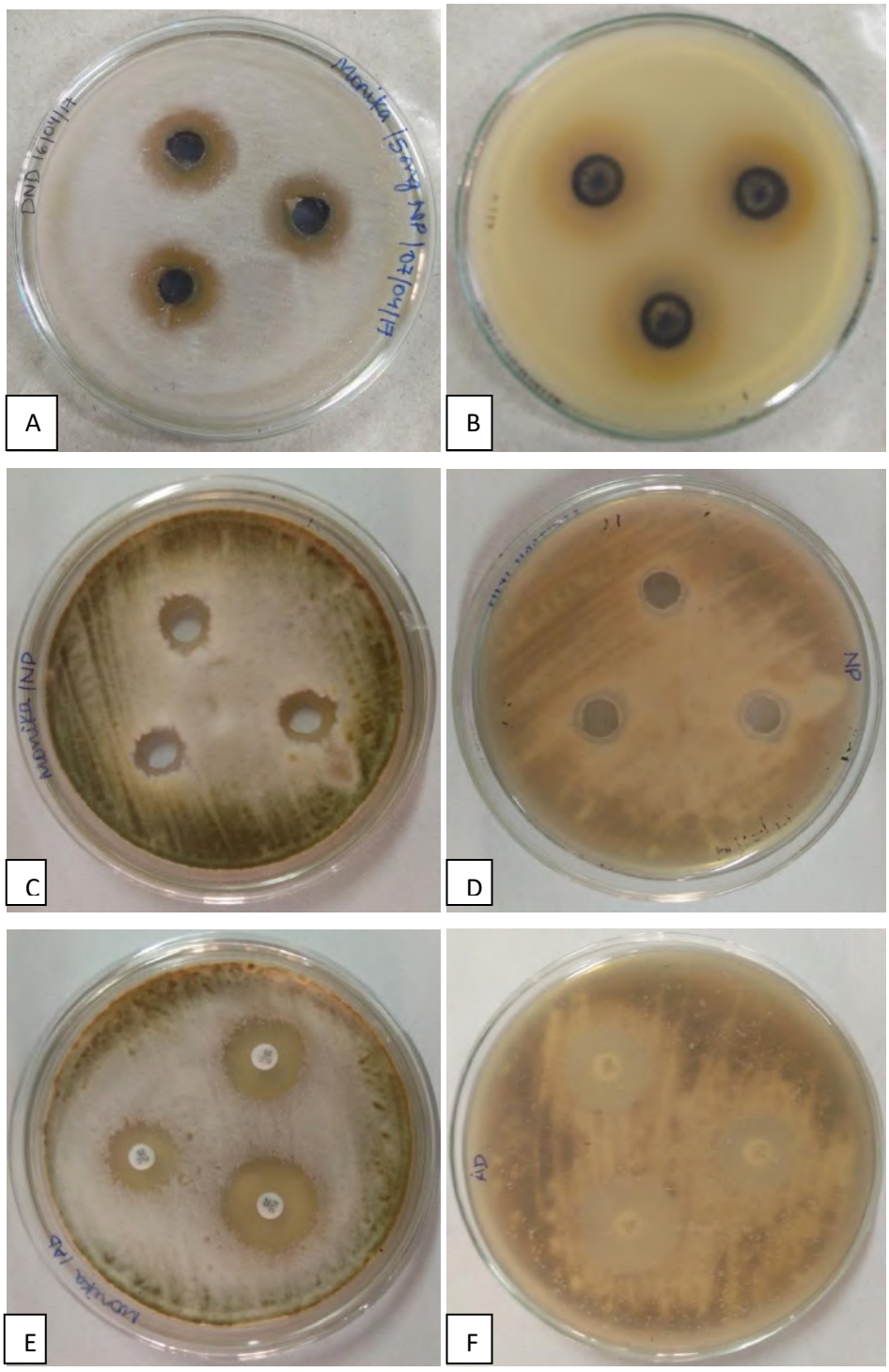

Figure 7.27: Antimicrobial Activity of Silver Nanoparticles (5mg) against *Curvularia lunata*.Here, S4D7 A) Front view of petri plate B) Back view of Petri Plate; S2D3 C) Front view of petri plate D) Back view of Petri Plate; Control (Fluconazole disc 25µg/disc) E) Front view of petri plate F) Back view of Petri Plate

7.7 Other Activities of AgNPs

7.7.1 Anti-Oxidant activity

7.7.1.1 DPPH Radical Scavenging Activity

Colour changes from deep purple to yellow which confirms the presence of antioxidant activity in all three Intracellular fungal extract, Extracellular fungal extract and Silver nanoparticles. It shows dose dependent increase according to the increase in concentration (125, 250, 500 µg/ml).

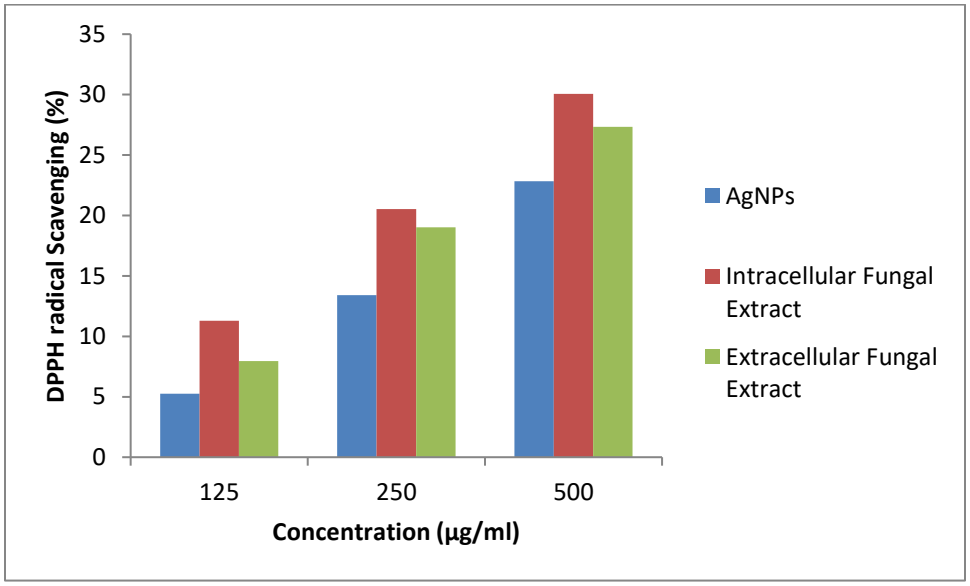

Figure 7.28 Graph representing DPPH Radical Scavenging Activity.

7.7.1.2 Iron chelating activity

The result obtained showed the Dose dependent increase with the increase in Concentration (250, 500,1000 µg/ml) i.e. Biogenic silver nanoparticles showed higher iron chelatingactivity followed by Extracellular fungal extract and Intracellular fungal extract. Data issummarized in figure

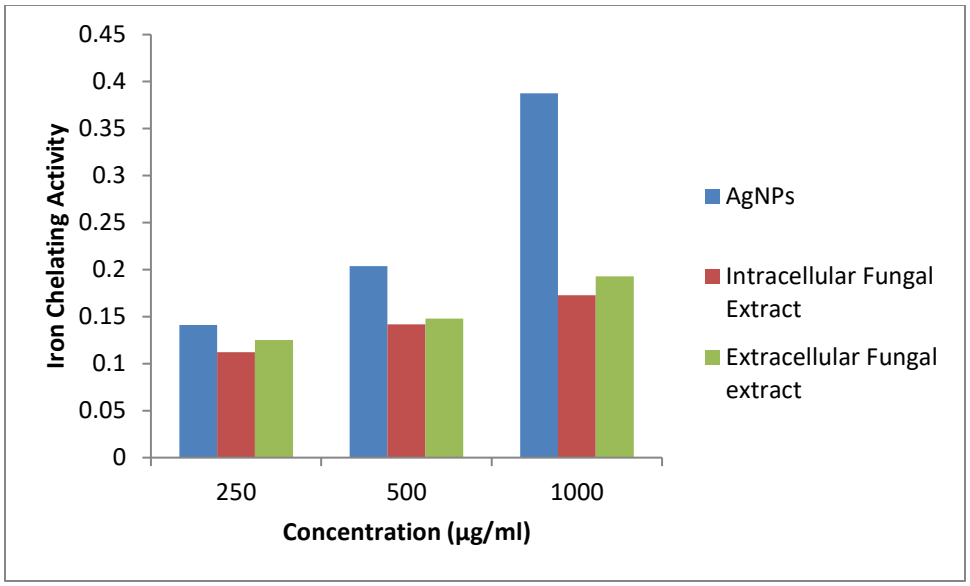

Figure 7.29 Graph representing Iron Chelating Activity.

7.7.2 Antibacterial Activity

Silver Nanoparticles shows maximum Zone of Inhibition against *Pseudomonas aeruginosa* (MTCC4673) i.e. 2.0±0.7mm; then *Streptococcus pyogens* (MTCC442); *E.coli* (MTCC1678) and minimum Zone of Inhibition was observed by *Bacillus cereus* (MTCC11456). It is presented in figure 7.30 and Table 7.5.

Table 7.5 Antibacterial Activity shown by different bacterial cultures.

Name of the organism	**Zone of Inhibition (mm)**
Bacillus cereus (MTCC11456)	1.6±0.9
Escherichia coli (MTCC1678)	1.7±0.5
Pseudomonas aeruginosa (MTCC4673)	2.0±0.7
Streptococcus pyogens (MTCC442)	1.8±1.0

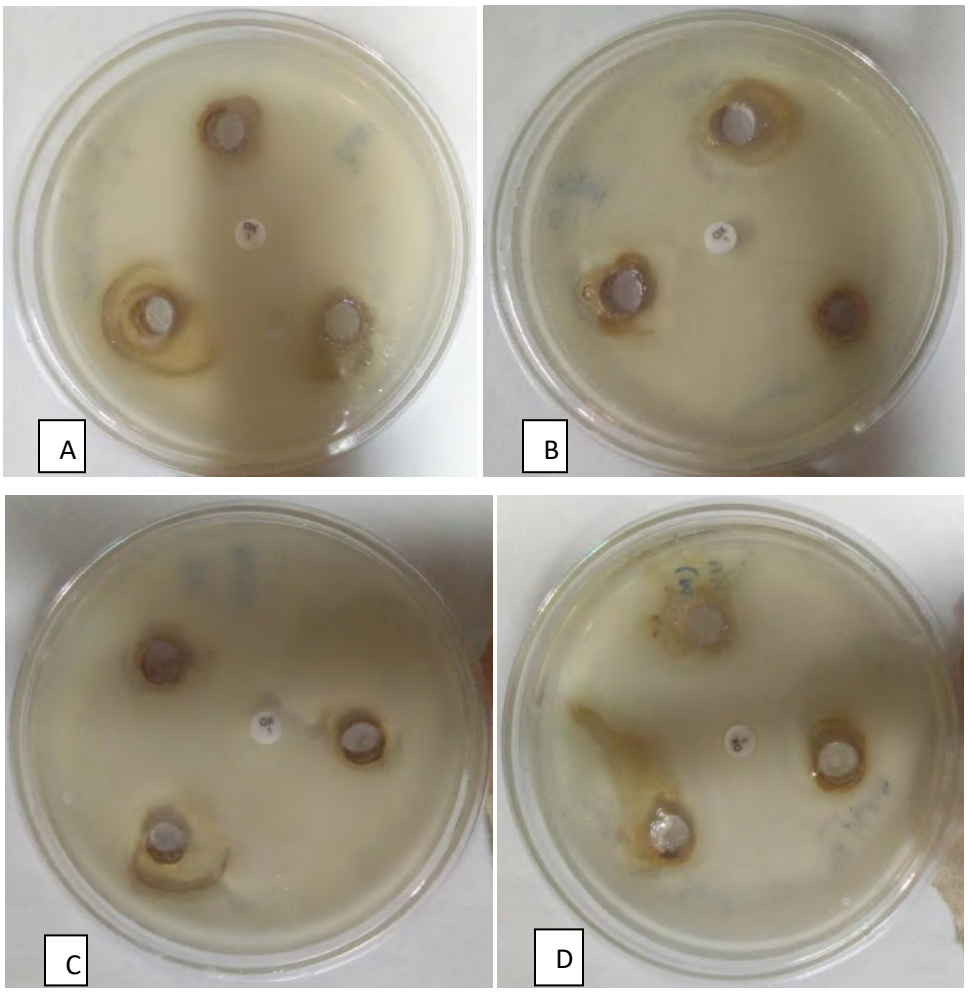

Figure7.30 Antibacterial Activity of AgNPs Here, A) *Bacillus cereus* (MTCC11456) B) *E.coli* (MTCC1678) C) *Pseudomonas aeruginosa* (MTCC4673) D) *Streptococcus pyogens* (MTCC442)

7.8 Effect of Stability

The effect of Stability of AgNPs is checked after 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs. It was observed that Silver nanoparticles gives best peak at 420nm after 48 hrs. Silver Nanoparticles were found to be stable even after 120 hrs. It is represented in figure 7.31.

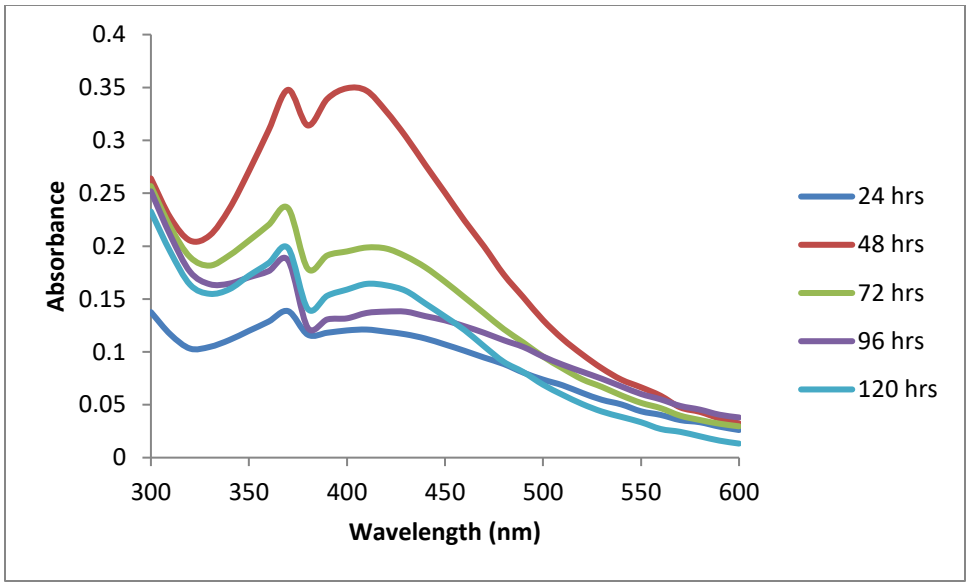

Figure 7.31 Effect of Stability

7.9 Effect of substrate concentration and physical factors on nanoparticle synthesis

7.9.1 Effect of Concentration of Silver Nitrate (AgNO3)

The Maximum Concentration involved i.e. 5mM shows dark brown colour and then colour intensity gets lighter as there is decrease in Conc. i.e 4mM, 3mM, 2mM, 1mM respectively. The UV-Visible spectra shows best peak at 5mM i.e. at 420nm while there is slight deviation of peak at 4mM, 3mM, 2mM, 1mM respectively. Change in Colour and UV-Vis Spectra are represented in figure 7.32 and 7.33 respectively.

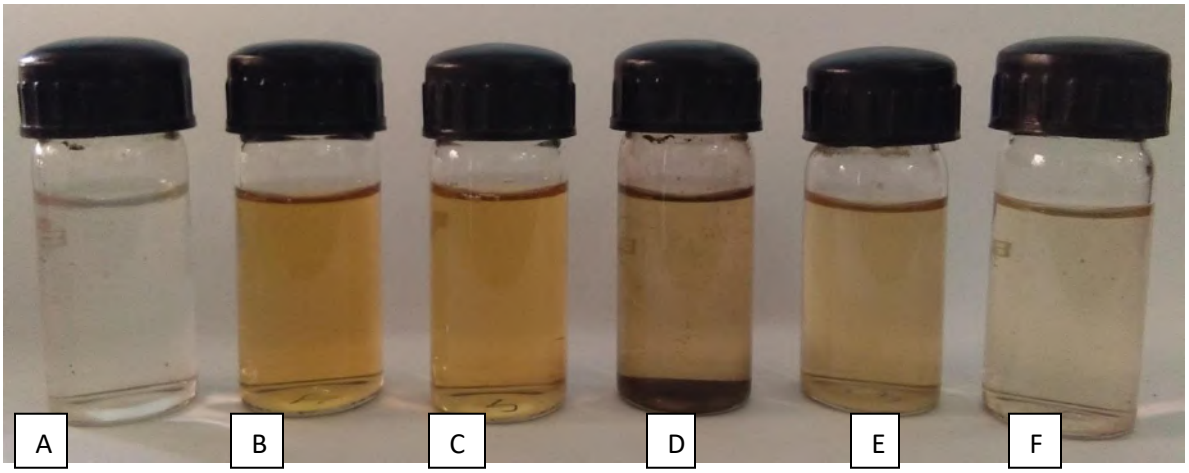

Figure7.32: Effect of Concentration of $AgNO_3$Here, A) Silver Nitrate Solution, B) 5mM C) 4mM D) 3mM E) 2mM F) 1mM

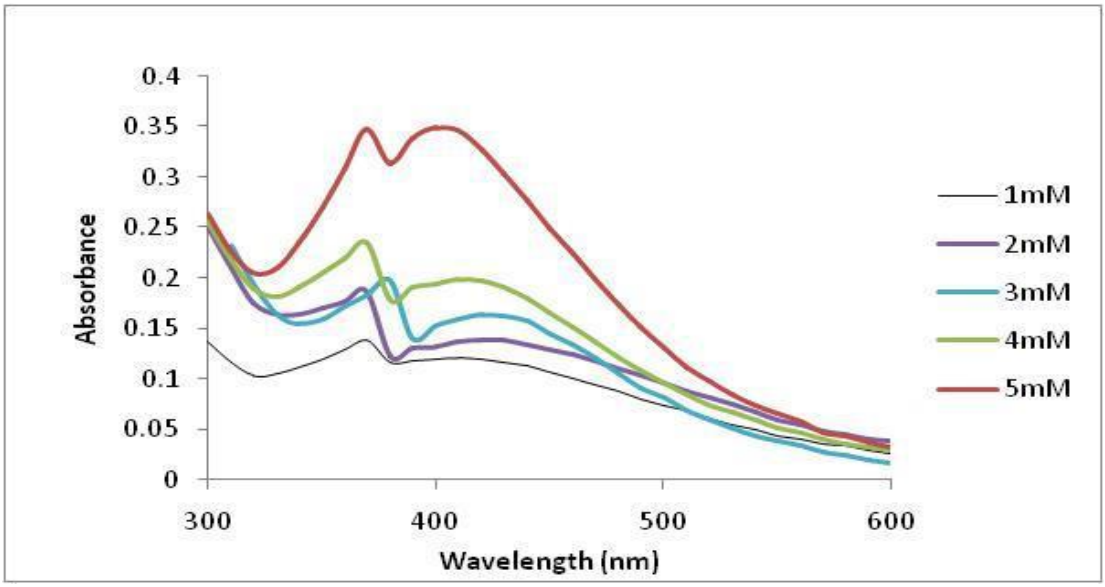

Figure 7.33: Graph representing the Different Conc. Of AgNO3(1mM, 2mM, 3mM, 4mM, 5mM).

7.9.2 Effect of Incubation temperature

The maximum colour intensity is shown at 27^0C i.e. it gives dark colour. While there is decrease in Colour Intensity at 37ºC and 47ºC. The UV-Visible spectra shows best peak at 27ºC i.e. at 420nm.Change in Colour and UV-Vis Spectra are represented in figure 7.34 and 7.35 respectively.

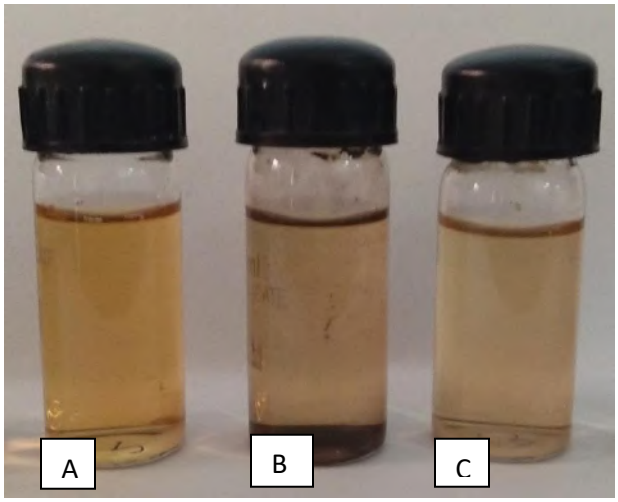

Figure 7.34 Effect of incubation temperature A) At 27ºC B) At 37ºC C) At 47ºC

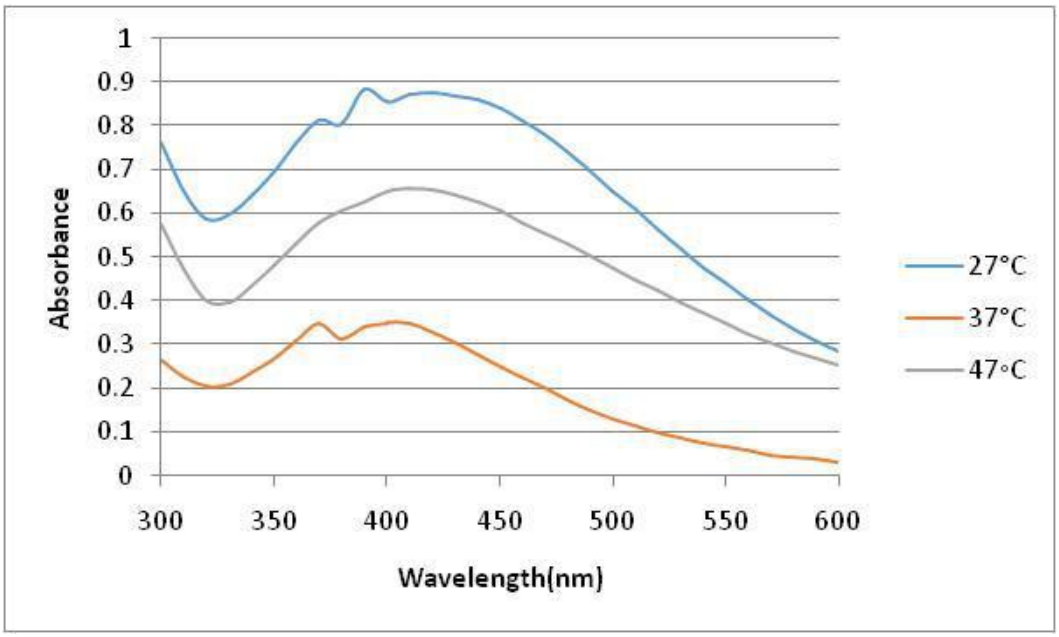

Figure7.35 :Graph representing Effect of different Incubation temperature

7.9.3 Effect of pH

The maximum colour intensity is shown at pH 7 i.e. it gives dark colour. While there is decrease in Colour Intensity at pH 4,6 and 8. The UV-Visible spectra shows best peak at pH 7 i.e. at 420nm. Change in Colour and UV-Vis Spectra are represented in figure 7.36 and 7.37 respectively.

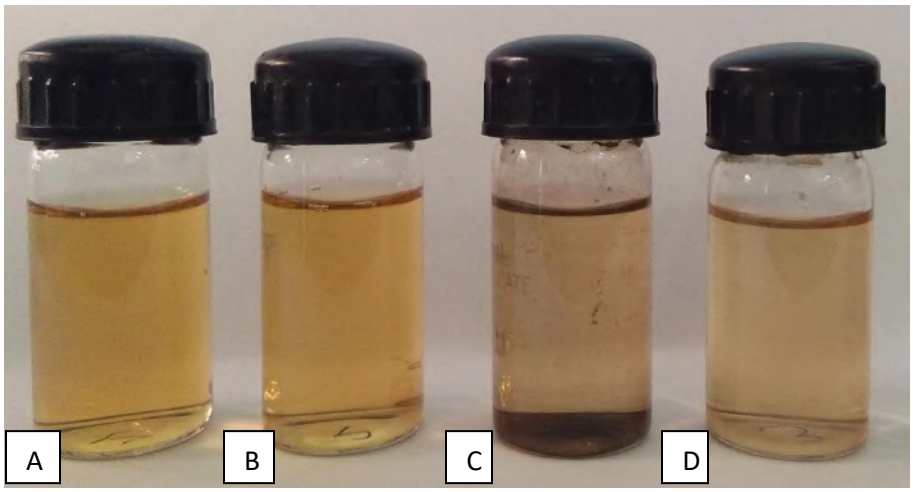

Figure 7.36 : Effect of pH A) pH4 B) pH6 C) pH7 D) pH8

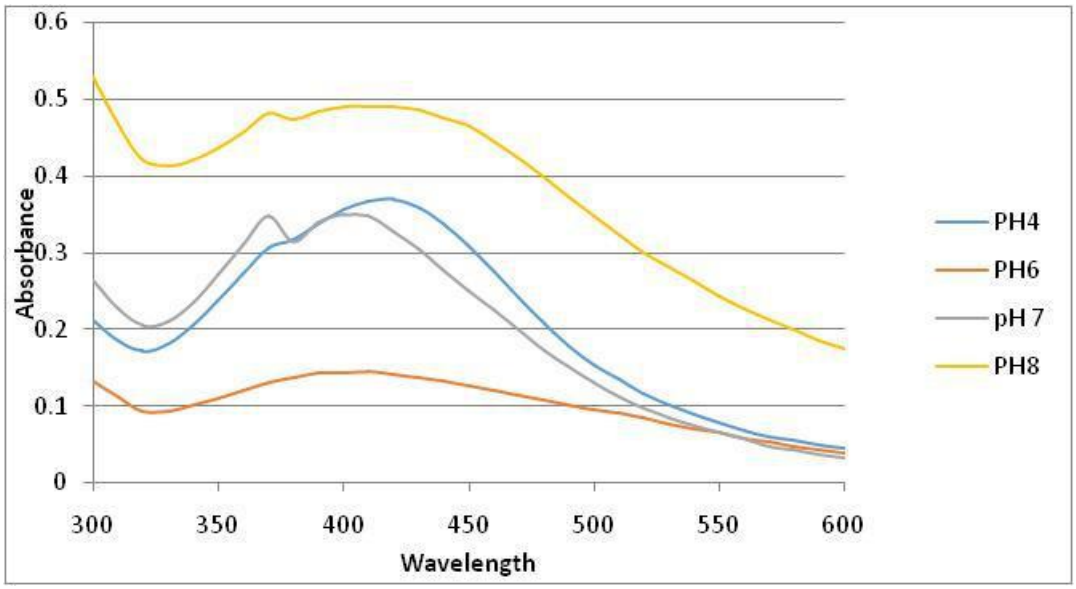

Figure 7.37 : Graph representing Effect of pH

7.10 Characterization of nanoparticles

7.10.1 FT-IR Spectra

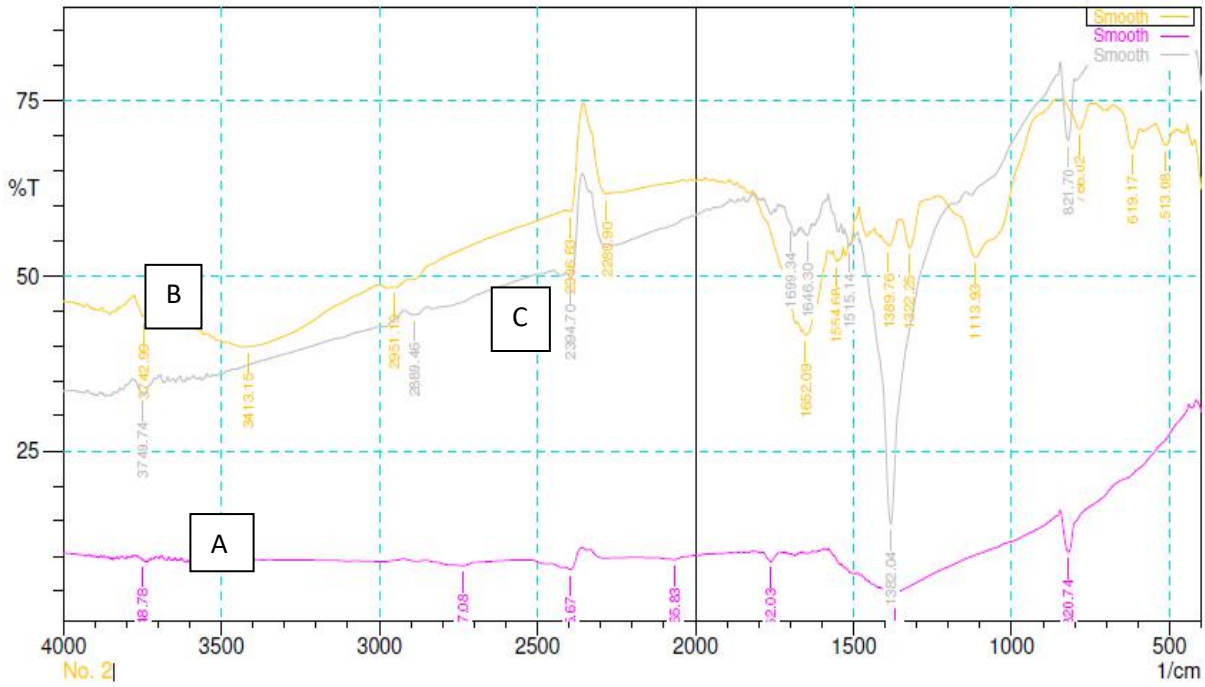

Figure7.38 FT-IR spectrum of S4D7 Sample

The FT-IR spectrum of samples of Silver nanoparticles is influenced by the particle size and morphology. The FTIR spectra for silver nitrate (Control), Intracellular and Synthesized Silver Nanoparticles are shown in figure 7.38. The synthesized silver nanoparticles has peaks at 3749.74 of functional group Alcohol (O-H); 2889.46.6 of C-H Stretch which shows methylene group; 1646.3 of C=C; 3413.15 shows N-H stretch; 1515.14 of functional group Aromatic skeletal (lignin and lignocelluloses); 2951.19 shows narrow stretching of C-H. These peaks have combined characteristics of Silver nitrate and fungal strain, therefore showing more importance of functional groups on the synthesis of silver nanoparticles. The intracellular was used in FTIR analysis, which concluded that the intracellular sample contained biological compounds responsible for the presence of fungus. Presence of the bands at 1699.34, 1646.3, 821.7 and 619.17 represents the presence of N-H functional group of proteins, which is the principle involved in the reduction of AgNO3 into SNPs and their capping. The intracellular has peaks at 3413.15 of functional group Amine (N-H); 2951.19 of Alkane (C-H); 2397.63 of Nitriles (C=N); 1652.09 of Alkene (C=C); 1113.93 of Alkyl halide (C-Cl); 619.17 of Alkyl halide (C-Cl).

7.10.2 Transmission Electron Microscopy (TEM)

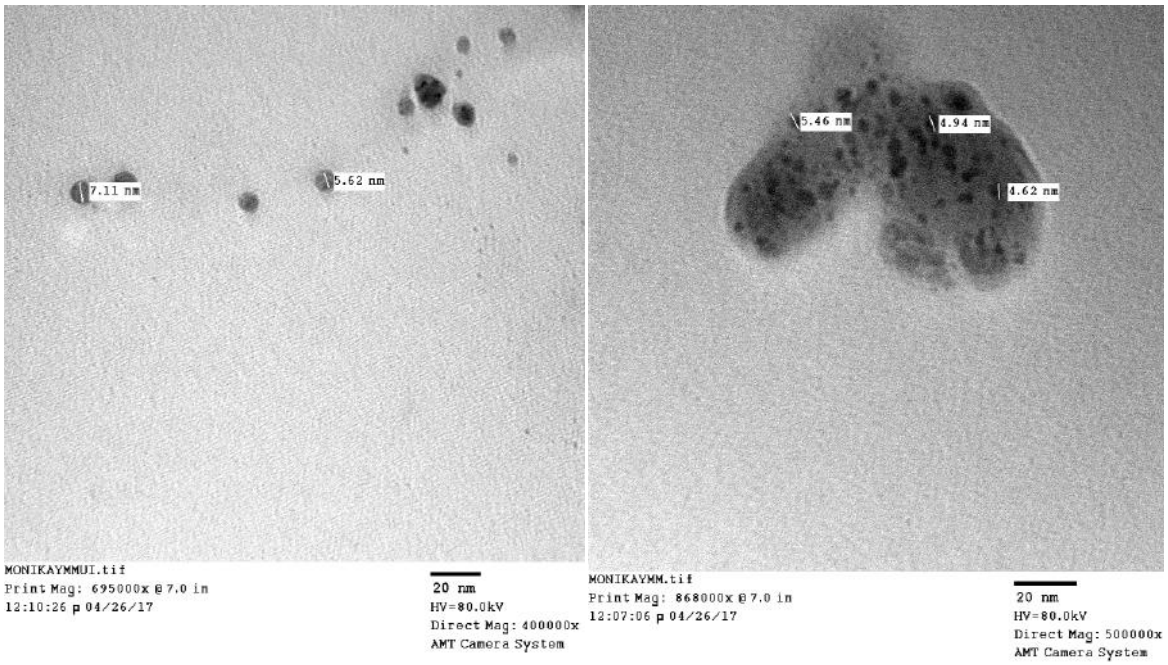

Figure 7.39: Transmission electron microscopic images of the obtained silver nanoparticles.

TEM microscopic images are shown in figure 7.39. It was found that the silver nanoparticles obtained were spherical in shape with particle size ranging from 3nm- 8nm.

7.10.3 Dynamic light scattering analysis (DLS)

The aggregation state of the silver nanoparticle was measured by Dynamic light scattering(DLS) to know their effective size particles in a solution. Results are presented in Figure.7.40. The data shows that the solution is having size well below 100nm while large particles were also observed that might be due to the aggregation of the particles.

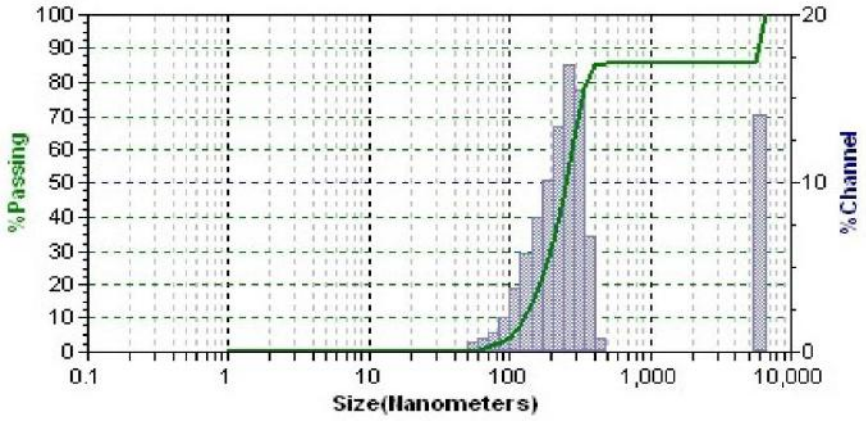

Figure7.40 DLS analysis of biologically synthesized nanoparticles

7.10.4 X-Ray Diffraction (XRD)

The powdered silver nanoparticles show a cubic structure showing peaks at 2Θ: 38.331 (111), 44.488 (200), 64.644 (220). The high intense peak for FFC material is (111), which is observed in the powdered sample. It can be represented in figure 7.41.

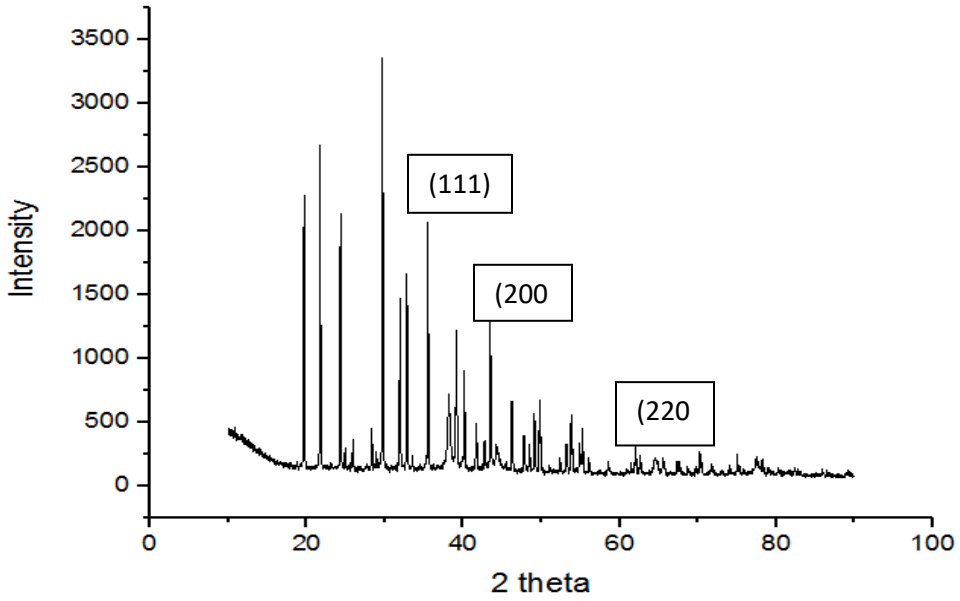

Figure 7.41 Graph representing X-Ray diffraction Pattern of AgNPs.

7.11 Effect on Plant growth

7.11.1 Seed germination test

Growth of Seedlings in Agar petri plates is observed in figure 7.41.

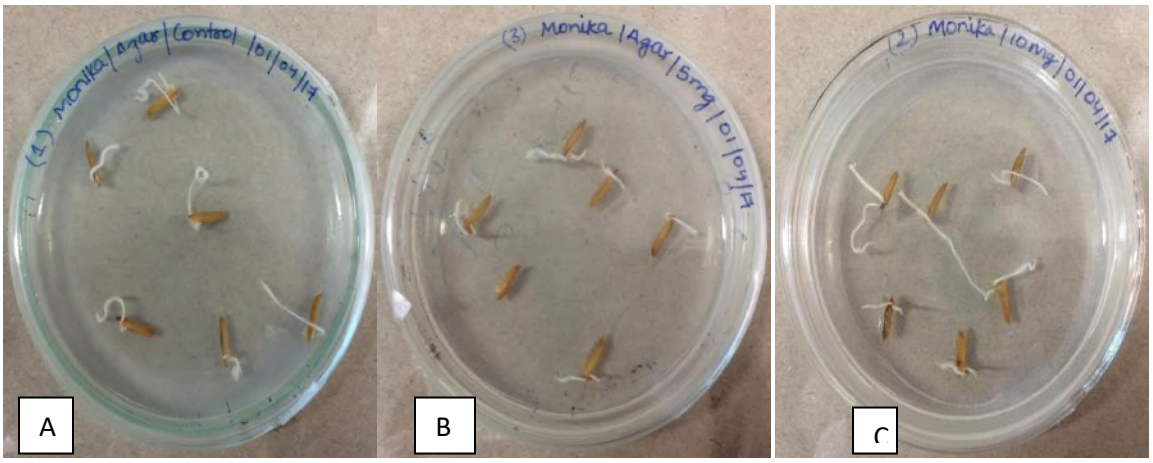

Figure 7.42 Seed Germination Test A) Control B) 5mg C) 10 mg

7.11.1.1 Germination %

The maximum % germination is shown in 10 mg/ml and then 5 mg/ml which is represented in Table 7.10.

Table 7.10: Represents the Germination % of Seedlings at different conc. of NPs

Days	**Seed germination (%)**		
	Control	**5mg**	**10 mg**
1	38.33	22.16	50
2	55	38.83	61
3	77.66	44.33	83.33
4	83.33	88.33	100
5	88.33	94.33	100
6	100	100	100

7.11.1.2 Physical Parameters of Germinated Seedlings

7.11.1.2.1 Root and Shoot length

Germinated Root and Shoot length is presented in figure 7.43. As there is increase in conc. of AgNPs, both root and shoot length increases. Maximum growth is shown in 10 mg/ml then 5mg/ml. Data is summarized in Table 7.11.

7.11.1.2.2 Fresh and Dry Weight

Dry weight of Silver Nanoparticles obtained in 10mg/ml is more as compared to 5mg/ml. It can be compared with the control values. It is represented in Table 7.11.

Table7.11: Physical Parameters of germinated seeds

Treatment Groups	**Shoot length (mm)**	**Root length (mm)**	**Fresh weight (mg)**	**Dry Weight (mg)**
Control	53.6±5.66	65.3±8.16	332.05±8.16	110.63±5.25
5mg/ml	76.8±7.17	82.8±9.75	402.03±6.88	120.46±4.72
10mg/ml	91.2±5.22	96.3±11.9	347.60±5.25	125.86±5.25

Figure 7.43 Germinated Root and Shoot length A) Control B) 5 mg/ml C) 10 mg/ml

7.9.2 Pot Studies

7.10.2 Effect of NPs on Plant growth

Silver nanoparticles showed increase in growth of Root length and Shoot length as compared to the Control as shown in figure 7.46.

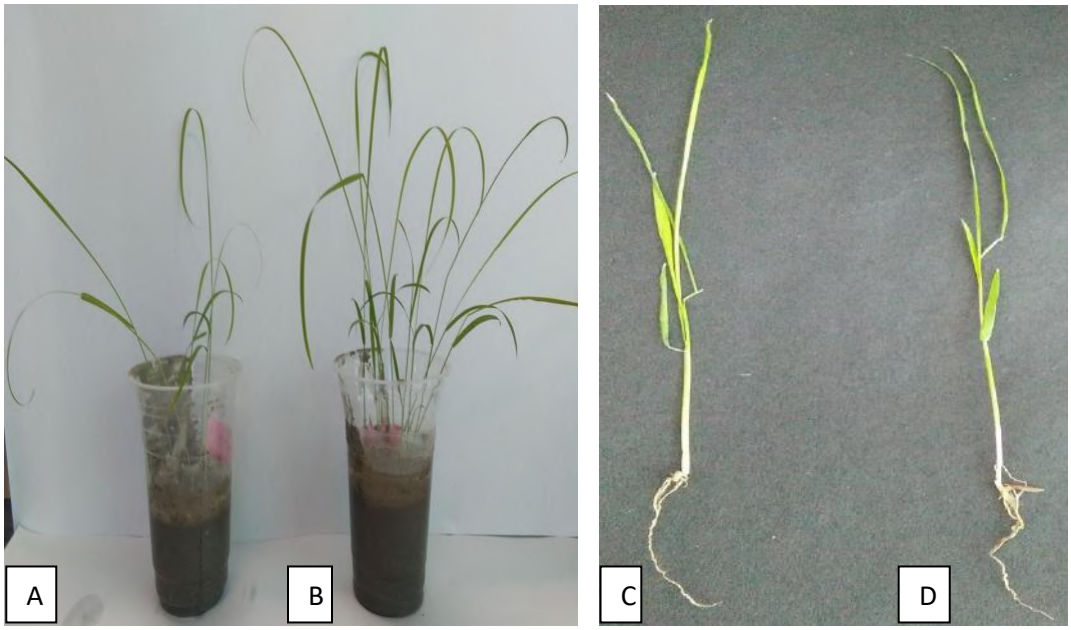

Figure7.43 Effect of NPs on Plant growth A) Control B) NPs ;Uprooted Plant C) Control D) NPs

Table 7.12 Physical Parameters

Treatment groups	**Root length (mm)**	**Shoot length (mm)**	**Fresh Weight (mg)**	**Dry Weight (mg)**
G-1	8.3±4.23	27.6±3.72	166.3±5.32	68.6±3.43
G-2	3.9±1.2	26.9±1.2	182.5±4.23	54.9±1.75

Where G-1 refers to Control group and G-2 refers to SNPs treated group.

7.10.3. Phytochemical Analysis

7.10.3.1Phenolic Compounds Test

The Nanoparticles treated group shows more Phenolic Content than Control group which means that the Silver Nanoparticle treatment is increasing the Phenolic Content of the plant which is considered as growth promoting activity as shown in Table 7.13 and figure 7.44.

Table 7.13 Represents Phenolic Content.

Treatment groups	Phenolic Content (mg/gm)
G-1	2.345562
G-2	2.736095

Where G-1 refers to Control group and G-2 refers to SNPs treated group.

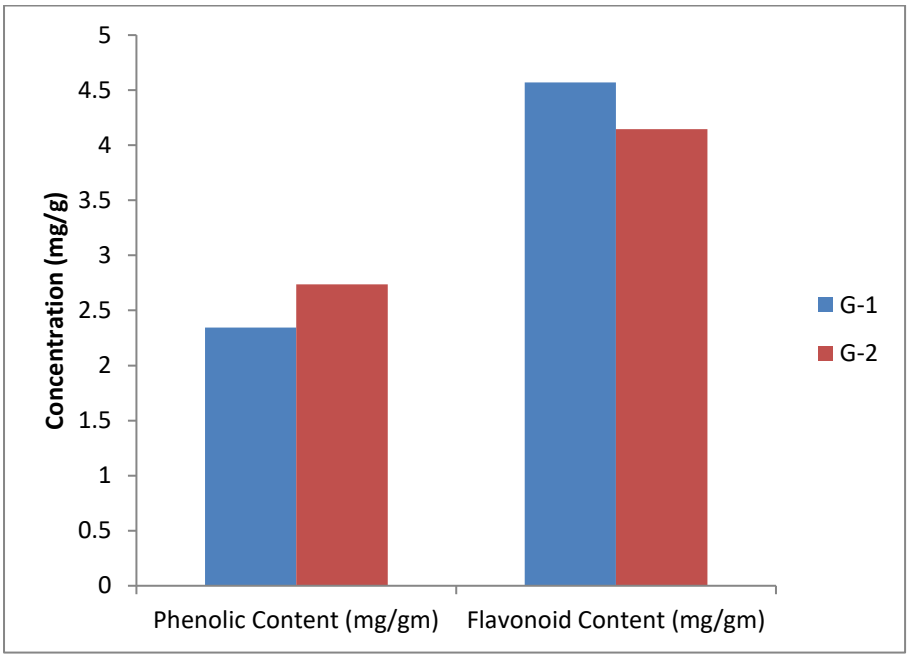

Figure 7.44 Graph represents Phenolic and Flavonoid Content where G-1 refers to Control group and G-2 refers to SNPs treated group.

7.10.3.2 Flavonoid Content

The Nanoparticles treated group shows lesser Flavonoid Content than Control group which means that the Silver Nanoparticle treatment is inhibiting the flavonoid Content as shown in Table 7.14 and figure 7.44.

Table 7.14 Represents Flavonoid Content

Treatment groups	Flavonoid Content (mg/gm)
G-1	4.570588
G-2	4.147059

Where G-1 refers to Control group and G-2 refers to SNPs treated group

7.10.3.3 Chlorophyll Estimation

The Nanoparticles treated group shows more Chlorophyll Content (Chl a, Chl b and Total Chl. Content) than Control group which means that the Silver Nanoparticle treatment is increasing the plant growth which in turn increases the flavonoid Content as shown in Table 7.15 and figure 7.45.

Table 7.15 Represents Chlorophyll Content

Treatment Groups	**Chlorophyll a**	**Chlorophyll b**	**Total Chlorophyll Content**
G-1	25.78	20.41	33.78
G-2	33.78	30.76	39.29

Where G-1 refers to Control group and G-2 refers to SNPs treated group.

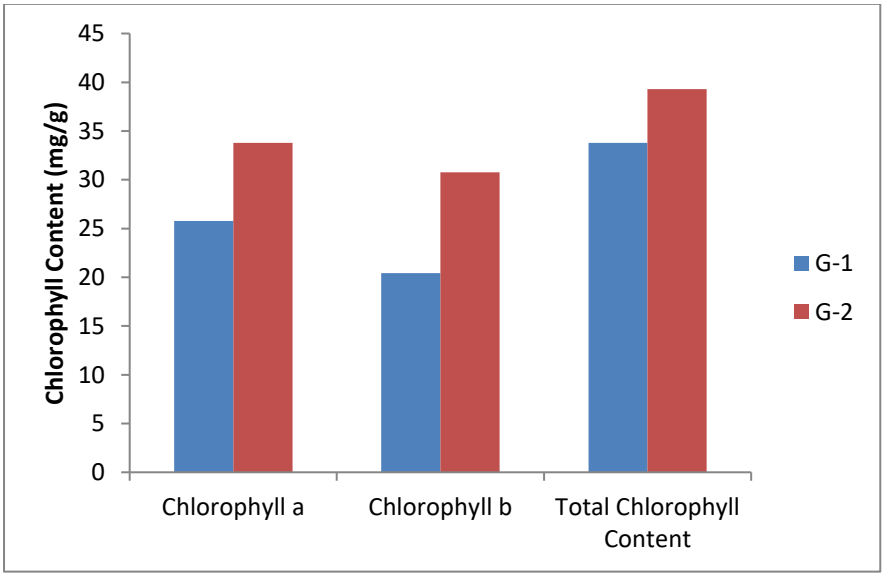

Figure 7.45 Graph represents Chlorophyll Content where G-1 refers to Control group and G-2 refers to SNPs treated group.

7.10.3.4 Carotenoid Content

The Nanoparticles treated group shows more Carotenoid Content than Control group which means that the Silver Nanoparticle treatment is increasing the plant growth which in turn increases the Carotenoid Content of the plant as shown in Table 7.16 and figure 7.46.

Table 7.16 Represents Carotenoid Content

Treatment Groups	Carotenoid Content (mg/g)
G-1	19.75
G-2	25.75

Where G-1 refers to Control group and G-2 refers to SNPs treated group.

7.10.3.5 Protein Content Estimation

The Nanoparticles treated group shows lesser Protein Content than Control group which shows that the Silver Nanoparticle treatment is toxic to the plant which in turn decreases the Protein Content of the plant as shown in Table 7.17 and figure 7.46.

Table 7.17 Represents Protein Content

Treatment Groups	Protein Content (mg/g)
G-1	96.378
G-2	86.543

Where G-1 refers to Control group and G-2 refers to SNPs treated group.

7.10.3.6 Carbohydrate Estimation Test

The Nanoparticles treated group shows lesser Carbohydrate Content than Control group which shows that the Silver Nanoparticle treatment is toxic to the plant which in turn decreases the Carbohydrate Content of the plant as shown in Table 7.18 and figure 7.46.

Table 7.18 Represents Carbohydrate Content

Treatment Groups	Carbohydrate Content (mg/gm)
G1	97.904
G2	88.416

Where G-1 refers to Control group and G-2 refers to SNPs treated group.

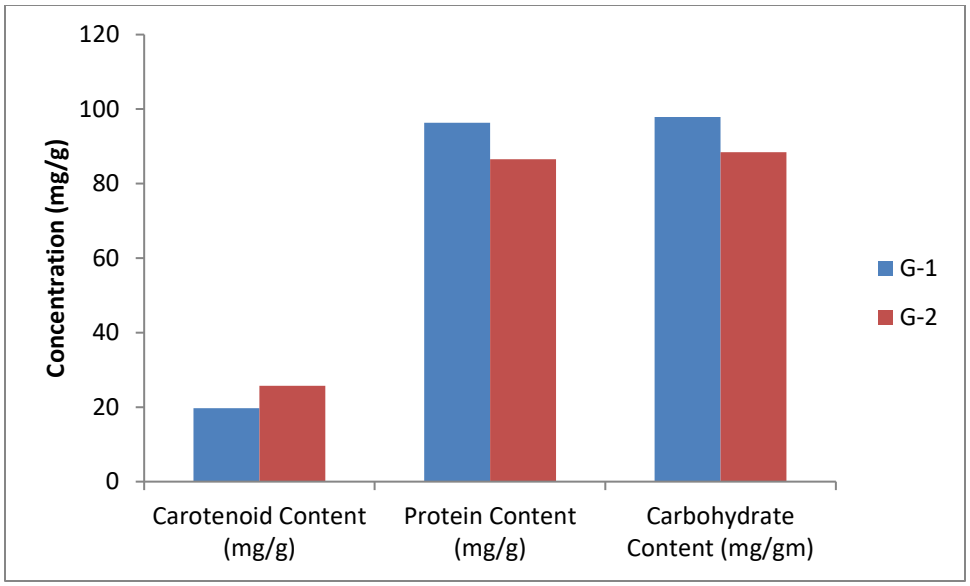

Figure 7.46 Graph represents Carotenoid, Protein and Carbohydrate Content where G-1 refers to Control group and G-2 refers to SNPs treated group.

7.10 Antifungal Activity by plant growth and its Physio-Chemical Analysis

7.11.1 Antifungal Activity of AgNPs

Silver Nanoparticles shows its Antifungal effect on the Plant growth as it is presented in the figure 7.47. *C.lunata* treated plant shows inhibitory growth as compared to the control. While the Nanoparticle treated plant shows more growth than diseased plant and When AgNPs are added to the *C.lunata* treated plant, Antifungal Activity is observed. It means AgNPs are inhibiting the effect of *C.lunata* and more plant growth is observed as compared to *C.lunata* treated plant.

Figure7.47 Antifungal Effect of AgNPs on Plant growth A) Control B) *C.lunata* C) NPs D) *C.lunata* + NPs; Uprooted Plant E) Control F) *C.lunata* G) NPs H) *C.lunata* + NPs

7.10.2 Physical Parameters

Root length shows maximum growth in Control, while least growth in *C.lunata* treated group. Shoot length shows maximum growth in Control. While In Nanoparticles treated group it shows growth more than *C.lunata* treated group. Both fresh weight and Dry weight groups shows maximum in Control. Dry Weight is more in *C.lunata* along with AgNPs treated group which means it shows maximum Biomass content.

Table 7.19 Physical Parameters

Treatment groups	**Root length (mm)**	**Shoot length (mm)**	**Fresh Weight (mg)**	**Dry Weight (mg)**
G-1	8.3±4.2	27.8±3.75	166.3±5.3	68.6±3.4
G-2	4.1±3.7	11.7±1.2	136.3±2.7	53.5±3.5
G-3	6.2±1.2	20.1±3.7	103.1±1.7	30.4±4.2
G-4	7.2±2.7	27.2±1.7	175.3±1.2	66.2±3.2

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

7.10.3 Phytochemical Analysis

7.11.3.1 Phenolic Compounds Test

The Control group shows more Phenolic Content than other groups while *C.lunata* group shows the least Phenolic Content. The Biocontrol fungus along with *C.lunata* treated group shows more Phenolic Content as compared to the *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata* as shown in Table 7.20 and figure 7.47.

Table 7.20 Represents Phenolic Content

Treatment groups	**Phenolic Content (mg/gm)**
G1	2.345562
G2	1.144379
G3	2.48284
G4	2.61538

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

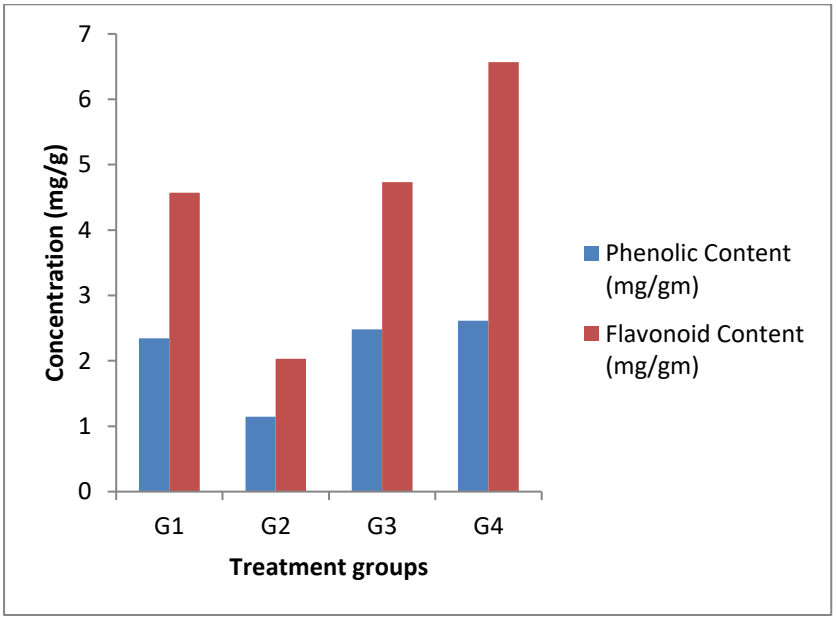

Figure 7.47 Graph represents Phenolic and Flavonoid Content; Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

7.11.3.2 Flavonoids Test

The Biocontrol fungus alongwith *C.lunata* treated group shows more Flavonoid Content than other groups which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Flavonoid Content as compared to the other groups as shown in Table 7.21 and figure 7.47.

Table 7.21 Represents Flavonoid Content

Treatment groups	Flavonoid Content (mg/gm)
G1	4.570588
G2	2.029412
G3	4.735294
G4	6.57

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group

7.11.3.3 Chlorophyll Estimation

The Biocontrol fungus alongwith *C.lunata* treated group shows more Chlorophyll Content than other groups which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Chlorophyll Content as compared to the other groups as shown in Table 7.22 and figure 7.48.

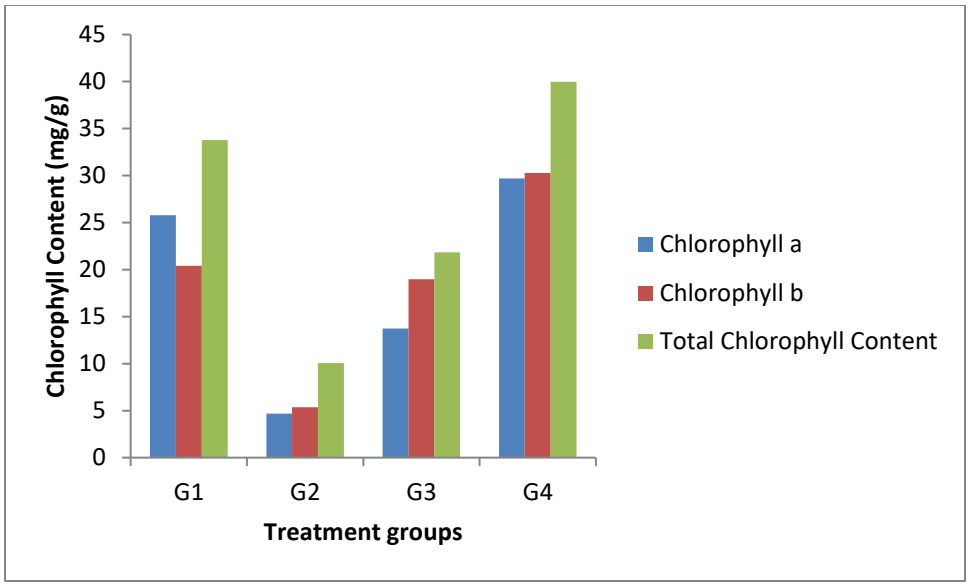

Figure 7.48 Graph represents Chlorophyll Content; Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

Table 7.22 Represents Chlorophyll Content

Different Treatment Groups	**Chlorophyll a**	**Chlorophyll b**	**Total Chlorophyll Content**
G-1	25.7891	20.41352	33.7861
G-2	4.679341	5.376262	10.05236
G-3	13.7341	18.975	21.837084
G-4	29.696631	30.27526	39.96557

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

7.11.3.4 Carotenoid Content

The Biocontrol fungus alongwith *C.lunata* treated group shows more Carotenoid Content than other groups which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Carotenoid Content as compared to the other groups as shown in Table 7.22 and figure 7.49.

Table 7.22 Represents Carotenoid Content

Different Treatment Groups	Carotenoid Content (mg/g)
G-1	19.7532
G-2	9.7325
G-3	10.1143
G-4	23.7561

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

7.11.3.5 Protein Content

The Control group shows more Protein Content than other groups. While the *C.lunata* treated group shows least Protein Content as compared to the other groups as shown in Table 7.23 and figure 7.49.

Table 7.22 Represents Protein Content

Different Treatment Groups	Protein Content (mg/g)
G-1	96.378
G-2	62.786
G-3	90.456
G-4	89.919

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group

7.11.3.6 Carbohydrate Estimation

The Biocontrol fungus alongwith *C.lunata* treated group shows more Carbohydrate Content than *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Carbohydrate Content as compared to the other groups as shown in Table 7.23 and figure 7.49.

Table 7.23 Represents Carbohydrate Content

Different Treatment Groups	Carbohydrate Content(mg/gm)
G1	97.904
G2	65.344
G3	93.328
G4	91.888

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group

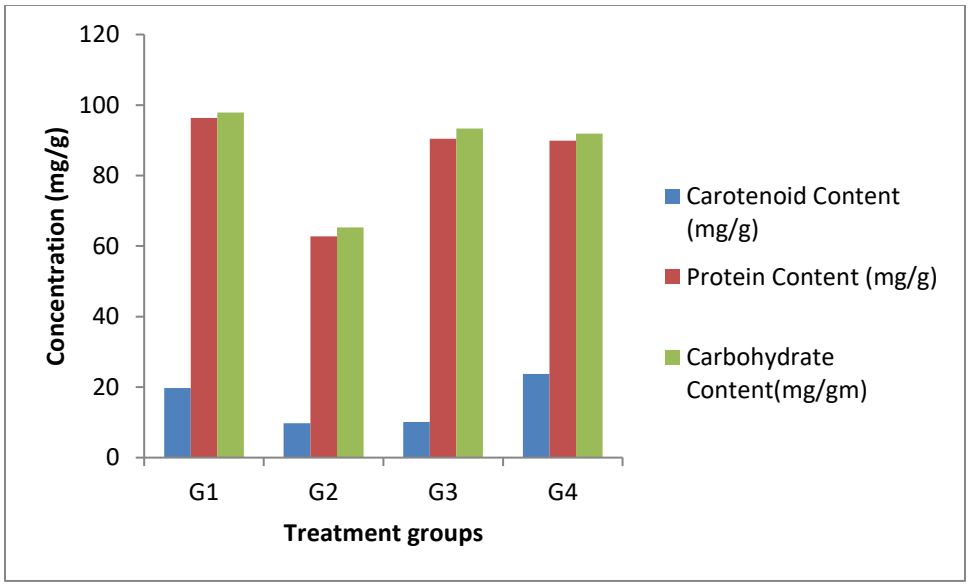

Figure 7.49 Graph represents Carotenoid, Protein and Carbohydrate Content; where Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to AgNPs treated group and G-4 refers to *C.lunata* alongwith AgNPs treated group.

7.11.3.7Antifungal activity of Biocontrol Fungus

Biocontrol Fungus shows its Antifungal effect on the Plant growth as it is presented in the figure 7.48. *C.lunata* treated plant shows inhibitory growth as compared to the control. While the Biocontrol fungus treated plant shows little more growth than diseased plant. When Biocontrol fungus is added to the *C.lunata* treated plant, Antifungal Activity is observed. It means

Biocontrol fungus is inhibiting the effect of *C.lunata* and more plant growth is observed as compared to *C.lunata* treated plant.

Figure 7.50 Effect of Biocontrol Fungus on Plant growth A) Control B) *C.lunata* C) Biocontrol Fungus D) *C.lunata* + Biocontrol fungus; Uprooted Plant E) Control F) *C.lunata* G) Biocontrol Fungus H) *C.lunata* + Biocontrol fungus

7.11.3.8 Physical Parameters

Both Root length and Shoot length shows maximum growth in C.lunata alongwith Biocontrol fungus treated group, while least growth in *C.lunata* treated group. Both fresh weight and Dry weight groups shows maximum in C.lunata alongwith Biocontrol fungus treated group which means it shows maximum Biomass content. Data is summarized in Table 7.24.

Table 7.24 Physical Parameters

Treatment groups	**Root length (mm)**	**Shoot length (mm)**	**Fresh Weight (mg)**	**Dry Weight (mg)**
G-1	8.0±4.2	27.6±3.	166.3±5.3	68.6±3.4
G-2	3.1±3.7	19.7±1.2	136.3±2.7	43.5±3.5
G-3	3.9±1.2	26.9±1.2	182.5±4.2	54.9±1.7
G-4	8.1±1.5	34.5±3.7	389.9±43	87.1±3.5

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group.

7.11.3.9 Phytochemical Tests

7.11.3.10 Phenolic Compounds Test

The Biocontrol fungus alongwith *C.lunata* treated group shows more Phenolic Content than *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Phenolic Content as compared to the other groups as shown in Table 7.25 and figure 7.51.

Table 7.25 Represents Phenolic Compounds.

Treatment Groups	**Phenolic Compounds (mg/gm)**
G1	2.34
G2	1.14
G3	2.68
G4	2.76

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

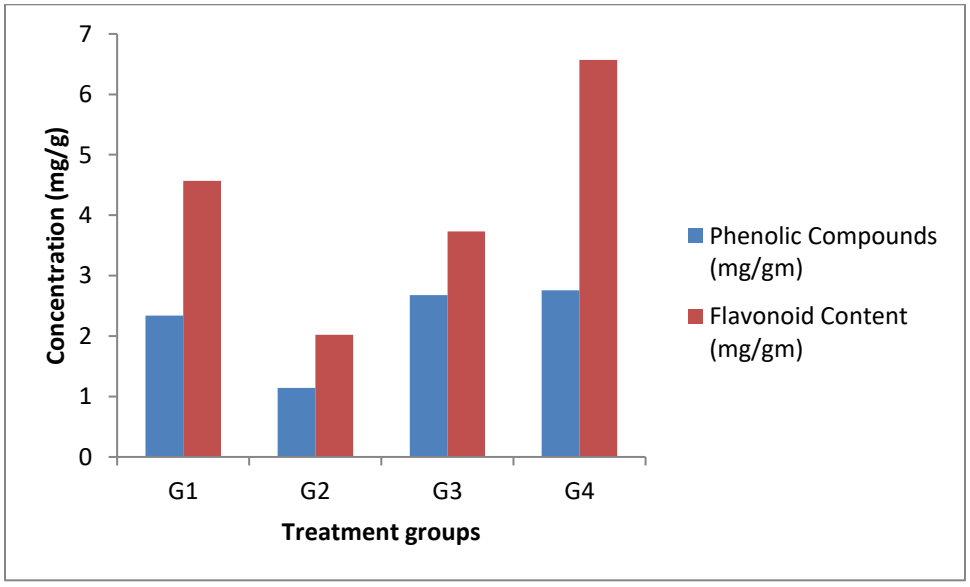

Figure 7.51 Graph represents Phenolic and Flavonoid Content; Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

7.11.3.11 Flavanoids Test

The Biocontrol fungus alongwith *C.lunata* treated group shows more Flavonoid Content than *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Flavonoid Content as compared to the other groups as shown in Table 7.26 and figure 7.51.

Table 7.26 Represents Flavonoid Content

Treatment Groups	Flavonoid Content (mg/gm)
G1	4.57
G2	2.02
G3	3.73
G4	6.57

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

7.11.3.3 Chlorophyll Estimation

The Biocontrol fungus alongwith *C.lunata* treated group shows more Chlorophyll Content than *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Chlorophyll Content as compared to the other groups as shown in Table 7.27 and figure 7.52.

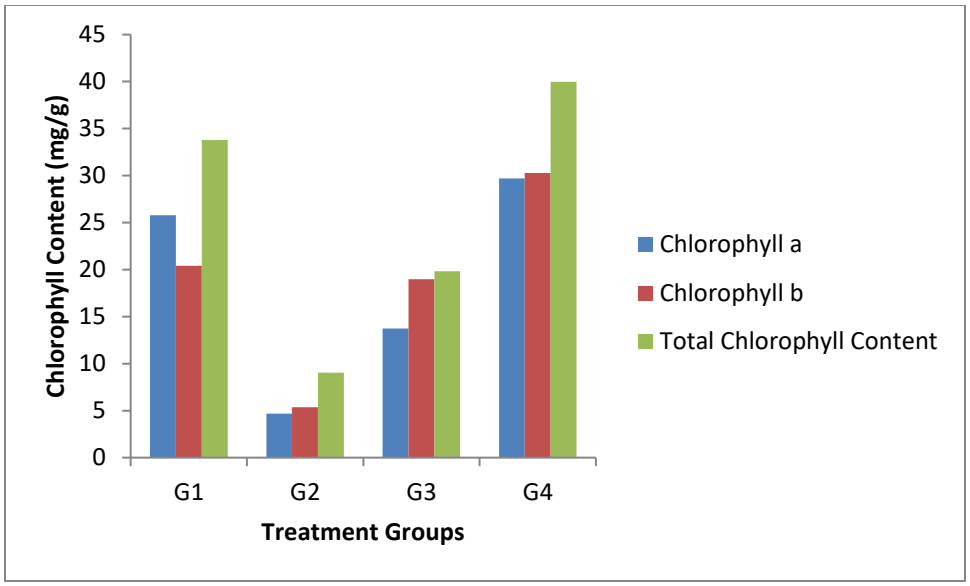

Figure 7.52 Graph represents Chlorophyll Content; Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

Table 7.27 Represents Chlorophyll Content.

Different Treatment Groups	**Chlorophyll a**	**Chlorophyll b**	**Total Chlorophyll Content**
G-1	25.7891	20.41352	33.7861
G-2	4.679341	5.376262	9.05236
G-3	13.7341	18.975	19.837084
G-4	29.696631	30.27526	39.96557

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

7.11.3.4 Carotenoids Content

The Biocontrol fungus alongwith *C.lunata* treated group shows more Carotenoid Content than *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Carotenoid Content as compared to the other groups as shown in Table 7.28 and figure 7.53.

Table 7.28 Represents Carotenoid Content.

Different Treatment Groups	Carotenoids
G-1	19.7532
G-2	9.7325
G-3	10.1143
G-4	23.7561

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

7.11.3.5 Protein Estimation Test

The Control group shows more Protein Content than *C.lunata* treated group .While the *C.lunata* treated group shows least Protein Content as compared to the other groups as shown in Table 7.29 and figure 7.53.

Table 7.29 Represents Protein Content

Different Treatment Groups	Protein Content (mg/g)
G-1	96.378
G-2	62.786
G-3	90.456
G-4	89.919

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

7.11.3.5 Carbohydrate Estimation Test

The Biocontrol fungus alongwith *C.lunata* treated group shows more Carbohydrate Content than *C.lunata* treated group which means that Biocontrol fungus is showing antagonistic activity against *C.lunata*. While the *C.lunata* treated group shows least Carbohydrate Content as compared to the other groups as shown in Table 7.30 and figure 7.53.

Table 7.30 Represents Carbohydrate Content

Different Treatment Groups	Carbohydrate Content (mg/gm)
G1	96.904
G2	75.344
G3	93.328
G4	97.888

Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

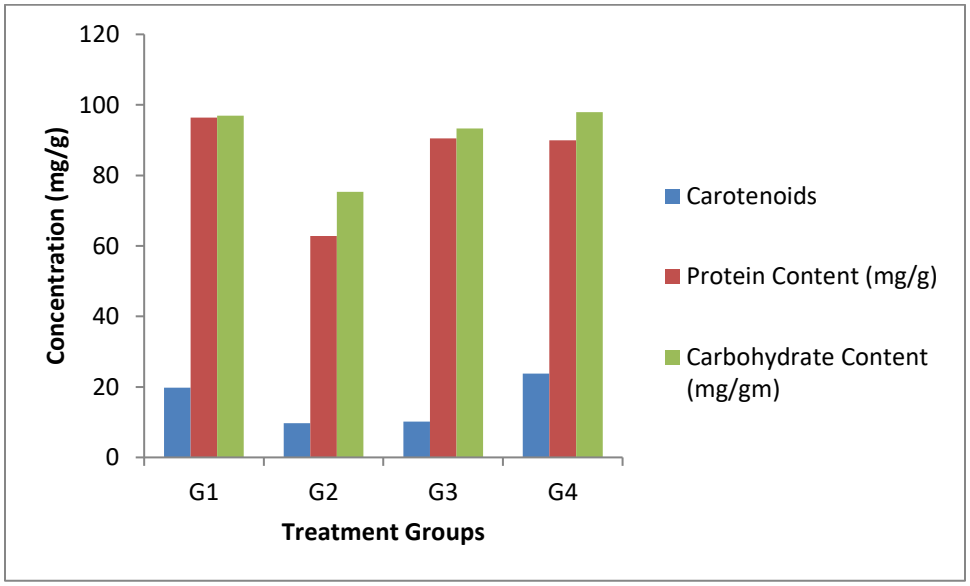

Figure 7.53 Graph represents Carotenoid, Protein and Carbohydrate Content Where G-1 refers to Control group, G-2 refers to *C.lunata* group, G-3 refers to Biocontrol fungus treated group and G-4 refers to *C.lunata* alongwith Biocontrol fungus treated group

7.12 Characterization of fungal isolate

Figure7.49 S4D7 Isolate A)Front View Of Petri Plate B)Back View of Petri Plate C) Growth In PDB D)LPCB Mount

7.12.1Morphological Characters: Green Coloured Round Sporulating Colonies with Yellow Colour at the base.

7.12.2 LPCB: Small brush like structure observed which are attached to Conidiophore.

Chapter 8

Conclusion and Future aspects

Among the fungal isolates obtained from the soil sample, 3 isolates shows synthesis of AgNPs. From these isolates antagonistic activity towards *Curvularia lunata* and AgNPs was checked; which act as biocontrol agent for controlling the Black kernel Disease. Those isolates which were showing antimicrobial activity were further assessed for its Antioxidant and Antibacterial Activity. Effect of AgNPs on Concentration of substrate, Incubation temperature and pH was checked and Characterization was done by UV-Visible Spectrophotometer, FTIR, XRD, SEM, TEM, DLS etc. These nanoparticles and biocontrol fungus were further assessed for their efficiency by Seed germination as well as Pot Studies. Potential Antagonist will be characterized on the basis of molecular sequencing.

Chapter 9

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Chapter 10

Appendix

Composition of Potato Dextrose Agar (PDA) Media:

Ingredients	**Gms / litre**
Potatoes infusion from	200.00
Dextrose	20.00
Agar	15.00

Composition of Potato Dextrose Broth (PDB) Media:

Ingredients	**Ingredients Gms / litre**
Potatoes infusion from	200.00
Dextrose	20.00

Composition of Mueller-Hinton (MH) Agar:

Ingredients	**Ingredients Gms / litre**
Beef, infusion form	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH	(at 25°C) 7.3± 0.1