

ESTABLISHMENT OF FUNGAL BIOCONTROL AGENTS AS ENDOPHYTES ON *Vigna mungo* (BLACK GRAM)

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

**Accredited with Grade "A" by NAAC | 12B Status by UGC | Approved by AICTE
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Vignesh A (37230094)**, **Kirubakaran V K (37230039)** who have done the project work as a team who carried out the project entitled “**Establishment of Fungal Biocontrol Agents as Endophytes on Vigna mungo (Black Gram)**” under our supervision from **October 2020 to March 2021**.

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We, **Vignesh A (37230094)** and **Kirubakaran V K (37230039)** hereby declare that the Project Report entitled “**Establishment of Fungal Biocontrol Agents as Endophytes on *Vigna mungo* (Black Gram)**” done by us under the guidance of **Dr. S. Karthick Raja Namasivayam**, Department of Biotechnology at **Sathyabama Institute of Science And Technology, Chennai** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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LIST OF ABBREVIATION

T.viride	Trichoderma viride
V.lecanii	Verticillium lecanii
V.mungo	Vigna Mungo
GSH Assay	Glutathione Assay
GST Assay	Glutathione S Transferase Assay
TBARS Assay	Thiobarbituric acid reactive substances Assay
PGP	Plant Growth Parameters

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ESTABLISHMENT OF FUNGAL BIOCONTROL AGENTS AS ENDOPHYTES ON *Vigna mungo* (BLACK GRAM)

ABSTRACT

Bio control agents based on fungi are the promising candidates in insect pests and disease management. Success of Bio control agents is based on various factors which determine the desired bio control effect. In this study, endophytic relationships of *Trichoderma sp* and *lecanicillium lecanii* also known as *Verticillium lecanii* were established on *Vigna mungo* (Black Gram). After the endophytic establishment, the various biochemical mechanisms of the fungal established plants were studied. Effect of fungal establishment on anti-oxidative enzymes, total chlorophyll content and qualitative protein profiling were studied. We marked difference on plant growth parameters was observed in *Trichoderma* treatment whereas some changes were recorded in *Lecanicillium lecanii* treatment. The present study will be helpful to understand the fungal bio control agents as endophytes.

Keywords: Bio control Agents, *Trichoderma sp*, *Lecanicillium lecanii*, Endophytes, Anti-oxidative enzymes, Chlorophyll content, Plant growth parameters.

CHAPTER 1

INTRODUCTION

1.1. Agriculture

It is the craftsmanship and study of developing the soil, developing yields, and raising Eutherian warm blooded animal. It incorporates the arrangement of plant and creature items for people to utilize and their conveyance to business sectors.

Agribusiness gives the majority of the world's food and materials. Cotton, fleece, and creature skin square measure every single agrarian item. Horticulture conjointly gives wood to development and paper items. These items, in like manner on the grounds that the farming ways utilized, could change from one piece of the globe to an alternate.

AGRICULTURE AROUND THE WORLD:

More than many years, the expansion of cultivating added to the augmentation of urban foundations. Before agribusiness got wide, individuals copied through by far most of their lives finding food—pursuing wild animals and collecting wild plants.

Around 11,500 years past, individuals bit by bit taught a way to deal with create oat and root crops and settled straightforwardly down to life-maintained developing.

By 2,000 years past, abundant of the Earth's general population had gotten subject to cultivating. Understudies aren't sure why this transition to developing showed up, in any case, it will have happened owing to temperature change. Exactly when individuals began creating harvests, they conjointly began social and raising wild animals. Changing wild plants and animals for individuals to use is named restraining. The initially prepared plant was doubtlessly rice or corn. Chinese farmers were creating rice as early as 7500 BCE.

The recently subdued animals were canines, which were used for looking. Sheep and goats were most likely prepared immediately. Individuals conjointly restrained bulls and pigs. Most of those animals had at whatever point been looked for stows away and meat. At present a couple of them moreover are wellsprings of milk,

cheddar, and margarine. At last, individuals used prepared animals like bulls for furrowing, pulling, and transportation.

Cultivation enabled individuals to give food. They may use this further food once crops unprofitable or trade it for a substitute thing. Food abundances allowed individuals to figure at different tasks separated to developing.

Cultivating strong past upset individuals close to their fields and semiconductor diode to the event of enduring towns. These got coupled through trade. New economies were in this manner palmy in specific spaces that metropolitan territories created and human advancements made. The most prompt human progressions that maintained heightened agriculture arose close to the Tigris River and Euphrates River Rivers in the geographic district (by and by Asian country and Iran) and on the Nile in Egypt. Improved Technology for centuries, agrarian headway was moderate. Quite possibly the most timely cultivating gadgets was the hearth. Neighborhood Americans used hearth to coordinate the improvement of berry-conveying plants, which they knew grew quickly when a damnation.

Farmer's grown little plots of land by hand, abuse hatchets to pull out trees and making by departure sticks to block up and until the earth. After some time, improved developing instruments of bone, stone, bronze, and iron were made. Better methodologies for limit progressed. Individuals began repositing food assortments in compartments and earth lined pits to be used amidst insufficiency. They conjointly began making earth pots and different vessels for passing on and cookery food.

Around 5500 BCE, farmers in the geographic region made clear water framework structures. By coordinating water from streams onto their fields, farmers had the choice to get settled zones once thought to be gravely masterminded to cultivating. In the geographic locale, and later in Egypt and China, individuals facilitated themselves and worked along to make and keep up higher water framework systems.

Early farmers conjointly made improved kinds of plants. For example, around 6000 BCE, a replacement sort of wheat arose in South Asia and Egypt. It was more

grounded than past oat grains; its bodies were less difficult to discard and it very well may be made into bread. As the Romans extended their domain, they hand created the best country techniques for individuals they won. They made manuals regarding the developing procedures they found incontinently and Asia, and hand made them to land in Europe.

The Chinese conjointly extraordinarily made developing gadgets and ways from near spaces. A spread of rice from Vietnam grow quickly and allowed farmers to acquire various collects all through one season. This rice quickly got wide all through China. Various ancient European farmers used Associate in the nursing open-field course of action of planting. One field would be planted in spring, another in fall, and one would be left wild, or incapacitated. this strategy shielded enhancements inside the soil, extending crop creation.

The tops of the Muslim Golden Age (which showed up at its stature around 1000) in North Africa and in like manner the Middle East made cultivating into a science. Muslim Golden Age farmers learned yield turn.

In the fifteenth and sixteenth many years, travelers introduced new sorts of plants and cultivating things into Europe. From Asia, they passed on home occasional, tea, and indigo, a plant that won't frame blue tone. From the solid ground, they took plants like potatoes, tomatoes, corn (maize), beans, peanuts, and tobacco. a portion of these became staples and extended people's weight control plans.

1.2. Asian Agriculture

India's farming comprises of numerous yields, with the first food staples being rice and wheat. Indian ranchers moreover develop beats, potatoes, sugarcane, oilseeds, and such non-food things as cotton, tea, espresso, elastic, and jute (a gleaming fiber wont to make packing and twine).

Asian nation might be a fishery huge comparably. A total catch of concerning 3,000,000 metric tons yearly positions Asian nations among the world's high ten fishing countries. Regardless of the staggering size of the horticultural area, in any

case, yields per region unit of harvests in the Asian nation are typically low contrasted with worldwide norms. Inappropriate water the executives is another downside contacting India's horticulture. During a period of speeding up water deficiencies and ecological emergencies, for instance, the rice crop in Asian nations is distributed lopsidedly high measures of water. One consequence of the wasteful utilization of water is that water tables in locales of rice development, as geographic territory, are on the expansion, while soil richness is on the decay. Increasing the horticultural situation is a partner degree in progress Asian dry season and nasty climate. In spite of the fact that all through 2000-01 a rainstorm with normal precipitation had been normal, possibilities of agrarian creation all through that sum weren't considered brilliant. This has part of the way been because of the relatively horrible conveyance of precipitation, bringing about floods in sure segments of the country and dry spells in some others.

Despite the fact that farming records for the greatest sum as 1/4 of the Indian economy partner debase utilizes a measurable sixty % of the working class, it's considered very wasteful, inefficient, and unequipped for deciding the craving and lack illness issues. Notwithstanding progress during this space, these issues have proceeding to disappoint Asian nations for a long time. It's measurable that the greatest sum as a fifth of the entire agrarian yield is lost on account of failures in gather home, transport, and capacity of government-sponsored crops.

1.3. Endophytes

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The term Endophytes was coined by De Bary in 1866, explains that the organism grows inside the plant tissues, in the form of fungal or bacterial provides a better environment to the host plant.

It is a microorganism (bacterium or fungus) present in every plant species. The main function of the endophytes is used to protect plants from the outside environment. It acts as a protective shield and also a biocontrol agent. It was the first organisms to be used for the biological control of pests. Endophytes can promote growth, protect the plant against harmful pathogens, and are used to reduce abiotic stress. Using Endophytes in disease control gives essential advantages while compare to other

biocontrol agents because they colonize the plant internally and thereby stay protected from abiotic stress and fluctuations.

The term “endophytes” includes a set of microorganisms that grow intra-and/or intercellular within the tissues of upper plants while not inflicting over symptoms on the plants within which they live, and have proved to be made sources of bioactive natural merchandise. Interdependence interaction between endophytes and host plants could end in fitness edges for each partner. The endophytes could offer protection and survival conditions to their host plant by manufacturing an excessiveness of gear that, once isolated and characterized, can also have the potential to be used in business, agriculture, and drug.

Approximately 300000 plant species growing in undiscovered space on the planet area unit host to 1 or a lot of endophytes, and also the presence of biodiverse endophytes in Brobdingnagian variety plays a crucial role on ecosystems with the greatest diversity, as an example, the tropical and temperate rainforests, that area unit extensively found in Brazil and possess nearly two-hundredth of its biotechnological supply. Considering that solely a low quantity of endophytes are studied, recently, many analysis teams are intended to gauge and elucidate the potential of those microorganisms applied on biotechnological processes specializing in the assembly of bioactive compounds.

The production of bioactive substances by endophytes is directly associated with the freelance evolution of those microorganisms, which can have incorporated genetic data from higher plants, permitting them to higher adapt to plant host and perform some functions like protection from pathogens, insects, and grazing animals. Endophyte’s area unit chemical synthesizer within plants, in different words, they play a job as a variety system for microbes to supply bioactive substances with low toxicity toward higher organisms.

Bioactive natural compounds created by endophytes are promising potential utility in safety and human health considerations, though there's still a major demand of drug business for artificial merchandise because of economic and long reasons. Issues associated with human health like the event of drug resistance in human unhealthful microorganism, plant infections, and life-threatening virus claim for

brand spanning new therapeutic agents for effective treatment of diseases in human, plants, and animals that area unit presently unmet.

Newman and Cragg conferred an inventory of all approved agents from 1981 to 2006, from that a major variety of natural medication area unit created by microbes and/or endophytes. Endophytes provide various types of bioactive secondary metabolites with distinctive structure, as well as benzopyrones, alkaloids, flavonoids, phenolic resin acids, chinones, quinones, xanthones, terpenoids, tetralones, steroids, etc. Such bioactive metabolites realize wide-ranging applications as agrochemicals, antibiotics, immunosuppressants, antiparasitic, antioxidants, and antineoplastic agents.

Endophytes play a key role in agriculture to progress production by acting as plant growth promoters (PGP). It can be treated into the plants in several ways. And it can be used as a combination of chemicals along with endophytes. In this study, we are using two fungal endophytes as a biocontrol agent named *Trichoderma viride* and *Lecanicillium lecanii* by seed treatment method.

4.1.1. *Trichoderma viride*:

Class: Sordariomycetes

Kingdom: Fungi

Family: Hypocreaceae

Scientific Name: *Trichoderma viride*

Order: Hypocreales

Trichoderma viride might be a high-effectiveness natural agent. It produces anti-microbials, supplement rivalry, parasitic, cell-divider corruption, proteins, and inspired plant obstruction components, that have threat result on a spread of plant embarrass organisms. Insurance and treatment will resultively oversee soil unfurl plant illnesses with twin impact. Use *Trichoderma viride* item, which may improve the endurance pace of phanerogam and relocate, keep the seedlings tough development. It could be will not stop dark mold.

Highlights of *Trichoderma viride*:

- Through the security and treatment, will viably stop and the executives plant illness, cataplexy, scourge, wither, organism shrivel, *Bacillus anthracis*, and distinctive soil-borne infections;
- Improve the dirt, break the bunch, improve the dirt porosity and give the essential framework;
- Promote root development, make crop development a ton of fiery and increment crop yield, and so forth

Component of Action of *Trichoderma viride*

- Competitive impact: *Trichoderma viride* contains a speedy development and duplicate rate, and may rapidly ingest and use supplements, water, territory, and inside the dirt, consequently deteriorating the living environment of plant microorganisms;
- Antibiosis: It secretes medication substances to restrain the development of plant microbes;
- Interdependency and opposition enlistment: the co-development of *Trichoderma viride* and plant roots actuates the plant's inner arms and improves the plant's disease obstruction;
- Serious parasitism: *Trichoderma viride* plant construction can develop, twine, and Pierce on the plant design of plant microorganisms and retain the supplements inside the plant design of plant microbes, bringing about the passing of plant microbes.
- Promote plant development:
 1. Increase plant development and yield.
 2. Increase the overall ailment opposition of plants.
 3. Increase root development and resistance to dry spell.
 4. Increase the ingestion of supplements and consequently the viable utilization of composts
 5. Improve the daylight and strength of plants.
 6. Increase the speed and portion of seed germination.

Considerations of exploitation *Trichoderma viride*

- Trichoderma viride might be a microorganism plant, essentially saprophytic, which may develop just if the dirt contains bound wetness and temperature.
- It should be used in the dirt, and try not to shower on the stems and leaves;
- Avoid exploitation compound pesticides with tough acids and bases the greatest sum as could be expected;
- Trichoderma itself is innocuous to crops, consistent use is better.

4.1.2. *Lecanicillium Lecanii*:

Order: Hypocreales

Family: Cordycipitaceae.

Rank: Species

Higher Classification: Lecanicillium

Scientific Name: *Lecanicillium lecanii* (*Verticillium lecanii*)

The term *Verticillium* comprised a division of Ascomycota a group of fungi. So, they are also called sac fungi. *Verticillium lecanii* is an entomopathogenic fungus, used as a biocontrol agent in agriculture. It acts as a parasite of various insects. Because of this function, it can even kill insects which is harmful to plants and become one of the best biocontrol agents against insect and pest control.

The life cycle of *Verticillium lecanii*:

It begins with the spores landing and attaching to the outermost layer of the insect. After attached to the outer layer, the spores begin germinating and form prolonged germ tubes to enter the hard layer. This process is done with the help of enzyme secretion. The enzymes are capable to lyse the outer layer and helps to enter into the covering and begin the further growth of the spores.

The enzymes which are helpful to the penetration are Proteases, chitinases, esterases, lipase. After the germ tube, it further develops into hyphae and colonizing the tissue of insects. This growth gives pressure on the insect and kills them.

V.lecanii as a pest control:

As it is an entomopathogenic fungus that grows inside the insects and kills them. It is used as a fungal biocontrol agent in agriculture for insect and pest control.

There are different methods to apply these biocontrol agents depends upon the nature of the crop. This agent may be formulated with different compounds, this allows the spores to be viable so that they can grow and kill the insects.

The most common method for applying these biocontrol agents to the plant is called the seed treatment method. In this method, *V.lecanii* is formulated with different compounds and directly applied to the seeds of the plants before they sow into the soil. This method ensures protection from soil-borne pathogens and also acts as a PGP (Plant Growth Promotor).

CHAPTER 2

REVIEW OF LITERATURE

S.W. Behie et al. (2012), studied the *Metarhizium* regular endophytic growths present in an enormous number of the biological system. It has a particular property

called microorganism for creepy crawlies (wellspring of nitrogen). So, they coupled the endophytic ability and creepy crawly pathogenicity of *Metarhizium* to guarantee dynamic exchange of nitrogen through parasitic mycelia to the host plant. They utilized soil microcosms to analyze the ability of *M.roberstii* to move creepy crawly inferred nitrogen to the host. To identify the movement development, they utilized (15) N-named nitrogen to the creepy crawlies and utilizing two plants as a host, for example, Haricot bean (*Phaseolus vulgaris*) and Switchgrass (*Panicum virgatum*). They have seen the movement of creepy crawly inferred nitrogen to the plants with the assistance of Liquid Chromatography and mass spectrometry techniques. They have presumed that the *M.spp* is utilized as an effective translocator of soil bug inferred nitrogen to the plant by utilizing root colonization.

Biswas C et al. (2012), established the fungal entomopathogen *Beauveria bassiana* as an endophyte in a fiber crop called jute (*Corchorus olitorius*) by utilizing the seed treatment technique. They have noticed plant development boundaries and separated by 30, 60, and 120 days. By utilizing PCR investigation, they analyzed the colonization of different pieces of the plant like leaf, case, seed, leaf, and roots. They have seen that the colonization happened uniquely in stems, cases, and leaves however not in seeds and roots. Also, colonization in leaves (55.87%) was higher than in all parts. The level of colonization fluctuates goes from 23.89% in 30 days, 43.34% in 60 days (higher one), and 35.39% in 120 days. They have inferred that *B.bassiana* move all through all pieces of the plant (jute) and set up the endophytic conduct to control the action of the irritations, for example, Bihar furry caterpillar and semi looper. It is the best methodology for biocontrol against bothers and guarantees an appropriate climate for the host plant.

Backman P et al. (2008), gave detailed information about endophytes and their biocontrol activity. Endophyte gives the colonized climate to the host plants and shields the plants from destructive microbes or bugs. Endophytic organisms and microbes are the regular kinds utilized as a biocontrol. We can treat these endophytes through seeds by seed treatment strategy and this gives an incredible effect on the plants. Since it can ready to lessen abiotic and biotic pressure. They likewise manage pH, supplement stream, dampness, and others. The future utilization of bio-synthetic blends of endophytes with business pesticides acquainted

with the seeds could shield from numerous sickness causing specialists. The biocontrol additionally focuses the yield creation.

Rojan P John et al. (2010) established *Trichoderma viride* as a biocontrol specialist against the two soil parasitic microorganisms named *Pythium arrhenomanes* and *Fusarium oxysporum*. While analyzing the biocontrol specialists against the two soil microbes, *T.viride* showed damaging control and mycoparasitism during an invitro biocontrol test. To check the pathogenic action, they have treated the *Trichoderma viride* alongside the microorganisms, for example, Control, *Fusarium* treated, *Trichoderma* treated, *Pythium* treated, *Fusarium* and *Trichoderma* treated, *Pythium* and *Trichoderma* treated plants by utilizing seed treatment technique. After the 12 weeks of development, the researchers saw that the *Trichoderma* advanced the development of root and shoot frameworks contrasted with others. *Trichoderma* treated natural product yield was 66% contrasted with the control was just 41%, *Fusarium* and *Pythium* treated with *Trichoderma* were just 53 and 43%. Subsequently, they have reasoned that the *Trichoderma* contaminated plants advance the development of the plants and secure the plants against irresistible microorganisms. *Trichoderma* likewise advances nodulation so it will assist with retaining more nitrogen which helps in the photosynthesis action.

Duy Nam Trinh et al. (2020) examined the in-vitro pathogenicity of different fungal biocontrol specialists, for example, *Beauveria bassiana* and *Verticillium lecanii* or by the mix of the two strains into the Bean aphid called *Megoura japonica* utilizing leaf plunge treatment. After the restraint of the strains into the plant, they have seen a huge impact by investigation of fluctuation. The mix of the parasitic strains shows critical change to the bean aphid. Subsequently, they have inferred that the *B.bassiana* and *V.lecanii* have diverse harmfulness potential. Also, the appropriate material control for the Bean aphid is Filtrate application. The most noteworthy mortality of around 90.5% has been given by the blend of B76 (*B.bassiana*) and L2 (*V.lecanii*) strains. So this shows that these strains can be utilized as a Biocontrol specialist for *M.japonica* (Bean aphids).

Christian joseph R. Cumagun (2014), explained the establishment of fungal *Trichoderma* as biocontrol agents in the different formulation and its different

delivery system to the plants. This is a vital process. He clarified the two most regular detailing techniques utilized broadly like Liquid plan: A methodology for biomass creation done by profound tank maturation. It is likewise useful to improve the creation and its quality. Strong detailing: It is another method of inoculum creation. Squanders, for example, rice straw, sugarcane, wheat are utilized as a substrate or consolidated for the Trichoderma development. He likewise acquainted the microencapsulation strategy with Trichoderma to expand the shelf life and its proficiency. Seed treatment innovation like fluid covering, twofold covering, and strong lattice preparing is the method of conveyance utilized in Trichoderma for better adequacy. In view of the harvest and its sorts, the extent of Trichoderma and its covering may contrast. Along these lines, he proposed that the advancement in the plan of Trichoderma upgrade the development and guarantee a sickness free climate for the harvest.

Deepak M. Kasote et al. (2015) proposed that the oxidative pressure occurred into the plants which may prompt different infection and it influences the advancement of the plant. Cell reinforcement plays a guard job to decrease these burdens. Like animals, plants additionally have the inborn capacity to incorporate cancer prevention agents which can be lessening the harm brought about by ROS-prompted oxidative.

CHAPTER 3

AIM AND OBJECTIVES

3.1. AIM AND SCOPE

- Endophytic establishment of fungal biocontrol agents is an important strategy of insect pests with pathogens control.
- The effect of endophytic establishment on the molecular mechanism of plant physiological processes should be analyzed.

- With their objective, the present study is undertaken to evaluate the endophytic establishment of fungal biocontrol agents on the molecular physiological mechanism was studied.

3.2. OBJECTIVES

The objectives of the present study are as follows,

- To establish an endophytic relationship of fungal biocontrol agents.
- To study the changes in Biochemical parameters of an established plant.

CHAPTER 4

MATERIALS AND METHODOLOGY

4.1. Selection of Bio control Agents



4.2. Endophytic establishment of Seed treatment Method:

Seed treatment method is defined as the application of pesticide, fungicide otherwise a combination of both, to seeds to disinfect them from seed or soil-borne pathogenic microorganisms and insects.

4.2.1. Types of seed treatment method:

1) Seed disinfestation method: Seed disinfestation is defined as the destruction of surface-borne organisms that have contaminated the seed surface but not infected the seed surface. Soaks, Chemical dips, fungicides applied in the form of dust, liquid, or slurry.

2) Seed disinfection method: Seed disinfection is defined as the penetration of fungal spores that have become established within the seed coat, or in more deep tissues. For effective control, the fungicidal treatment must eradicate the seed to kill the fungus which is present.

3) Seed Protection method: The importance of seed protection is to protect the seedlings and seeds from organisms in the soil which might otherwise cause decay of the seed before germination.

4.2.2. Precautions:

Most products which are used in this treatment of seeds are harmful to humans as well as seeds. So, Extreme care has to be provided to ensure that treated seed is never used as human or animal food. To limit this chance, treated seed ought to be plainly marked as being perilous, whenever devoured. The temptation to use unsold treated seed for human or animal feed can be avoided if care is taken to treat only the quantity for which sales are assured.

Care should likewise be taken to treat seed at the right dose rate; applying excessively or too minimal material can be just about as harming as never treating. Seed with exceptionally high dampness content is truly defenseless to injury when treated with a portion of the concentrated fluid items.

Benefits of Seed Treatment method:

- 1) Improves germination
- 2) Controls soil insects
- 3) Prevents spread of plant diseases Improves
- 4) Protects from storage insects
- 5) Protects seed from seed rot.

In this study, we are using Bio fungicides such as *Trichoderma viride* and *Lecanicillium lecanii* directly into the seeds by using the seed treatment method.

4.2.3. Methodology:

- Seeds of *Vigna mungo* (Black Gram) were taken.



Figure 4.5 Image of Vigna mungo (Black Gram) seeds

- Bio control agents such as *Trichoderma viride* and *Verticillium lecanii* taken in a separate container and mixed with Jaggery water in the proportion of 1:3.



Figure 4.6 Image of *T.viride* and *V.lecanii* mixed with Jaggery water

- In order to establish our endophytes into the seeds, the must be immersed into the above solution for about 30 minutes.



Figure 4.7 Seed Treatment of *Vigna mungo* (Black Gram)

- After the treatment, the seed has to be dried for 2 to 3 hrs in shade and sowed within 24 hrs into the soil.

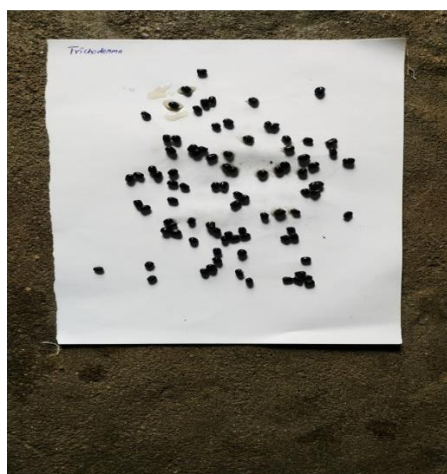


Figure 4.8 Shade Drying of Endophyte Treated seeds

4.3. Phytotoxicity Assessment of endophytic established plant:

4.3.1. Estimation of photosynthetic pigments (mg/g/f. wt.)

Chlorophyll a and b contents were extracted from the respective dose of leaves and estimated according to the method of Arnon (1949).

Two hundred mg of fresh young leaves were ground with 10ml of 80 percent acetone Pestle mortar and spun at 2500 rpm for 10 min at 200 c in the centrifuge. The homogenate was re-extracted with 80 percent acetone until the green color

disappeared in the residue and the extract was pooled and made up to 20 ml with 80 percent acetone. Two ml of extract was transferred into a cuvette and the absorbance/ optical density (OD) was read at 663, 645 in a spectrometer (Elico Made) against 80 percent acetone as a blank, to determination chlorophyll a and band carotenoids content.

Chlorophyll content was calculated using the formula of Arnon (1949).

Chlorophyll 'a' (mg/g f wt) = $(1.27) \times (A.663) - (0.269) \times (A.645)$

Chlorophyll 'b' (mg/g f wt) = $(2.29) \times (A.645) - (0.468) \times (A.663)$

Total chlorophyll (mg/g f wt) = $(2.02) \times (A.645) + (0.802) \times (A.663)$

4.3.2. Anti-oxidant assay

Catalase assayCAT activity was assayed by the method of Goth (1991) with slight modifications. The enzyme extract (0.5mL) was added to the reaction mixture, containing 1mL of 0.01mol/L phosphate buffers (pH 7.0), 0.5mL of 0.2 mol/L H₂O₂ or 30% H₂O₂, 0.4mL H₂O, and incubated for 10mins. The enzymatic reaction was stopped with 1ml of 32.4mmol/l ammonium molybdate and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm against blank 3.

$$\text{Sample CAT activity (kU/l)} = \frac{A (\text{Blank 1}) - A (\text{Sample})}{A (\text{Blank 2}) - A (\text{Blank 3})} \times 271$$

A (Blank 2) – A (Blank 3)

Where:

Blank 1 contained 1.0 ml substrate, 1.0 ml molybdate and 0.2 ml sample; blank 2 contained 1.0 ml substrate, 1.0 ml molybdate and 0.2 ml buffer; blank 3 contained 1.0 ml buffer, 0.2 ml buffer and 1.0 ml molybdate.

Estimation of reduced glutathione (GSH):

Estimation of reduced glutathione was done according to the method of Jollow et al. With slight modification plant sample homogenate (500µL of 0.1M potassium

phosphate buffer (pH7.4)) was precipitated by the inclusion of 4% sulfosalicylic acid (500 μ L). After keeping for 1 h at 4°C, the samples were centrifuged for 20min at 1200g. The supernatant (33 μ L) was gathered and added to aliquots consisting of 900 μ L of 0.1M potassium phosphate buffer (pH7.4) and 66 μ L of 100mM dithiobis (2-nitrobenzoic acid) (DTNB). GSH reacts with DTNB and produces a yellow-colored complex. Absorbance was taken at 412 nm within 10min 100 μ L of the extract was treated as above and the absorbance was taken at 412 nm. Blank tubes having all the reagents except glutathione solution and the extract were also included.

Glutathione S-Transferase assay (GST):

30 mg of each sample was weighed and dissolved in 3ml of DMSO solution and mixed well. This extract was further used. A 2ml of phosphate buffer (pH7.4) and 2ml of plant extract was added to blank and sample wells, respectively. The reaction was initiated by adding 1ml of CDNB (1mM/ml) to both the wells and mixed well. The absorbance was read at 340nm. The extinction coefficient for CDNB is 9.6 mM⁻¹ cm⁻¹ at 340nm. Protein was estimated using Bradford's assay.

Quantification of Lipid Peroxidation:

Thiobarbituric acid-reactive substances (TBARS) assay is used to detect lipid peroxidation. In this assay, malondialdehyde forms a complex with Thiobarbituric acid, which can be quantified spectrophotometrically. The plant sample was homogenized with ice-cold KCl Buffer then the homogenized sample was collected in Eppendorf. Then added 0.2ml of 8.1% SDS, 1.5ml of 20% acetic acid mixed and incubated for 10min, then added 1.5ml of 0.8% TBARS and 0.7ml of distilled water to the solution. This reaction mixture was incubated for 1 h at 95°C and then cooled to 25°C and centrifugation at 5000g for 15min to remove debris. Absorbance was recorded at 532nm. The untreated sample was taken as a control. The standard was performed with MDA (Malondialdehyde). The sample values compared with standard and the concentration expressed as μ M.

4.4. Protein Profiling Study Using SDS PAGE:

Measurement of soluble protein content in the sample

The sample was grained with liquid nitrogen and extracted with 1x PBS buffer pH-7.4. Soluble proteins in the extract were measured with the BCA kit method and assessed by SDS-PAGE.

SDS PAGE:

Make the separating gel: Prepare the gel solution as 12% (with 40% Acrylamide, 1.5M Separating buffer, 30% APS, 20% SDS, and 8 μ l TEMED). Pipette suitable measure of isolating gel arrangement into the hole between the glass plates.. Fill in water into the gap until an overflow. Wait for 20-30min to let its polymerase.

Make the stacking gel: Prepare the gel solution as 5% (with 40% Acrylamide, 1.5M Stacking buffer, 30% APS, 20% SDS, and 8 μ l TEMED). Pipette appropriate amount of stacking gel solution into the gap between the glass plates and place the comb. Remove the glass plates from the projecting casing and set them in the cell support dam. Pour the running buffer (electrophoresis support) into the inward chamber and continue to pour after flood until the buffer surface arrives at the necessary level in the external chamber.

Set up the samples: Mix your samples with test buffer (stacking support). Warmth them in bubbling water for 5-10min.

Performing electrophoresis: Load prepared samples into wells and make sure not to overflow. Load protein marker into the first lane. At that point cover the top and interface the anodes. Set an appropriate volt and run the electrophoresis. As for the total running time, stop SDS-PAGE running when the down most sign of the protein marker almost reaches the foot line of the glass plate.

Staining protocol:

Fixer	Stainer	Destainer
45ml H ₂ O	45ml H ₂ O	45ml H ₂ O
45ml Iso propanal	45ml Methanol	45ml Methanol

10ml Acetic acid	10ml Acetic acid	10ml Acetic acid
	0.1% Coomassive brilliant blue	

Table 4.1 Composition of Staining

Place the gel in fixer for 15-20minutes then drain the fixer add stainer to the gel leave for 15-20minutes. Drain the stainer adds destainer for 15–20minutes. Drain the destainer, Preserved the gel in 2% Acetic acid.

4.5. Plant Growth Parameters:

Difference between endophytic plants and control has been determined by plant growth parameters such as Leaf Height, Leaf Ratio, Plant Height, Flowers, Number of Branches, Dry matter etc.,

The observation of these parameters takes place by every 7 days.

4.6. SEM Analysis:

SEM (Scanning Electron Microsocpe) is an analytical technique used to scan an electron beam on the surface to create an SEM image. The electrons from the beam interacts with the sample, gives numerous signals in order to obtain information about the composition of the sample and surface topography.

We are using T.viride established in a leaf of V.mungo as a sample to view its surface topography.

CHAPTER 5 RESULTS

5.1. PLANT GROWTH OBSERVATION AND ITS PARAMETERS:

After the establishment of fungal endophytes as biocontrol agents in plants through seeds. We have observed the growth and other parameters every 7 days.

DAYS	OBSERVATION
DAY 1	
DAY 5	
DAY 14	
DAY 21	

DAY 28	
DAY 35	

PLANT GROWTH PARAMETERS:

PARAMETERS	CONTROL					TRICHODERMA VIRIDE					VERTICILLIUM LECANII				
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 7	Day 14	Day 21	Day 28	Day 35	Day 7	Day 14	Day 21	Day 28	Day 35
Shoot Length(cm)	4.2	8.3	12	16.7	18.2	7	18.7	21.4	25.6	29.2	8	17.2	20.3	24	27.5
Number of Branches	2	2	3	4	5	2	3	4	6	6	2	4	5	6	7
Number of Leaves	6	10	13	15	18	5	12	17	24	29	4	10	12	17	26
Number of Flowers	-	-	1	2	4	-	-	1	5	9	-	-	1	5	7
Leaf size (cm)	2.1	2.6	3.3	4	4.5	2.1	2.6	3.6	4.1	5.3	2.3	2.8	3.4	4.2	4.8
Number of fruits	-	-	-	2	3	-	-	1	3	5	-	-	1	3	4

PARAMETERS	CONTROL	TRICHODERMA VIRIDE	VERTICILLIUM LECANII
Root Length (cm)	7	17	12
Dry matter (g)	0.002	0.003	0.004
Chlorophyll "A" (mg/g)	0.8	0.54	0.56
Chlorophyll "B" (mg/g)	0.43	0.22	0.35
Total Chlorophyll (mg/g)	1.34	0.78	0.94

5.2. PHYTOTOXICITY ASSESSMENT:

5.2.1. Chlorophyll pigmentation Assay

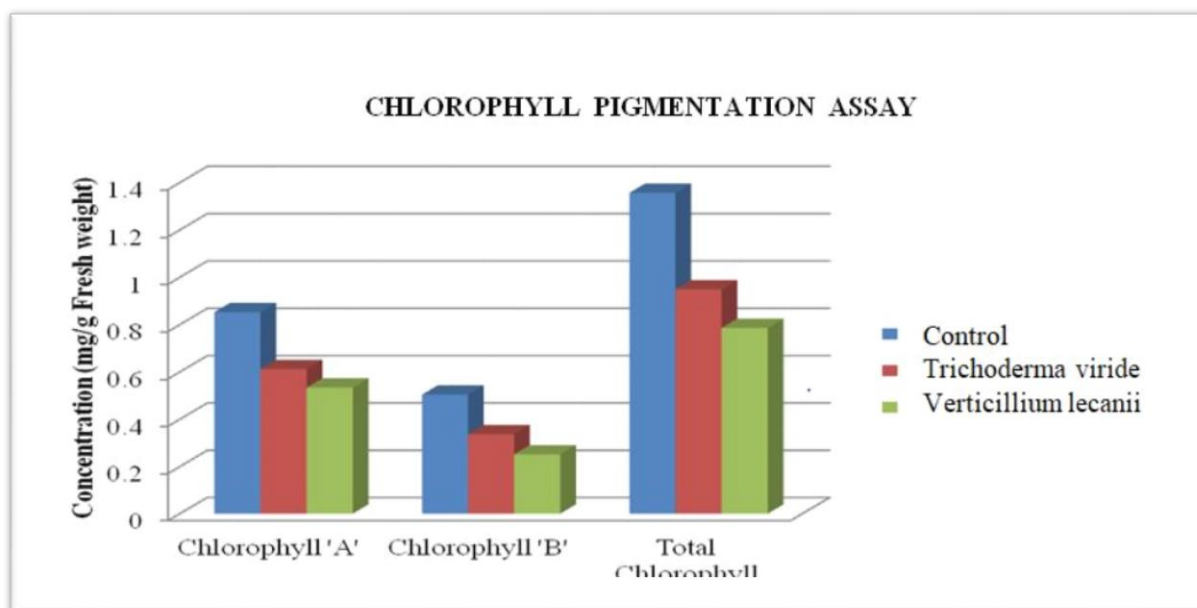


Table 5.1 Chlorophyll Pigmentation Assay

The photosynthetic pigment such as chlorophyll a, b and total chlorophyll (Chl-a, b and Total-chl) content were decreased in treated groups compared to control group.

5.2.2. Anti-oxidant assay

I. Catalase activity

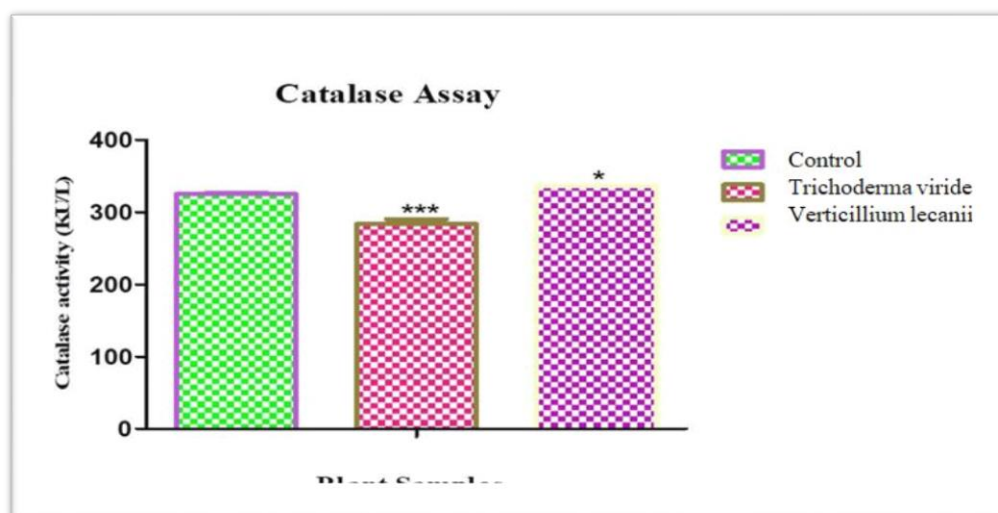


Table 5.2 Catalase Assay

The total content of catalase among the treated and untreated plants values are expressed as mean ($\pm$ SD) analyzed by One-way analysis of variance. The values are highly significant (***) between the samples ($p < 0.0001$). The graphical representation shows a slight difference compared to the control group; these results indicate that there was increased Catalase activity in the *Verticillium lecanii* treated plant compared to the control plant.

II. Glutathione Assay

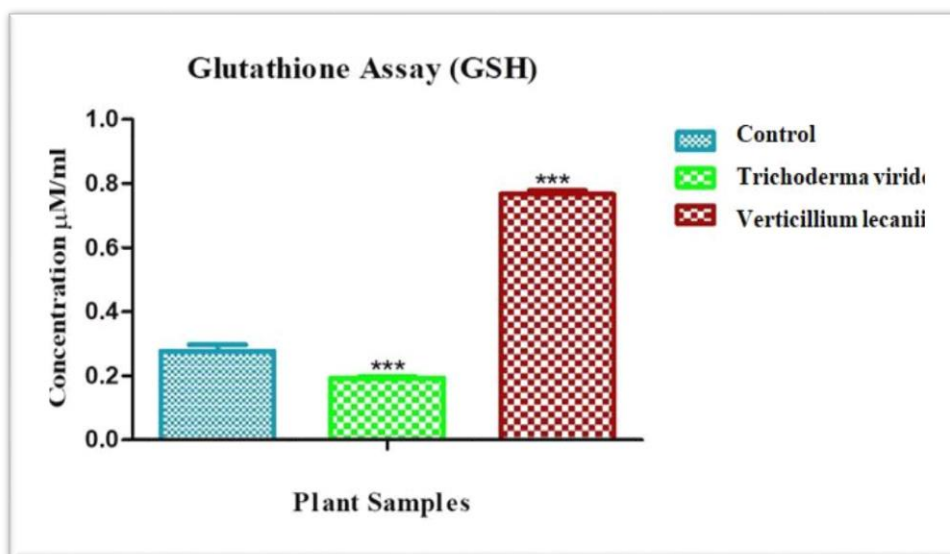


Table 5.3 GSH Assay

The total content of Glutathione (GSH) among the treated and untreated plants values are expressed as mean ($\pm$ SD) analyzed by One-way analysis of variance. The values are highly significant (***) between the samples ($p < 0.0001$). The graphical representation shows the significant differences compared to the control group, these results indicate that there was increased GSH content in the *Verticillium lecanii* treated plant compared to the control plant.

III. Glutathione-S-Transferase Assay

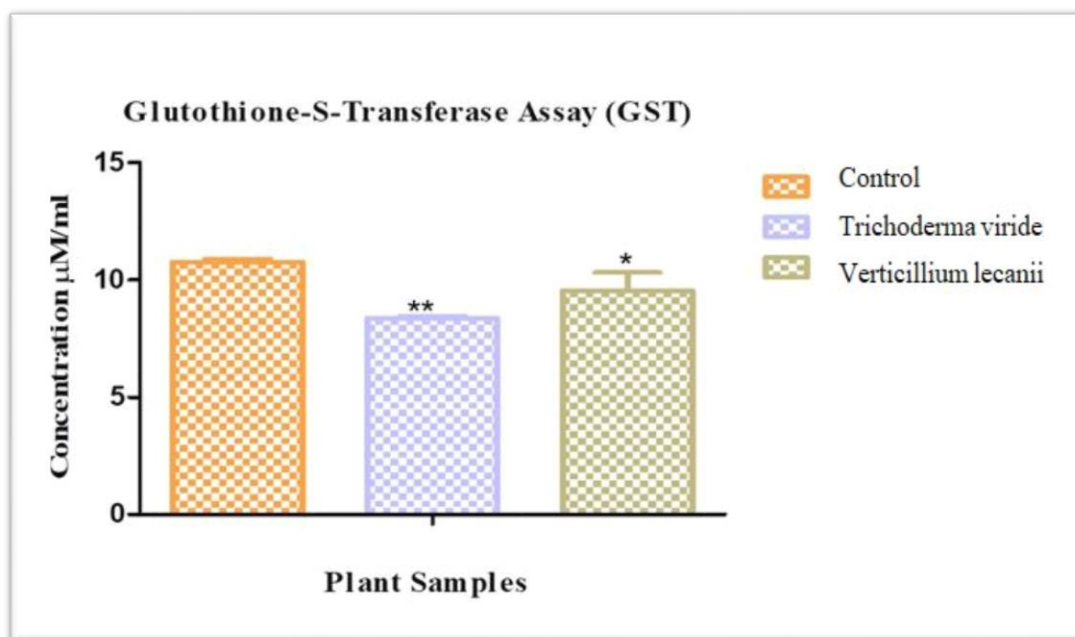


Table 5.4 GST Assay

The total content of Glutathione-S-Transferase (GST) among the treated and untreated plants values are expressed as mean ($\pm$ SD) analyzed by One-way analysis of variance. The values are significant (**) between the samples ($p = 0.0018$). The graphical representation shows a slight difference compared to the control group, these results indicate that there was decreased GST content in the treated groups compared to the control.

IV. TBARS Assay

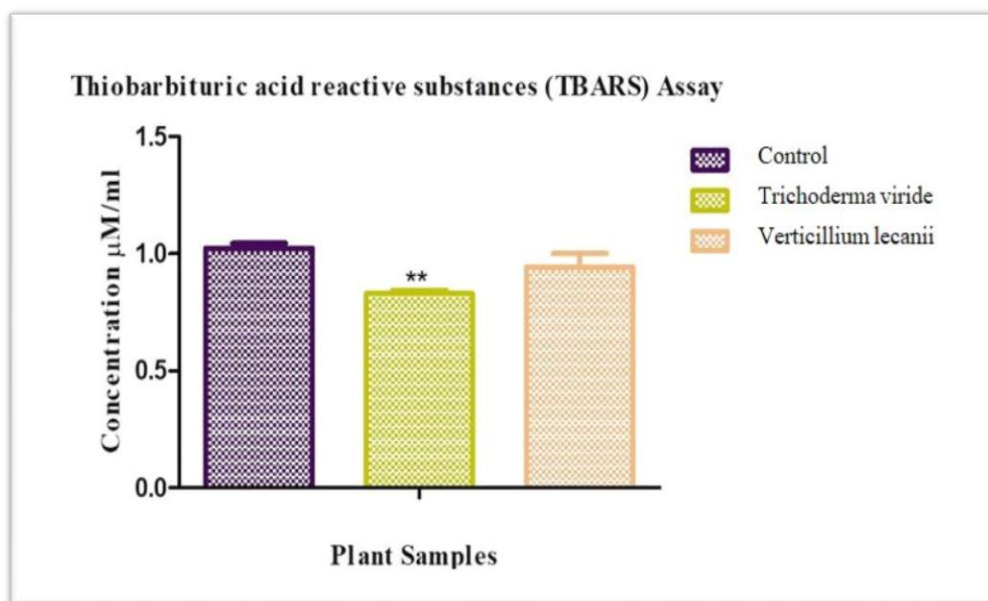


Table 5.5 TBARS Assay

The total content of Thiobarbituric acid reactive substances (TBARS) among the treated and untreated plants values are expressed as mean ($\pm$ SD) analyzed by One-way analysis of variance. The values are significant (**) between the samples ($p = 0.0019$). The graphical representation shows a slight difference compared to the control group, these results indicate that there was decreased oxidative stress in the treated groups compared to control.

5.3. Measurement of soluble protein content

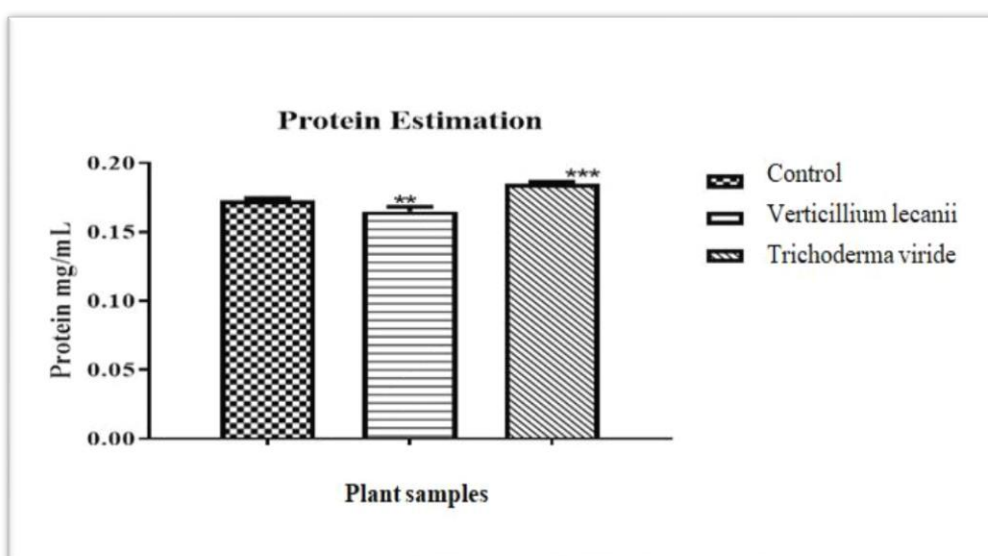


Table 5.6 Protein Estimation Graph

The total content of Protein (Bradford estimation method) among the treated and untreated plants values are expressed as mean ($\pm$ SD) analyzed by One-way analysis of variance. The values are highly significant (***) between the samples ($p < 0.0001$). The graphical representation shows a slight difference compared to the control group, these results indicate that there was increased protein content in the *Trichoderma viride* treated plant compared to the control plant.

5.4. SDS-PAGE

The protein content was observed in SDS-PAGE by visualizing the size of the protein band in 12% Acrylamide Gel. The overall quality of the protein was good with less vertical streaking and smearing in *Verticillium lecanii* treated plant whereas in the *Trichoderma viride* shown equal band quality compared to control.

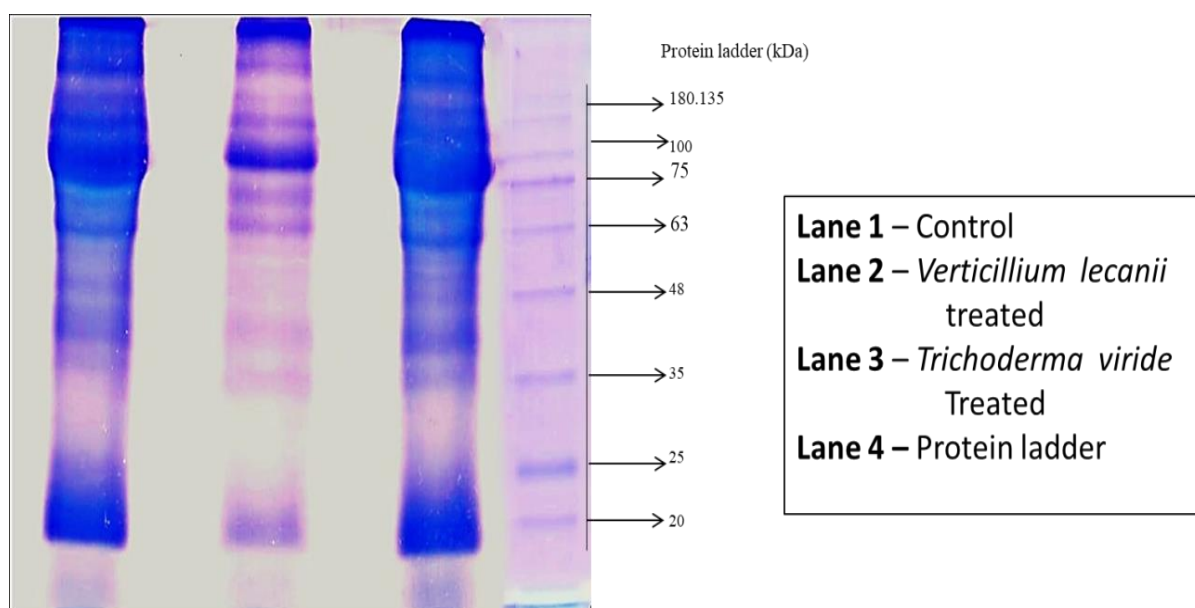


Figure 5.7 Protein Bands of SDS Page

5.5. SEM ANALYSIS:

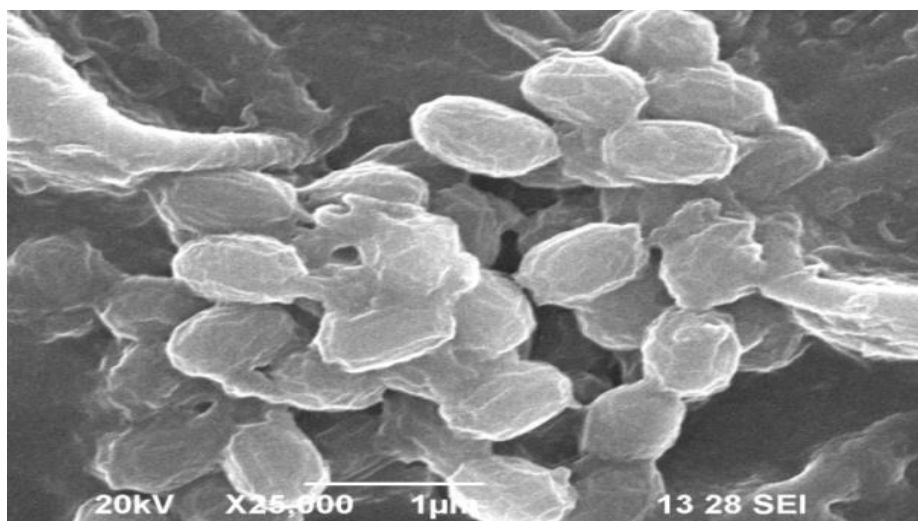


Figure 5.8 SEM Image of *Trichoderma viride*

The SEM microscopy indicates the fungal mycelia with fruiting bodies of fungi which were observed in leaf tissue treated with *Trichoderma viride*.

CHAPTER 6

SUMMARY & CONCLUSION

After we established the Fungal endophytes as a biocontrol agent into the seed of *Vigna mungo*, we observed the growth and other biochemical properties by using various methods such as enzyme analysis method, chlorophyll pigmentation, Protein profiling study, SEM analysis. Based on the observation, we have concluded that *T.viride* has a great tendency to act as biocontrol agents and also plant growth promoters compare to the control and other biocontrol agents. This research gives a wide range of knowledge about the endophytes and their role in agriculture. In the future, we will do more research based on these endophytes.

CHAPTER 6

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