

***Trichoderma* AS POTENTIAL STIMULATOR
OF YIELD, SOIL HEALTH AND DEFENCE
GENE IN MAIZE (*Zea mays* L.)**

Thesis

submitted to

Uttar Banga Krishi Viswavidyalaya

In partial fulfillment of the requirements for the degree

of

Master of Science Agriculture

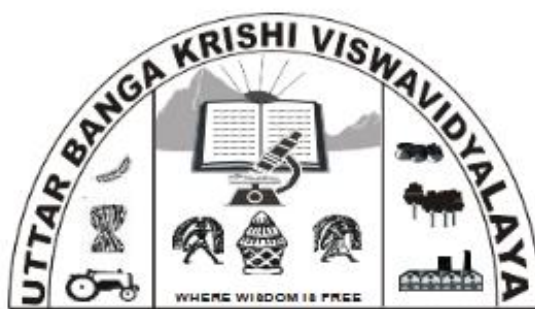
in

PLANT PATHOLOGY

By

SEERAM PHANINDRA

(A-2020-035-M)



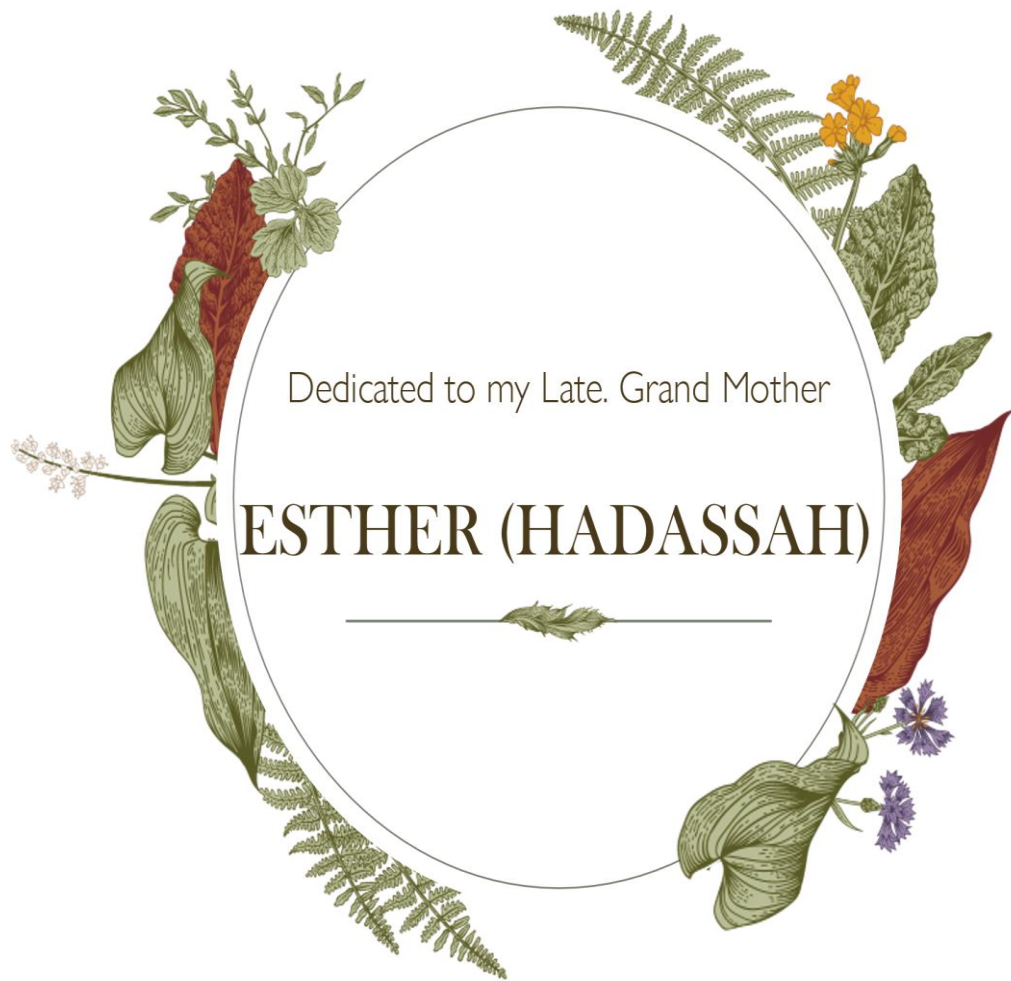
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Dedicated to my Late. Grand Mother

ESTHER (HADASSAH)

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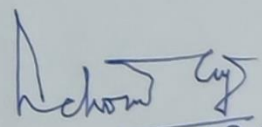
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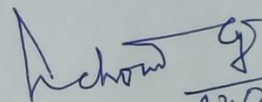
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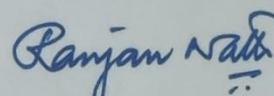
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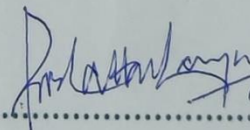
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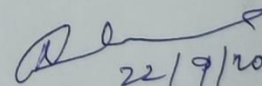
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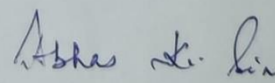
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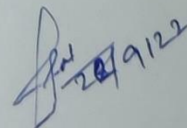
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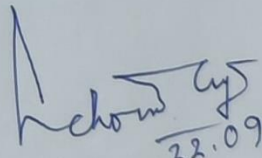
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With regardful memories.....

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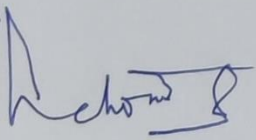
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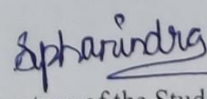
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ABSTRACT

Banded leaf and sheath blight caused by *Rhizoctonia solani* f. sp. *sasakii* is one of the most destructive disease that results in 10-40% yield loss with an economic loss of 1456.6 million rupees annually. The management of the disease is very difficult due to its nature and wide host range. *Trichoderma* spp. have emerged as the most promising agents among the generally utilized BCAs due to their distinct biocontrol activities and extensive range of desirable plant interactions (Benitez *et al.*, 2004), high reproductive potential, adaptability to a wide range of climatic situations, effectiveness in utilising nutrients, ability to alter the soil rhizosphere, high aggressiveness over disease causing fungus, enhancing plant development and defence activities. Considering the above facts, a study was undertaken to determine the biostimulant activities of the different strains of *Trichoderma* maize yield, the population of beneficial microbes and induction of defence genes against *Rhizoctonia solani* infection. The different strains of *Trichoderma* have been shown to enhance the yield of maize by stimulating the main and lateral roots for obtaining nutrients from different layers of soil when used as seed treatment. Beside this, *Trichoderma* also increased the population of antagonistic microflora viz., *Trichoderma* and *Pseudomonads*. Further, the soil dehydrogenase enzyme activity was found to be higher in *Trichoderma* treated soil. The tamarind seed powder amended *Trichoderma* has better potential to protect *R. solani* infection which is responsible for banded leaf and sheath blight disease. Finally, the expression of PAL1 and PAL 2 genes as well Salicylic acid gene in *Trichoderma* treated plants were higher that indicates that *Trichoderma* has the potential to stimulate the defence genes to protect it against *R.solani* infection.


Signature of Chairman (Advisory Committee)


Signature of the Student

CONTENTS

Chapter	Topic	Page No.
I.	INTRODUCTION	1-4
II.	REVIEW OF LITERATURE	5-24
III.	MATERIALS AND METHODS	25-42
IV.	RESULTS AND DISCUSSION	43-61
V.	SUMMARY AND CONCLUSION	62-63
VI.	REFERENCES	I-XX
VII.	PLAGIARISM DOCUMENT	-1-
VIII.	VITA	-2-

LIST OF TABLES

Table No.	Table Description	Page No.
1.	Effect of seed treatment with <i>Trichoderma</i> on harvesting index of Maize.	43
2.	Effect of seed treatment with <i>Trichoderma</i> on main and lateral roots of Maize.	45
3.	Estimation of Soil <i>Trichoderma</i> , <i>Pseudomonas</i> and Soil Bacterial populations in <i>Trichoderma</i> treated Maize (<i>Zea mays L.</i>) plots by Agar plate method.	48
4.	Soil dehydrogenase activity in <i>Trichoderma</i> treated soil from Maize plots.	52
5.	Effect of <i>Trichoderma harzianum</i> formulations on <i>Rhizoctonia solani</i> .	53
6.	Estimation of PAL enzyme activity in maize by seed treatment with different strains of <i>Trichoderma</i> .	56
7.	Gene expression analysis of defence signalling genes (PAL and SA) in maize plants inoculated with <i>R. solani</i> :	59

LIST OF FIGURES

Figure No.	Figure Description	Page No.
1.	Graphical representation of Yield and Harvesting Index (%) of Maize as influenced by <i>Trichoderma</i> .	44
2.	Graphical representation of Root Architecture analysis of <i>Trichoderma</i> treated Maize plant for total no. of main and lateral roots, length of main and lateral roots.	45-46
3.	Graphical representation of soil population dynamics of <i>Trichoderma</i> , Soil Bacteria and <i>Pseudomonas spp.</i> in <i>Trichoderma</i> treated maize plots.	49
4.	Graphical representation of Soil Dehydrogenase Enzyme Activity of <i>Trichoderma</i> treated soil from Maize plots.	52
5.	Graphical representation of Growth Inhibition (%) of <i>Trichoderma harzianum</i> formulations against <i>R. solani</i> .	53
6.	Graphical representation of Phenylalanine Ammonia Lyase (pal) enzyme analysis in <i>Trichoderma</i> inoculated maize plants at different time intervals (0, 24, 48 & 72 HAI).	56-57
7.	Graphical representation of defence signalling genes (pal 1, pal 2 & Salicylic) in maize plants inoculated with <i>R. solani</i> at different time intervals (0, 24, 48 & 72 HAI).	59

LIST OF PLATES

Plate No.	Plate Description	Page No.
1.	Root system of Maize plants treated with different strains of <i>Trichoderma spp.</i>	47
2.	Root architecture analysis by using Rootnav v1.8.1x64 software.	47-48
3.	<i>Trichoderma spp.</i> culture in TSM for estimation of Total Soil <i>Trichoderma</i> population in soils collected from root surface of <i>Trichoderma</i> inoculated maize plots.	50
4.	<i>Pseudomonas spp.</i> culture in King's B Broth for estimation of Total Soil <i>Pseudomonas spp.</i> population in soils collected from root surface of <i>Trichoderma</i> inoculated maize plots by MPN Technique.	50
5.	Soil Bacterial culture in Soil Agar extract for estimation of Total Soil Bacterial population in soils collected from root surface of <i>Trichoderma</i> inoculated maize plots by Agar plate method.	51
6.	Dual culture of <i>Trichoderma harzianum</i> (UBT 18) and <i>R. solani</i> .	54
7.	Different <i>Trichoderma</i> strains culture on <i>Trichoderma</i> specific medium (TSM).	54
8.	Microscopic pictures of Different <i>Trichoderma</i> strains used in the Research studies.	55
9.	<i>R. solani</i> isolation and culturing on PDA with match sticks. Inoculation of <i>R. solani</i> and symptoms of Banded leaf and sheath blight of maize.	57-58

ABBREVIATIONS

μL	Microlitre
μM	Micromolar
°C	Degrees Celsius
ABC	ATP- Binding cassette
ATP	Adenosine triphosphate
BARC	Baba atomic research centre
BCA	Biocontrol agent
BLSB	Banded leaf and sheath blight
BOD	Biological oxygen demand
cDNA	Complementary Deoxyribose nucleic acid
CHS	Chalcone synthase
cm	Centimetre
CT	Cycle threshold
DEPC	Diethyl pyro-carbonate
DHA	Dehydrogenase
DNA	Deoxyribose nucleic acid
DNTP's	Deoxynucleoside triphosphates
ET	Ethylene
FDA	Food and drug administration
g	Gram

ha	Hectares
hai	Hours after inoculation
HCl	Hydrochloric acid
HI	Harvesting index
HR	Hypersensitive response
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
JA	Jasmonic acid
L	Litre
mg	Milligram
ml	Millilitre
mm	Millimetre
MPN	Most probable number
MT	Metric tonnes
nm	Nanometre
PAL	Phenylalanine ammonia lyase
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PF	Pseudomonas fluorescens
PGPF	Plant growth-promoting fungus
PGPR	Plant growth-promoting rhizobacteria
POX	Peroxidase
PPO	Polyphenol oxidase

PR	Pathogenesis related proteins
psi	Pound per square inch
qRT-PCR	quantitative Real Time - Polymerase Chain Reaction
RBD	Randomized block design
RNA	Ribonucleic acid
RSA	Root system analysis
RT	Reverse transcriptase
Sal	Salicylic acid
SAR	Systemic acquired resistance
TPF	Triphenyl formazan
TSM	Trichoderma specific medium
TTC	2,3,5- triphenyl tetrazolium chloride

CHAPTER- I



INTRODUCTION



I. INTRODUCTION

As a source of food, animal feed, and industrial products, maize (*Zea mays* L.) is one of the most significant cereal crop in the global agricultural economy with world's average yield of 47.19 quintals/ha. It has high yield potential and ranks top among cereals ahead of rice, wheat, and millets. No other cereal has such tremendous potential thus acquires the title of "queen of cereals". The crop requires less water than other cereals besides being C4 and day neutral one, it has wider adaptability and cultivated throughout the year, it is rightly called as a "miracle C4 crop".

Maize is grown in an area of 193.7 million hectares worldwide with a production of 1147.6 million metric tonnes and a productivity of 5.75 tonnes/ha. India stands fourth in terms of area and seventh in terms of production, accounting for about 4 percent of the global maize area and 2 percent of global production. It is mostly cultivated during the *kharif* and *rabi* seasons with an area of 9.56 m ha, yields 28.76 million metric tonnes with a productivity of 3.07 tonnes per hectare. As of third December 2021, 6.17 lakh hectares of *rabi* maize has been sown in India, which is less than the 6.33 lakh hectares cultivated during the comparative period of last year. Rabi maize is gaining much faster spread in West Bengal in recent past and major states for maize cultivation include West Bengal (0.31 lakh ha), Gujarat (0.50 lakh ha), Telangana (0.82 lakh ha), Bihar (0.86 lakh ha), Maharashtra (1.21 lakh ha), Tamil Nadu (1.60 lakh ha) (Anon., 2021).

In India sixtyone diseases have been recorded in maize that are infected by fungi, bacteria, viruses, and nematodes. Among them, sixteen diseases are regarded as prime constraints in reducing the yield of crop. The major diseases are downy mildews, foliar diseases, pre-flowering and post-flowering stalk rots, and sheath diseases (Payak & Sharma, 2008). Among the diseases that occur in India, the pathogen *Rhizoctonia solani* f. sp. *sasakii* causing Banded leaf and sheath blight in maize is one of the most destructive disease that results in 10-40% yield loss with an economic loss of 1456.6 million rupees annually (Hooda *et al.*, 2017). In 1927, Bertus mentioned it as a sclerotial disease from Sri Lanka (Bertus, 1927). At earlier it was found to be a minor maize disease. Currently, it was identified to be one of the major disease emerging not only throughout the India but also in a majority of other tropical Asian countries where maize

is cultivated. During 1960s, the disease was identified in western central foothills of the Himalayan areas of India. Only in the early 1970s, when an outbreak appeared in the warm, moist foothill regions of Himachal Pradesh's Mandi district, the disease became significantly evident (Payak and Renfro, 1968). The disease is more prominent in humid conditions with optimum temperature of 28°C and due its soil borne nature, lack of disease resistant varieties poses a huge threat for maize farmers.

The symptoms were observed on the leaf sheath, stalks, leaves and ears. The symptoms include characterized appearance of bands on lower leaf sheaths, blighting of leaves and sheath, rind spotting, breakage of stalks, cracking and clumping of silk fibre, rotting of ear, lesions of horse shoe-shaped with banded caryopses etc., The disease occurred generally at the stage of pre flowering on 30 to 40 days old maize plants under normal field conditions but it may also infect new emerging plants, which can lead to severe blighting and necrosis of apical part of developing plants (Saxena, 2002). In *R. solani*, the mycelium and sclerotia are often used for identification generally as they do not generate spores. During initial stages the mycelium appears as colourless but on maturation they become brown coloured. The hyphae are hyaline, septate, multinucleate (coenocytic) and branched at 90 degrees angle when observed under a microscope. On highly infected plant portions and in culture, the sclerotia are formed in huge amounts. Sclerotia are normally round with a diameter of 1 to 5 mm and are dark brown to black coloured. The pathogen is generally found in soil and can endure in soil from months to few years (Sneh *et al.*, 1991).

Most hybrids and composites of maize that are cultivated on a commercial basis are discovered to be moderately vulnerable to BSLB disease. One of the prime most reason is lack of durable resistance may be resulted due to existence of variability in maize populations (Zhang *et al.*, 2012). In the absence of resistant cultivars, many farmers rely on application of systemic fungicides for managing BLSB disease.

However long-term usage of chemical pesticides polluted water and atmosphere, leave hazardous residues that can stimulate the growth of particular resistant organisms. In recent years there is a rise in public awareness to hazardous effect of pesticides and consequent damage to environment. As a result, search is on for environmentally safe, non-hazardous, and sustainable plant protection approaches.

iological control is one of the promising method that relies on microbial antagonism and are crucial alternatives for controlling plant diseases (Baker,1987).

Trichoderma spp. is one among fungal BCAs that was most widely studied and promoted commercially as biopesticides (Kamala and Devi, 2012). In controlling the plant fungal infections *Trichoderma* was discovered as the genus with great potential for biocontrol amongst 25 fungal antagonists (Thambugala *et al.*, 2020). Weindling (1934) first reported the culture of *Trichoderma lignorum* (*Gliocladium virens*) culture filtrate are harmful to *R. solani* and other disease causing fungi. *Trichoderma spp.* have emerged as the most promising agents among the generally utilized BCAs due to their distinct biocontrol activities and extensive range of desirable plant interactions (Benitez *et al.*, 2004), high reproductive potential, adaptability to a wide range of climatic situations, effectiveness in utilising nutrients, ability to alter the soil rhizosphere, high aggressiveness over disease causing fungus, enhancing plant development and defence activities. *Trichoderma* applied plants have exhibited enhancement in plant biomass and yield (Chang *et al.*, 1986), augmented nutrient uptake, and improved seed germination rates (Yedidia *et al.*, 2001), Induced systemic resistance (ISR) to infections (Yedidia *et al.*, 1999; Djonovic *et al.*, 2006; Viterbo *et al.*, 2007). Application of *Trichoderma* as a biocontrol of plant diseases has gained farmers acceptance due to its efficiency, environmentally safe and alternative to fungicide application for minimizing the use of chemicals in farming ecosystems (Papavizas, 1985).

Trichoderma spp. colonises the rhizosphere or rhizoplane on interaction with the plant roots. Bipartite interaction occurs during root colonization causes morphological variations in plant roots (Contreras-Cornejo *et al.*, 2009) and stimulate plant development through enhanced root density and improved nutrient uptake, mineral solubilization, and immune reaction to abiotic and biotic stresses (Mastouri *et al.*, 2010). In several studies, it was found that *Trichoderma* inoculation on maize plants changed the root architecture, enhanced root hair formation and increased root biomass production and increased production. (Harman *et al.*, 2004).

Trichoderma species are soil-borne (free-living), non-pathogenic fungus that also invade the roots of various plants (Harman *et al.*, 2004). *Trichoderma* in association with roots strengthens the plant's defence system for increased resistance to diseases. *Trichoderma spp.* induces the systemic acquired resistance (SAR), induced

systemic resistance (ISR), and the hypersensitive response in plants (Benitez *et al.*, 2004; Vinale *et al.*, 2008). Numerous studies demonstrated that *Trichoderma* species may indirectly contribute to systemic resistance (Ahmed *et al.*, 2000; Lo *et al.*, 2000). *Trichoderma spp.* are able to defend plants against some foliar infections in addition to soil-borne pathogens due to induced systemic resistance. Plants are able to identify fungus secreted proteins, and such molecular patterns activate systemic resistance. The systemic regulation of gene expression in distal leaves is one of the most intriguing effects of plant *Trichoderma* relationships. Proteomic and transcriptomic method of analysis have shown that *Trichoderma* colonises plant roots and causes a systemic change in the expression of genes in those plants that are involved in stress reactions, isoprenoid oxylipins, scavenging of reactive oxygen species, photorespiration, and ethylene biosynthesis. Induced resistance is a result of increase in the quantities of defensive phytochemicals and enzymes, these mostly contain phytoalexin biosynthesis (HR) and mainly involves the participation of enzymes involved phenylpropanoid pathway, especially phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). Jasmonic acid and ethylene acts as signalling molecules in the main signal transduction pathway of *Trichoderma*-mediated induced systemic resistance in cucumber (*Cucumis sativus*), and a similar model has been exhibited in maize (*Zea mays*) (Djonovic *et al.*, 2007).

Objectives:

With this background, a research work entitled "*Trichoderma* as potential stimulator of yield, soil health and defence gene in maize (*Zea mays L.*) " was carried out in the Department of Plant Pathology, UBKV with the following objectives.

1. To measure the yield attributes in maize as influenced by *Trichoderma*.
2. To estimate the soil dehydrogenase enzyme and population of beneficial microbes in *Trichoderma* treated soil.
3. To measure the specific defence related gene in maize after application of *Trichoderma*.

CHAPTER- II



REVIEW OF LITERATURE



II. REVIEW OF LITERATURE

Maize Scenario:

In India corn or maize (*Zea mays L.*) ranks third among the cereal crops after rice and wheat. It has high value across the world for its multiple uses as food, fodder, feed and raw materials for numerous industrial commodities. With its wide range of adaptability, maize can be grown in varied environmental conditions with elevations ranging from more than 3000 m high above mean sea level and with annual rainfall of 250 mm to more than 5000 mm. The global production of maize is 1423 million MT, which is grown over 188 million ha of land in more than 170 nations.

Traditionally, in India maize is grown as a kharif or rainy season crop in northern part of India. However, there has been a significant change in maize farming since from the mid 1980's, where maximum area under maize cultivation was shifted to peninsular part of India. At present conditions, peninsular India accounts for over 40 percent of overall maize cultivating area and 50 percent of total maize yield. On the other hand, non-traditional belts like coastal Andhra Pradesh, Bihar, Telangana, West Bengal, and others states have observed substantial growth in rabi or winter maize. In recent years, cultivation of Rabi maize in West Bengal has gained significant importance and spread faster.

As of third December 2021, 6.17 lakh hectares of *rabi* maize has been sown in India, which is less than the 6.33 lakh hectares cultivated during the comparative period of last year. Rabi maize is gaining much faster spread in West Bengal in recent past and major states for maize cultivation include West Bengal (0.31 lakh ha), Gujarat (0.50 lakh ha), Telangana (0.82 lakh ha), Bihar (0.86 lakh ha), Maharashtra (1.21 lakh ha), Tamil Nadu (1.60 lakh ha) (Anon., 2021).

Banded leaf and sheath blight of Maize:

The plant pathogens such as viruses, bacteria, fungi and nematodes infect the plants under field conditions or during post-harvest storage, significantly reducing production loss of agricultural products that poses a serious threat to food production throughout the world (Savary *et al.*, 2019). Plant pathogens cause a major threat for maize cultivation and among the diseases that occur in India, Banded leaf and sheath blight caused by *Rhizoctonia solani f. sp. sasakii* is one of the most destructive disease that results in 10-40% yield loss with an economic loss of 1456.6 million rupees

annually (Hooda *et al.*, 2017). Banded leaf and sheath blight of maize caused by *Rhizoctonia solani* f. sp. *sasakii* is a soil borne disease that have been found in major maize growing parts of the world and most commonly seen in an optimum temperature of 28°C and humid weather conditions. It is one of the major diseases of maize which can damage entire crop within a short span of time. Under favourable environmental conditions it spreads from lower basal portion of sheath to developing ears or inflorescence. The growing ear is severely destroyed and dries up prematurely making the husked leaves caked.

One of the major diseases of maize, banded leaf and sheath blight (BLSB) caused by *Rhizoctonia solani* f.sp. *sasakii* Exner (*Thanatephorus sasakii*) is becoming more prevalent and economically significant in major parts of the world. Depending on the severity of the disease, the loss in grain production ranges from 11 to 40% (Singh and Sharma, 1976).

Symptoms:

Bertus (1927) reported that BLSB symptoms on infected leaf sheath as minute brown purplish spots or olive greenish continuous areas, below the infected leaf sheath the stalks and the stem at base of silk appears as pale olive brown spots or lesions. BLSB symptoms include prominent and characterized appearance of distinct bands on maize leaf sheaths and sclerotial bodies on infected plant portions. Except tassel part, all aerial parts of a plant exhibit symptoms on infection. The disease is observed on leaf sheath, leaves, ears and stalks as rind spotting or lesions on stalk, leaf and sheath blight, breakage of stalks, cracking and clumping of styles and formation of horseshoe-shaped lesions with distinct banding of the caryopses which results in complete rotting of ears. Every part of maize especially aerial plant parts are more prone to a wide range of pathogenic infections that significantly lower crop yield and quality (Shurtleff, 1980).

Host susceptibility to pathogenic infection differ from maturity or age of the host (Agrios, 1997 and Singh, 1998). Ahuja and Payak (1978) found that 30 to 45 days old maize plants were very susceptible to infection by *Rhizactonia solani* sp. *sasakii* due to high humidity prevalent in the months of July and August. The development of diseases has been significantly correlated with plant age that ranges from 30 to 60 days. Before pre-flowering stage to silking, when inoculation was done it resulted in infection up to the inflorescence completely and grain formation is hampered with no grain

development. Plant stage with more than sixty days did not encourage the infection of cob because the grains became progressively harder as they mature (Lal *et al.*, 1980).

The disease can be managed by different approaches like cultural, chemical, biological as well as disease resistance. Among the biological methods, *Trichoderma* has received more attention among the researchers.

***Trichoderma* as biocontrol agent:**

The fungal genus *Trichoderma* is found all over the world and are widespread in many diverse regions including soil, wood, forests, and paper (Harman *et al.*, 2004a).

Forty percent of plant photosynthates exudates from roots i.e., they generate carbon-rich resources by secreting substances like sugars, amino acids and organic acids into the soil rhizosphere so it becomes relatively high in nutrients and also develop a molecular connection with soil microorganisms that promotes root colonisation. As a result, the rhizosphere is inhabited by numerous microbial communities that can influence growth of plant in a positive, neutral, or negative way. (Bais *et al.*, 2006).

Trichoderma species are non-pathogenic, free living, facultative anaerobes widespread in root and soil environments where they interact with other fungus and plant roots. *Trichoderma spp.* have been reported to be symbiotic with plants (Harman *et al.*, 2004a). They infect plant roots in a symbiotic process, but chemical communication signals enable the plant to wall off the invading hyphae of *Trichoderma* so that it is limited only to root's outer layer. So that their hyphae are confined to the cell epidermal layers and a few cells above this level, where they proliferate mostly in the intercellular spaces of roots (Yedidia *et al.*, 1999; Harman *et al.*, 2004). In recent times, they are extensively studied for their ability to promote plant development, generate antibiotics, parasitize on other fungus, and compete with pathogenic plant microbes (Chang *et al.*, 1986; Harman *et al.*, 2004a; Yedidia *et al.*, 2001).

Trichoderma belongs to Deuteromycetes subdivision, in which most of the members do not have or show sexual reproduction rather they adapted more to asexual form of life cycle (Harman, 2004a). *Hypocrea* is the teleomorph stage of *Trichoderma spp.* with Hypocreales order and Hypocreaceae family (Samuels, 1996). *Trichoderma spp.*, are widespread saprophytic filamentous fungi found nearly in all soils and soil

rhizosphere microflora. They are well known for their biocontrol against several plant pathogenic fungi that led to numerous soil-borne and post-harvest infections in several crops (Hajieghrari *et al.*, 2008; Howell, 2003). In addition to biocontrol mechanism, some *Trichoderma* species can directly stimulate plant growth through rhizosphere colonisation and alter the microflora competition on roots, as opportunistic competence in soil rhizosphere (Ahmad and Baker, 1987).

Trichoderma spp. are well known for their significant utility to agriculture, such as their potential to defend crops from disease causing pathogens and enhances agriculture production under field areas (Harman *et al.*, 2004). *Trichoderma* isolates effect on growth and development of plants have significant economic results including minimising the growth period of plants and in nurseries the time is reduced, enhances vigour of the plant to withstand abiotic and biotic stresses, increased plant productivity. As a result, use of *Trichoderma* strains in plant enhanced development is crucial for sustainable agriculture, since chemical fertiliser is not economically viable over the long term due to its high cost and environmental damage. Therefore, it is extremely desired to reduce or eliminate the use of synthetic chemical fertilisers in agriculture system.

Numerous biotic and abiotic factors namely plant species, form of inoculum applied, inoculum concentration, the isolate of *Trichoderma* utilised, the soil environment and the microflora of soil rhizosphere have significant influence on activity of *Trichoderma* in soil.

Trichoderma spp. are endophytic organisms, they are efficient biological control agents of fungi in the soil rhizosphere, releasing antimicrobial substances, promoting plant growth and vigour by solubilizing soil minerals, and supplying vital nutrients and growth-regulating substances to crops (Altomere *et al.*, 1999).

In 1794, *Trichoderma spp.* was first isolated from soil and decomposing organic material (Persoon *et al.*, 1794). *Trichoderma spp.* encourage prolific root growth in symbiotic association with plants so that the plants have abundance of roots well developed. *Trichoderma* species are utilised as biofertilizers and biological agents (Adams *et al.*, 2007; Bais *et al.*, 2006). Use of *Trichoderma* species is economical, environmentally safe and beneficial biocontrol agent. They can establish themselves in different patho-systems, have less effect on soil ecology, and do not harm beneficial

microbes that results in control of plant pathogens. This symbiotic relationship with plants enhances plant defence, resistance to pathogens, developmental processes and yields, fertiliser use efficiency and increased nutrient uptake.

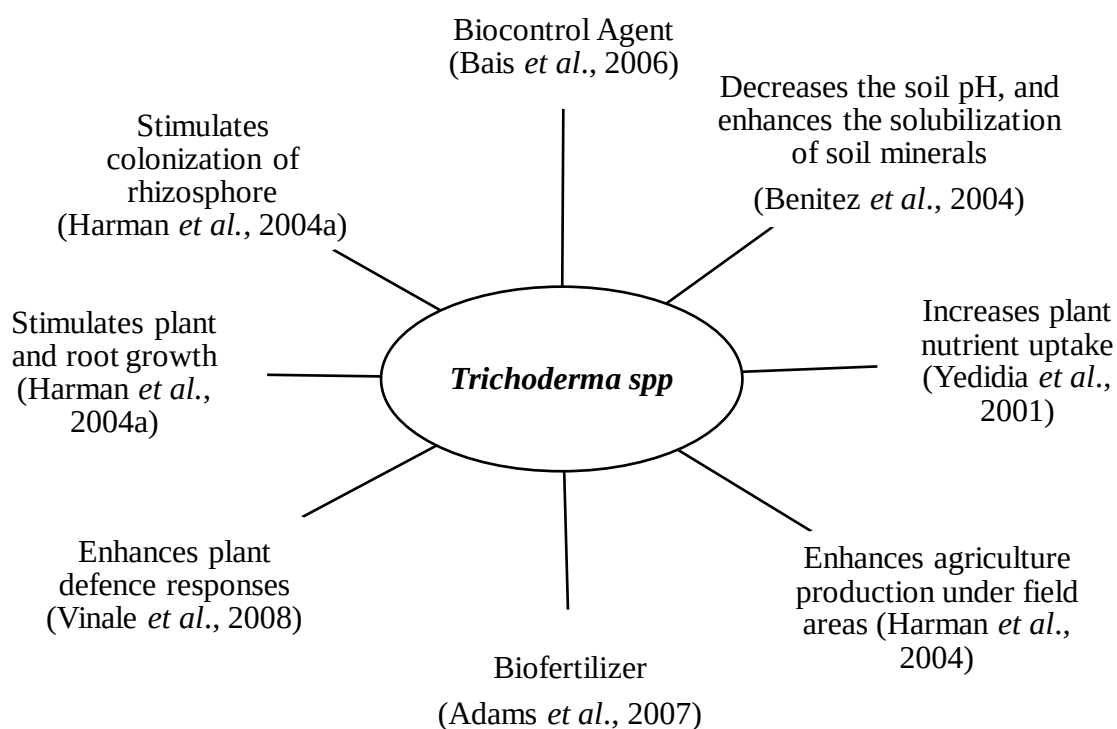
The performance of using *Trichoderma spp.* as biofertilizer and biocontrol agent depends on a varied factors such as the isolate's virulence, environmental conditions, application time, and ability to survive in specific environments rather than their native place and colonise plant roots for a specific period of time to manage and control soil borne plant pathogens (Kredrics *et al.*, 2003; Nemec *et al.*, 1996; Stephan *et al.*, 2005; Vinale *et al.*, 2008) suggesting that increasing the local virulent would be more effective and successful. Among the fungal biocontrol agents used for plant disease management, 90 percent belongs to various *Trichoderma* strains (Hermosa *et al.*, 2012).

Plants support a wide range of soil microbes in root rhizosphere and agronomic practises like crop rotation increases the soil microbial diversity to a greater level by supplying various organic resources in great amounts. Further, it was stated that the presence of soil microbes enhances the availability of nutrients to plants, particularly nitrogen and phosphorous. (Turmuktini *et al.*, 2012). Because of their association with soil biology, soil enzymes serve as an essential indicators of soil quality and are referred to as "biological fingerprints" of previous soil management practises (Bandick and Dick, 1999). Differences in soil enzyme activity often reflect changes in soil microbial dynamics (Dick and Kandeler, 2005).

Application of chemical fungicides adversely affects the other non-target microorganisms, therefore biological control strategies are viewed as important approaches for management of plant diseases (Kohl *et al.*, 2019). There are numerous research analysis that show certain microbes can impede the growth of plant pathogens by interfering with their metabolisms or by developing a direct parasitic interaction (Panth *et al.*, 2020). It has been recommended that use of soil enzyme analysis as a technique for monitoring the changes in ecology of soil brought down by interaction between native soil microbes and biocontrol inoculants (Doyle and Stotzky, 1993). Dhruva Kumar *et al.*, (1992) reported that activity of soil enzyme is frequently employed as a measure of total soil microbial activity and soil fertility. This assessment can help us to understand the nature of disturbances soil ecosystem.

Culture filtrates of strains of *Trichoderma* suppressed the mycelial growth of *R. solani* under invitro situations. Tang Jiabin et al., (2002) tested six strains of *Trichoderma* against *R. solani* by using dual culture method. Cultural filtrate of T3 (*T. aureoviride*) recorded the greatest inhibition by 52.54% against *R. solani*. Specific soil microbial communities that act against the plant pathogens in soil results in disease suppressiveness. *Trichoderma*, *Bacillus*, *Pseudomonas*, *Actinomyces* and *Streptomyces* spp. are some of the microbes responsible for the disease suppressiveness in soils (Mendes et al., 2011).

Formulations of *T. harzianum* based on talc and cmc decreased the disease incidence by 24% and severity by 52% respectively (Khan and Sinha, 2007). Performances of *Trichoderma viridae*, *Trichoderma harzianum* and *Pseudomonas fluorescens* were evaluated for their potentiality to suppress mycelial growth of *R. solani* (isolated from infected soybean plant). Ninety six hours after incubation the biocontrol agents suppressed the mycelia of *R. solani* (Anjana et al., 2007).



Importance of *Trichoderma* spp.

Mechanism of Biological control:

Trichoderma lingnorum (viride) is a fungal biocontrol agent whose biocontrol activity on soil-borne fungal plant pathogen of *Rhizoctonia solani* was first demonstrated by Weindling (1932). After that the myco-parasitic activities of the similar strains of *Trichoderma* on *Sclerotium*, *Pythium*, *Phytophthora*, and *Rhizopus* spp. were reported. *Trichoderma* spp. has gained a lot of emphasis as a biocontrol organism and today most of the fungal biocontrol research and foundations are rooted on *Trichoderma* spp. (Chet, 1993).

Trichoderma species are reported as fungal biocontrol agents that either they produce antimicrobial substances or parasitize fungi that infect the plant species (Handelsman and Stabb, 1996). *Trichoderma* spp. are successful biocontrol agents that are found highly effective towards virulent fungal plant pathogens. They have indirect mechanism by striving for space and nutrients to live, altering the environment, or enhancing growth of a plant, plant defence systems and antibiosis through release of antimicrobial compounds or directly by mycoparasitism action (Papavizas, 1985; Howell, 2003; Vinale *et al.*, 2008).

Trichoderma can function in various ways for biological control of plant pathogens:

- They utilize food resources more effectively and grows rapidly than the pathogen, as a result the pathogen is crowded out and this mechanism is called as Nutrient competition.
- They may ingest and feed directly in or on a pathogen and this mechanism is called as Parasitism.
- They may secrete a substance that inhibits or kills plant pathogens that are close to it and this mechanism s called as Antibiosis.
- They may stimulate the plant to produce a substance that eliminates or kills the plant pathogen and this mechanism is called as Induced resistance. In fact, the plant fights back. In this way, the pathogen is destroyed and killed.

Although some biocontrol agents utilize only one of these mechanisms but most of the advanced and successful biocontrol agents such as *Trichoderma* utilize multiple mechanisms (Monte E, 2001).

Nutrient Competition:

Competition is one of conventional biological control mechanism where competition is seen between the biocontrol agent (antagonist) and the plant pathogen for nutritional resources and space (Chet *et al.*, 1997). *Trichoderma spp.* are quick multiplying fungi with persistent conidia and they have a wide range of substrate utilization. They are fierce and efficient competitors for nutrient resources and living place (Hjeljord *et al.*, 2000).

The biocontrol agent competes with the other plant pathogens for nourishment and this competition provides disease control. *Trichoderma spp.* are highly aggressive that they can survive in nutrient deficient situations by forming chlamydospores and conidia. Rhizosphere competence is significant because a biocontrol agent that has the ability to grow and proliferate in the soil rhizosphere can effectively compete for nutrients and space. On application of *Trichoderma spp.* to soil or as seed treatment, they can grow and multiply along with the advancing root system of the treated plant (Harman, 2000; Howell *et al.*, 2000; Zhang *et al.*, 1996).

Trichoderma generates ATP from various nutritional resources in soil environment and they include cellulose, hemi-cellulose, glucan, chitin, and other sugars into glucose (Chet *et al.*, 1997). Proteins plays a significant role in *Trichoderma spp.* by the activity of colonizing plants root system and aid in competition with other root colonizers (Saloheimo *et al.*, 2002; Viterbo *et al.*, 2004; Brotman *et al.*, 2008) and some develop symbiotic relationship with host plant species (Samolski *et al.*, 2012). The biocontrol agent preoccupies the infection area by competing directly with plant pathogens for space in that particular time frame (Papavizas, 1985).

Trichoderma spp. competes with the pathogens for carbon in soil which is necessary for viability (Alabouvette *et al.*, 2009). *Trichoderma spp.* produce efficient siderophores under low iron condition in soil, which are low molecular weight and ferric iron chelating compounds. These substances can impede the growth of pathogenic fungus at plant roots (Chet and Inbar, 1994). Germination of pathogenic fungal spores is inhibited at low Iron reserves in soil rhizosphere and rhizoplane. Therefore, soil physical and chemical properties influence the activity *Trichoderma* as a biocontrol agent in soil.

Trichoderma species are inherently resistant to a various harmful toxic substance, such as phenolic compounds, some fungicides, and herbicides. As a result, they have the ability to multiply quickly and have an influence on infectious plant

pathogens by generating metabolic substances that prevent germination of spore (fungistasis) or alter the soil rhizosphere (by creating acidic soils so that the pathogens cannot thrive) (Benitez *et al.*, 2004). Resistance to harmful toxic substances may be connected with the presence of ABC transport systems in *Trichoderma* strains (Harman *et al.*, 2004). As a result, *Trichoderma* strains are effective in controlling various plant pathogens like *Sclerotium rolfsii*, *R. solani*, *P. ultimum* when they are alternated with benomyl, methyl bromide, captan or other chemicals.

Parasitism:

Trichoderma spp. are well-known for their use in agricultural biocontrol utilisation that they invade and kill the fungal hosts, this is known as mycoparasitism. *Trichoderma* species are antagonistic against a wide range of soil-borne phytopathogens, including *Pythium ultimum* and *Rhizoctonia solani* that makes *Trichoderma spp.* as efficient biocontrol agent. Mycoparasitism signifies the direct activity between *Trichoderma* and the fungal plant pathogen. As indicated previously, *Trichoderma spp.* was initially identified as a biocontrol agent by Weindling (1932), who also identified mycoparasitism mechanism of *T. lignorum* (*viridae*) hyphae against *R. solani* by hyphal coiling and destruction.

It is a complicated process that involves series of actions such as host identification, invasion, and thereafter host penetration and host destruction. *Trichoderma spp.* parasitize a wide range of pathogenic fungi, detects other fungal pathogens that grow towards them and generate cell wall-degrading hydrolytic enzymes. The successive expression of pathogenesis-related proteins, mainly chitinases, proteases and glucanases contributes to distant sensing in plants (Harman *et al.*, 2004).

According to Chet *et al.* (1998), there are four sequential phases of the mycoparasitism process, and they are are:

- i. chemotropism and identification,
- ii. adhesion and hyphal coiling,
- iii. penetrating the cell wall and
- iv. host cell digestion.

Trichoderma spp. adheres to surface of the host fungi, forms appressoria on the surface of the host, enters inside the host cell, and destroys the hyphae of host (Steyaert *et al.*, 2003). In 1994, the molecular level initiation of mycoparasitism was first

described (Carsolio *et al.*, 1999) and it was based on the scientific study of endochitinase-encoding gene (ech42) regulation. In myco-parasitic interaction between *T. harzianum* and *Rhizoctonia solani*, the gene (ech42) was expressed. In other studies, it was demonstrated that P1 mutants of *T. atroviride* strain the endochitinase (ech42) or exochitinase (nagI) expression was required to initiate mycoparasitism action, that was seen in the treatments comprising pure colloidal chitin isolated from the cell wall of fungi (Vinale *et al.*, 2008).

In mycoparasitism mechanism, it involves a series of interaction between the biocontrol agent and pathogenic fungi. They include accumulation of parasite organelles in the parasitized cells, hyphal coiling, producing hyphal sheath matrix, extracellular fibrillar deposition and formation of haustoria (Chu and Wu, 1981; Elad *et al.*, 1983).

In *Trichoderma spp.* lectin plays an important role in host identification. (Barak *et al.*, 1985; Inber and Chet. 1994; Elad *et al.*, 1999). Durell (1968) investigated the haustorial formation and penetration of hyphae in phycomycetes fungal hyphae especially *T. viride*. The cDNA sequence and expression of two possible proteins associated with mycoparasitism by *T. harzianum* during its growth on *R. solani* cell wall have molecular weights of 62,853 Da and 37,010 Da (Vasseur *et al.*, 1995).

Trichoderma species plays a significant role in the mycoparasitism mechanism by producing and controlling lytic enzymes such as proteases, cellulases, glucanases and chitinases (Mukherjee *et al.*, 2008). Chitin and β 1,3-glucans are the primary components of the fungal cell walls (Fesel *et al.*, 2016). *Trichoderma* species produces these lytic enzymes 1,3-glucanases (EC 3.2.1.39) and chitinases (EC 3.2.1.14) which are anticipated to have myco-parasitic effect that result in breakdown of pathogenic fungal cell wall. All of these enzymes target and attack the pathogen's hyphal tips and it results in thinned cell wall, imbalance in turgor pressure, and eventually the protrusion and rupture of hyphal tips. Gajera *et al.*, (2013) reported the suppression of fungal pathogen is because of exochitinases secretion which causes the degradation of fungal cell walls at reduced levels of enzyme production.

Antibiosis:

Antibiosis is one of the biocontrol mechanism by which low-molecular weight diffusible substances react and lowers the growth and multiplication of other microbes. Antibiosis, mainly focuses on synthesis of secondary metabolites, that have an antagonistic or lethal effect on pathogenic or parasitic fungus.

Trichoderma spp. produce secondary metabolites and antimicrobial compounds which are crucial for antagonistic activity of biological control (Ajith & Lakshmidēvi, 2010; Vinale *et al.*, 2008). *Trichoderma* defence mechanism against *R. solani* was first described by Weindling in 1934, and he later gave the name "gliotoxin". *Trichoderma* produces antibiotics that are unique in their action and prevent the growth of pathogens (Benitez *et al.*, 2004). *Trichoderma* strains produces a wide range of secondary metabolites that includes polyketides, terpenes, peptaibols, alkyl pyrones, isonitriles, diketopiperazines and some steroids which are antibacterial and antifungal in nature.

Antibiotics including secondary metabolites are chemically distinct from natural substances and they do not directly act in development, natural growth, or reproduction and they are crucial for the plant defence response, differentiation, metal transport, symbiosis, and promoting or preventing spore initiation and germination of pathogens (Demain & Fang, 2000; Vinale *et al.*, 2008). Mutation studies showed that mutant *Trichoderma spp.* lack the ability of producing antibiotics and also have very less antagonistic activity (Wilhite *et al.*, 1994).

In biocontrol activity, antibiotics are the most commonly associated ones. For instance, the synthesis of an antibiotic similar to pyrone from *T. harzianum* shown biocontrol action against *Ganumannomyces graminis* (Ghisalberti *et al.*, 1990). The first identified secondary metabolite in *Trichoderma spp.* was the peptide antibiotic paracelsin (Bruckner & Graf, 1983). *Trichoderma spp.* produces three different types of secondary metabolites such as water-soluble compounds (heptelidic acid), volatile chemicals (6-pentyl- α -pyrone) and peptaibol substances (Ghisalberti and Sivasithamparam, 1991). Polyketides are one of the largest group of secondary metabolites produced by fungi. They lower the competitive ability by promoting competition for nutrient resources and establishes effective chemical communication with other nearby surrounding microbes (Mukherjee *et al.*, 2012).

The proportion of volatile antibiotics (acetaldehyde, pyran and ethanol etc.) relies on the nitrogen and carbon sources availability in media. Viridin, gliovirin,

tricholin, harzianic acid, glisoprenin, heptelidic acid and massoilactone are toxic metabolites produced by the *Trichoderma* species that prevent the invasion of other pathogens (Reino *et al.*, 2008)

Various *Trichoderma* species produce a wide range of antibiotics. Trichodecenins, trichocellins, trichorovins, trichotoxins A and B are produced by *T. viride*. Trichorzianins A and B, trichorzins HA and MA were detected from *T. harzianum* culture filtrate. Neoatroviridins A-D and atroviridins A-C were isolated from culture filtrate of *T. atroviride*. Moreover, several other antifungal and antibacterial substances such as lignoren, viridin, trichoviridin, koningins, dermadin and koningic acid were extracted from *T. lignorum*, *T. viride*, *T. harzianum*, *T. koningii*, *T. virens* and *T. aureoviride* cultures (Reino *et al.*, 2008).

Induced resistance:

Plants in their native habitats are accompanied by various harmful or beneficial microbes. Plants have the capability to localise and decrease the effect of the pathogenic invasion by evolving various mechanisms that help in identification of the pathogenic invader preceded by synthesis of an arsenal of anti-herbivory or antimicrobial substances. Subsequently, numerous studies have been done for *Trichoderma* application as a bio-stimulant of plant growth enhancer, establishment of seedlings and stimulation of defence response in plants (Shanmugaiah *et al.*, 2009).

Trichoderma species grows rapidly, produces spores in a large number and opportunistic invaders. They synthesize antibiotics and contain enzymes that degrade the cell wall (chitinases, glucanases and celluloses) of fungi (Vinale *et al.*, 2008). Further, *Trichoderma* species induces the generation of induced systemic resistance (ISR), systemic acquired resistance (SAR) and the hypersensitive response in plants (Vinale *et al.*, 2008; Benitez *et al.*, 2004). Various research studies revealed that *Trichoderma* species may indirectly lead to systemic resistance (Lo *et al.*, 2000). *Trichoderma spp.* are effective in controlling plant diseases by eliciting systemic or localised resistance (Harman *et al.*, 2004a). Therefore, root-colonizing *Trichoderma spp.* employs a complex interaction between the pathogen, host plant, the biocontrol agent and various environmental conditions to control plant diseases. (Harman, 2004a; Alfano *et al.*, 2007).

Induced resistance is a result of elevated quantities of defensive metabolic substances and enzymes. They contain enzymes for biosynthesis of phytoalexin (HR), phenylpropanoid pathway involved participating enzymes (phenylalanine ammonia lyase and chalcone synthase). It is well known that *Trichoderma spp.* stimulate plant gene expression for defence response against antagonistic pathogens that includes peroxidase, chitinase and glucanases (Yedidia *et al.*, 2003; Hanson *et al.*, 2004). Further, it has been demonstrated that pre-treated plants with *Trichoderma* species enhanced resistance in plants to pathogen invasion (Harman, 2004a).

Transcriptomic and proteomic research studies have showed that *Trichoderma spp.* colonises roots of a plant and stimulates change in systemic gene expression that are involved in responses towards stress, biosynthesis of ethylene, reactive oxygen species scavenging, isoprenoid oxylipins and photorespiration (Djonovic *et al.*, 2006; Alfano *et al.*, 2007; Segarra *et al.*, 2007; and Harman, 2008). *Trichoderma spp.* stimulates localised resistance to pathogenic invasions, further they stimulate systemic based interactions that are seen within the plants. As a result, the foliage develops resistance to plant diseases even though the *Trichoderma* species are found confined to plant roots (Yedidia *et al.*, 2003; Harman *et al.*, 2004a).

Further, interaction of *Trichoderma spp.* with roots of a plant induces sensitized condition in plant, enabling it to react more effectively to a later pathogenic invasion. This sensitization is clear for both decrease in plant disease symptoms and the systemic augmentation of defence related gene expression (Shoresh *et al.*, 2005; Yedidia *et al.*, 2003). Jasmonic acid and ethylene acts as signalling molecules in the main signal transduction pathway of *Trichoderma* mediated induced systemic resistance in cucumber (*Cucumis sativus*), and a similar model has been demonstrated in maize (*Zea mays L.*) (Djonovic *et al.*, 2007).

Through artificial inoculation of *Trichoderma spp.* several terpenoid phytoalexin synthases are produced in cotton against the pathogen *R. solani*. The synthesis of several enzymes and Pathogenesis Related Proteins (PRs) for development of resistance is triggered by oxidative stress in plants (Gajera *et al.*, 2013). Cellulose complexes found in *Trichoderma spp.* stimulated induced systemic resistance (ISR) against *Curvularia* leaf spot in maize plant by enhanced gene expression associated with jasmonate/ethylene signalling pathway (Saravanakumar *et al.*, 2016).

Various genera of micro-organisms that are associated with rhizosphere of soil, were entitled as Plant growth-promoting rhizobacteria (PGPR) and fungus (PGPF) can promote plant growth and enhance plant health (Lugtenberg *et al.*, 2009; Shores *et al.*, 2010). Several research studies showed that application of PGPR and PGPF to planting material or soil promotes induced systemic resistance in plants under normal field situations (Kloepper *et al.*, 2004).

When the beneficial microorganisms colonise the roots of host plant species effectively, it results in induction of ISR. By reacting to reciprocal signals, the soil microorganisms and host plants form a symbiotic connection thus benefiting each other (Lugtenberg *et al.*, 2009).

Induced systemic resistance is formed by a result of network of interlinked signalling pathways, where plant hormones act a key role in regulation (Pieterse *et al.*, 2012). The two phytohormones, ethylene (ET) and jasmonic acid (JA) are crucial in regulation of the plant defence system (Thomma *et al.*, 2001). Variations in the biochemical parameters such as phenylalanine ammonia lyase, chlorophyll content and total phenol content can be employed as biochemical markers for selection of resistant varieties because of their increased activity is typically recognized as an early reaction to pathogenic infection (Dickerson *et al.*, 1984; Macardo *et al.*, 2003).

Plant growth promotion by *Trichoderma* species:

Trichoderma spp. promotes plant growth and development of roots in addition to biological control of pathogens and activate plant defence system (Harman, 2004a). Some *Trichoderma* species have been demonstrated to colonise the root surfaces by establishing long lasting and robust colonisation after penetrating the epidermis of plant roots (Harman, 2004a). Research studies on maize plants showed that the plant roots of treated plants with *Trichoderma harzianum* strain T-22, after several months were almost twice longer than the untreated plants (Harman, 2004a). Secondary metabolites synthesised by *Trichoderma* species such as koningii (*Trichoderma koningii*) and 6-pentylalpha pryone (*Trichoderma harzianum*) acts a growth regulator in plant (Cutler 1986). Further, *Trichoderma* species synthesises citric and gluconic acids, lowers the soil pH, enhances phosphate solubilization, mineral and micronutrient substances such as manganese, iron and magnesium (Harman *et al.*, 2004b; Benitez *et al.*, 2004; Vinale *et al.*, 2008).

Trichoderma application increased the plant biomass, plant size and root length along with reduced intensity of sheath blight through various mechanisms like plant hormones production (IAA), solubilization of soil phosphates and induces systemic resistance in plants (Da Silva *et al.*, 2012).

Howell (2003) showed that seed biopriming with *Trichoderma spp.* has enhanced the seed performance and improved the levels of plant growth promoters in various crops. *Trichoderma spp.* also promotes plant growth through various mechanisms such as expansion of primary and secondary root lengths, solubilization of soil nutrients (Kapri and Tewari, 2010) and production of phytohormones like cytokinin, gibberellins, indole acetic acids and zeatin (Gravel *et al.*, 2007; Contreras-Cornejo *et al.*, 2009). *Trichoderma spp.* also enhanced the plant development by producing siderophore and hydrogen cyanide (antifungal compounds) that these compounds protect the plant from different plant pathogens (Anke *et al.*, 1991; Whipps, 2001).

From decades the *Trichoderma* species have been known to promote growth of a plant, including root and shoot biomass, enhanced crop productivity (Lindsey and Baker, 1967; Harman, 2000), improved nutrient uptake (Yedidia *et al.*, 2001) and utilisation of fertilisers (Harman, 2000), increased growth rate of plant and greenness that results higher rate of photosynthesis (Harman, 2006). Further, *Trichoderma spp.* exudates growth stimulating factors and plant hormones such as indole acetic acid and its analogues (Vinale *et al.*, 2008a; Windham *et al.*, 1986), vitamins (Inbar *et al.*, 1994) that promote plant growth and development.

Trichoderma spp. on colonisation with plant roots both in monocotyledonous and dicotyledonous plant have resulted in significant variation in metabolism of a plant. They include growth regulator biosynthesis, amino acids, phenolic substances and compatible osmolytes, other physiological functions such as leaf water potential, transpiration and photosynthesis (Bae *et al.*, 2009). A binding protein of cellulose named swollenin has the ability to breakdown the cellulose crystalline structure in cell wall of plants (Meng *et al.*, 2019). It has a sequence similar to that of expansins (plant protein), that helps in expanding the cell wall in plant roots and root hairs. *Trichoderma spp.* may increase the surface area of plant roots by producing swollenin protein, which also helps in enhanced establishment in the rhizosphere of plants (Meng *et al.*, 2019).

Numerous studies have shown that *Trichoderma* application to rhizosphere of plant improved several morphological characteristics of a plant that includes plant

biomass, root-shoot length, number of leaves, height, branches, tillers, and fruits etc. (Sajeesh *et al.*, 2015; Halifu *et al.*, 2019). For instance, soil application of *T. atrovirde* increased the root hairs and lateral roots number in *A. thaliana* (Cornejo *et al.*, 2015). Improved chlorophyll content was observed when *T. harzianum* T969 was treated to seed (Rasool *et al.*, 2011). Application of *T. harzianum* to cucumber roots enhanced the formation of lateral roots (Cornejo *et al.*, 2009) and also biomass (Yedidia *et al.*, 2001).

Use of *Pseudomonas* as Biocontrol agent:

Pseudomonas fluorescens are gram negative, rod shaped, aerobic and non-pathogenic saprophytic bacterium and most commonly found better adapted to grow in rhizosphere of agricultural soils. The name infers, that under particular situations especially under low availability of iron *Pseudomonas spp.* secretes fluorescein (a soluble greenish fluorescent pigment). It has been discovered that some species of *P. fluorescens* were found to serve as biocontrol agents, by suppressing the plant diseases by defending plant roots and seeds against infections caused by fungus, primarily root pathogens. Among the plant-growth promoting rhizobacteria, fluorescent *Pseudomonas sp.*, are the most efficient ones in lowering the incidence of soil-borne plant pathogens in naturally occurring suppressive soils (Raaijmakers and Weller, 1998).

They promote growth of a plant and lowers the severity caused by fungal infections in plants (Hoffland *et al.*, 1996). Their widespread distribution in rhizosphere and agricultural soils, their potentiality to use the exudates from plant roots as source of nutrients makes *P. fluorescens* ideal for use as successful agent for biological control of plant pathogens. It possess crucial characters for their capacity to bind with the rhizoplane and soil particles, motility, prototrophy, bacterial fitness and synthesis of antimicrobial compounds.

The biocontrol mechanism is due to synthesis of various secondary metabolites such as siderophores, hydrogen cyanide and antibiotics (O Sullivan & O Gara, 1992).

P. fluorescens promotes growth enhancing characters of a plant and they include production of plant hormones, nitrogen fixation, iron chelation, solubilization of phosphates in soil. Because of their multifaceted utility, it makes *Pseudomonas spp.* as successful biocontrol in the field of agriculture for reducing the fungal infections (Panpatte *et al.*, 2016).

In France, Hebbar *et al.*, (1991a) reported that bacteria from roots of sunflower suppressed the growth of *Rhizoctonia solani*, *Sclerotium rolfsii*, *Macrophomina phaseolina* and *Alternaria helianthi*.

PAL Enzyme:

The first main enzyme in flavonoid pathway and phenyl propanoid pathway is phenylalanine ammonia-lyase (E.C.4.1.3.5). Phenyl propanoid pathway is a secondary metabolic pathway that occurs in higher plants and is mostly associated with plant defense mechanisms (Koukol and Conn, 1961). In higher plants the PAL enzyme is extensively distributed thus enabling them to utilize L-Phenylalanine as a carbon and nitrogen source (Fritz *et al.*, 1976).

PAL enzyme catalyses the conversion process of L phenylalanine to trans-cinnamic acid and ammonia, in turn promotes the production of polyphenolic substances like phenylpropanoid, flavonoid, and lignin in plants (Zhang and Liu, 2015). In plants, the phenylpropanoid pathway is one of the three main secondary metabolic pathways. Phenylalanine ammonia-lyase (PAL) enzyme uses tyrosine or L-phenylalanine as substrates to produce trans-cinnamic acid, later results in production of coumarin-CoA. Coumarin-CoA is then utilized as a substrate in the synthesis of coumarins, flavonoids and lignins (Fraser & Chapple, 2011). Similar to other polyphenols, flavonoids contain one or more phenolic hydroxyl groups that are highly reducible and capable of removing ROS generated by environmental stressors (Treutter, 2006).

PAL enzymes are amenable to plant injuries, nutritional stress, pathogenic invasions, UV and elevated temperature (Dixon and Paiva, 1995; Payyavula *et al.*, 2012). Further, PAL enzyme activity follows the rapid identification of plant pathogenic invaders and results in increased accumulation of phytoalexins phenolic compounds and lignin in plants (Mandal and Mitra, 2007). It acts as a signalling molecule and triggers the Systemic acquired resistance (SAR) response as a defence mechanism against pathogen infection (Kavitha *et al.*, 2012). In both compatible and incompatible host pathogen interactions the PAL level rises. The primary precursor of trans-cinnamic acid is Salicylic acid.

Kashyap and Dhiman, (2009) reported that enhancing the plant's inherent defence system by earlier application of abiotic and biotic inducers is one of the most successful method of plant protection. When plants are inoculated with a virulent

pathogen, the defence related enzymes like phenylalanine ammonia lyase (PAL) gets increased in a significant amount in all the treatments that differ in successive time intervals when compared with control. Through inferred biochemical analysis it showed the enhanced enzymatic activity of phenylalanine ammonia lyase (PAL) in the *T. harzianum* treated plants (Madhavi *et al.*, 2018).

Quick reaction of plants to pathogen infection is crucial for developing resistance (Maleck *et al.*, 2000). After being infected by a pathogen, the susceptible plants take longer period to activate their defence system, or occasionally they may not react at all (Moerschbacher *et al.*, 1999). Higher concentrations of enzymes, phenolics and lignins near the infection tissue are found in resistant plants, results in reduced or inhibited germination of spores, formation of appressoria and delayed penetration prevents the development of disease (Angra and Sharma, 1994).

Hypersensitive response (cell necrosis) of infected plant cells is a result of increased PAL accumulation (Ralton *et al.*, 1988). Numerous studies have demonstrated that the expression of PAL gene is responsible for a wide range of external environmental stimuli such as wounding, pathogen infection, extremely high temperatures, nutrition deprivation, UV radiation and other stress related situations (Lawton *et al.*, 1983; Dixon and Paiva, 1995). Further, it has been discovered that the involvement of PAL gene in resistance and direct influence on synthesis of salicylic acid, expression of PR proteins and NPR1 gene activation (Fitzgerald *et al.*, 2004).

Slusarenko (1996) reported that resistance to downy mildew in *Arabidopsis* plant is due to synthesis of Salicylic acid precursors that plays a major role in PAL gene function. The quadruple mutants *pal1*, *pal2*, *pal3* and *pal4* in *Arabidopsis* plant after inoculation with a pathogen resulted in lowered amounts of salicylic acid accumulation and lignin content (Rohde *et al.*, 2004). Signalling coordinated by the plant hormones that includes jasmonic acid (JA), ethylene and salicylic acid (SA) is the biochemical mechanism by which plants react to infection by a pathogen. Plants that are incapable of generating SA response becomes susceptible to pathogen infection, where as in resistant plants there is an increased accumulation of SA elicited by plant pathogen. The growth of pathogen is inhibited by various secondary metabolites in addition to accumulation of SA (Pieterse *et al.*, 2009).

After the inoculation, PAL enzyme activity in leaves treated with *T. atroviride* SG3403 were augmented and reached at 9.60 U/mg in 24 hours, but the control

produced the enzyme activity of 7.56 U/mg. These findings imply that *Trichoderma* strain induced PAL activity in leaves (Wang *et al.*, 2015).

Maize seedlings treated with strain of *Trichoderma harzianum* enhanced the activity of PAL enzyme. When compared to control, the activity of defence related enzymes such as phenylalanine ammonia lyase (PAL) and peroxidase significantly increased in different intervals of time in all the treatments after inoculated with *R. solani*. *P. fluorescens* and *T. harzianum* applied treatments enhanced the action of the phenylalanine ammonia lyase (PAL) and peroxidase (PO) enzymes, through biochemical analysis as a result systemic resistance was induced. The activity of Phenylalanine ammonia lyase enzyme often rose from 24 to 72 HAI and then decreased thereafter and in control, the enzyme activity was not changed. When compared with the control, after 24 HAI the phenylalanine activity was increased significantly. At 96 hours, the combination of seed treatment and foliar spray of *T. harzianum* and *P. fluorescens* resulted in considerably greater PAL activity (1.72 change in cinnamic acid/min/g) than seed treatment alone (1.29 change in cinnamic acid/min/g) (Madhavi *et al.*, 2018).

Effect of Tamarind seed powder and chitin on *Trichoderma* strains:

Mishra and Malhotra (2009) reported that the tamarind seeds are sticky in nature because they contain a large amount of xylo-glucan substances. Both in Wild and mutant types, the growth and conidia spore formation were enhanced by use of broken tamarind seeds. After two hours of incubation, the tannase enzyme activity of the *Trichoderma harzianum* strain efficiently used most of the tannin substances. The seeds of tamarind (*Tamarindus indica*) are one of the tannin based substrates used for *Trichoderma harzianum* strain and the tannin level is 1.02 and relative activity (%) after 1hour and 2hours is 83 and 119 respectively (Iqbal & Kapoor, 2012).

Kishore *et al.*, (2005) reported that the use of chitin with biocontrol agent has significant importance for the management of plant diseases because of their potential inhibition on growth and proliferation of pathogenic fungi in soil rhizosphere (Benhamou & Theriault, 1992; Bell *et al.*, 1998; Bade and Wick, 1988 and Sultana *et al.*, 2000). Application of *Trichoderma* formulation along with chitin lowered the disease incidence complex caused by the combined action of *Sclerotinia sclerotiorum* and *Meloidogyne incognita*, resulted in increased activities of chitinase, phenylalanine

ammonia lyase (PAL), polyphenoloxidase (PPO), and peroxidase (POX) in cabbage (Loganathan *et al.*, 2010). Howell (2003) found that the hyphal lysis of *R.solani* cells was due to the action of lytic enzymes (proteases, chitinases and β -1,3 glucanases) by digesting its major components of cell wall.

Chitin application in soil under field conditions reduced the incidence of disease. Application of chitin induced the potential antagonistic action of *T. harzianum* antagonistic activity against *Phytophthora capsici* and *R. solani* in pepper plants (Sid-Ahmed *et al.*, 2003).

CHAPTER- III



MATERIAL AND METHODS



III. MATERIAL AND METHODS

The detailed field and laboratory experiments, materials and methodologies done during the research work have been elucidated in this chapter under the following headings.

1. Media used:

1.1. *Trichoderma* Specific Medium (TSM, Elad *et al.*, 1981; modified by Saha and Pan, 1997): TSM is used for selective isolation of *Trichoderma* spp. from collected soil samples.

Composition	g/L
1. Distilled Water	1L
2. Glucose	3.00
3. Pottasium Chloride (KCl)	0.15
4. Magnesium Sulphate (MgSO ₄ .7H ₂ O)	0.25
5. Ammonium Nitrate (NH ₄ NO ₃)	1.00
6. Di-pottasium Hydrogen Phosphate (K ₂ HPO ₄)	0.90
7. Agar Agar	15.00
8. Captan	0.10
9. Methyl Orange	0.30
10. Rose Bengal	0.033
11. Chloramphenicol	0.25
12. pH	5.0-5.5

1.2 Kings B medium (*Pseudomonas* broth): It is used for selective culturing of *Pseudomonas* spp. from collected soil samples.

Composition	g/L
1. Distilled water	1L
2. Glycerol	15 mL
3. Protease peptone	20.00
4. Pottasium Dihydrogen Phosphate (KH ₂ PO ₄)	1.50
5. Magnesium Sulphate (MgSO ₄ .7H ₂ O)	1.50
6. Antibiotics (Chloramphenicol, Ampicillin, Actidione)	0.25

1.3 Potato Dextrose Agar (PDA) medium: A semi-synthetic media used for culturing of fungus including *Trichoderma spp.*

Composition	g/L
1. Distilled water	1L
2. Peeled Potato	200
3. Agar	20
4. Dextrose	20
5. Chloramphenicol	0.25
6. Final pH (at 25°C)	5.6±0.2

1.4 Potato Dextrose Broth (Downes *et al.*, 2001): PD Broth (without agar) was used for mass multiplication of *Trichoderma spp.*

Composition	g/L
1. Distilled Water	1L
2. Potatoes, Infusion form	200
3. Dextrose	20
4. Chloramphenicol	0.25
5. Final pH (at 25°C)	5.1 ±0.2

1.5 Soil extract agar (readily available) medium: It is used for culturing of soil bacteria from collected soil samples. It is ready made media (manufactured by Hemidia).

Add 34.25 g of soil extract agar in 1000 mL of distilled water later heated in oven for complete dissolving of chemicals in the media.

Composition	g/L
1. Distilled Water	1L
2. Glucose	0.50
3. Dipotassium phosphate	0.50
4. Soil extract	17.75
5. Agar	15.00
6. Final pH (at 25 °C)	6.8±0.2

1.6 Sterilisation of Media and Glassware:

Sterilization refers to method in which microorganisms found in any substance/material are destroyed and killed. At high temperature microorganisms are permanently killed due to irreversible denaturation of the proteins found in the microbial cells. Sterilization is done in two ways.

- **Sterilisation by dry heat**

The equipment hot air oven was used to sterilize glassware by using dry heat treatment. This technique inhibits the growth of any pathogen by denaturation of proteins and oxidation of biomolecules. Prior to sterilization, all glassware (Petri plates, Conical flasks, microscopic slides, Test tubes, Beakers etc.,) were wrapped in paper and later heated at 160 - 180° C in a hot air oven for one to two hours.

- **Sterilisation with steam under pressure (moist heat)**

All the growth medias, broths, distilled water, pipette tubes, cotton plugs were sterilized in autoclave at 121⁰c 15psi for 15-20 minutes.

Chromic acid and detergents are used for cleaning the glassware like Petri plates, Conical flasks, microscopic slides, beakers, and pipette tubes etc., were done regularly after use to avoid any kind of contamination.

Inoculation needles, tips of tubes and flasks, forcep tips were sterilised in flame after being dipped in ethanol. 70% ethyl alcohol is used to sterilise hands and also used for surface sterilisation of inoculation chamber.

Experimental details:

Location of the field : Experimental farm, College of agriculture, UBKV,
Pundibari, Cooch Behar.

Crop : Maize (Variety DKC 9081)

Date of sowing : 03-11-2021

Season : Rabi 2021-2022

Treatments : 4 (3 *Trichoderma* strains and one untreated control)

T1 - Untreated (control)

T2 - *Trichoderma ernacium* (collected from ICAR-NRRI)

T3 - *Trichoderma atroviride* (collected from ICAR-NRRI)

T4 - *Trichoderma virens* mutant G2 (collected from BARC)

Seeds are treated with different *Trichoderma* strains @ 10g/kg of seeds and sown in field.

Design : RBD

Plot size : 5.0 x 4.0 m

2. Collection of soil samples:

Six composite soil samples from a depth of 0-5 cm were collected from 10 different treated plots and the samples were collected two times in Rabi season (Before sowing and 45 Days after sowing) during the year 2021-22.

For further analysis, the soil samples were air dried, passed through 2 mm sieve, processed and stored in plastic bags properly.

2.1 Preparation of serial dilution for soil:

Qualitative and quantitative analysis of soil microbes is essential to evaluate the performance of various microbiological communities occurring in the soil, which in turn facilitate and encourage the nutrition of plants. Regarding this, prepare serial dilutions of soil from where particular dilutions are taken for analysis of soil microbial studies. The specific steps followed for serial dilution is listed below:

- Following the 10-fold dilution principle, 10 g of the soil sample were weighed in an aseptic environment before being transferred to 90 ml of sterile distilled water. After that, it was vigorously shaken to ensure even mixing.
- From there, more dilutions were done by adding 1 ml to 9 ml of sterilized distilled water in aseptic conditions to any required level, like 10^{-4} , 10^{-5} , 10^{-6} and so forth.

2.2 Agar-Plate Method for Total Microbial Count (Francis E. Clark, 1965):

Introduction

Soil encompasses a wide range of microbes that are so sparsely found or vary in number in millions per gram of soil. They decay organic material present in soil and mobilisation of nutrients from soil to plants. They enhance soil physical and chemical characteristics while some have an impact on plant health by prevention of plant diseases especially from soil borne plant pathogens.

Preparation of poured media plates

Pour 1 ml of freshly mixed soil suspension into each of two sterile petri dishes from the highest prepared serial dilution (10^{-6}). Similar transfers were done from the next two lower dilutions (10^{-4} and 10^{-5}) into other petri dishes. Later pour 12 ml of liquid media (Soil agar extract and TSM). While pouring media into petri dishes, make sure they are not so hot (more than 40°C) is not advised and as soon as the agar is added, swirl it around in the petri-dish by hand to make sure the inoculant is thoroughly mixed with it.

Incubation and counting of Microbial population from Plates

Until the media gets properly settled, let the poured plates stand upright. Then flip them over, place in polythene and put them in a 28°C incubator. The plates are left undisturbed for at least 7 days, ideally 10 to 14 days. Later take them from incubator and examine each plate made from every single sample for presence of microbial colonies formed. Count the total number of colonies formed in each dilution.

Calculations

To calculate the total viable count per gramme of soil sample, multiply the average number of colonies formed per plate for a specific dilution by the dilution factor.

2.3 Methods of counting Bacteria:

Most Probable Number (MPN) technique

Serial dilution of soil was made and used for subsequent microbiological analysis.

Procedure

- Five dilutions 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} were selected and inoculated by adding 1.0 ml of the soil suspension from each of these selected dilutions into 3 tubes containing liquid specific media (PF, King's B broth).
- The inoculated test tubes were incubated at 25–30°C for 5 days, and then analysed for the presence of turbidity.

Observations

- The three test tubes that exhibited turbidity was recorded as positive and the dilutions were marked as positive.
- The positive test tubes for each of the subsequent dilutions were marked similarly. The code in table represents the number of positive tubes for bacterial growth. The first, second, and third numbers in the code indicate positive tubes number at various levels of dilution in increasing manner.

Calculations

The FDA's Bacterial Analytical Manual's MPN table was used to calculate the results for a three-replicate design.

3. Preparation of *Trichoderma* talc formulation:

For preparation of *Trichoderma* talcum powder formulations, the *Trichoderma* strains are cultured in PDB (potato dextrose broth). Conical flasks were filled with 50 ml of potato dextrose broth and sterilized in autoclave at 15psi and 121°C for 20 minutes. Next day *Trichoderma* mycelia from pure cultures strains were inoculated into broth and the conical flasks are incubated in BOD at 28±1°C for 12- 15 days. Later the *Trichoderma* strains produce a mat of mycelia over the broth and this broth with mycelium is finely grinded (homogenized) and mixed well in sterilized talcum powder under aseptic conditions. For 50g of talc formulation 10ml of finely grinded broth with mycelium is used. Later it is packed in a polythene and heat sealed.

Along with talc 1g of Chitin and 2g of Tamarind seed powder was used for 50g talc formulation to know the better composition that increases the efficiency of the *Trichoderma* formulation.

3.1 *Trichoderma* treatment combinations

- i. Untreated
- ii. *Trichoderma* talc formulation
- iii. *Trichoderma* talc formulation with chitin
- iv. *Trichoderma* talc formulation with tamarind seed powder
- v. *Trichoderma* talc formulation with chitin and tamarind seed powder were used for the experiment.

These treatment combinations were used to determine the antagonistic potential of different *Trichoderma* strain.

3.2 Determining the antagonistic potential of different *Trichoderma* strain formulations:

To determine the growth inhibition of the test pathogenic plant fungus under the presence of *Trichoderma* strain the "Bangle method" was used (Singh *et al.*, 2004). The *Trichoderma harzianum* strain was used in this experiment. Pour 12ml of sterilized PDA media into sterile petri plates, later sterilized bangles were treated in different *Trichoderma* formulations mixed in sterilized water and placed carefully inside the disc of petri plates. Three replicates were done for each formulation and are incubated in an incubator (BOD) at 28±1°C. After one day on appearance of *Trichoderma* mycelium, they were again reinoculated with 6 mm mycelia disc of an actively growing *Rhizactonia solani* at the centre of petri plate. The test pathogen inoculated in separate petri plate poured with sterilized PDA media serves as control. They were incubated in BOD at 28±1°C. Radial growth of plant pathogen (*R. solani*) and inhibition of *Trichoderma* strain with respect to control was studied. The growth inhibition percentage was calculated by using the formula given by Vincent (1947):

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

Where,

I = Inhibition Percentage (%)

C = Diameter of colony in control (cm)

T = Diameter of colony in treatment (cm)

4. Strains of *Trichoderma* used:

T1 - Untreated (control)

T2 - *Trichoderma ernacium* (collected from ICAR-NRRI)

T3 - *Trichoderma atroviride* (collected from ICAR-NRRI)

T4 - *Trichoderma virens* mutant G2 (collected from BARC)

T5 - *Trichoderma harzianum* (collected from UBKV, Dept. of Plant Pathology.

T6 - *Trichoderma asperellum* (collected from UBKV, Dept. of Plant Pathology.

5. Root system analysis by Rootnav v1.8.1x64:

In recent years quantitative study of root growth has gained practical importance as a way to investigate the influence of biotic and abiotic stress. Characterization and segmentation of plant roots through image becomes a notable computer vision challenge. Images of root contain complex structures such as size variations, number of primary, secondary and other lateral roots, variation in angle of emergence and tip angles, hull area etc. Rootnav is a software analysis method for root image analysis that fully automates the extraction of intricate root system structure for a wide range of plant species. Rootnav v1.8.1x64 used to do RSA (Root System Analysis) and measurements.

Maize plants grown in pots of 15-18 days old seedlings are collected and washed in water to remove the soil. Later placed in trays filled with ordinary tap water and kept overnight to remove the traces of soil from the roots without no damage to root system. After that root systems from each treatment were properly placed on moist black paper and finely distributed and separated with the aid of a painting brush. Images were captured and saved as JPEG format. Images taken were saved on the device and then moved to a desktop computer, stored separately in a folder for further use. The images were processed in Rootnav software, they were analysed for primary and lateral roots. In nested structure, the root system of each is stored in a Rootnav and gives the result in the form of a table.

6. Estimation of Harvesting index of Maize:

The harvest index (HI) is the percentage ratio of economical yield and biological yield, which measures production capacity of a crop. Expressed in percentage.

The total biomass developed above ground portion is referred to as the biological yield. It was calculated by combining the economic yield (threshed seed yield) of the maize grown for seed purpose. After harvest, the maize plants are left to stand in the field and allowed to dry. After complete drying the plants were cut to base level and weighed from each plot to obtain the total biomass. Expressed as kilograms per plot.

For estimation of economical yield, the cobs of the maize plants were harvested from each plot and are dried in sun for at least one week and then by a thresher they are shelled to obtain seeds. The obtained seeds from each plot were weighed by using a weighing balance and analysed for knowing Harvesting index.

Calculations

The Harvesting index of maize is expressed as:

$$\text{Harvesting Index (\%)} = \frac{\text{Economical yield}}{\text{Biological Yield}} \times 100$$

7. Study of Biological activity of Soil:

Soil samples were collected from the experimental site and examined for the biological activity of soil namely soil dehydrogenase enzymatic activity is analysed. The reagents, procedures and its calculations described below:

Determination of soil dehydrogenase activity (DHA)

Reagents

- 3% 2,3,5- triphenyl tetrazolium chloride (TTC) solution - Dissolve 3g of TTC in 100ml distilled water and later stored in amber/ brown colour bottle.
- Methanol (Molecular graded).
- 1% Glucose solution - Dissolve 1g of glucose in 100ml distilled water.
- 100ppm Standard triphenyl formazan (TPF)- Dissolve 100mg of TPF in 100ml distilled water.

Procedure

1. Add 1g of air dried representative soil sample in an air tight screw-capped test tube.
2. Add 0.2 ml of 3% TTC (2,3,5- triphenyl tetrazolium chloride) solution in each of the test tubes to saturate the soil.
3. Later add 0.5ml of 1% glucose solution in each tube. Gently tap bottom of the tube to liberate the trapped oxygen bubbles this ensures a water seal formed above the soil so that no air bubbles are seen.
4. The test tubes were incubated in incubator for 24 hours at $28 \pm 0.5^{\circ}\text{C}$.
5. After that add 10ml of methanol to each test tube, shaken vigorously and left to stand for six hours.
6. Clear pink coloured supernatant was removed and by using a spectrophotometer, readings were taken at a wavelength of 485nm (blue filter).
7. Calculate the amount of TPF formed from the standard curve taken in the range of 10milligrams to 90milligrams TPF millilitre.
8. Results were represented as mg TPF formed/hour/g soil.

8. Inoculum preparation and Inoculation:

Preparation of inoculum

Potato Dextrose agar medium (200g peeled potato + 20g dextrose + 20g agar agar) was utilized for pathogen multiplication. It was prepared and sterilized in autoclave at 15 psi and 121°C for 15-20 minutes. Addition of 25 mg Chloramphenicol L^{-1} medium avoid bacterial contamination. Under aseptic conditions after media gets solidified in the petri-plates, with the help of cork borer 5 mm disc of culture was inoculated at the center of medium. The inoculated petri-plates were incubated at $26 \pm 1^{\circ}\text{C}$ for 1 week.

Preparation of mass culture of *Rhizactonia solani*

Match sticks with their ends (Red phosphorous) removed are double sterilized in autoclave and later placed along with PDA so that the match stick absorbs PDA.

Next day, mycelial plug of 5 mm diameter was cut from one week old culture of *R.solani* with help of a cork borer and was inoculated in the center of petri-plate poured with PDA along with match sticks. The petriplates were incubated at $26\pm 1^{\circ}\text{C}$ for 8-10 days. After 8-10 days, the mycelium of *R.solani* grows on the match sticks and these infected match sticks with mycelium is used as inoculum.

Pot preparation and sowing

Sterilized soil, thoroughly mixed with farmyard manure, urea, SSP and MOP were used to fill the experimental pots. Variety ADV 757 (Hybrid Corn), a susceptible variety of maize for banded leaf and sheath blight was used in this experiment. Seeds were treated with *Trichoderma* strains (*Trichoderma ernacium*, *Trichoderma atroviride*, *Trichoderma virens*, *Trichoderma harzianum* and *Trichoderma asperellum*) at the rate of 10g/kg of seed 24 hours before sowing. 2-3 seeds were sown in each pot and covered with soil.

Inoculation of the pathogen in potted wheat plants

The cultured mycelium (*R.solani*) infected match sticks are placed at the lower sheaths of maize plants which are of one month old. Place the match stick inside the sheath and cover it with cotton dipped in sterilised water for providing enough moisture for growth of pathogen. Later cover the entire portion with an aluminium foil and the entire inoculation process is done during evening hours. Light irrigation was provided immediately after inoculation to ensure proper humidity.

Sample collection

At 0, 24, 48 and 72 hours after inoculation (hai) with *R.solani*, leaf samples were collected. The collected leaves were wrapped in aluminium foil, tagged with cryotags and immediately dipped in liquid nitrogen. Later stored at -80°C for further analysis.

7.1 Estimation of defense related enzymes:

Estimation of Phenylalanine ammonia lyase (PAL) enzyme:

L-Phenylalanine is transformed into trans-cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL). Cinnamic acid acts as a precursor for biological synthesis of isoflavonoids, coumarins, and lignin. These substances play a crucial response in disease and pest resistance strategies. It has been documented that bacterial, viral, and fungal plant infections alter PAL enzyme activity.

Principle

Spectrophotometer is used to measure the Phenylalanine ammonia lyase enzyme activity and is determined by following the generation of trans-cinnamic acid that exhibits an increase in absorbance at 290 nm.

Materials

- 0.2 M Borate Buffer (pH 8.7).
- 0.1 M L-Phenylalanine - Dissolve 165 mg of L-Phenylalanine in 10 ml distilled water and pH is adjusted to 8.7 with 0.1 N KOH.
- 1 M Trichloro Acetic Acid- Dissolve 16.3g in 100 ml water.
- Enzyme extract- Weigh 500 mg of plant leaves and homogenize it in 5 ml of cold 25 mM borate-HCL buffer (pH 8.8) containing 5 mM mercaptoethanol (0.4ml/L). The homogenate was centrifuged at 12,000g for 20 min. The supernatant was collected and used as enzyme source.

Procedure

1. In a test tube, pipette out 0.5 ml of borate buffer, 0.2 ml of enzyme solution and 1.3 ml of water.
2. Add 1 ml of L-phenylalanine solution to start the reaction.
3. Later, it was incubated at 32 c for 30 to 60 minutes.
4. Add 0.5 ml of 1 M trichloroacetic acid to stop the reaction.
5. Run a control in which add phenylalanine after trichloroacetic acid.

6. At 290 nm, the absorbance was measured.
7. Prepare a standard curve for Trans-cinnamic acid.

Calculation

The amount of trans-cinnamic acid generated per milligram of protein per minute was used to express the reaction rate.

7.2 Analysis of comparative gene expression:

The quantitative Real Time - Polymerase Chain Reaction (qRT-PCR) procedure was performed to analyse the gene expression levels of specific selected differentially expressed genes, which was evaluated from distinct resistant and susceptible varieties. At different intervals of time, total RNA was isolated from the inoculated and healthy leaf samples, and then cDNA was synthesised by using random oligo dT primers and PCR analysis was done.

RNA isolation:

Introduction

Ribonucleic acid (RNA) is a polymeric molecule crucial for several biological functions in regulation and expression of genes. Messenger RNA (mRNA) synthesis is a dynamic expression of the genome of an organism. As a result mRNA is central key to information that flow within a cell.

Material required

1. Leaf tissues of maize were collected freshly and stored in -80°C.
2. Autoclaved, oven dried and cleaned the mortar and pestles, eppendorf tubes, pipette tubes, glass wares, plastic wares, and bottles.

(DEPC treatment is not mandatory. In case treated, they must be thoroughly washed earlier to drying so that traces of DEPC is removed. Clean gloves are mandatory to avoid RNA contamination).

3. RNA Purification Kit (Cat. No. 12183-555)
4. Liquid Nitrogen

5. TRIzol

6. Chloroform

7. Ethanol

Note: RNA purification Kit (Invitrogen Cat. No. 12183-555) can be procured separately.

Procedure

1. The total RNA was extracted from 100 mg of leaf tissues collected from 45 days old infected maize plants.
2. Prior to isolation of RNA the following steps were followed; all the mortar and pestles, eppendorf tubes, pipette tubes, glass wares, plastic wares, and bottles were treated by DEPC water (1ml/ 1000ml of Milli-Q water) for overnight. By wearing protective gloves all the dipped materials are taken out and kept in an autoclave (15psi, 121°C for 45 minutes). After finishing the autoclave, all the materials were again kept in hot air oven (25°C) overnight for drying. After the materials become ready, they are wrapped in aluminium foil and stored in ultra-low refrigerator at -80°C.
3. Grind the 100mg maize leaves with a mortar and pestle in presence of liquid nitrogen. Add 1 mL TRIzol for 100 mg young tissue used. Be careful that the content must not undergo fridge-thaw cycles while grinding process.
4. The ground material was incubated for 5 min at room temperature as a result powder melts to a homogenous liquid condition.
5. Use 200 µL of chloroform per mL of homogenate mixture and mix well by using low speed vortex mixing or vigorous hand mixing.
6. Incubate the homogenate mixture at room temperature for two-three minutes and centrifugation is done for fifteen minutes at 12000 x g.
7. In a fresh Eppendorf tube, collect the top aqueous layer among the three layers formed and add equal volume of 70% ethanol, mix it by using a vortex.
8. Transfer 700 µL of mixture in a spin column and centrifugation is done at 12000 x g for 15 secs in room temperature. Reinsert the column to the collection tube after discarding the flow through. If necessary reload the column with same sample and repeat the process.

9. Use 700 μL of Wash Buffer I in spin column and centrifugation is done at 12000 x g for 15 secs in room temperature. Reinsert the column to the collection tube after discarding the flow through.
10. Use 500 μL of Wash Buffer II (ethanol added) in spin column and centrifugation is done at 12000 x g for 15 secs in room temperature. Reinsert the column to the collection tube after discarding the flow through.
11. The empty column is centrifuged at 12000 x g for 1 min at room temperature and keep it in laminar air flow chamber for 30 min for drying.
12. Place the dried spin columns to new Eppendorf tubes. At the center of the column's membrane add 30 μL of RNase-free water and let it stand idle at room temperature for 1 minute later centrifuge it for two minutes at 12000 x g. Second elution step can be done if necessary.

RNA quality analysis:

In Nanodrop, the quantity and quality of total RNA in samples were evaluated. Two microlitre of each sample were dropped in designated spots of the μDrop plate by using a pipette. Later the plate was inserted into the equipment and after selecting the proper program for RNA sample analysis then it gets initiated. The analysis report gives the sample concentration (mg/ml) along with sample purity (260/280) for each designated sample.

cDNA Preparation:

Introduction

Complementary DNA strand or cDNA refers to the region of the genome that is actually expressed. Reverse transcriptase (RT) is the enzyme that converts mRNA to DNA. Oligo dT is used as a primer and mRNA (poly A) as template strand. Differentially expressed cDNA is a useful research tool to isolate, identify and analyze up and down regulated ones.

Method

M-MLV reverse transcriptase, RNaseH minus (200U/ μL) enzyme (NEB) and their protocol was used to generate cDNA from mRNA templates. The protocol includes:

1. First, the RNA template was thawed in room temperature and kept in ice, and all other materials, such as Oligo dT primer, RT buffer, Reverse Transcriptase (RT), RNase free water, DNTP's were also thawed at room temperature and later kept in ice.
2. Next, based on the amount of RNA samples taken into consideration, the DNA removal process, master mix was made using the following chemicals:
 - RNA Template - 4 μ L
 - 50 M Oligo dT Primer - 1 μ L
3. The mixture tubes are placed in PCR machine and incubated at 70°C for five minutes, after completion they are kept in ice.
4. After that, prepare master mix of Reverse Transcriptase by using the following materials:
 - RT Buffer - 2 μ L
 - DNTP's - 1 μ L
 - RT enzyme - 1 μ L
 - RNase free water - 6 μ L
5. Prior to adding the RNA samples, all the chemicals were properly mixed and placed in PCR machine for precise incubation.
6. The incubation process starts with 42°C for 60 minutes and then at 70°C for 15 minutes. L lastly, they are hold at 4°C.
7. The obtained cDNA samples quality and quantity were analysed in Nanodrop and stored in a refrigerator at -20°C for further use.

Primers used:

For gene expression experiment the primers used were –

Gene name	Forward Primer	Reverse Primer	Annealing Temperature (°C)
<i>pal 1</i>	TCGATCGAGCGAGAGATCAA	CGATAGGTGTGCCCTGGAAGT	54
<i>pal 2</i>	CAACGGTCTTCCTTCCAACCT	TCCGCACTTTGGACATGGT	52
<i>Sal</i>	CGAAATAATGTTCCATGGTGTT	TGTACTACGGATCGGTGCAA	46

Phenyl ammonia lyase (*pal*) primers were of wheat and Salicylic acid (*sal*) were of rice are used in this experiment. 100 μ M stock concentration of each primer was made by adding Milli-Q water (amount formulated by the synthesising firm). Working concentration was made at 1:10 ratio.

qRT- PCR analysis:

A Quant Studio 3 (Applied Biosystems) was used for the gene expression analysis. The following components used for real-time experiments are as follows-

○ cDNA template (25 ng)	- 1 µL
○ ROX (passive) reference dye (100X)	- 2 µL
○ Nuclease-free water	- 1 µL
○ SYBR master mix (2X)	- 5 µL
○ Forward primer (40 nM)	- 0.5 µL
○ <u>Reverse primer (40 nM)</u>	- 0.5 µL
Total	- 10 µL

The reagents were thawed before use and placed in ice. 1X (0.3 µL/30µL) ROX dye was made from (100X) ROX dye before using in the master mixture. The cDNA samples were made almost to an equal level (concentration) by adding nuclease-free water. The primers used were validated by conventional PCR method followed by gel electrophoresis before going for qRT-PCR. Subsequently, the SYBR Green qRT-PCR was performed by using the above listed reagents and the experiment followed was as follows-

Hold Stage	- 50.0 ⁰ C (2 minutes) & 95.0 ⁰ C (10 minutes)
PCR Stage	- 95.0 ⁰ C (15 seconds) Variable annealing temp. (1.0 minute) 95.0 ⁰ C (15 seconds)
Melt Curve stage	- 95.0 ⁰ C (15 seconds) 60.0 ⁰ C (1 minute) 95.0 ⁰ C (1 second)

The real-time experiments were carried out in triplicate, and the CT values were recorded. To validate the genes expressed agarose gel electrophoresis was carried out.

Analysis of relative gene expression:

The real-time PCR instrumentation's triplicate C_T (Threshold cycle) results were exported into a spreadsheet and the mean values were computed for each one. The gene that was targeted was normalised with the house keeping (reference) genes such as actin, GAPDH, and GLDH. The efficiency of PCR (slope of the log-linear phase of amplification: -3.58 to -3.10) was kept at two. Using the formula $2^{-\Delta\Delta C_t}$ (comparative C_T technique), the fold change was calculated where $\Delta\Delta C_t = (C_T \text{ target} - C_T \text{ reference gene}) \text{ infected} - (C_T \text{ control} - C_T \text{ reference gene}) \text{ healthy}$.

CHAPTER- IV



RESULTS AND DISCUSSION



IV. RESULTS AND DISCUSSION

Effect of seed treatment with *Trichoderma* on yield parameters of maize:

Initially, the maize seeds of cv. DKC 9081 were treated with different species of *Trichoderma* @ 10 g/kg of seed and planted in the experimental field of the university as per methodology described in Materials and Methods. The results are presented in Table-1 and Figure-1.

Table-1: Effect of seed treatment with *Trichoderma* on harvesting index of Maize (cv. DKC9081):

Treatments	Yield (Kgs)	Total shoot dry matter (Kgs)	Harvesting Index (%) of Maize
1. Control	11.4 ^B ± 4.99	30.72 ^A ± 3.35	26.26 ^A ± 6.23
2. <i>Trichoderma virens</i> mutant G2	15.58 ^{AB} ± 0.13	22.86 ^B ± 0.18	40.52 ^A ± 20.78
3. <i>Trichoderma ernacium</i>	16.9 ^A ± 0.4	24.84 ^B ± 2.88	40.66 ^A ± 2.24
4. <i>Trichoderma atroviride</i>	17.85 ^A ± 0.55	24.84 ^B ± 2.16	41.9 ^A ± 1.37

From Table-1, it indicates that the seed treatment with *T. atroviride* showed highest harvest index (41.9) among the other treatments followed by *T. ernacium*. The seed treatment with *T. virens* showed lowest effect among the three *Trichoderma* strains though it is significantly higher than untreated.

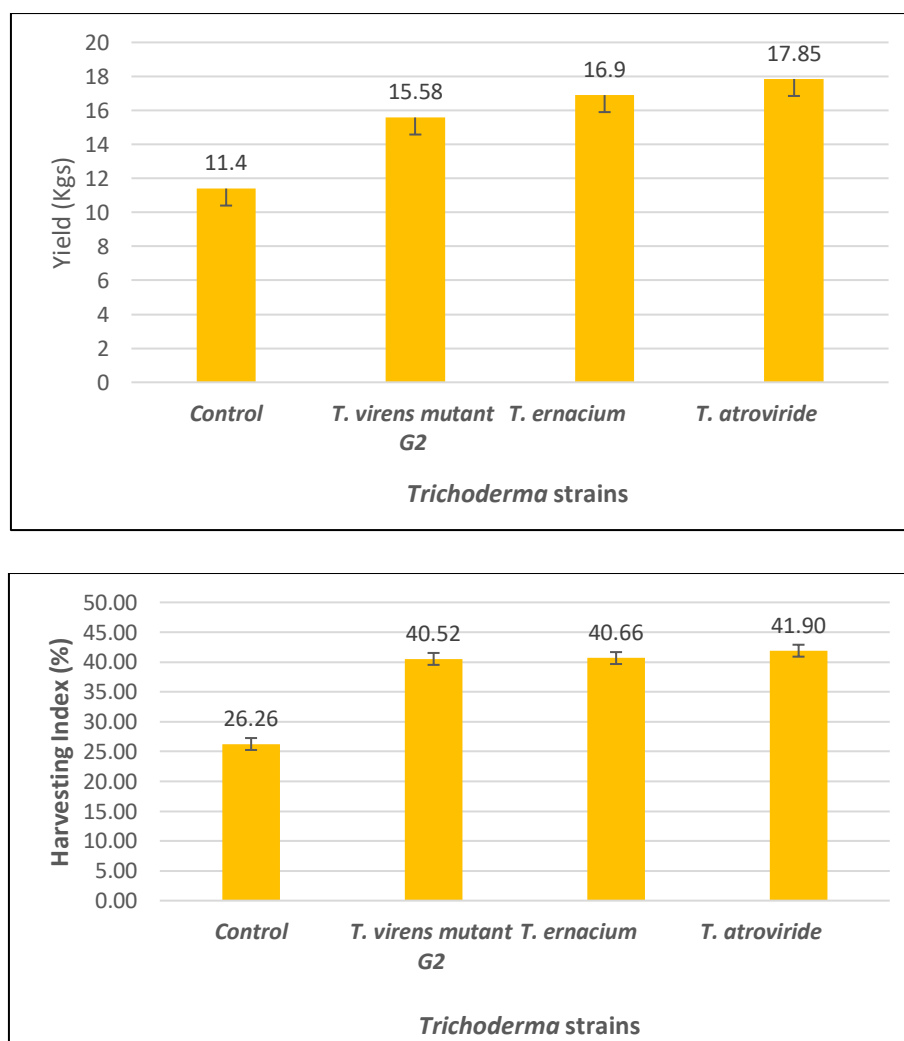
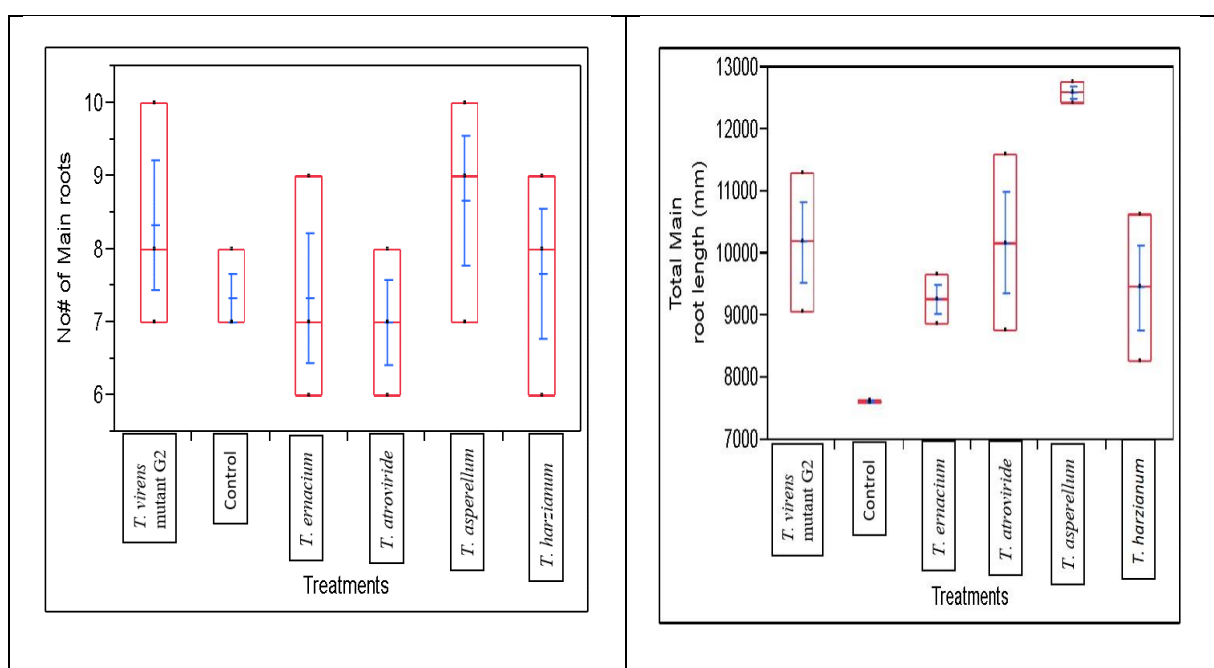


Figure-1: Graphical representation of Yield and Harvesting Index (%) of Maize as influenced by *Trichoderma*.

The yield of maize was higher in *Trichoderma* treated soil as indicated in the earlier experiment. The increased growth response induced by *Trichoderma spp.* has been reported for various crops such as beans (*Phaseolus vulgaris*) cucumber (*Cucumis sativus*), pepper (*Capsicum annum*), carnation (*Dianthus carophyllus*), maize (*Zea mays*), and wheat (*Triticum aestivum*) (Lo and Lin, 2002). To measure the root characteristics in maize *Trichoderma* treated seeds, the main and lateral roots were analysed. Beside the above three strains of *Trichoderma*, another two strains viz. *T. asperellum* and *T. harzianum* were also included in this study and the results are presented in Table-2 and Figure-2.

Table-2: Effect of seed treatment with *Trichoderma* on main and lateral roots of Maize.

Treatments	No. of Main roots	Total Main root length (mm)	No. of Lateral roots	Total Lateral root length (mm)
1. Control	7.34 ^A	7619 ^C	31 ^C	717.78 ^C
2. <i>Trichoderma virens</i> mutant G2	8.34 ^A	10184 ^B	41.67 ^{AB}	896.97 ^B
3. <i>Trichoderma ernacium</i>	7.34 ^A	9259.7 ^B	44 ^A	1179.69 ^A
4. <i>Trichoderma atroviride</i>	7.00 ^A	10180.2 ^B	33.67 ^{BC}	765.54 ^C
5. <i>Trichoderma asperellum</i>	8.67 ^A	12588.7 ^A	41 ^{AB}	1203.84 ^A
6. <i>Trichoderma harzianum</i>	7.67 ^A	9454 ^B	37 ^{ABC}	755.38 ^C
Std Error	0.77	520.53	3.22	31.52
CD (P=0.05)	2.37	1603.91	9.92	97.12



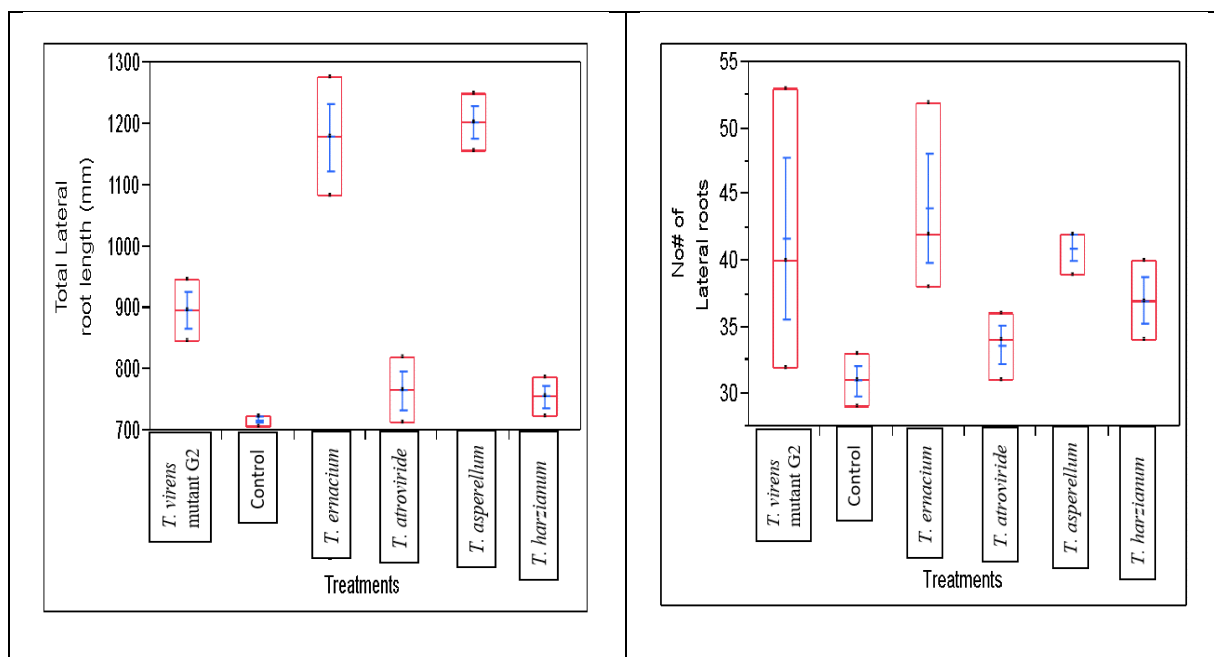


Figure-2: Graphical representation of Root Architecture analysis of *Trichoderma* treated Maize plant for total no. of main and lateral roots, length of main and lateral roots.

The results showed that the main and lateral root number and length in maize with antagonists were always higher than the untreated one. The main root and lateral root number and length were recorded in *T. asperellum* treated maize seeds followed by *T. virens* and the difference in between the treatments are statistically not significant ($P=0.05$). The number of main roots in *T. asperellum* treated seeds was 8.67 and root length was 12588 mm and the corresponding values in case of lateral roots are 41 and 1203 respectively. The root system in different *Trichoderma* treated plants show a considerable variation in their architecture (Plate-1 & 2)

Plate-1: Root system of Maize plants treated with different strains of *Trichoderma* spp.

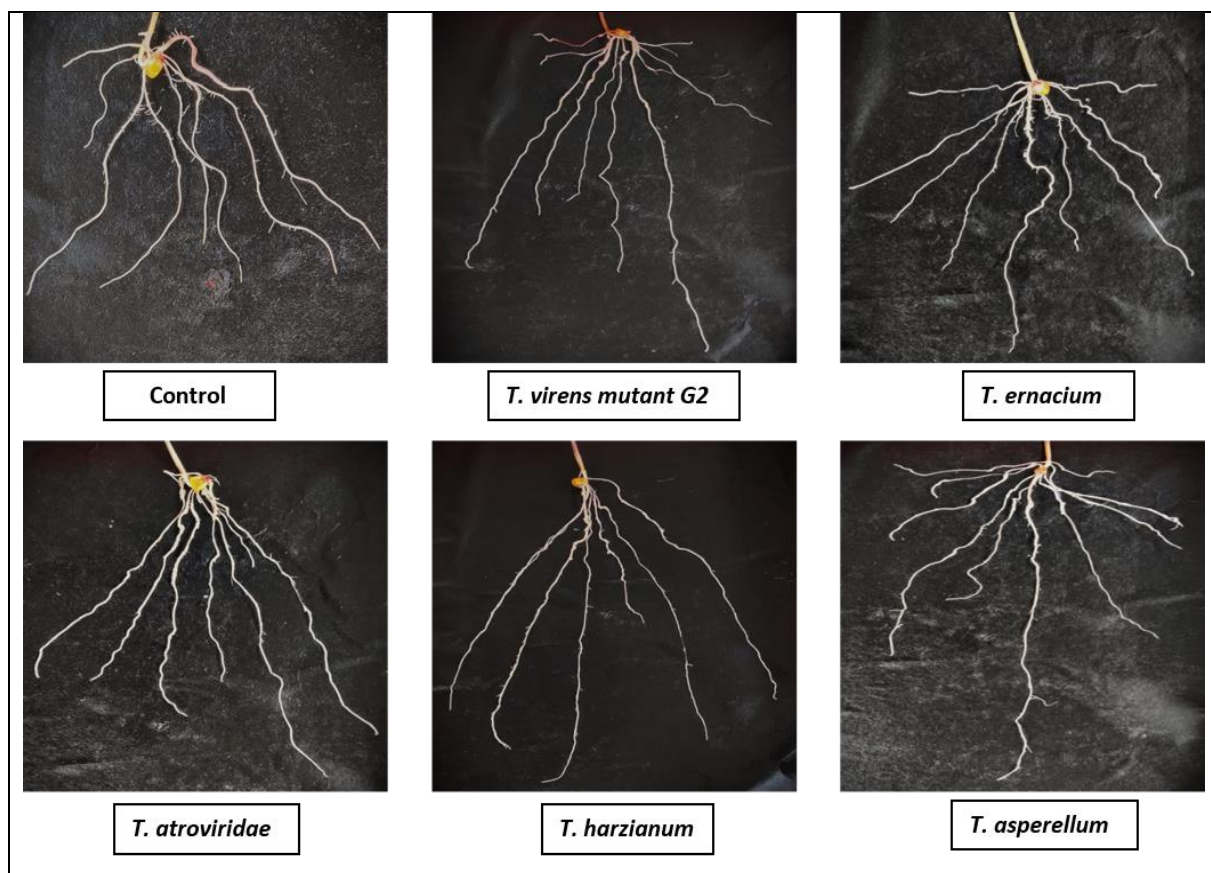
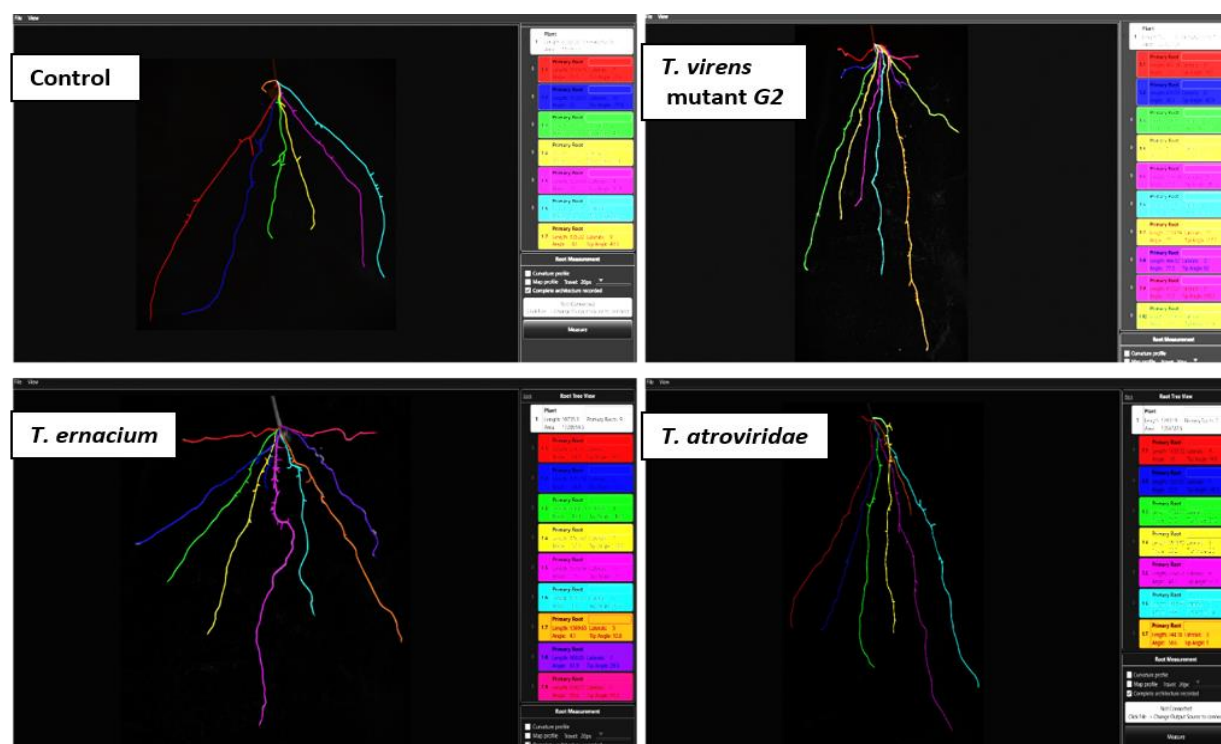
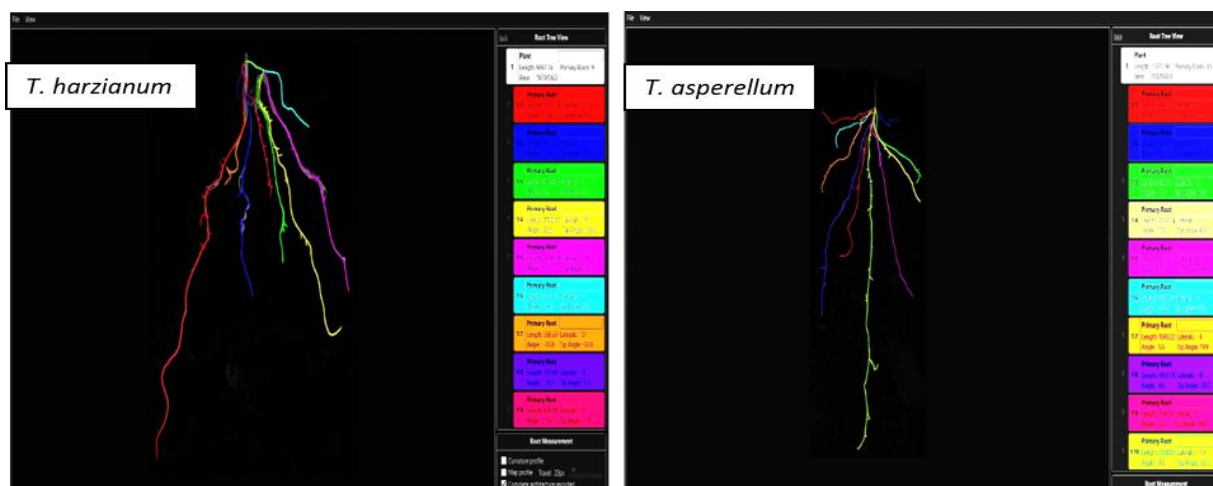


Plate-2: Root architecture analysis by using Rootnav v1.8.1x64 software.





As indicates that the different strains of *Trichoderma* have differentially influence the yield of maize, so it was of interest to measure the population of *Trichoderma* as well as *Pseudomonas spp.* and soil bacteria in different treatments. To carry out this study, soil of the different treatments were collected and the population was estimated in specific media as per protocol described in methodology. The results were presented in Table-3.

The population of *Trichoderma*, Soil Bacteria and *Pseudomonas* were higher in *T. artoviride* and *T. ernacium* than *T. virens* (Table-3) which supports the earlier observation where same trends were recorded in yield of the crop. The graphical representation was given in Figure-3.

Table-3: Estimation of Soil *Trichoderma*, *Pseudomonas* and Soil Bacterial populations in *Trichoderma* treated Maize (*Zea mays L.*) plots by Agar plate method.

Treatments	Total Soil <i>Trichoderma</i> Populations (Log10) Values	Total Soil Bacterial Populations (Log10) Values	Total Soil <i>Pseudomonas</i> Populations (g ⁻¹ soil)
1. Control	4.75 ^B ± 0.04	7.05 ^A ± 0.02	2.4
2. <i>Trichoderma virens</i> mutant G2	4.94 ^A ± 0.14	7.17 ^A ± 0.06	4.27
3. <i>Trichoderma ernacium</i>	5.08 ^A ± 0.06	7.13 ^A ± 0.13	9.33
4. <i>Trichoderma atroviride</i>	5.00 ^A ± 0.1	7.2 ^A ± 0.11	11.98

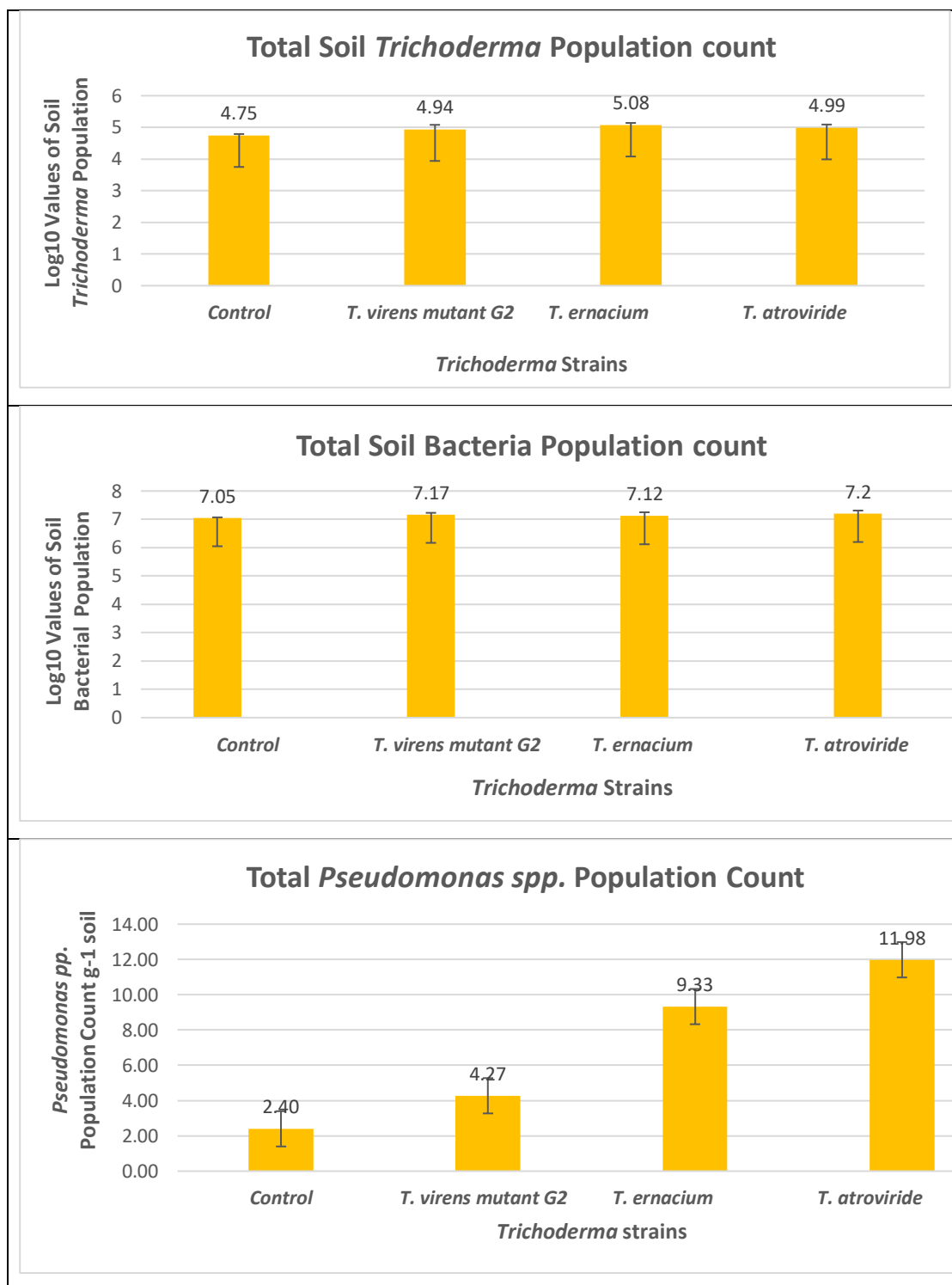


Figure-3: Graphical representation of soil population dynamics of *Trichoderma*, Soil Bacteria and *Pseudomonas* spp. in *Trichoderma* treated maize plots.

Plate-3: *Trichoderma* spp. culture in TSM for estimation of Total Soil *Trichoderma* population in soils collected from root surface of *Trichoderma* inoculated maize plots.

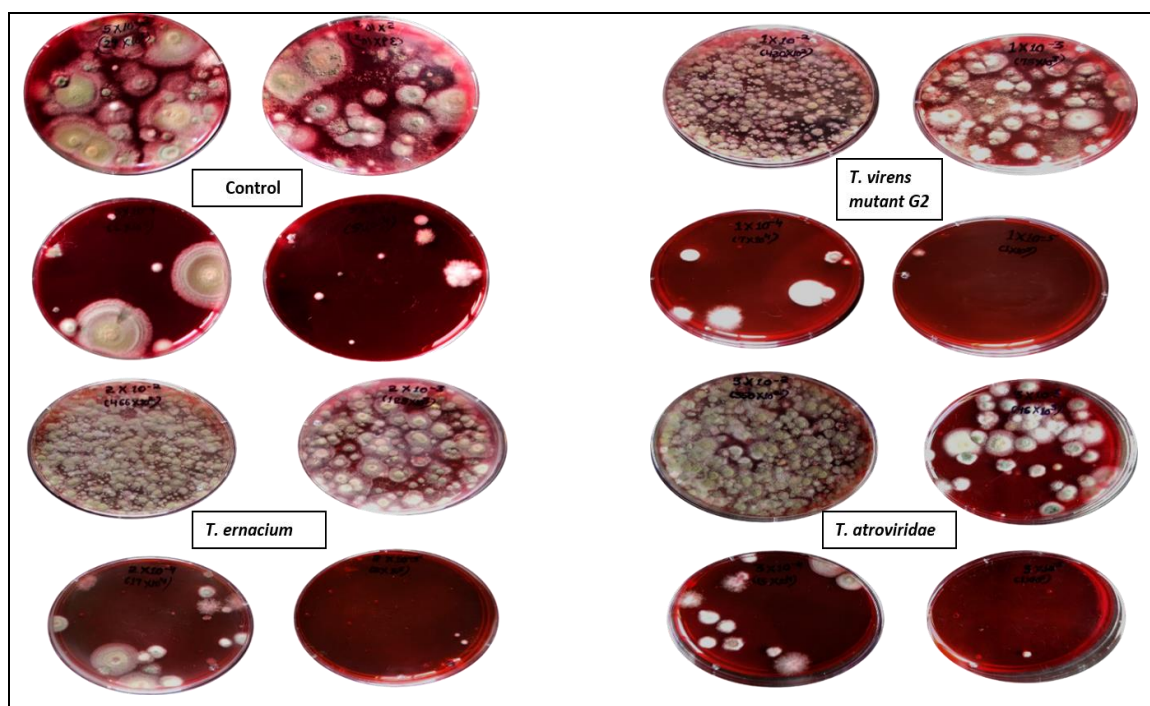


Plate-4: *Pseudomonas* spp. culture in King's B Broth for estimation of Total Soil *Pseudomonas* spp. population in soils collected from root surface of *Trichoderma* inoculated maize plots by MPN Technique.

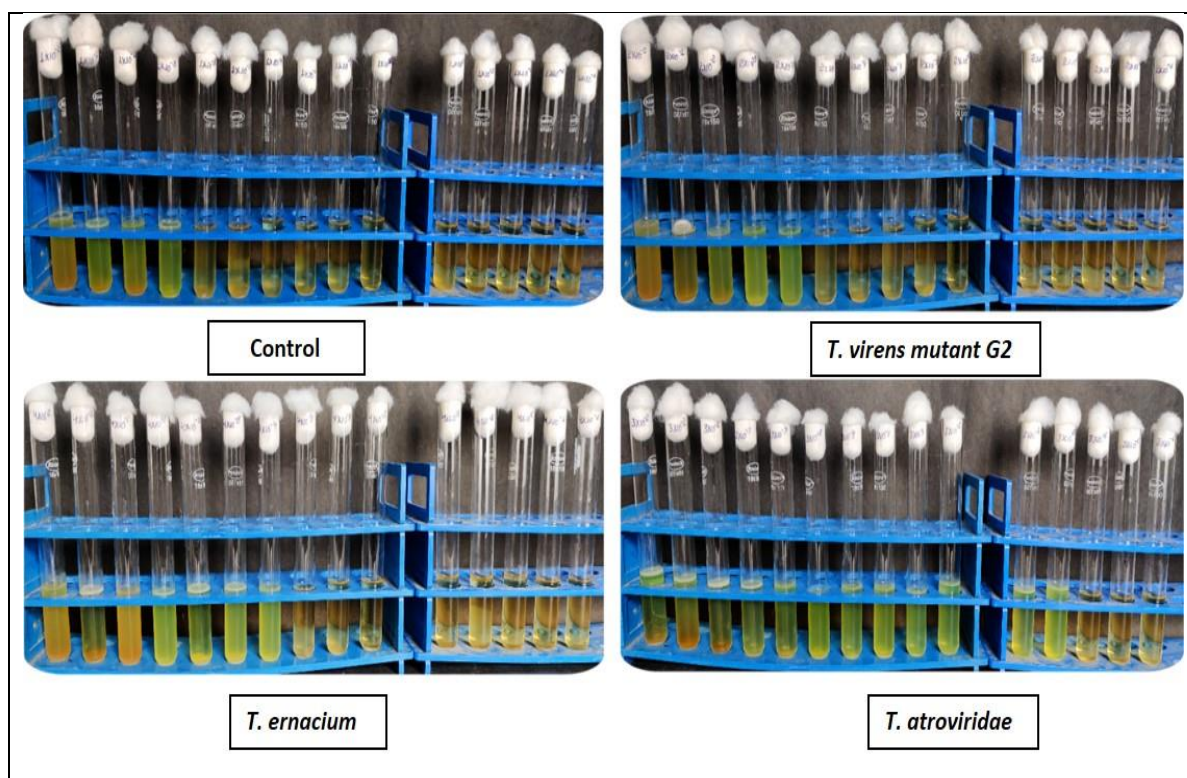
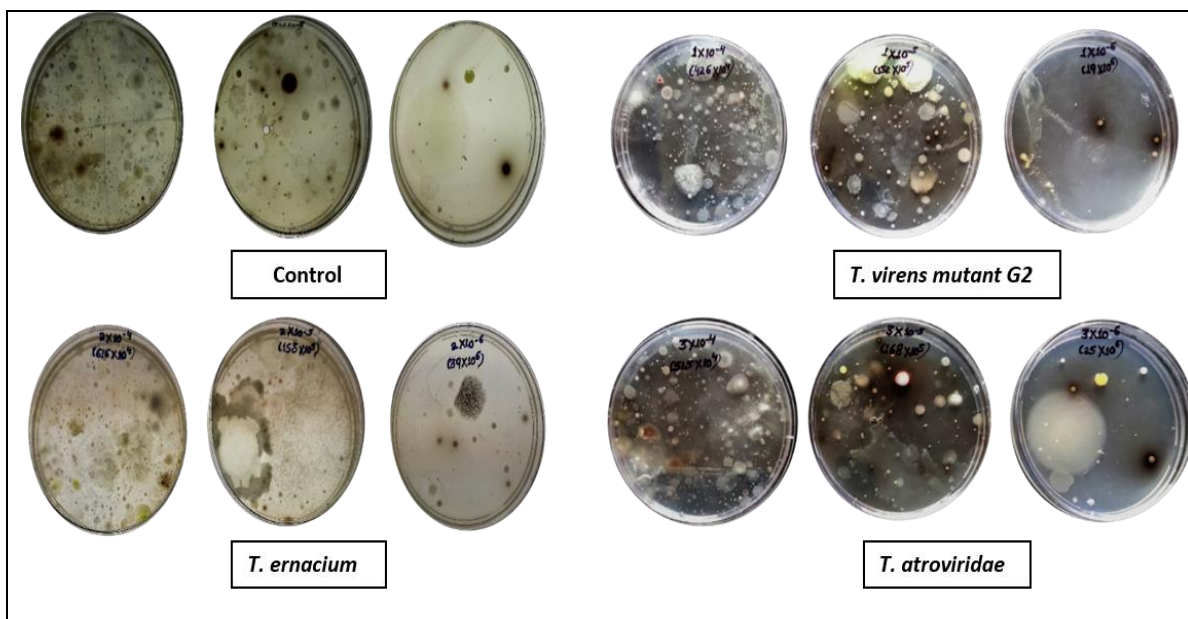


Plate-5: Soil Bacterial culture in Soil Agar extract for estimation of Total Soil Bacterial population in soils collected from root surface of *Trichoderma* inoculated maize plots by Agar plate method.



Determination of enzymatic activity of Soil dehydrogenase of given soil samples reveals a huge information about the soil's biological activity. The anaerobic microbes in the soil synthesizes most of the dehydrogenase enzymes that utilises electron acceptors and oxygen i.e., under anaerobic situations, the enzymatic activity of soil dehydrogenase rises significantly. A number of environmental factors such as availability of oxygen, reduction and oxidation potential, soil moisture, soil pH, content of organic matter, soil profile depth, soil temperature, seasons of the year, soil fertilization, contamination of heavy or use of pesticides have a significant impact on soil DHA.

In the next step, the soil dehydrogenase activity was measured after application of different strains of *Trichoderma*. The results are presented in Table-4 and Figure-4.

The treated soil has higher (53% to 55%) DHA than control (Table-4). The highest dehydrogenase activity of soil was recorded in *T. virens* treated soil recording 19.09 ug, followed by *T. ernacium*. The results supported the earlier observation though least effective treatment i.e., *T. virens* had higher DHA than other *Trichoderma* treated soil. It needs further exploration.

Table-4: Soil dehydrogenase activity in *Trichoderma* treated soil from Maize plots.

Treatments	Mean Soil Dehydrogenase ($\mu\text{g TPF h}^{-1} \text{g}^{-1}$)
1. Control	7.47 ^C $\pm$ 1.12
2. <i>Trichoderma virens</i> mutant G2	19.09 ^A $\pm$ 1.98
3. <i>Trichoderma ernacium</i>	13.63 ^B $\pm$ 4.57
4. <i>Trichoderma atroviride</i>	11.42 ^{BC} $\pm$ 1.92

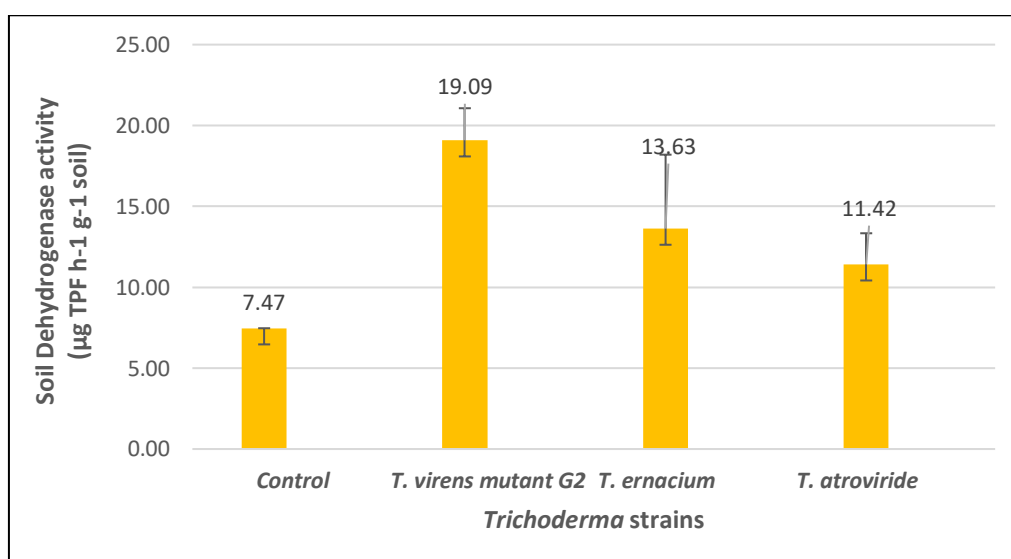


Figure-4: Graphical representation of Soil Dehydrogenase Enzyme Activity of *Trichoderma* treated soil from Maize plots.

In vitro screening of *Trichoderma* on *Rhizoctonia solani*:

Biological control is an integral component of organic farming, but almost all the commercial *Trichoderma* formulations contain synthetic additives like the carboxymethyl cellulose (CMC). Here an attempt has been made to produce organic based formulation with tamarind powder to assay the efficacy of the antagonist against *Rhizoctonia solani* pathogen, a devastating soil borne fungus inciting banded leaf and sheath blight (BLSB) disease is a constraint in maize production.

Results of screening test of *Trichoderma harzianum*, collected from the department of Plant Pathology in different formulations on *Rhizoctonia solani* on PDA plates are summarized Table-5 and Figure-5.

The result presented in Table-5 depicted that different formulation have different efficacy against the growth of *R. solani* and all formulation of *Trichoderma* significantly reduced the growth of *R.solani* as compare to control. All the isolates of antagonists caused 44-53% inhibition of radial colony diameter of the test pathogen. Among the tested isolates, *T. harzianum* formulation with tamarind seed powder showed the highest inhibition of colony diameter of *R. solani* followed by *T. harzianum* with chitin. An interesting observation was found when chitin is mixed tamarind seed powder, the efficacy was reduced as compared to individual application.

Table-5: Effect of *Trichoderma harzianum* formulations on *Rhizoctonia solani*.

Treatments	Growth of the Pathogen (cms)	Mean Growth inhibition (%) of pathogen
1. Control	5.67 ^A ± 0.12	-
2. <i>T. harzianum</i>	3.07 ^B ± 0.25	45.92 ^{BC} ± 3.62
3. <i>T. harzianum</i> + Chitin	2.8 ^{BC} ± 0.10	50.60 ^{AB} ± 1.03
4. <i>T. harzianum</i> + Tamarind seed powder	2.53 ^C ± 0.06	55.30 ^A ± 0.11
5. <i>T. harzianum</i> + Chitin+ Tamarind seed powder	3.13 ^B ± 0.31	44.75 ^C ± 4.61

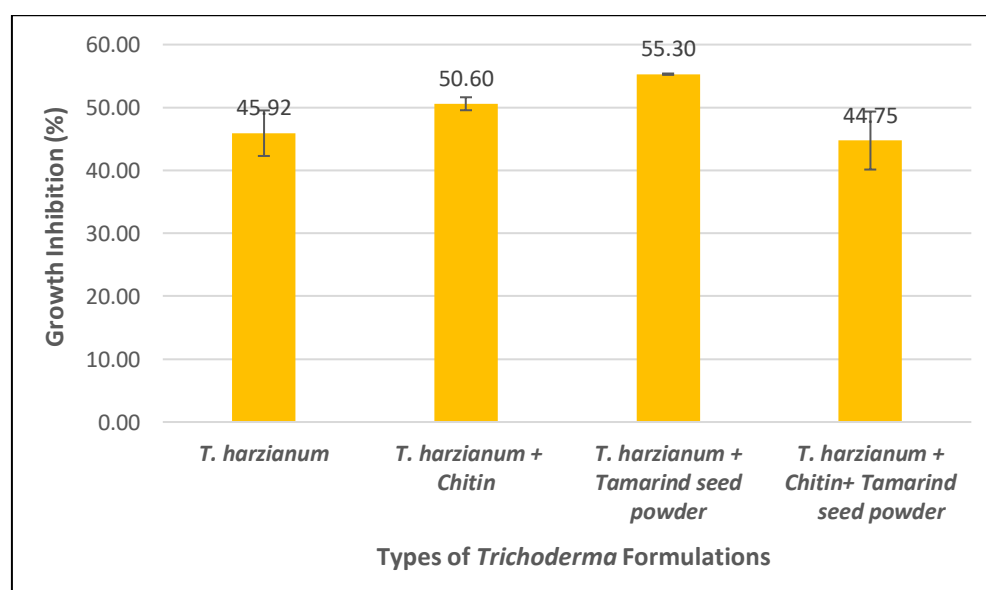


Figure-5: Graphical representation of Growth Inhibition (%) of *Trichoderma harzianum* formulations against *R.solani*.

Plate-6: Dual culture of *Trichoderma harzianum* (UBT 18) and *R. solani*.

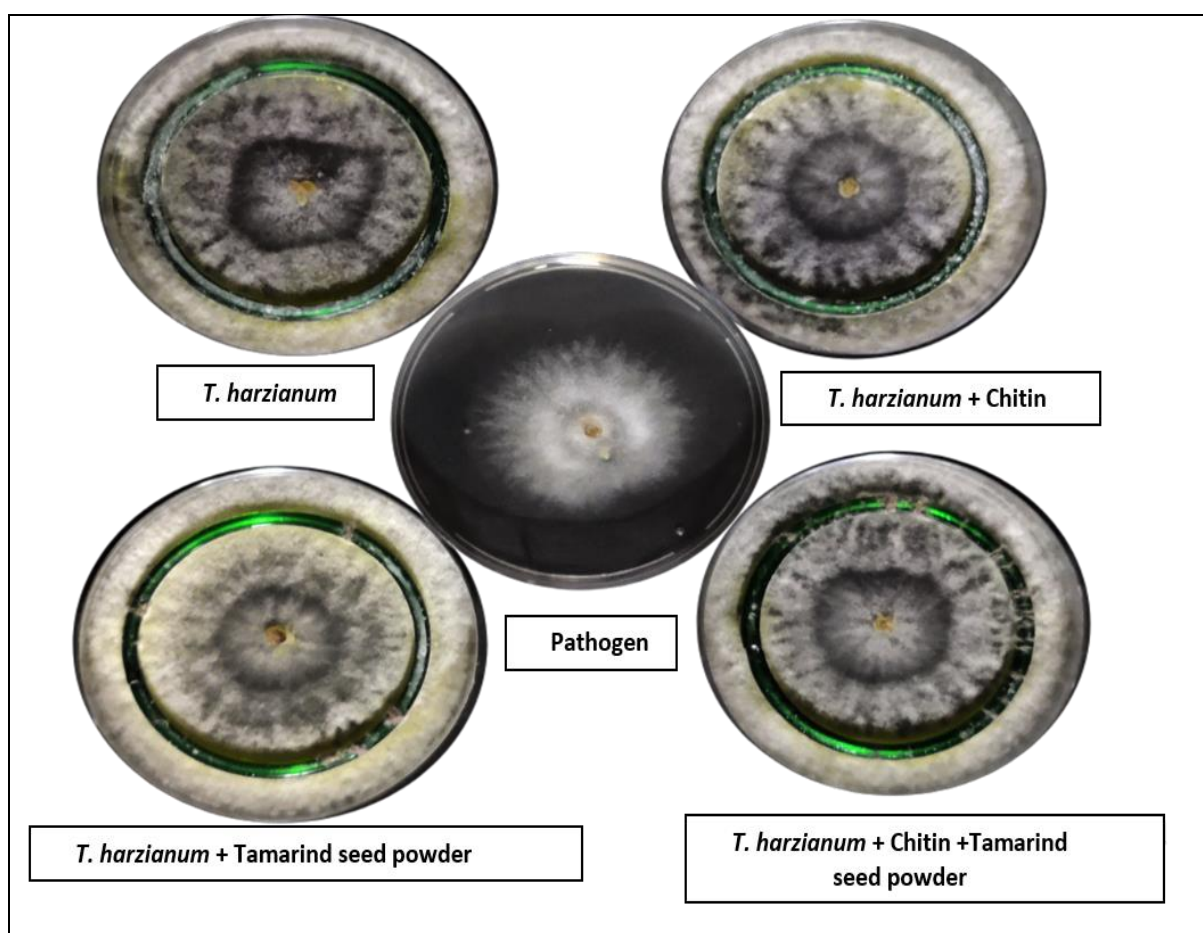


Plate-7: Different *Trichoderma* strains culture on *Trichoderma* specific medium (TSM).

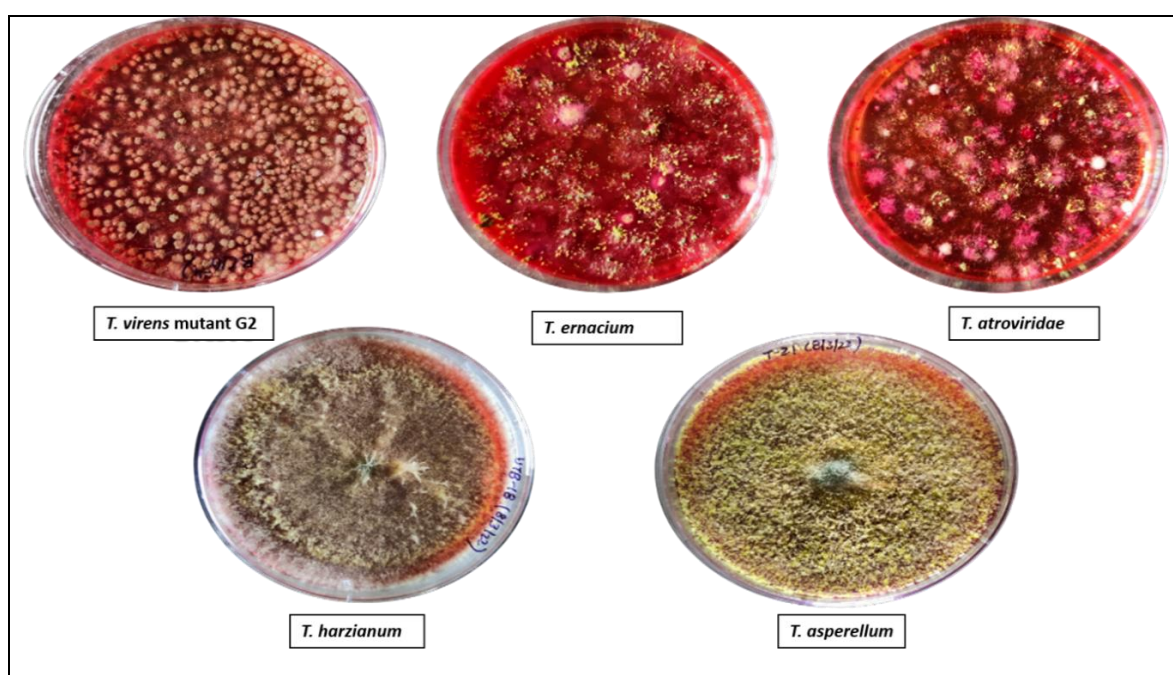
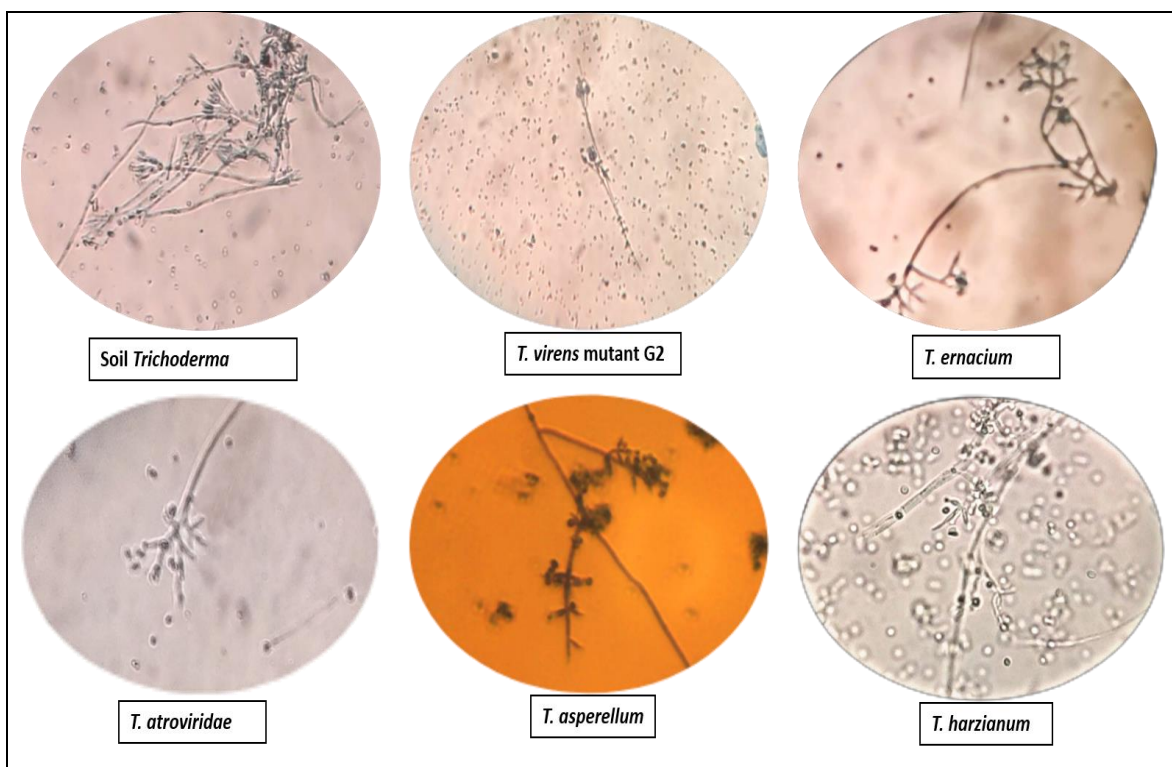


Plate-8: Microscopic pictures of Different Trichoderma strains used in the Research studies.



Expression of Phenylalanine ammonia lyase activity in Maize as influenced by *Trichoderma*:

Trichoderma spp. have ultimate functions in promoting the plant beneficial microbial community and decreasing the pathogen attack through the specific interactions with host-pathogen. However, the effect of *Trichoderma* on changes of maize rhizosphere microbiome is poorly understood.

One of the most widely investigated enzymes in terms of plant responses to abiotic and biotic stress is phenylalanine ammonia-lyase (PAL), which plays an important role in plant's defence system through secondary phenylpropanoid pathway.

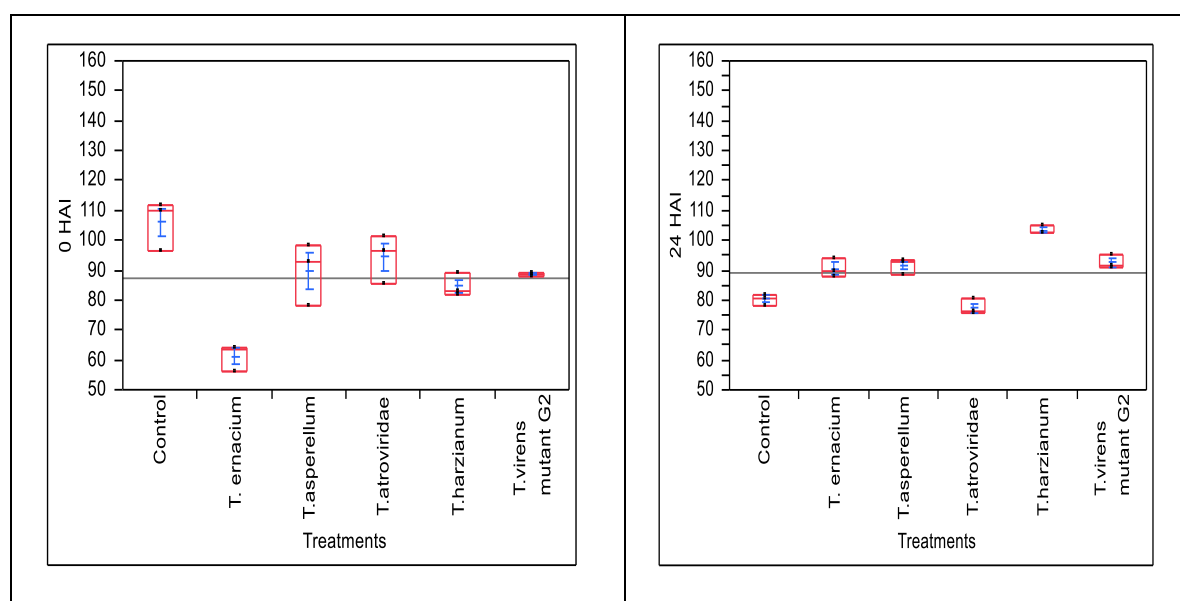
To investigate the efficacy of *Trichoderma spp.* on plant defence with respect to expression of PAL enzyme, the experiment was conducted by using the different species of *Trichoderma* as seed treatment followed by artificial infection with *R. solani* as methodology described earlier. The results are shown in Table-6 And Figure-6.

Table-6: Estimation of PAL enzyme activity in maize by seed treatment with different strains of *Trichoderma*.

Treatments	pal enzyme activity (mg trans Cinnamic acid min-1g-1 fresh weight)			
	0 HAI	24 HAI	48 HAI	72 HAI
1. Control	105.97 ^A	80.39 ^C	76.16 ^A	81.42 ^B
2. <i>Trichoderma virens</i> mutant G2	88.66 ^B	92.59 ^B	82.11 ^A	85.43 ^B
3. <i>Trichoderma ernacium</i>	61.17 ^C	90.55 ^B	82.21 ^A	90.74 ^B
4. <i>Trichoderma atroviride</i>	94.51 ^{AB}	77.45 ^C	87.72 ^A	136.17 ^A
5. <i>Trichoderma asperellum</i>	89.77 ^B	91.57 ^B	79.45 ^A	85.35 ^B
6. <i>Trichoderma harzianum</i>	84.7 ^B	103.33 ^A	82.44 ^A	125.34 ^A
Std Error	3.93	1.42	5.39	6.62
CD (P=0.05)	12.11	4.38	16.61	20.4

HAI: Hours after inoculation

The result shows that the highest stimulation of PAL enzyme activity was recorded with *T. artoviride* followed by *T. harzianum* after inoculation and final post-infectional value was 136 mg trans cinnamic acid after 72 hours of inoculation. In untreated plants, the level of activity was highest and inoculation resulted a marked decrease (24.1% and 28.1%) after 24 and 48 hrs of inoculation whereas, the *Trichoderma* treated plants showed a gradual increase in the enzyme activity irrespective of treatments and the final post infectional value is 4.6 % to 67.2 % higher PAL enzyme activity, the highest induction was found with *T. artoviride*.



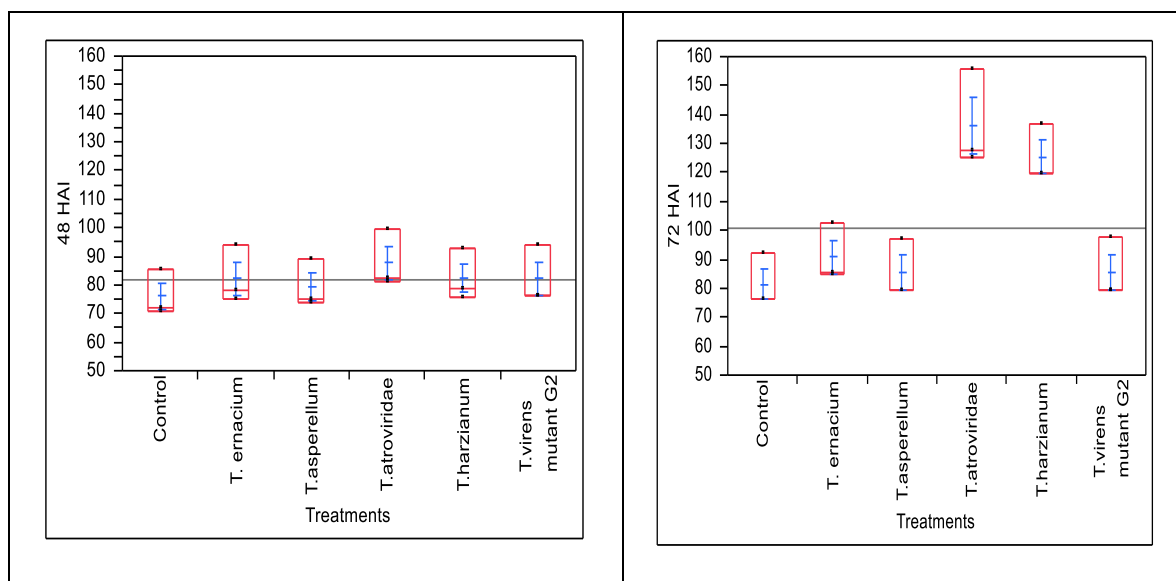
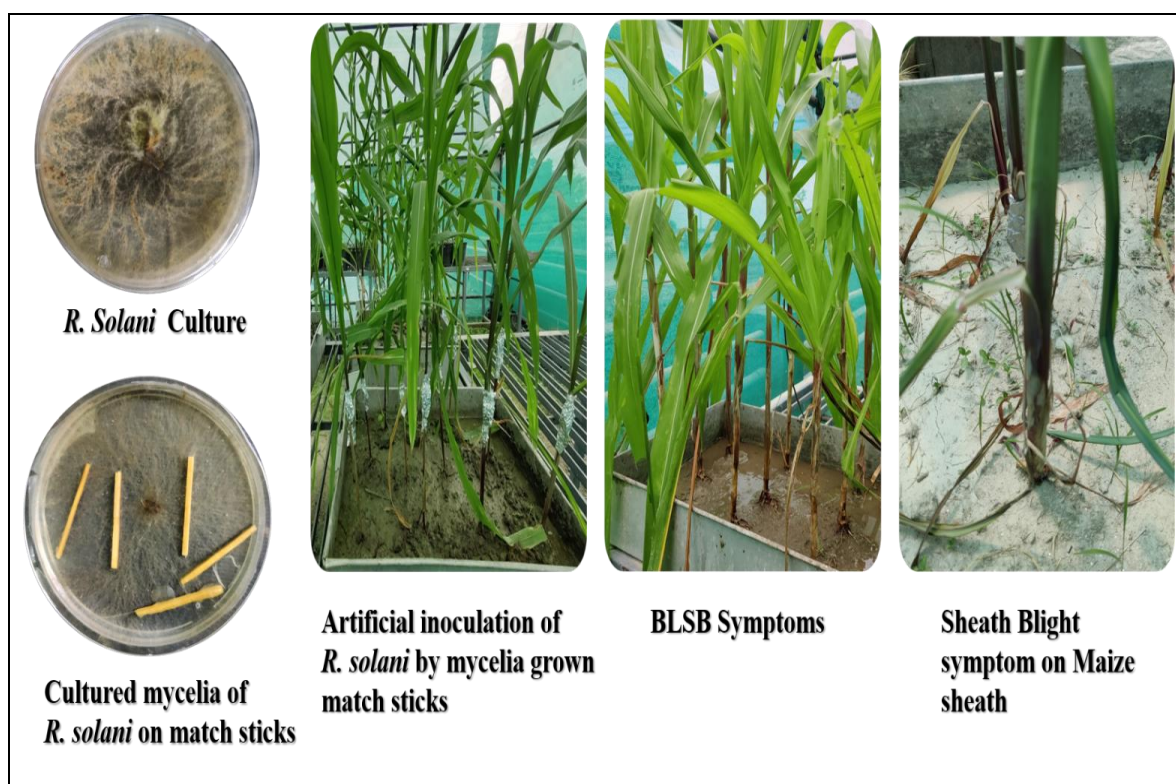


Figure-6: Graphical representation of Phenylalanine Ammonia Lyase (pal) enzyme analysis in *Trichoderma* inoculated maize plants at different time intervals (0, 24, 48 & 72 HAI).

Plate-7: *R. solani* isolation and culturing on PDA with match sticks. Inoculation of *R. solani* and symptoms of Banded leaf and sheath blight of maize.





Expression pattern of two PAL genes and Salicylic gene in Maize by *Trichoderma*:

The phenylpropanoid compounds are precursors to a broad range of phenolic substances with many functions in plants. The initial step in phenylpropanoid pathway is deamination of Phenylalanine to cinnamic acid by Phenylalanine ammonia lyase and this step is crucial transition point between primary and secondary metabolism in plant's defence system. PAL is encoded by a small gene family, PAL 1-PAL4. Beside these, the small phenolic compound, Salicylic acid (*sal*) responsive genes plays a main role in plant defence signalling. It is required for recognition of pathogen derived components and subsequent establishment of local resistance in the infected region as well as systemic resistance throughout the whole plant level.

To determine the PAL genes viz., PAL1 and PAL2 and also Salicylic acid (*sal*) responsive genes were analysed in seed treated maize plants with *Trichoderma* after inoculation with *R. solani*, the tissue samples were collected at different intervals and analysis were done as described in the methodology.

The result in Table-6 shows that the expression of PAL 1 was higher in maize plants treated with *T. ernacium* after 72 hours of inoculation whereas the PAL 2 was recorded with *T. harzianum*. However, in all treatments PAL 1 were higher at 72 hours of inoculation. The Salicylic acid (*sal*) was higher in *T. ernacium* and *T. virens* treated plants as compared to the *T. harzianum*.

Table-7: Gene expression analysis of defense signalling genes (*pal* and *sal*) in maize plants inoculated with *R. solani*:

Treatments	Relative fold expression of defense signalling genes											
	<i>pal</i> 1 (HAI)				<i>pal</i> 2 (HAI)				Salicylic (HAI)			
	0	24	48	72	0	24	48	72	0	24	48	72
<i>T. harzianum</i>	1.75	0.42	2.35	5.93	1.64	0.33	1.86	7.15	1.56	0.61	0.77	0.56
<i>T. virens</i> mutant G2	0.5	0.33	0.03	6.86	0.04	0.06	0.03	0.48	2.08	0.45	0.18	5.26
<i>T. ernacium</i>	0.06	1.9	0.57	7.96	0.06	0.66	0.2	3	0.83	0.84	0.17	5.52

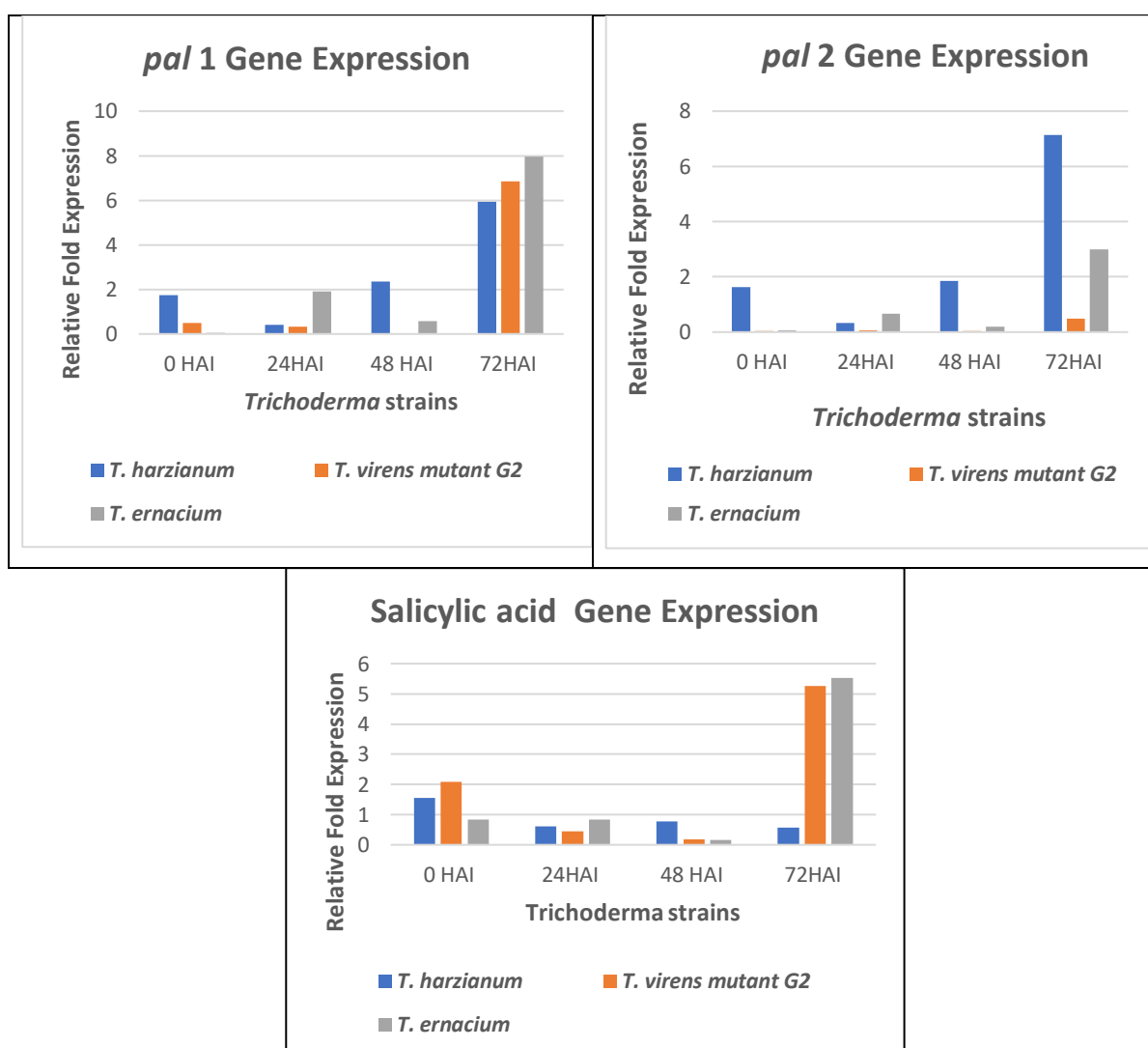


Figure-7: Graphical representation of defense signalling genes (*pal* 1, *pal* 2 & Salicylic acid (*sal*)) responsive genes) in maize plants inoculated with *R. solani* at different time intervals (0, 24, 48 & 72 HAI).

Trichoderma species are ubiquitous component of soil and play an important role in decomposing plant material, with several species of the genus *Trichoderma* identified as etiologic agents of infection in immunocompromised hosts, including *T. longibrachiatum*, *T. citrinoviride*, *T. harzianum*, *T. virens*, *T. ernacium*, *T. atroviride*, *T. koningii*, *T. pseudokoningii*, and *T. viride*.

The biocontrol of *Trichoderma* is achieved through several mechanisms with a combination of two or more mechanisms acting together, probably responsible for the versatility of its biocontrol. It is a well-known mycoparasite that releases enzymes that degrades cell walls as well as other substances that can attack the pathogen directly. Additionally, it synthesizes peptaibols, antibiotics, and other bioactive substances with antibiosis mechanism. It is an efficient colonizer of soil rhizosphere and may compete with other microbes there for nutrients and available space. However, in recent years it was found that they have the capacity to trigger local and induced systemic resistance in various plants. *Trichoderma* spp. could effectively increase the soil enzyme activity and improve plant growth (Zhang *et al.*, 2018).

Trichoderma spp. proliferate in the rhizosphere, establishing a symbiotic association, thus improving plant nutrition and growth in a natural way. Apart from *Trichoderma*'s capacity to attack plant pathogens and inhibit its growth, many reports have shown the stimulation of systemic and local resistances against a broad range of pathogens. (Shoresh *et al.*, 2010). From the high-density oligonucleotide microarrays and 2D electrophoresis results it was proved that The *Trichoderma* spp. treated plants showed the differential expression of gene and stimulation of proteome (Segarra *et al.*, 2007). Various chemical inducers such as jasmonic acid (JA), salicylic acid (SA) and methyl jasmonate (MeJA) constitute the major signal molecules that are crucial for the growth and development of plants as well as for the stimulation of defensive mechanisms in response to various biotic and abiotic stresses (War *et al.*, 2011; Sticher *et al.*, 1997).

In phenylpropanoid metabolism, phenylalanine ammonia lyase enzyme plays a significant role in the production of anti-microbial secondary metabolites in the plants. PAL is an important enzyme in the synthesis of important phenolics. The reaction pathway consists in the conversion of phenylalanine to t-Cinnamic acid catalysed by PAL then, t-Cinnamic acid is converted into p-Coumaric acid after addition of hydroxyl group and after another addition p-Coumaric acid gives ferulic acid. These sequential steps of conversion of phenylalanine are the responsible for the formation of lignin,

tannin, flavonoids and isoflavonoids, which are the plant's weapons working against pathogen invasion. Treatment of plant with bioagents and their metabolites stimulates the phenylpropanoid pathway for the synthesis of different chemicals helping to produce higher amount of phenolics which act as precursor molecules for lignin synthesis (De Ascensao & Dubery, 2000; Gallou *et al.*, 2009) reported the stimulation of PAL gene expression on potato plant treated with *T. harzianum* Rifai MUCL 29707, against *R. solani* during early hours of post-infection. In another study, it was observed that *Trichoderma* upregulated PAL1 gene which is responsible for encoding PAL (Shoresh & Harman, 2008; Shoresh *et al.*, 2005). In this investigation, a rise in the level of PAL in the *Trichoderma* treated plants which is in agreement with the various other reports (Harman *et al.*, 2004; Karthikeyan *et al.*, 2006; Singh and Singh, 2015).

The present investigation showed that the seed treatment of maize with *Trichoderma* induced the defense system against *Rhizoctonia solani* infection with increased PAL enzyme and PAL genes as well Salicylic acid (*sal*) responsive gene expression leading to augmented defence response in addition to direct antagonism. In conclusion, it can be concluded that the *Trichoderma* application as seed treating agent can be useful and better promising management method for suppressing banded leaf and sheath blight disease of maize more efficiently due to the involvement of wide defence mechanisms beside direct antagonism.

CHAPTER- V



SUMMARY AND CONCLUSION



V. SUMMARY AND CONCLUSION

An investigation was done with the different strains of *Trichoderma* collected from UBKV, BARC (Mumbai) and ICAR-NRRI (Cuttack) on maize as seed treatment to observe their reaction against *Rhizoctonia solani* and yield parameters of maize, elicitation of defence genes as well soil population of antagonistic microflora. The summarized findings are as follows.

1. Initially, the three different strains of *Trichoderma* were treated with maize seeds and the harvesting index was calculated. The seeds treated with *T. atroviride* showed highest harvesting index followed by *T. ernacium*.
2. The stimulation of main root and lateral roots in maize were higher *T. asperellum* followed by *T. virens*.
3. The population of *Trichoderma*, Soil Bacteria and *Pseudomonas fluorescens* in soil were also higher in *T. atroviride* and *T. ernacium* treated maize plots.
4. The soil dehydrogenase activity in the maize fields indicate higher activities than control and maximum stimulation of DHA was recorded with *T. virens* followed by *T. ernacium*.
5. The in vitro assay shows that the talc formulation of *Trichoderma* with Tamarind seed powder inhibited the *Rhizoctonia solani* pathogen significantly than chitin amended *Trichoderma*.
6. The Phenylalanine ammonia lyase activity indicates that all the strains of *Trichoderma* had higher PAL enzyme activity than untreated and maximum stimulation was recorded with *T. atroviride*.
7. Finally, the defence genes viz., PAL 1 and PAL 2 as well Salicylic acid (sal) responsive genes were estimated in *Trichoderma* treated maize seeds. The results clearly established that PAL 1 and PAL2 were higher after 72 hours of inoculation. The stimulation of Salicylic acid shows the similar trend.

In conclusion, it is clearly established that the different strains of *Trichoderma* can enhance the yield of maize and also stimulated higher main and lateral roots for obtaining nutrients from different layers of soil when used as seed treatment. Beside this, *Trichoderma* has the potential to encourage the growth of antagonistic microflora viz., *Trichoderma* and *Pseudomonads*. The activity of soil dehydrogenase enzyme was

found to be higher in *Trichoderma* treated soil which indicates the better soil health. The tamarind seed powder amended *Trichoderma* has better potential to protect *Rhizoctonia solani* infection which is responsible for banded leaf and sheath blight disease. Finally, the expression of PAL1 and PAL 2 genes as well Salicylic acid gene in *Trichoderma* treated plants were higher which indicates that *Trichoderma* has the potential to stimulate the defence genes to protect it against *R.solani* infection. Among the *Trichoderma* species, variations were found to have several beneficial effect on various functions of plant.

CHAPTER- VI



REFERENCES



VI. REFERENCES

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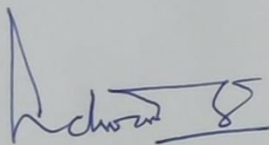
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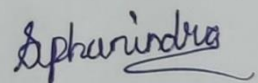
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