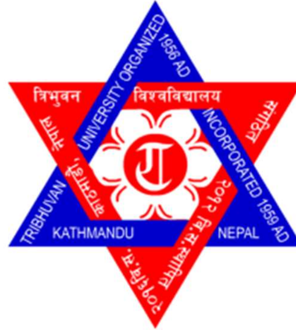
EVALUATION OF ANTAGONISTIC POTENTIAL OF *TRICHODERMA* ISOLATES AGAINST SELECTED PHYTOPATHOGENIC FUNGI



A Dissertation

Submitted to the **Department of Microbiology, Central Campus of Technology**, Tribhuvan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology

(Agriculture)

By

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2018

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RECOMMENDATION

This is to certify that **Mr. Nepoliyan Teyung** has completed this dissertation work entitled "**EVALUATION OF ANTAGONISTIC POTENTIAL OF *TRICHODERMA* ISOLATES AGAINST SELECTED PHYTOPATHOGENIC FUNGI**" as a partial fulfillment of the requirements of M.Sc. Degree in Microbiology (Agriculture) under our supervision. To our knowledge this work has not been submitted for any other degree.

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ACKNOWLEDGEMENTS

I express my sincere and deepest gratitude to my supervisor, Mr. Shiv Nandan Sah and Mr. Nabin Kumar Dangal who ploughed through several preliminary versions of my text, making critical suggestions and posing challenging questions. Their expertise, invaluable guidance, constant encouragement, affectionate attitude, understanding, patience and healthy criticism added considerably to my experience. Without their continual inspiration, it would have not been possible to complete this study.

I owe my special thanks to Nepal Agricultural Research Council (NARC), Tarahara for providing necessary laboratory facilities. I take this opportunity to express my deep sense of gratitude and respectful regards to Miss. Shasikala Rai, Mrs. Rita Joshi and Mrs. Sita Pokhrel; Technical officers of RARS for their unconditional support and gave me untiring help during my study.

I am highly thankful to honorable teachers and staffs of Department of Microbiology, Central Campus of Technology and NARC, Tarahara, for the collection of plant pathogens samples throughout different geolocations.

I owe my most sincere gratitude to Lab technician of microbiology department Mr. Prajwal Bhandari, Mr. Ain Bahadur Karki, Mrs. Saru Ghale and our campus librarian Mr. Om Nath Khatiwada, Mr. Singhman Tamang, Mr. Yaghya Prasad Adhikari and store keeper Mr. Sunil Sherestha. I sincerely admire the contribution of all my friends specially to Mr. Pradip Rai; juniors Miss. Deepika Rai, Miss. Junita Rai and all NARC’s intern student extending their unstinted support, timely motivation, sympathetic attitude and unfailing help during the course of entire study.

Words prove a meagre media to write down my feelings for my mother Smt. Man Kumari Teyung, brother Piter Teyung and sister Mery Teyung and all my relatives and friends for providing me constant encouragement, divine presence and supporting me spiritually throughout.

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ABSTRACT

Phytopthogen are simply an organism parasitic on a plant host resulting the serious problems regarding crop losses in agriculture sector. To facing such a threat, it is very wise to use Biological Control Agents (BCA's) like *Trichoderma*, a soil-borne filamentous fungi that are capable of parasitizing several plant pathogenic fungi. It is a potential fungal BCA's against a range of plant pest and pathogens. Unfortunately, popularization of bio-pesticides is very slow as compared to chemicals and only 0.035% bio-pesticides are available in pesticides market share of Nepal as of 2011/12. *Trichoderma harzanium* and *Trichoderma viride* have curved a niche for themselves in Nepal as important BCA's for management of various diseases. In this study, *Trichoderma* and phytopathogens were isolated from soil samples and diseased plant parts using soil dilution plating methods and tissue culture techniques respectively. Thereafter, antagonistics properties of eight T-isolates coded as Tar, T22, I2, I3, TV, T-gel, TH3 and Dar were evaluated against fourteen phytopathogenic fungi (*Acremonium* spp., *Alternaria brassisicola*, *Exsirohilum turcicum*, *Fusarium* spp., *Macrophomina phaseolina*, *Penicillium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*) tested in vitro study using dual culture techniques volatile and non-volatile compounds methods whereas in vivo study includes germination percentage and percentage increased or decreased of diseases severity over control. In dual culture techniques and non-volatile compound effects, it is found that T-isolates has maximum growth inhibition of *R. solani* (as much as 100% overgrowth) whereas *M. phaseolina* has lowest effect on it. Generally volatile organic compounds of T-isolates shows moderate and similar effect on phytopathogen except *M. phaseolina* which has almost no effect on them. Study conducted on vivo trail gives satisfactory results to proof T- isolates as a very potent BCA's. Overall results analysis shows *Trichoderma* isolates Tar and I2 are very potent BCA's and could be recommended for mass production of *Trichoderma* based biopesticides from them. Present study concludes the uses of *Trichoderma* and assessment of their suitability as bio-pesticides for control of selective fungal phytopathogen.

Key words: *Trichoderma*, Phytopathogen, antagonistics, biocontrol.

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LIST OF ABBREVIATIONS

ABM	-	Acibenzolar-s-methyl
BCA	-	Biological Control Agent
BDD	-	Banana Disease, Dharan
BOD	-	Biological Oxygen Demand
CDL	-	Chilli Disease, Lahan
CDT	-	Cabbage Disease Tarhara
CIAT	-	International Center for Tropical Agriculture
CWDE’s	-	Cell Wall Degrading Enzymes
DAI	-	Days After Inoculation
DAS	-	Days After Sowing
DAT	-	Days After Transplantation
DI	-	Disease Incidence
DSI	-	Diseases Severity Index
FAO	-	Food and Agriculture Organization
FO	-	*Fusarium oxyporum*
FS	-	*Fusarium solani*
IK1	-	Ilam, Kulbang 1
IK2	-	Ilam, Kulbang 2
IMA	-	International Mycological Association
IRRI	-	International Rice Research Institute
ISTA	-	International Seed Testing Association
LPCB	-	Lactophenol cotton blue
MB	-	Maize blight
MBCA	-	Microbial Biological Control Agent
NARC	-	Nepal Agricultural Research Council
NVOC	-	Non- Volatile Organic Compounds
PAN	-	Pesticide Action Network
PDA	-	Potato Dextrose Agar
PDB	-	Potato Dextrose Broth
PDT	-	Potato Disease, Tarahara
PGPF	-	Plant Growth Promoting Fungi
PGPR	-	Plant Growth Promoting Rhizobacteria

RARS	-	Regional Agriculture Research Station
RBA	-	Rose Bengal Agar
RS	-	*Rhizoctonia solani*
RSR	-	*Rhizoctonia solani,* rayo
ROS	-	Reactive Oxygen Species
SAR	-	Systemic Acquired Resistance
SOM	-	Soil Organic Matter
SR	-	*Sclerotium rolfsii*
TCD	-	Tarahara, Citrus Disease
TVD	-	Tomato Disease, Dhankuta
T-isolates	-	*Trichoderma* isolates
TSM	-	*Trichoderma* Selective Medium
VOC	-	Volatile Organic Compound
ZOI	-	Zone of Interaction

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CHAPTER I

INTRODUCTION

1.1 Background

Phytopathogen are simply an organism parasitic on a plant host to causes diseases in plant (Merriam-Webster Dictionary 2018; Collins English Dictionary 2018). There are various types of phytopathogens such as fungi, fungus-like organisms, bacteria, viruses, viroids and virus-like organisms, nematodes, protozoa, algae and parasitic plants. Among them, fungi are spore-forming, non-chlorophytic, eukaryotic organisms and most of the true fungi are filamentous and branched. Over 100,000 species of fungi are saprophytes in which over 20,000 species of fungi are parasites and cause disease in crops and plants (USEPA 2005). Fungal parasites are the most prevalent plant pathogenic organism thereby all plants are attacked by one species or another of phytopathogenic fungi. Individual species of fungi can parasitize one or many different kinds of plants (Cooper and Gardner 2007). However, there are several fungal genera containing species that cause disease (e.g., infections, allergies, toxicity) in plants, animals and man. These fungi can be categorized into two groups in regards to infection, one is saprophytic fungi which can be opportunistic pathogens that enter via wounds or due to a weakened state of the host and second is true pathogens that may depend on living plant or human tissues for nutrients but can also sur survive outside of the hosts. There are some fungal species that cause not only disease in plants and but also in humans. These fungi include *Aspergillus* and *Fusarium* as well as other phytopathogenic fungi now considered members of the "emerging pathogen" group in humans (Lucca 2007).

To facing such a threat, it is very wise to use Biological Control Agents (BCA's) like *Trichoderma*, a genus of asexually reproducing fungi that are often the most frequently isolated from soil. These fungi colonize woody and herbaceous plant materials, in which the sexual Teleomorph (Genus: *Hypocrea*) has most often been found. However, many strains, including most biocontrol strains, have no known sexual stage. In nature, the asexual forms of the fungi persist as clonal,

often heterokaryotic, individuals and populations that probably evolve independently in the asexual stage (Harman et al 1998a). They show a high level of genetic diversity, and can be used to produce a wide range of products of commercial and ecological interest. They are prolific producers of extracellular proteins, and are best known for their ability to produce enzymes that degrade cellulose and chitin, although they also produce other useful enzymes (Harman and Kubicek et al 1998). For instance, different strains produce more than 100 different metabolites that have known antibiotic activities (Harman et al 1998b). *Trichoderma* species used as agents for the control of plant disease and for their ability to increase plant growth and development, high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficacy in promoting plant growth and defense mechanisms. They are becoming widely used in agriculture, and the most useful strains show a property that is known as 'rhizosphere competence' (Chet et al 1997). *Trichoderma* is more efficient in acidic than alkaline soils. *Trichoderma* BCAs control ascomycetous, deuteromycetous and basidiomycetous fungi, which are mainly soil borne but also air borne pathogens (Monte 2001).

With gradual development of techniques and methods involved in agricultural sector, the goal of all of them is to produce agricultural yield in increased state. In doing so several factor affects on agricultural yield such as soil nutrient, water supply, fertilizer and other additive supplement season and so on. Beside this pathogen and pest is the most serious problems regarding crop losses in agriculture pathogen and pest is the most serious problems regarding crop losses in agriculture (Oerke 2006).

To manage pathogen in field, different approaches gradually develops with series of success and failures pattern and managing pathogen with single tactics and strategy never work too long as they develops resistances capacity to overcome them easily relatively in short time as we applied on them in the field. Now the point must be considered is; to develop a type of chemical insecticides will cost billions of rupees (in contrast to few lakhs rupees required for development of bio-pesticides) which will kill pest specially designed for along with number of natural enemies existing in same niche. In case, number of

natural enemies survive they also forced to migrate from there due to either complete loss of food sources on pesticides treated area or altered predation or parasitism behavior of them viz; loss of ability to recognize host or pest insect, loss of coordination, reduction of predation efficiency, temporary paralysis or knockdown from natural habitat, termination of feeding and repelling from treated host or pest insect. This will disrupts the natural agroecosystem on chemical insecticides treated area if uses directly without the principal based on Integrated Pest Management (IPM) which states chemicals are the last viable options in pest managements. Even biorational approach has been avoiding over biological control; as much as possible because this approach also has many disadvantages out of which most notable one is that pest slowly but surely adapt themselves to these because they have no sensory organisms and tactics to fight against pest or pathogen whereas fungal BCA's like *Trichoderma* does have (Atwal and Dhaliwal 2015).

Now, considering long term sustainable techniques to manage pest in field thus one can approach BCAs. There are number of option in biological control including use of parasitoids, predator and pathogen. Among them microbial pathogenic agent such as fungal bio-pesticides can be used in IPM strategy and among all BCAs it is reported that insecticides resistance against fungal mycobio-pesticides is lower than other BCAs since their mode of action is quite complex and have different pattern of action (Leo and Rathore 2015).

Thus considering number of factors such as ecological and aesthetical values, human health, cost vs benefit, effects of chemical insecticides on non-target species, recalcitrant properties of synthetic pesticides; one can suggest to any farmers to use and encourage them to utilize bio-pesticides over them. Even considering if farmer only use synthetic ones, in such a cases one day such time will reach where no chemical shows pesticide selectivity over them though the farmers use different doses and combination of them to kill pest will become waste efforts (Margarita 2011). Therefore, it was honor to study the evaluation of *Trichoderma* isolates against selected phytopathogenic fungi.

As FAO (2002) defined, "pesticide is any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest, including

vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies". A pesticide may be a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest. The use of chemicals in modern agriculture has significantly increased productivity. But it has also significantly increased the concentration of pesticides in food and in our environment, with associated negative effects on human health (Margarita 2011).

About two million tons of pesticides are used globally among which 45 percent is consumed in Europe followed by 24 percent in the USA and remaining 25 percent in the rest of the world. The domestic consumption of pesticide in Nepal is very low (0.142 kg/ha) comparing with other countries like India (0.5 kg/ha), Mexico (0.75 kg/ha), Germany (3 kg/ha), UK (5 kg/ha), USA (7kg/ha), Netherlands (9.4kg/ha), Japan (12 kg/ha), China (14 kg/ha) and Taiwan (17 kg/ha) (Dhital et al 2015). Uses of pesticides come with adverse effects on the health of the people land and environment. Studies have already confirmed that the use of pesticides might results in the cancer non-cancer and improper neural development, dermatological effects acute and chronic neurotoxicity, birth defects fetal death altered growth and genotoxicity destroys habitat. These health effects are different depending on the degree, and the type of exposure. Typically, the effects are different for farmers who are directly exposed to pesticides, compared to those for farmer's relatives or people living in rural areas who are less directly exposed. There are also effects on consumers through pesticide residues in food. Even though the effects of the pesticides are already pronounced, people in developing countries are still using the pesticides (Margarita 2011). Thus the rationale for the development and deployment of bio-pesticides is for pest management due to their environmental safety, specificity, and biodegradability.

1.2 Objectives

1.2.1 General Objective

To evaluate the antagonistic potentiality of *Trichoderma* spp. as Biological Control Agents against some selected phytopathogenic fungi.

1.2.2 Specific Objectives

a) To identify antagonistic *Trichoderma* spp. from soil sample of forest area of Tarahara and Dharan.
b) To identify phytopathogenic fungi from diseased plant parts from selected geolocation of eastern region of Nepal.
c) To know the antagonistic potential of *Trichoderma* spp. on selected pathogen in vitro and vivo.
d) To study cultural and morphological characteristics of isolated strains of *Trichoderma* spp. and phytopathogen.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction of *Trichoderma* spp.

Trichoderma is easily identified in culture media, which produces large number of small green or white conidia from phialids present on the profusely or meagerly branched conidiophores. However, the identification of the isolates to species level is difficult and confusing due to the complexity and closely related character of the species. Species concept within the *Trichoderma* is very wide and this has resulted in the establishment of many specific and sub-specific taxa (Samuels 1996). *Trichodrema* spp. (*T*. spp.) are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available (Kubicek et al 2009) as well as in the rhizosphere of plants, where they can induce Induced Systemic Resistance (ISR) against pathogen (Harman 2000). *T*. spp. are highly successful colonizers of these habitats, which is reflected both by their efficient utilization of the substrate as well as capacity for antibiosis and enzymes secretion. Besides they have effects on growth, reproduction, and secondary metabolite biosynthesis (Schuster et al 2010).

2.2 Taxonomic Classification

The taxonomic classification of *T.* spp. and soil born phytopathogens used as model organism in this study are listed in Appendix A.

2.3 Morphological Characters

Morphological Characters are used to distinguish different species of *Trichoderma*. Conidia shape may vary from globose, ellipsoidal, obovoidal or short cylindrical with the basal end truncate and tapering type. Size of conidia may also differ. There is also variation in the culture color. Some species show dull yellow pigmentation, other reddish color and still others produce no pigment at all. Branching of conidiophores is either regular or verticillate or irregular. Conidiophores are erect or straggling, branched at regular intervals, branches divergent, solitary or aggregated into floccose tufts. Conidiogenous cells are

phialids, borne singly or in clusters terminally, lageniform, ampulliform, sub-globose or cylindrical, conidium bearing neck. Conidia are single celled, may be colorless, green, grey, or brownish; smooth walled to roughened, wing-like projections, obovoid, oblong or short cylindrical, sub-globose bound in globoid heads or may be enclosed in a sac. Morphologically, *Trichoderma* somewhat resembles *Verticillium* and *Gliocladium*. In *Trichoderma* the branching pattern is less regular as compared to *Verticillium*, whereas in *Gliocladium* branching arrangement is terminal (Schuster et al 2010). *Trichoderma* is characterized by fast growing hyaline colonies bearing repeatedly branched conidiophores in tuffs with divergent, often irregularly bent flask shaped phialids. *Trichoderma harzianum* conidiophores side branches are long and slender without sterile hyphal elongation, phialids not crowded, rather slender, colonies yellowish, bright, dull to dark green, floccose or with compact conidiophores tufts. Conidiophores with complicated dendroid branching system, phialids regularly disposed in numbers of 3 or more. Conidia (2.8 to 3.2 × 2.5 to 2.8 µm) globose, sub-globose or short obovoid, with length: width ratio of less than 1.25; colony reverse uncolored; colonies reaching >9 cm diameter in 5 days at 20°C on Oat Meal Agar Media (Hui et al 2013). *Trichoderma* viride's conidiophores side branches are long and slender without sterile hyphal elongation, phialids not crowded, rather slender, colonies yellowish, bright, dull to dark green, floccose or with compact conidiophores tufts. Conidia roughened 3.6 to 4.8 µm × 3.5 to 4.5 µm. (Nagmani et al 2006).

2.4 Biodiversity of *Trichoderma*

The habit, habitat and biodiversity of *Trichoderma* are described in Appendix B.

2.5 *Trichoderma* spp. as Biocontrol Agent

2.5.1 Disease Control: *Trichoderma* is a potent BCA's and used extensively for soil borne diseases. It has been used successfully against pathogenic fungi belonging to various genera, viz., *Fusarium, Phytopthara, Rhizoctonia* etc (Singh 2010).

2.5.2 Plant Growth Promoter: *Trichoderma* strains (*T.* strains) application solubilize phosphates and micronutrients and increases the number of deep roots in plants, thereby increasing the plant's ability to resist drought (Singh 2010).

2.5.3 Biochemical Elicitors of Disease: *T.* strains are known to induce resistance in plants. Three classes of compounds that are produced by *Trichoderma* and induce resistance in plants are now known. These compounds induce ethylene production, hypersensitive responses and other defense related reactions in plant cultivars (Singh 2010).

2.5.4 Transgenic Plants and Bioremediation: Introduction of endochitinase gene from *Trichoderma* into plants such as tobacco and potato plants has increased their resistance to fungal growth. Selected transgenic lines are highly tolerant to foliar pathogens such as *Alternaria alternata, A. solani* and *Botrytis cirerea* as well as to the soil-borne pathogen, *Rhizoctonia* spp. (Singh 2010). Similarly, *T.* strains play key role in the bioremediation of soil that are contaminated with pesticides and have the ability to degrade a wide range of insecticides such as organochlorines, organophosphates and carbonates (Singh 2010).

2.6 Modes of Action of *Trichoderma*

2.6.1 Mycoparasitism and Antibiosis: It is a direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. *T.* spp. may exist direct biocontrol by parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing in the partially due to the sequential expression of the cell degrading enzymes mostly chitinase, glucanase and protease. It involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host and contained high concentration of osmotic solutes such as glycerol (Benitez et al 2004).

Antibiosis occurs during interaction involving low molecular weight (MW) diffusible compounds or antibiotics produced by *T.* strains that inhibits the growth of other microorganisms (Benitez et al 2004). Most *T.* strains produce volatile and non-volatile toxic metabolites that impede colonization by

antagonized micro-organism, among these metabolites the production of harzianic acid, alamenthicins, tricholin, peptaibols, antibiotics, 6-pethyl-α–pyron, viridian, gliovirin, gliosoprenins, heptelidic acid and other have been described. *T.* spp. also produces siderophores and a large number of peptaibiotics known as peptaiboles, which contain with a high frequency non- standard amino acids (Degenkolb et al 2006).

2.6.2 Competitive Saprophytic Ability: Competition is the phenomenon in which the pathogen and the introduced *Trichoderma* compete for the availability of space and nutrients. *Trichoderma* is capable of degrading straw with mycoparasitic ability against several plant pathogenic fungi. It is found that the higher the amount of inoculum of *T. harzianum* strain no 94-016, the higher percent colonization of rice straw in the soil. Further tests will be carried out to compare the decomposition of rice straw on the surface and buried in the soil and the effect of moisture on soil decomposition. The depth of rice straw and moisture content are factors identified as significant in affecting crop residue decomposition (Cumagun et al 2009).

2.6.3 Fungistasis: Few antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites produced by other species, including plants, and to survive under very extreme competitive conditions. *T.* strains grow rapidly when inoculated in the soil, because they are naturally resistant to many toxic compounds, including herbicides, fungicides and insecticides such as DDT and phenolic compounds and the strains recover very rapidly after the addition of sub-lethal doses of these compounds due to presence of *T.* strains of ABC transport systems within *Trichoderma*. For this reason, preparations of *T.* strains are very efficient in controlling several phytopathogens, such as *P. ultimum, R. solani* or *S. rolfsii*, when alternated with methyl bromide, benomyl, captan or other chemicals (Gajera et al 2013).

2.6.4 Induced Systemic Resistance (ISR): A variety of strains of *T. virens, T. asperellum, T. atroviride and T. harzianum* induce metabolic changes in plants that increase resistance to a wide range of plant-pathogenic microorganisms and viruses. Moreover, this response seems to be broadly effective for many plants; for example, *T. harzianum* strain *T-22* induces resistance in plants as diverse as

tomatoes and maize, which indicates that there is little or no plant specificity. When spores or other propagative structures are added to soil and come into contact with plant roots, they germinate and grow on root surfaces, and at least some infect the outer few root cells. They produce at least three classes of substance that elicit plant defense responses that prevent further infection of roots by plant pathogens. These elicitors include peptides, proteins and low-molecular weight compounds (Harman 2004).

2.6.4.1 Cell wall Degrading Enzymes (CWDEs): The cell wall of either BCA's – *Trichoderma* or host plant is the first barrier encountered by most plant pathogens, and thus it must be degraded to allow their (pathogen) penetration and tissue colonization. Necrotrophic fungal pathogens degrade the structural polymers in plant host cell wall and colonized inter cellular spaces facilitated by the production of extra cellular Cell wall Degrading Enzymes (CWDEs). Pectinase and cellulase were breakdown into pectin and cellulose, the two major polymers that maintain the firmness and structure of host cell walls. Production of CWDEs often determines the pathogenecity of necrotrophic pathogen (Collmer and Keen 1986). Among the different pectinolytic enzymes, Polygalactouronase (PG) facilitates the penetration of primery cell walls and onset the production of other isoenzymes involved in pectin catabolism. Inhibition of CWDEs production leads to the reduction in virulence of fungal pathogens. Several bacterial and fungal BCA's were known to inhibit the in vitro production of fungal pathogen. *T. harzianum* and *Bacillus megaterium* inhibited the production of cutin esterase and different pectinases which otherwise produced by *R. solani* (Bertagnolli, Soglio and Sinclair 1996). Inhibition of CWDEs of *Botrytis cineres* by *T. harzianum* on bean leaf surface leads to reduced disease incidence (Elad and Kapat 1999).

2.6.4.2 Pathogenesis Related Protein/Enzymes (Chitinases): Pathogenesis related proteins (PPR) have been described in plants infected with various types of potential pathogens: fungi, bacteria, viruses, and viroids (Sivan and Chet 1993). PPR is defined as the polypeptides with relatively low molecular weights (MW, 10,000 - 40,000) that accumulate extracellularly in infected plant tissue, exhibit high resistance to proteolytic degradation and generally possess the extreme isoelectric points (Sivan and Chet 1993). PPR have been studied in

several systems with respect to physical properties, relationship to the corresponding mRNAs and cDNAs, and gene activation following pathogen infection (Monte 2001).

Systemic acquired resistance (SAR) is a plant resistant response to a microbial challenge as a result of induced signal transduction pathway results in broad spectrum resistance (Chet et al 1997) and can be induced by chemicals like ABM (Harman et al 2004), by the use of plant-associated bacteria (Vyas and Vyas 1995) or by inoculation of avirulent strain of the same species (Eisendle et al 2004). Several studies have established the role of selected strains of nonpathogenic PGPR and PGPF in enhancing plant resistance (Tjamos et al 1992). An example of PGPF are *T.* spp., which have recently been shown to induce local and systemic defense responses in cucumber (Delgado-Jarana et al 2003) and other agricultural crops, such as cotton, tobacco, lettuce, and bell pepper (Mach and Zeilinger 2003). ISR, (Howell 2003) mediated by such nonpathogenic rhizospheric microorganisms, has been demonstrated in several plant species and shown to be effective against bacterial, viral, and fungal disease (Rey et al 2001). Plants have developed an arsenal of defense mechanisms to protect themselves against pathogen attacks. These include synthesis of PPR and phytoalexins, accumulation of reacting oxygen species (ROS), rapid alterations in cell walls and enhanced activity of various defense-related enzymes (Stacey and Keen 1999). Plant peroxidases (POs) have been implicated in a variety of defense-related processes, including the hypersensitive response, lignification, crosslinking of phenolics and glycoproteins, suberization and phytoalexin production (Dana et al 2001). Close relationships have been found between enhanced levels of POs and resistance of plants upon infection with pathogens (Walters et al 2005).

2.7 Biological Control in Practice

T. harzianum when applied either to seed or soil, remain in soil, multiplies and offers protection to the plant roots by improving the biological soil suppressiveness (Chet and Baker 1981; Jayaraj and Ramabadran 1999; Prasad et al 2002; Sharma et al 2002 and Yobo et al 2004). Spore suspension and dry spore powder were used to coat the seeds with fungal antagonists (Harman et al 1981;

Hadar et al 1984; Mukhopadhyay and Mukherjee 1991). Krishnamurthy (1987) suggested the use of carboxymethylcellulose (CMC), gumarabica, wheat flour paste, pidivyl and rice gruel as effective adhesives for pelleting of seeds with *T. harzianum.* Seed treatment with *T.* spp. significantly reduced the incidence of several diseases of crops (Pande 1985; Samiyappan, 1988; Kheri and Chandra 1991 and Dinakaran et al., 1995). Successful biological control of several diseases have been achieved by soil application of *T.* spp. in several hosts under glass house and field studies (Jacob 1989; Kousalya and Jeyarajan 1991; Rukmani and Mariappan 1994 and Sangeetha 2009). Hadar et al. (1979) found wheat-bran-grown cultures of *Trichoderma* when added to soil in green house plantings reduced damping off caused by *R. solani* in beans, tomatoes and egg plants. Costa et al (2015) evaluate *T. harzianum* isolates for biological control of white mold in common bean (*Phaseolus vulgaris*). Monte and Llobell (2003) had developed a biocontrol formulation, based on *Trichoderma* conidia, that was tested with satisfactory results against the main avocado root pathogens: *Phytophthora cinnamomi* and *Dematophora necatrix* in plantations maintained in the ecological conditions of Motril (Granada, Spain). *D. necatrix* is more resistant than *P. cinnamomi* to the action of *Trichoderma* biocontrol strains. However, being more difficult, the control by *Trichoderma* of root diseases caused by *D. necatrix* is effective (Freeman et al 1986; Monte and Llobell 2003).

MBCA’s currently used in agriculture can be classified in two types based on the mechanisms of action used to directly kill or inhibit the causal agents of plant disease and interact with the plant. The group defined as the “generalists”, includes species of *Bacillus, Pseudomonas*, Streptomyces, *Trichoderma*, Clonostachys, yeasts, etc., that are capable of controlling a large spectrum of taxonomically diverse pathogen hosts by using a variety of mechanisms of action. The group of the “specialists” includes biocontrol species of *Agrobacterium, Ampelomyces, Coniothyrium*, non-pathogenic *Fusaria,* atoxigenic *Aspergillus*, etc. that can counteract only one or few targeted pathogens (Benitez 2004; Hermosa 2012; Druzhinina 2011; Lorito 2010 and Mukherjee 2013).

According to the PAN, Pesticide Database (http://www.pesticideinfo.org), a pesticide is grouped according to: Use Type (i.e. fungicide) and Chemical Class

of the active substance/ingredient, whereby a microorganism is categorized as a "Microbial". A MBCA can be classified as Chemical Use Type of "Fungicide" or "Plant Growth Regulator" (Sheridan et al 2014).

2.8 Soil Borne Phytopathogens Used as Model Organism

Soil borne phypathogens considerably reduce crop yields worldwide and are difficult to control due to their "masked" occurrence in the heterogeneous soil environment. This hampers the efficacy of chemical - and microbiological control agents. Outbreaks of crop diseases are depend on the presence of pathogen and complexity of the soil environment in which pathogen propagules reside. Many pathogens form survival structures adapted to harsh conditions that can survive for years and thus remain present as a source of infection for a very long time (Bruehl 1987) however biotic and abiotic factors can contribute to irreversible loss of viability of pathogenic propagules. This dissertation focusses on application of *Trichoderma* in biological control of following phytopathogen:

2.8.1 *Fusarium* spp.: *Fusarium* is a large genus of soil fungi widely distributed in the world. Most species are harmless saprobes and are relatively abundant members of the soil microbial community. Nonetheless, some *Fusarium* species are economically important due to their devastating impact on crops. (CANNA 2018). In the beginning there was confusion on its taxonomy with more than 1,000 species, varieties and races. People started to realize that *Fusarium* causes serious diseases and became relevant to make a precise classification system. Nowadays, scientists still debate the classification system from nine to about 50 species, 0 to 29 varieties and 0 to 12 forms. Due to the confusion in identifying many *Fusarium* species, the classification is also based on the plant symptomatology. Therefore, most of the species have been divided in groups that are represented by the type of disease, like the *Fusarium* stem canker, the *Fusarium* foot rot and *Fusarium* wilt species groups (CANNA 2018). *Fusarium* stem canker group is a soil fungus caused by six species *(F. sulphureum, F. graminearum, F. lateritium, F. sambucinum, F. avenaceum and F. culmorum)*. *Fusarium* foot rot and root rot group is a soil fungus caused principally by *F. solani*. *Fusarium* wilt group is a vascular fungus caused by a xylem pathogen called *F. oxysporum* (CANNA 2018).

F. oxysporum has several specialized forms – known as formae specialis (f. sp.) - that infect a variety of hosts causing various diseases. *F. oxysporum* f. sp. *cubenses* is a well-defined sub-species that causes the Panama disease of *Musa spp*. This pathogen has caused the disappearance of the banana cultivar 'Gross Michael'. Afterwards, a new banana variety Cavendish was introducing due to its resistance to *Fusarium*. Like in banana, extensive breeding for resistance to *Fusarium* spp. is in progress in a wide range of crops, notably cereals, cotton, potato and tomato (CANNA 2018).

In brinjal *F. oxysporum* f. sp. *melongenae* causes wilting, discoloration of the stem, and yellow leaves that eventually drop. *F. solani* f. sp. *melongenae* causes stem canker diseases on brinjal (Vujanovic et al 1999). *Fusarial* dry rot (*Fusarium* spp.) develops most rapidly under conditions of high relative humidity (rh) and that the optimum temperature lies between 15-25°C. Relative humidities of at least 50% do not alter *Fusarium* dry rot development, but lower humidities retard disease development. Disease development will even continue at the coldest temperatures safe for potato storage. As potato dry rot cause by different *Fusarium* species. *F. solani* has been most intensively investigated. Theron and Holz (1989) found that South African isolates grew optimally between 25-35°C. High rh (90-98%) was found to be more favourable for growth than low rh (70-80%) (Weiss et al 1928; Moore 1945).

F. sambucinum can infect potatoes over a wide range of temperatures (2-30°C) and grows optimally at temperatures of 15-25°C (Weiss et al 1928), although it penetrates potato tubers more rapidly at 10-12°C under dry rather than moist conditions (Weiss et al 1928; Seppänen 1981). A temperature of 25°C and a high rh are reported to favour dry rot, but the fungus could also cause dry rot at lower temperatures (Goss 1921).

Tomato is one of economically the most important vegetable crops and are parasitized by a number of pathogens, including *F. oxysporum* f. sp. *lycopersici* (Sacc.), the causal agent of *Fusarium* wilt of tomato (Maja Ignjatov et al 2012). *Fusarium* that are the causal agents of tomato wilt cause root and basal stem deterioration and result in the wilting of vegetable plants. Browning of the vascular tissue is strong evidence of *Fusarium* wilt (Snyder and Hans 2003).

Some isolates of this fungus are pathogenic only to specific plant species (forma specialis) and there is also a large number of physiological races within each of these specialized forms, all of which make the selection for resistance to this pathogen more difficult (Armstrong and Armstrong 1981).

Rhizome rot of ginger is reported to be incited by species of *Pythium* and *Fusarium*. *Pythium* spp. thrives well in moist environment whereas *Fusarium* spp. in dry conditions. Both the pathogens incite the disease in field and storage as well. However, soil and storage environment influences the specific association of the pathogens with the disease. Haware and Joshi (1973) and Sharma and Dohroo (1990) reported *F. oxysporum* f. sp. *zingiberi* as the cause of rhizome rot in ginger. Fungal pathogens are associated with rhizome rot of ginger by Bhardwaj et al (1988) found *Pythium aphanidermatum* associated with wet rot, whereas *F. equiseti, F. solani, Cladosporium cladosporioides* and *Mucor hiemalis* were isolated from rhizomes showing dry rot symptoms. According to Rana (1992), yellows disease of ginger, prevalent in Himachal Pradesh is mainly caused by *F. oxysporum* f. sp. *zingiberi* and causes upto 100% damage under farmer's storage conditions (Gangrade 2006).

2.8.2 *Penicillium* spp.: *Penicillium* Rots: *P. digitatum* Sacc. and *P. italicum* Wehmer are the two most significant and widely reported postharvest pathogens in citrus. The major menace of these pathogens is due to their spores, which appear as fine powder and are airborne. The stem end is the most common entry site for the *Penicillium* species (Kaul and Lall 1975). In infected fruit, very profuse sporulation can be seen – fruit is completely covered by white mycelium followed by green and bluish spores of *P. digitatum* and *P. italicum* respectively. The typical terpenous odor spreads in the surrounding area where these fungi infect the fruit. It is quite possible that these fungi produce ethylene in sufficient quantities, resulting in the rapid senescence of adjacent fruits. Citrus volatiles and even the synthetic mixtures of ethanol, limonene, acetaldehyde, and CO_2 at certain concentrations stimulate the growth of *P. digitatum* (Eckert et al 1992).

Blue mold is more harmful because it spreads in the box and healthy fruits are directly attacked, regardless of injury. Blue mold is a nesting-type pathogen, meaning that it produces enzymes that soften the adjacent fruit and thus allow

fungus to enter. Green mold does not spread by nesting; thus, if a single fruit is affected it remains as such without contaminating adjacent fruit. However, spores lead to soiling of fruits and thus require repacking with a box change (Ladaniya 2008).

Green mold (*P. digitatum*) is quite common in India and grows rather slowly at lower temperatures. At higher temperatures (25–30°C) it grows very rapidly. Green mold infects fruit through wounds. Orchard and packinghouse sanitation is required to restrict sporulation of *Penicillium* on fruits in orchards and packinghouses to minimize decay losses. Benomyl is used as pre harvest spray in South Africa and many other citrus-growing countries to prevent *Penicillium* rots (Ladaniya 2008).

2.8.3 *Alternaria* spp.: *Alternaria* generally attacks the aerial parts of its host. In the leafy vegetables, symptoms of *Alternaria* infection typically start as a small, circular, dark spot. As the disease progresses, the circular spots may grow to 1⁄2 inch (1 cm) or more in diameter and are usually gray, gray-tan, or near black in color. Due to fluctuating environmental conditions, the pathogen does not have a uniform growth rate, thus spots develop in a target pattern of concentric rings. Where host leaves are large enough to allow unrestricted symptom development, the target spots are diagnostic for *Alternaria* as there are few other pathogens that cause this type of diagnostic expression (Laemmlen 2001).

Many *Alternaria* species also produce toxins that diffuse into host tissues ahead of the fungus. Therefore, it is not uncommon to see a yellow halo that fades into the healthy host tissues that surround the target spot (Laemmlen 2001). Dark, sunken lesions are usually the expression of *Alternaria* infections on roots, tubers, stems, and fruits. The fungus may sporulate in these cankers, causing a fine, black, velvety growth of fungus and spores to cover the affected area (Laemmlen 2001). The spores of *Alternaria* species are often beaked and always multicelled. The cells are divided longitudinally and transversely. Spores are dark and borne singly or in chains. Some *Alternaria* species and the diseases they cause on specific hosts include *A. dauci* (carrot leafblight); *A. radicina* (black rot of carrot); *A. brassicae* and *A. brassicicola* (leaf spot of crucifers); *A. solani* (tomato early blight and fruit rot); *A. brassicae* or *A. brassicicola*

(broccoli headrot); *A. tenuis* and *A. alternata* (fruit spot on peppers) (Laemmlen 2001).

2.8.4 *Acremonium* spp.: They are usually slow-growing and are initially compact and moist. Their hyphae are fine and hyaline, and produce mostly simple phialides. Their conidia are usually one-celled (i.e. ameroconidia), hyaline or pigmented, globose to cylindrical, and mostly aggregated in slimy heads at the apex of each phialide. The *Acremonium* genus (formerly known as *Cephalosporium*) depending on the different authors, the genus *Acremonium* currently contains between 20 to 200 recognised named species. The taxonomic fungal database administered by the IMA has 205 registered strains of which 197 are named species. This genus comprises moulds that lack any known sexual state or teleomorph forms and thus belongs to the Fungi imperfecti group. However, because it possesses structural characteristics similar to those of the Ascomycota group, it is often included in the Ascomycota phylum (*Acremonium* spp. 2018)**.**

Cephalosporium stripe is a vascular wilt disease of winter wheat and other small grains and grasses; common in the higher rainfall areas, it can occur in the intermediate- and low-rainfall wheat-growing areas on suitable conditions. Yield loss can be 100% when disease is severe in case of susceptible variety is grown (Wheat & Small Grains 2018). The pathogenicity of *Acremonium cucurbitacearum* to 31 cucurbits, 18 crop plant species, and 15 weed species was evaluated under greenhouse conditions (Armengol et al 2007).

2.8.5 *Exserohilum* spp.: *Turcicum* leaf blight of maize caused by *Helminthosporium turcicum* was first reported by Passerini (1876) in Perma, Italy. The causal agent of *turcicum* leaf blight on maize is normaly identified by its imperfect stage *E. turcicum*. The disease starts at first as small elliptical spots on the leaves, greyish green in color and water soaked lesions. The spots turn greenish with age and get bigger in size, finally attaining a spindle shape. Individual spots are usually ¾ inch wide and 2 to 3” long. Spores of the fungus develop abundantly on both sides of the spot. Heavily infected field present a scorched appearance (Chenulu and Hora 1962). Ullstrup (1966) described the symptoms of the disease in USA. The disease is recognised by long elliptical

grayish or tan lesions. When fully expanded, the spots may be 1½× 6 inches in size. These lesions appear first on the lower leaves and as the season progresses, the lesion number increases and all the leaves are covered. The plants look dead and grey (Reddy 2012).

2.8.6 *Rhizoctonia* spp.: *Rhizoctonia* is a soilborne fungus found naturally in outdoor soils from fields, landscapes, gardens, etc. It produces sclerotia, (tough, brownish-black structures) that allow it to survive in the soil or infected plant tissue for years. With a wide host range, it can cause a variety of diseases including stem-rot, root rot, damping-off in seedlings and aerial blight of leaves. It is often the cause of rot in cuttings, especially those under mist. The most common species that infects plants is *R. solani*. Although there are other species known to cause plant disease, not all species of *Rhizoctonia* are plant pathogens (Lance 2018). Leaves that come in contact with the soil can become infested with *Rhizoctonia*, causing aerial blight, can spread quickly if the leaves are wet, plants are too close together and/or the rh is high in the greenhouse. When *Rhizoctonia* causes root rot, it attacks roots that are in the upper layer of the growing medium. Seeds can be infected before germination or after emergence, leading to damping-off. Older plants' roots can also become infected, but *Rhizoctonia* is more virulent in young, tender plants. Roots diseased by *Rhizoctonia* turn brown and mushy, like other root rot pathogens. It is often introduced as a natural contaminant from mineral soils. (Lance 2018).

Sheath blight of rice disease is favoured by highly humid and warm temperatures. Symptoms of the disease usually appear when plants are in the late-tillering or early internode elongation stages. Small, water-soaked spots first appear on the leaf sheath within 3 inches above the water line. These spots enlarge rapidly under favorable conditions, are longer up and down the plant than they are wide, and have grayish-white centers with a tan-to brown margin. If unchecked, the disease progresses up the plant causing white-to-gray lesions on the leaves. Lesions may be as much as three-fourths of an inch long and involve the entire width of the leaf. As the disease spreads and lesions coalesce, areas of diseased rice 1.5 to 3 feet in diameter may develop throughout the rice canopy. Lodging may occur in severely diseased plants, particularly in taller varieties (Rangaswamy and Mahadevan 2005). Singh et al 2003 reported about

the growth of the mycelium on the affected parts of the plant under humid conditions and this aids in the spread of the disease to a considerable distance in the field through irrigation water. Sclerotia may be produced loosely externally on the sheaths or between the sheath and culm (Gangopadhyay and Chakrabarti 1982; Soujanya 2011).

2.8.7 *Sclerotium* spp.: *Sclerotium rolfsii* is a very common soil-borne fungus infecting a wide range of vegetable, ornamental and field crops. It is most active during warm, wet weather in tropical and subtropical regions. The fungus causes rots of the lower stem, roots and crown. It can also cause rot of fruit in contact with the soil.

Symptoms develop on plant parts in or near the soil. The most common symptom is a brown to black rot of the stem near the soil line. The stem becomes girdled and the plant wilts suddenly and dies. A coarse, white, cottony fungal growth, containing white, spherical resting bodies (sclerotia) covers the affected area. The sclerotia soon become light brown and resemble cabbage seed. Fruit symptoms usually develop where there has been contact with the soil. Decay may progress rapidly, eventually causing complete collapse. The fungus can survive for years as sclerotia in the soil or in host plant debris. Sclerotia spread with soil movement, infested plant material and contaminated equipment. Infection and disease development are favoured by warm, moist conditions. *Sclerotium* diseases often develop on crops produced under sub-optimal growing conditions, when plant vigour and quality has been compromised by other factors. Vegetable crops commonly affected include bean, beetroot, capsicum, carrot, cucurbits, sweet potato, potato and tomato (Queensland Government 2018).

2.8.8 *Macrophomina* spp.: *Macrophomina* crown rot (charcoal rot), is most severe when the infected plant is subject to stresses due to weather extremes, water stress, poor soil conditions, or heavy fruit loads. Confirmation of *Macrophomina* crown rot requires diagnostic procedures in a pathology lab. This is a soilborne fungus and persists in the soil in the form of small, black survival structures called microsclerotia. In California, research suggests that most of the isolates of *M. phaseolina* that infect strawberry have a narrow host range and

only infect strawberry. Symptoms of *Macrophomina* crown rot in strawberries consist of wilting of foliage, plant stunting, and drying and death of older leaves, while the youngest leaves in the center of the plant often remain green and alive. Symptoms usually first appear well after plants are established and after plants begin bearing fruit or are subjected to stress. Plants can eventually collapse and die completely. When internal tissues of plant crowns are examined, vascular and cortical tissues are dark to orange brown. Internal tissues of the main roots may also be discolored and show the same dark brown coloration (Koike et al 2018).

Dry Root Rot (*Macrophomina phaseoli, Fusarium* spp. and *Diplodia natalensis)* is characterized by moist decay of the bark in the early stages and a dry shredded condition of the bark with hard, dead wood underneath in later stages. The affected roots emit bad odour. Affected tree defoliates, and produces heavy crop of small-sized fruits (Citrus Diseases 2018).

CHAPTER III

MATERIALS AND METHODS

This study was carried out at the Department of Plant Pathology, NARC (RARS), Tarahara, Sunsari, Nepal. The laboratory techniques were in accordance with the standard methods described by Goldman and Green (2009).

3.1 Materials required

The materials, equipments, culture media and reagents used and their application in this study are systematically accounted in Appendix C.

3.2 Soil Sampling

Soil samples (50g each) were collected from different ecological habitat forest area of Sunsari district for the isolation of *Trichoderma* spp. The samples were collected from top 2-5cm depth of tree root rhizospheric soil. The composite soil samples were collected from a particular field in the polythene bag labeled separately. Samples were brought to laboratory and stored at 4°C until used.

3.3 Isolation of Microorganisms

One gram of the soil sample was taken and added to 1ml of sterilized distilled water to make a dilution of 10^{-1}. Five-fold serial dilutions of each soil samples were prepared in sterilized distilled water and 0.5 ml of each dilution viz., 10^{-2} to 10^{-4} was poured on to PDA, RBA and TSM (Elad 1982), contained in petri plate and spread uniformly by adopting spread plate method. The Petri plates were incubated at 28 ± 2°C for 96h. Morphologically different colonies appearing on the plates were purified in the Potato Dextrose Agar (PDA). The purified isolates were preserved at 4°C.

3.4 Phenotype Characters of the *Trichoderma* Isolates

The morphological and cultural characteristics of *Trichoderma* isolates were studied in two different media viz., PDA and TSM following the protocol of Samules et al (2002). Mycelial discs (4 mm) of young growing culture of

respective isolates of *Trichoderma* was inoculated in the periphery of the petri plates containing PDA and TSM media and incubated at 28 ± 2°C for one week. Colony radius was measured at 48, 72 and 96 h each growth rate experiment was repeated three times in triplicate and the results were averaged for each isolate. Additional characters include presence of pigments, green conidia, odor and colony appearance were also noted.

Morphological observations were recorded from cultures grown on PDA plates. The following characters were measured; Phialide width at the widest pint, phialide length, conidium length and width and presence of chlamydospores.

3.5 Isolation of Plant Pathogenic Fungi

The diseased plant samples showing typical wilt or disease symptoms were collected from the field and brought to the laboratory, washed repeatedly with tap water. Thereafter, small pieces of diseased portion were cut using sterilized blade for isolation. Care was taken that each cut piece should have some healthy parts as well. The pieces were then surface sterilized in mercuric chloride ($HgCl_2$) solution (1:1000) for 20- 30 sec. followed by thorough rinsing in sterilized distilled water, thrice. The surface sterilized pieces were then aseptically transferred separately to the plates containing PDA medium and then incubated at 27 ± 2°C. After 48-72 h of incubation, the growing mycelium from the margin of apparently distinct colonies was sub-cultured on fresh PDA slants. In this way, the culture of phytopathogenic fungi were isolated.

3.6 Purification and Identification of Isolates of Phytopathogenic Fungi

The cultures of phytopathogenic fungi were purified by frequent sub culturing by the hyphal tip method as described by Dhingra and Sinclair (1985). Isolated fungus was identified according to their morphological characters based on Tsuneo Watanabe (2010) and stored at 4°C until use.

3.7 Screening of Antagonistic *Trichoderma* spp. against Soil Borne Phytopathogen in vitro

Trichoderma isolates were evaluated for their potential to antagonize twelve economically important plant pathogens viz., *Fusarium oxysporum* f. sp. *cubense (Foc), Penicillium* spp., *Alternaria brassicicola, Fusarium oxysporum* f. sp. *melongenae, Fusarium solani* f. sp. *melongenae, Acremonium* spp., *Fusarium oxyporium* f. sp. *zingiberi, Exserohilum turcicum, Fusarium sambucinum, Rhizoctonia solani* Kuhn*, Rhizoctonia solani* (Musrard green), *Sclerotium rolfsii, Macrophomina phaseolina, Fusarium oxysporum* f. sp. *lycopersici* using three different tests in vitro.

3.7.1 Dual Culture (DC's) Methods

Antagonistic activity of T-isolates against fourteen phytopathogenic isolates was determined through dual culture technique (Dennis and Webster 1971c). Petri dishes (90 mm) containing PDA were inoculated with a 4 mm disc of 7 day old pure culture of antagonistic fungi and pathogen. One mycelial disc of each fungus was placed at opposite sides on PDA plates and incubated at 25 ± 2°C, 12 h/12 h darkness/light in completely randomized design (CRD). Control Petri plates were inoculated with phytopathogen and a sterile agar plug. A number of five replications were assigned to each treatment. The experiment was repeated once. Growth of each phytopathogen isolates in dual culture and in control (without antagonist) was measured after different intervals i.e. 1, 2, 3, 4 and 5 DAI and percent inhibition of radial growth was determined with following formula:

Growth inhibition (%) = (C-T)/C X 100%; where, (C = growth radius of pathogen in control plates, T = growth radius of pathogen in dual culture plates).

3.7.2 Non-volatile Organic Compound (NVOC'S) Methods

To determine the effects of non-volatile metabolites of *Trichoderma* on mycelial growth of the pathogen, three 4 mm discs of mycelial agar plugs (4 mm diameter) were removed from the edge of the young *Trichoderma* culture

and inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 25 ± 2°C on a rotary shaker set at 100 rpm for 25 days. The control conical flasks were inoculated with three 4 mm discs of sterile PDA medium. The culture was filtered through Whatman filter paper of pore size 20-25 µm for removing mycelia mats then further filtered through solvent filter filtration devices using membrane filter having pore size pore size, 0.22–0.45 µm. The cultural filtrate was added to molten PDA medium (at 40 ± 5°C) to obtain a final concentration of 100%, 50% and 25% (v/v). The medium was poured in Petri dishes at 15 ml per plate and inoculated with 4 mm mycelia plugs of the pathogens in the centre of the plates and incubated at 25 ± 2°C (Dennis and Webster 1971a). In control plate sterilized medium was used. There were three replicates for each treatment. Radial growths of the pathogens were recorded at regular interval i.e. at 3, 5 and 7 DAI. Percent inhibition of average mycelial growth in relation to growth of the control was calculated by the following formula:

Growth inhibition (%) = (C-T)/C X 100%; where, (C=growth radius of pathogen in control plates, T = growth radius of pathogen in dual culture plates).

3.7.3 Volatile Organic Compound (VOC'S) Methods

The effect of the *Trichoderma* isolates of released volatile metabolites was evaluated on the mycelial growth of the pathogen isolates by following the methods of Dennis and Webster (1971b). The 4 mm diameter mycelial disc of 7 days-old culture obtained from the margin of each the *Trichoderma* isolates was centrally placed on the PDA plates and incubated in 25 ± 2°C for 24 h. In control plates, a 4 mm diameter of sterile PDA medium was placed in the dish as done above. At the end of incubation period, the top of each plate replaced with bottom of the PDA plate inoculated centrally with 4 mm diameter mycelial plug of the pathogen isolates and held together with adhesive tape. The experimental design used was completely randomized with three replicates. Radial growths of the pathogens were recorded at regular interval i.e. at 5 and 7 DAI and percent inhibitions of average mycelial growth in relation to growth of their controls were calculated by the following formula:

Growth inhibition (%) = (C-T)/C X 100%; where, (C=growth radius of pathogen in control plates, T=growth radius of pathogen in dual culture plates).

3.8 Application of Antagonistic *Trichoderma* spp. Against Soil Borne Phytopathogen in vivo

3.8.1 Mass Multiplication of Fungal Inoculum

3.8.1.1 Mass Multiplication of *Sclerotium rolfsii*

S. rolfsii was multiplied on sand corn meal medium. The medium consisted of sand and corn powder at the ratio of 95: 5 w/w and moistened with distilled water to 30 percent (Abeygunwardhana and Wood 1975). Two hundred grams of medium was taken in 500 ml conical flasks and mixed with 60 ml of distilled water and it was sterilized in autoclave at 121°C and 1.1 kg/cm^2 pressure for 25 min. On cooling the pure culture of the pathogen was inoculated to flasks under aseptic condition and incubated at 27 ± 2°C for 30 days. The flasks were shaken frequently to get uniform growth of the pathogen. The mass culture thus obtained was used in further studies for in vivo trail (Siddanagoudar 2005).

3.8.1.2 Mass Multiplication of *Rhizoctonia solani*

Wheat bran (100 g) and distilled water (200 mL) were mixed well in a 500 mL conical flask, autoclaved at 121℃ for 40 min. Each flask was inoculated with 10 discs (0.4 cm diameter) containing the pathogen cultured for 4 days in Potato Sucrose Agar (PSA). These were cultured at 25°C under dark light condition. After 30 days, the wheat bran with hyphae and a small amount of sclerotia was collected, and naturally dried. Most of the wheat bran culture became very soft and fragile, and was grounded into powder by hands. A small part of culture is still very hard, which is grinded with mortar and pestle lightly (Xiao-Yu Zhang et al 2014).

3.8.1.3 Mass Multiplication of *Fusarium oxyporum* and *Fusarium solani*

The sorghum grains were soaked partially for one hour in warm water (40° to 45° C) and then spread on the clean blotting paper for air drying. About 150 g

moistened grains were filled in each 250 ml flask with 10 ml water and autoclaved at 15 lbs psi pressure. The mycelium bit of pure culture of *Fusarium oxysporum* f. sp. *melongenae* and *Fusarium solani* f. sp. *melongenae* were inoculated under aseptic condition in the flasks containing grains and incubated at 28 ± 2º C for 10 days. Meanwhile flasks were shaken to avoid clumping of grains and to facilitate early growth of the fungus. The grains turned whitish due to mycelial growth of the test fungus. These inoculum served as master culture and uses for further investigation.

3.8.1.4 Mass Multiplication of *Trichoderma* isolates

White sorghum grains were washed and soaked in water for 12 hrs, shade dried for 30 min and later were placed in 250 ml Erlen Meyer conical flasks. Fifty ml of two percent glucose solution was added to the grain, and the flasks were plugged with non- absorbant cotton and autoclaved at 15 psi pressure for 15 min. After cooling, the flasks were shaken vigorously to separate any grains tending to cling together. The flasks were autoclaved a second time after 24 hrs. Before re-autoclaving, the flasks were checked for moisture content, and if required additional 10 ml of water was added. After cooling, small fragments of agar-plate culture of the selected isolate of *Trichoderma* were placed in each flask and the medium was shaken to distribute the inoculum. The flasks were incubated for 15 days. The flasks were shaken on alternate days to break up the mycelium, to prevent matting of the sorghum grain, to improve the entry of oxygen into the grain culture and for uniform colonization of sorghum grains by the inoculated fungus (Whitehead 1957). These inoculums served as master culture and uses for further investigation.

3.8.1.5 Biopriming of Seeds with *Trichoderma* isolates

Seeds were pre-soaked in water for 12 hours. Prepared inoculum of T-isolates were mixed with pre-soaked seeds at the rate of 10 g per kg seed. These treated seeds were putted as a heap. Heap were covered with a moist jute sack to maintain high humidity and seeds were incubated under high humidity for about 48 h at room temperature. T-isolates adhered to the seeds were grown on the seed surface under moist condition to form a protective layer all around the seed coat. These seeds are sowed in the sick soil pot as described in this

section. The pots containing inoculum were frequently stirred, watered at room temperature to allow the colonization of fungus in the soil. Control were taken as brinjal variety plus pathogen inoculum on sterilized soil pot. Treatments were taken as rice variety plus T-isolates plus pathogen inoculum on sick soil pot. These bioprimed seeds were subjected to vivo trail and data analysis were done as described in this section.

3.8.1.6 Procedure of Biopriming of Rice Seed

There are various methods such as Talc based powder formulation, wheat bran method etc, exist for biopriming of rice seed. In this investigation experiment was carried out by using modified methods of wheat bran method to wheat flour method:

Wheat flour: water 1:1 (w/v) was autoclaved in 500 ml flask for one and half hour at 121°C at 1.5kg/cm2 pressure for 20 minutes. Then, 4mm plug of T.-isolates was inoculated after cooling of the medium and incubated at 22 ± 2°C for 5 days. After T-isolates mycelial grown over the surface of liquid medium of wheat bran, flask were stirred and allowed mixed T-isolates and wheat-bran liquid becoming homogenious mixtures of both masses. Rice variety named Chaite-2 and Hardinath were chosen for vivo trail to test pathogenicity of *S. rolfsii a*nd *R. solani* Kuhn after application of T-isolates. These, rice variety were weighed and putted into the beaker separately. Now prepared T-isolates plus wheat liquid medium solution were mixed with rice into the beaker and stirred well i.e. Seed treatment with T-isolates was done at the rate of 25ml of mixture/100gms of rice. Control were taken as rice variety sowed on sterilized soil tray plus pathogen inoculum whereas treatments were taken as rice variety plus T-isolates on sick soil tray. These prepared trays are subjected to vivo trail and from vivo trail data, rice diseases were recoded and analysed on further investigation.

3.9 Preparation of Sick Soil

The preparation of sick soil for different phytopathogens are as follows:

3.9.1 Preaparation of sick soil for *Fusarium oxyporum* and *F. solani*

The inoculum was prepared by growing culture of *Fusarium oxysporum* f. sp. *melongenae* and *Fusarium solani* f. sp. *melongenae* in a modified liquid broth Czapek's Dox (CD) (Esposito and Fletcher, 1961) on a rotary shaker with indirect fluorescent light at room temperature (22º C). After 15 days, the contents of the flask were filtered through eight layers of sterile cheesecloth. One hundred milliliters of the filtrate, which was predominantly microcondia, was mixed with 4 L of a twice-autoclaved sand / sorghum grains (4:1, v/v) mixture. The mixture was incubated for 8 weeks at room temperature to allow the fungus to colonize the medium extensively. To verify that *Fusarium oxysporum* f. sp. *melongenae* and *Fusarium solani* f. sp. *melongenae* had been established successfully. The infested soil was incorporated into the top 24.5 X 26.2 cm of 90 polythene pots. Plastic pots were planted with seeds of the wilt tolerant brinjal variety. Moderate percentage of wilt had occurred in all infested plastic pots while no wilt occurred in uninfested ten plastic pots. The remaining aboveground plant material was cut off at the soil line and removed. All plastic pots were provided to light irrigation and avoided the direct sunlight.

3.9.2 Preaparation of sick soil for *Rhizoctonia solani* and *Sclerotium rolfsii*

Mass multiplication of *S. rolfsii* and *R. solani* was carried out in Potato Dextrose broth at room temperature for 3 weeks (Ordentlich et al 1988) and then the numbers of sclerotia produced were used for the preparation sick trays. This experiment was carried out in trays and pentaplates. All trays were first disinfected with 5 % $CuSO_4$ solution. All soils in trays are then filled with 250 gms sclerotia of *Sclerotium* and *Rhizoctonia*. The trays containing inoculum were incubated for 15 days at room temperature, frequently stirred and watered for colonization of fungus in the soil. Then local varieties of rice named chaite-2 and Hardinath were inoculated on sick soil over trays while only Hardinath is choosen for *Rhizoctonia solani* infection. These two varieties of rice were subjected to bio-priming of *Trichoderma* isolates and then allowed to dry overnight. Then each trays were labelled as per *Trichoderma* isolates (treatment no.), replication no. and phytopathogen and then bio-primed rice were sowed as per label on the trays and watered on regular interval of time.

Then, Germination Percentage (G%), Diseases Incident (DI) and Percent Disesases Severity (DSI) were estimated on 9th, 18th, 27th, 36th and 45th days (Botha 2007) for (SR modified) and for *R. solani* (IRRI 2002).

3.10 Thermostability of the Culture Filtrates of *R. solani* and *S. rolfsii*

The thirty days+ old culture filtrate of respective bioagents was subjected to fractionation by centrifugation at high speed (15,000 rpm for 10 minutes). The top 50 percent of filtrate was decanted as top filtrate and remaining 50 per cent of filtrate was considered as bottom filtrate. The culture filtrate of *T.-isolates* which were grown on potato dextrose broth were taken for this study. After separation of the two fractions they were exposed to 121°C for 15 minutes. The filtrates subjected to heat treatment were evaluated against *R. solani* and *S. rolfsii* by 'Poison food technique'.

For this, 50 ml of respective culture filtrate was mixed with 50 ml of sterilized, molten potato dextrose agar and the contents were mixed thoroughly. The mixture was poured in sterile Petri plate. In control treatment sterile distilled water was substituted in the place of culture filtrate. The plates were allowed to cool and they were inoculated with a sclerotial body at the centre of the plate. The plates were incubated at room temparature (27 ± 2°C) for seven days. The colony diameter of *R. solani* and *S. rolfsii* was recorded separately for each treatment and data was analyzed statically (Siddanagoudar 2005).

3.11 Assessment of Biocontrol Efficiency of T-isolates against Test Phytopathogen in vivo

3.11.1 Standard Germination Test (%)

Twenty-five seeds of each genotype in three replicates were placed in between sufficient moistened rolled towel papers and kept at 25°C in room enviroment. The final count was taken on 9th day and only normal seedlings were considered for percent germination according to the rules of International Seed Testing Association (ISTA 1999 and 2002).

Germination percentage (G%) = (G/TS) X 100%; where, G = Numbers of seed germinated, TS = Total numbers of seed.

3.11.2 Assessment of Disease Incidence:

The disease was observed on vivo trail of experimental plant and incidence of each disease was calculated by the following formula:

Diseases incidence percent= (Numbers of plant infected/Numbers of plant observed) X 100%

Each treatment was divided into five equal replications to assess the disease incidence and severity of each disease. The incidence and severity of disease were recorded at 10 days interval for brinjal and 9 days for rice, commencing from the appearance of each disease on plants in field upto maturity i.e. 45 days trail.

3.11.3 Assessment of Disease Severity

Assessment of the Seedling blight disease symptom of *S. rolfsii* on rice was made with the help of the descriptive scale described by Botha (2007), using 0-5 scale rating. Where, rating description. Assessments commenced after 9 days. The disease rating scale was as follows;

0 = no visible symptoms on leaves; 1 = leaves showing small symptoms (spots, browning of leaves and curling up); 2 = Some leaves yellow, large brown lesion visible or lesion visible underneath the straw, moving downward on the stem; 3 = infected leaves and petioles are brown, mycelium growth visible in some cases, lesions moved further down stems, leaves start to die; 4 = 90% of leaves are dead, curled up or have been shed. Leaves and apical meristem wilted and lodging occurs. Mycelial growth visible; 5 = all leaves infected, dead or shed, mycelial growth and sclerotia visible whole plant dead in some cases.

Wilting was assessed as follows;

0 = no wilting visible; 1 = only infected leaves show signs of wilting; 2 = top part of plants wilted (apical meristem and upper leaves); 3 = infected leaves and petioles wilted, wilting of trifoliate leaves underneath straw visible. Lesion

moved downwards towards the middle of plant stem; 4 = main stem and primary leaves wilted; 5 = whole plant and main stem wilted, leaves dead and shed;

Assessment of the Sheath blight disease symptom of *R. solani* on rice was made with the help of the descriptive scale developed by Patro and Madhuri, (2014) where

Scoring Scale	**Plants parts affected**
0	No infection
1	Lesions limited to lower 20% of the plant height
3	Lesions limited to lower 20 to 30% of the plant height
5	Lesions limited to lower 31 to 45% of the plant height
7	Lesions limited to lower 46 to 65% of the plant height
9	Lesions more than 65% of the plant height

Similarly, assessment of damping off disease symptoms of *R. solani* was made with the help of rating scale mentioned by Jiang and Tam 2016.

0 = healthy, exhibiting no lesions on the hypocotyl; 1 = minor discoloration of the hypocotyl; 2 = discoloration plus small necrotic lesions (<1mm) on the hypocotyl or primary root; 3 = discoloration plus large necrotic lesions (>1mm) on the hypocotyl or primary root; 4 = collapsed hypocotyl exhibiting wilted leaves or dead seedlings.

Assessment of the *Fusarium* wilting (*Fusarium oxysporum* f. sp. *melongenae*) disease symptom of brinjal diseases was made with the help of Winstead and Kelman (1952) and Zakir Hussain et al (2005), with some modification.

The modified rating scale was taken as: 0 - Highly Resistant (HR) with no wilt symptom; 1- Resistant (R), with 1 - 10% wilted plants; 2 - Moderately Resistant (MR) with 11 -20% wilted plants; 3 - Moderately Susceptible (MS), with 21-30% wilted plants; 4 - Susceptible (S) with 31- 40% wilted plants, and, 5 - Highly Susceptible (HS) with > 40% wilted plants.

Assessment of the *Fusarium* wilting (*Fusarium solani* f. sp. *melongenae*) disease symptom of brinjal diseases was made with the help of CIAT, (1987)

Scale	Root parts affected
1	No visible disease
3	Light discolouration either without necrotic lesions or with approximately 10% of the hypocotyl and root tissues covered with lesions.
5	Approximately 25% of the hypocotyl and root tissues covered with lesions but tissues remain firm with deterioration of the root system. Heavy dis-coloration symptoms may be evident.
7	Approximately 50% of the hypocotyls and root tissues covered with lesions combined with considerable softening, rotting, and reduction of root system.
9	Approximately 75% or more of the hypocotyl and root tissues affected with advanced stages of rotting, combined with severe reduction in the root system.

Computation of disease severity

Disease severity or Infection index (DSI/DII) = {Sum of all disease rating / (Total no. of rating x maximum diseases grade)} X 100%

DI and DSI percent increase or decrease over control (PDIC) was calculated using the formula:

PDIC = {(Disease in control plot - Disease in treatment plot)/ Disease in control plot} X 100%

CHAPTER IV

RESULTS

In the present investigation, 7 isolates of *Trichoderma* spp., *T. harzianum* viz: Tar (Isolated from Tarahara); T22, TH3 and T-gel (Strains isolated by NARC); I2 and I3 (Standard strains obtained from NARC) and Dar (Isolated from Dharan) and 1 isolate of *T. viride* viz: (TV isolated from Tarahara) were screened against 14 isolated phytopathogenic fungi (PF) in vitro while only 5 isolates were subjected into vivo trail. These 14 isolated PF were isolated from diseases plant parts by tissue culture techniques and identified by microscopy on LPCB staining and *Fusarium* spp. were identified with the help of Daniela et al (2013).

Table 1: Diseased plants and their respective phytopathogen

S.N.	Plants					Isolated phytopathogen	
	CN	SN	PPS	LA	RCN	Organisms	CDN
1	Banana	S1	Leaf	L1	BDD	Foc	Banana *Fusarium* wilt disease
2	Chilly	S2	Leaf/fruit	L2	CDL	Ps	*Penicillium* rot
3	Cabbage	S3	Leaves	L3	CDT	Ab	*Alternaria* Leaf Spot
4	Brinjal	S4	Leaves	L3	FO	Fom	*Fusarium* wilt
5	Brinjal	S5	Leaves	L3	FS	Fsm	*Fusarium* wilt/Root rot
6	BC	S6	Rhizome	L4	IK1	As	Rhizome rot disease
7	Ginger	S7	Rhizome	L4	IK2	Foz	Rhizome rot disease
8	Maize	S8	Leaf	L3	MB	Et	Northern corn leaf blight
9	Potato	S9	Tuber	L3	PDT	Fs	Dry Rot of Potatoes
10	Rice	S10	Stem	L5	RS	Rs	Sheath blight of rice
11	BLM	S11	IRSP	L3	RSR	Rsr	RSD
12	Rice	S12	DSDP	L6	SR	SR	Seedling blight diseases of rice
13	Lemon	S13	Stem	L3	TCD	Mp	Gummosis disease
14	Tomato	S14	SAL	L7	TVD	Fol	Tomato *Fusarium* Wilt

Where, CN = Common Name; SN = Scientific Name; BC = Black cardamom; DSDP = Direct soil dilution plating on PDA; SAL = Stem and Leaf; S1 = *Musa acuminate,* S2 = *Capsicum annuum*, S3 = *Brassica oleracea*, S4 = S5 = *Solanum melongena*, S6 = *Amomum subulatum*, S7 = *Zingiber officinale*, S8 = *Zea mays*, S9 = *Solanum tuberosum*, S10 = S12 = *Oryza sativa*, S11 = *Brassica juncea*, S13 = *Citrus limon*, S14 = *Solanum lycopersicum*; IRSP = Infected root-shoot; portions PPS = Plant Parts Selected. LA = Location Area; L1 = Dharan, Sunsari; L2 = Siraha, Lahan; L3 = Tarahara, Sunsari L4 = Kulbang, Ilam; L5 = Itahari, Sunsari; L6 = Ghapa rice superzones; L7 = Tamor, Dhankuta. RC = Research Coding Name. CDN = Common Disease Name. GS = Gummosis's stem. RSD (*R. solani* disease for mustard green) = Damping-off, Wirestem, Brown Girdling Root Rot; Foc = *Fusarium oxysporum* f. sp. *cubense*, PS = *Penicillium* spp., Ab = *Alternaria brassicicola,* Fom = *Fusarium oxysporum* f. sp. *melongenae,* Fsm = *Fusarium solani* f. sp. *melongenae*, As = *Acremonium* spp., Foz = *Fusarium oxyporium* f. sp. *zingiberi,* Et = *Exserohilum turcicum,* Fs = *Fusarium sambucunum,* Rs = *Rhizoctonia solani* Kuhn, Rsr = *Rhizoctonia solani,* SR = *Sclerotium rolfsii,* Mp = *Macrophomina phaseolina,* Fol = *Fusarium oxysporum* f. sp. *lycopersici*

4.1 In Vitro Antagonism

4.1.1 Antagonistic Efficacy of T- isolates against Phytopathogen in Dual Culture Method

A) Comparative study of T-isolates vs *F. oxysporum* f. sp. *cubense* (BDD).

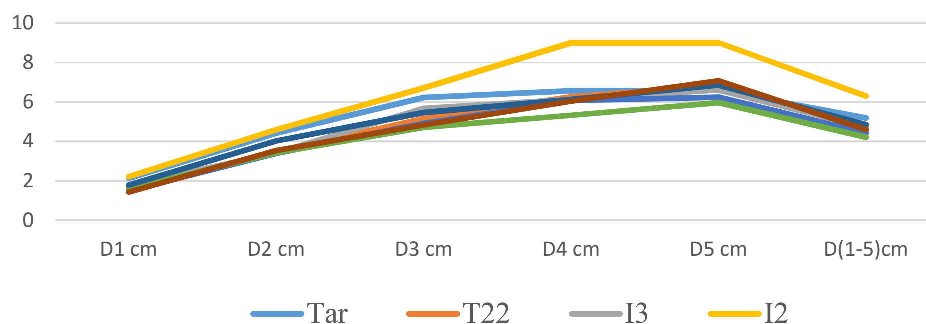


Figure 1: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Fusarium oxysporum* f. sp. *cubense*

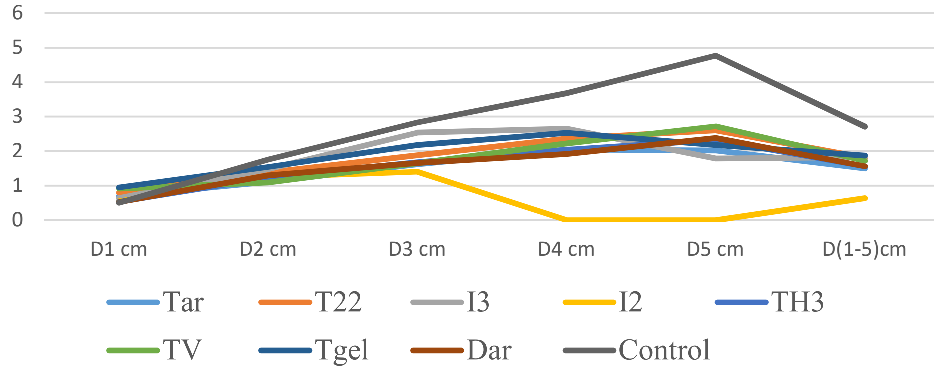


Figure 2: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Fusarium oxysporum* f. sp. *cubense*

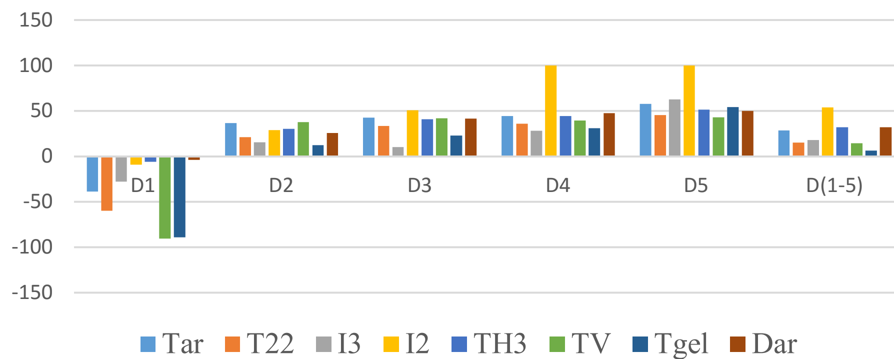


Figure 3: Growth inhibition percentage of *Tricoderma* isolates vs *Fusarium oxysporum* f. sp. *cubense*

In this study, out of chosen 8 isolates I2 has been found to be most potent isolates as it has very low negative GI% on day1 than others and become positive by day2 to day5 while only it can suppress completely BDD by days 4. Next better isolates found were local isolates coding Tar excluding day1 performance. The overall position of potent isolates in decreasing order of strength were found to be I2>Tar≥Dar≥TH3>TV>T22>Dar>T-gel.

B) Comparative study of T-isolates vs *Penicillium* spp. (CDL)

Here, out of chosen eight isolates, antagonistic potential of each isolates vary with time as it shows the most potent isolates were T-gel on day1, T22 on day 2 and 3, Tar on day 4 and 5 while overall days 1 to 5 average performance goes to again T-gel. As days increases interaction between

them is more crucial than before i.e. days 4 and 5 have greater importance than days 1, 2 and 3, the result clearly shows that the most potent isolates were local isolates Tar and weakest one is another local isolates Dar.

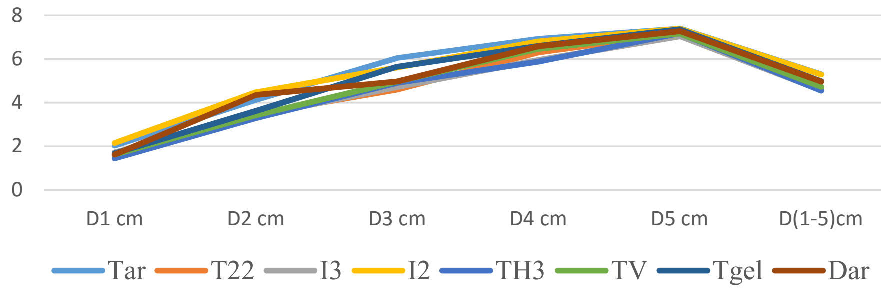


Figure 4: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Penicillium* spp.

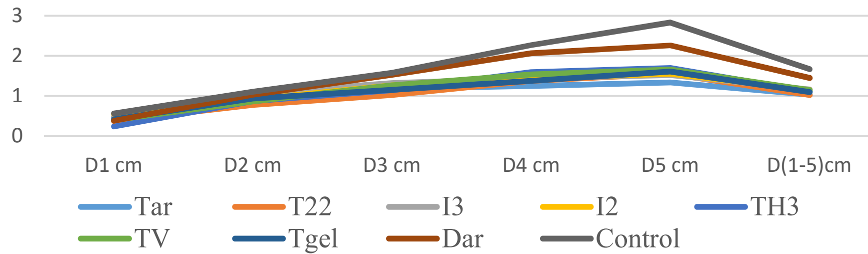


Figure 5: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Penicillium* spp.

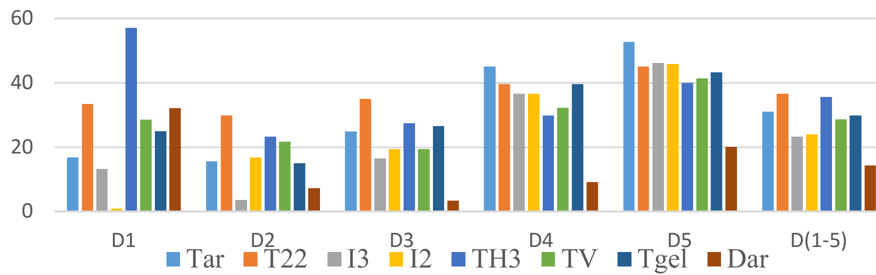


Figure 6: Growth inhibition percentage of *Trichoderma* isolates vs *Penicillium* spp.

C) Comparative study of T-isolates vs *Alternaria brassicicola*.

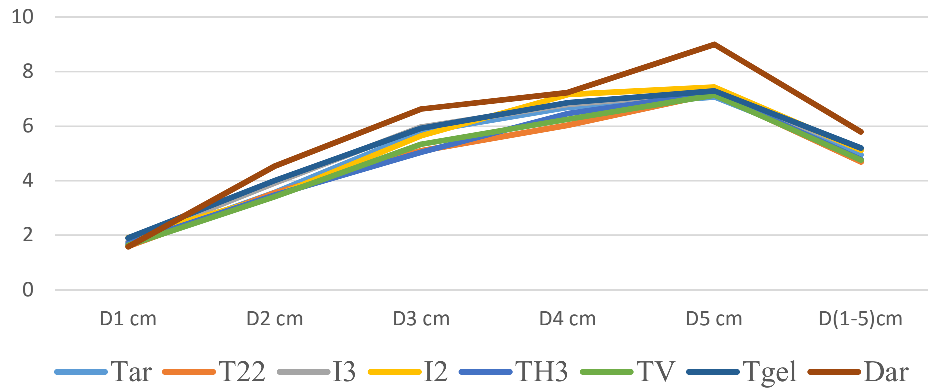


Figure 7: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Alternaria brassisicola*

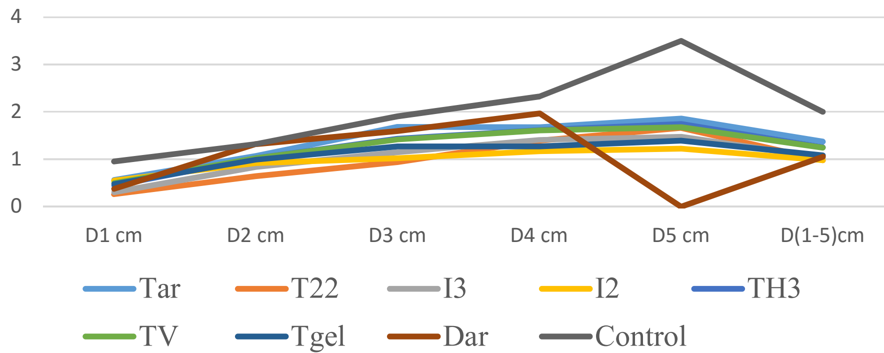


Figure 8: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Alternaria brassisicola*

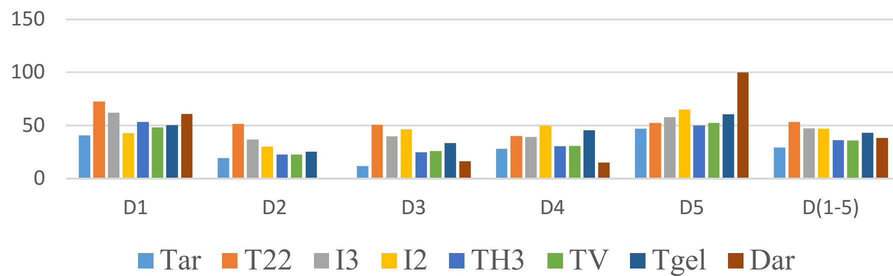


Figure 9: Growth inhibition percentage of *Trichoderma* isolates vs *Alternaria brassisicola*

Here, among eight isolates, the most potent isolates found were T22 on day 1, 2, 3 and day (1-5) but loses first position by day 4 to I2 and I2 again loses its first position to Dar by day5. This results found that most potent isolates against CDT were found to be Dar as it completely suppressed growth (100% GI) by day 5 and overlapping over the growth on CDT in vitro. Brief analysis of this study shows following order of strength in decreasing order for bio-controlling *Alternaria* Leaf Spot of Cabbage: Dar>T22>I2>I3>TH3>TV>T-gel>Tar.

D) Comparative study of T-isolates vs *F. oxysporum* f. sp. *melongenae.*

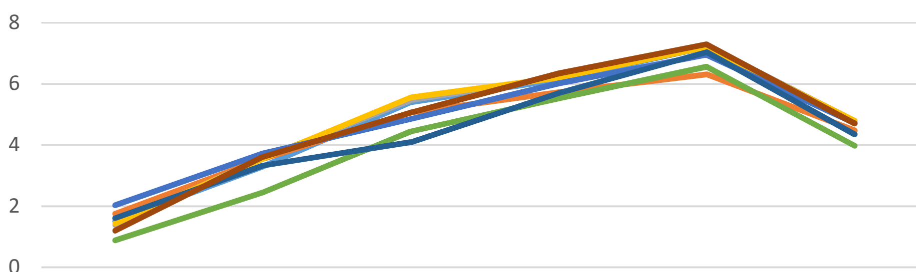


Figure 10: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *melongenae*

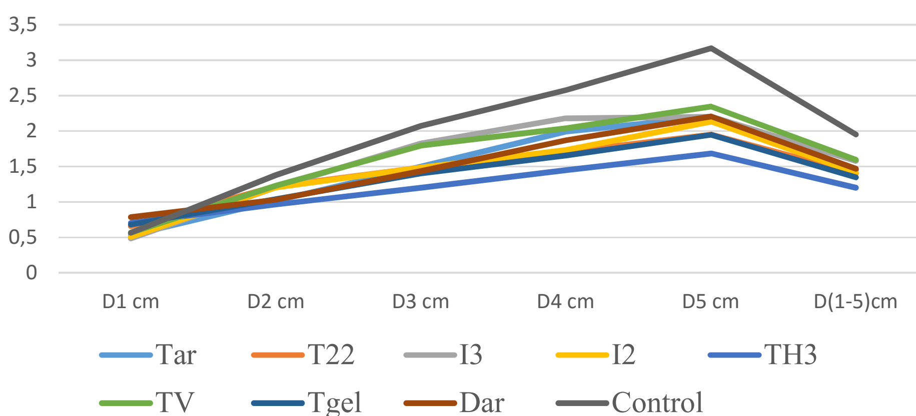


Figure 11: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *melongenae*

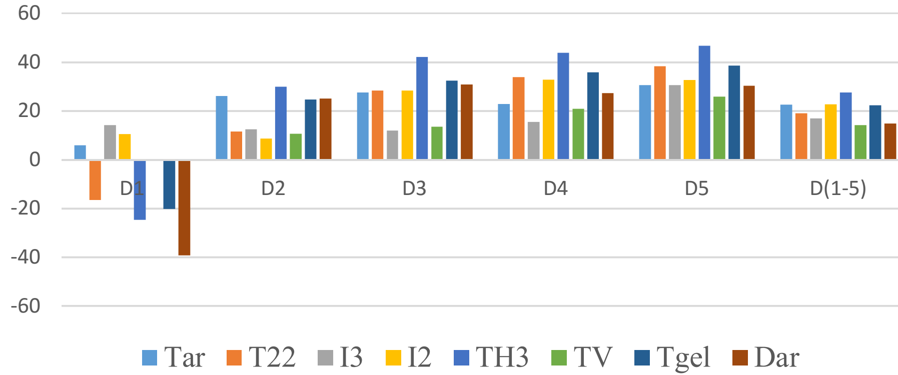


Figure 12: Growth inhibition percentage of *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *melongenae*

From result data, it is showed that our NARC'S isolates coding TH3 show maximum antagonism in DC and has positive biocontrol efficiency from day 2, and it continuously rank same position to day 5. Next better isolates should be T22 and T-gel in equal range on viewing day 5 performance alone but T-gel exceed T22 capacity of antagonism on day 2, 3, 4 and in overall day average performance thus it is concluded that T-gel is rank 2nd while Dar to 3rd and others performance are very little difference from each other.

E) Comparative study of T-isolates vs *Fusarium solani* f. sp. *melongenae.*

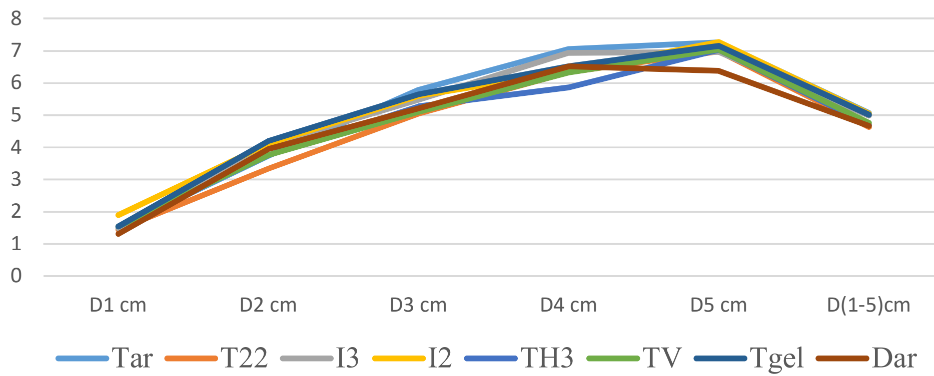


Figure 13: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Fusarium solani* f. sp. *melongenae*

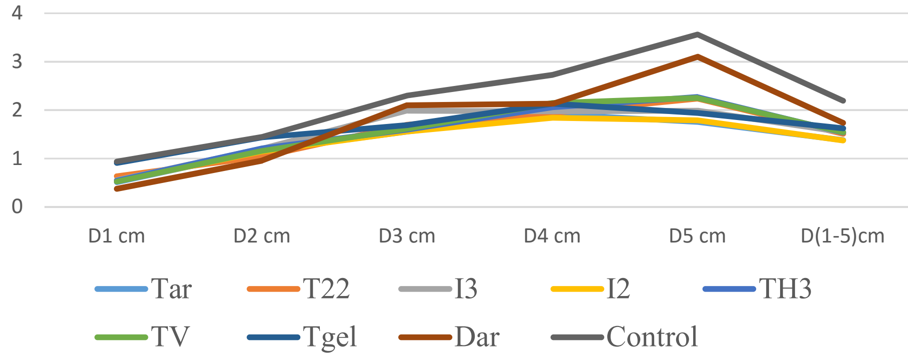


Figure 14: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Fusarium solani* f. sp. *melongenae*

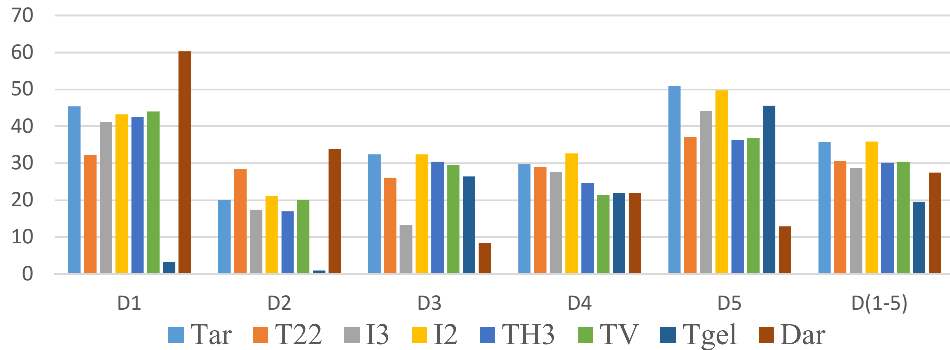


Figure 15: Growth inhibition percentage of *Trichoderma* isolates vs *Fusarium solani* f. sp. *melongenae*

In present result, out of two local isolates one (Tar) rank 1st and another (Dar) rank last. Dar shows inconsistency in its day to day performance i.e. highest antagonism on day 1 and 2 and rank lowest by day 3 and 5. Tar shows consistency continuously and top performer on day 5. DC's method for T – isolates vs FS shows following order in decreasing antagonistic efficiency: Tar>I2>T-gel>I3>T22>TV>TH3>Dar.

F) Comparative growth study of T-isolates vs *Acremonium* spp.

Comparative studies of T-isolates vs IK1 in DC gives the rough ideaof choosing T-isolates for biocontrolling the Cardamom rhizome rot diseases. On viewing on day 4 and 5 GI%, Tar I2 and I3 has similar range potency, if adding to D(1-5) Values, order should be in Tar>I2>I3>T-gel>Dar>TV>TH3>T22.

Results showed mycoparasitism capicity of local isolates is best among all while another local isolates Dar only had positive GI% on day 1 showing better lag phase than others.

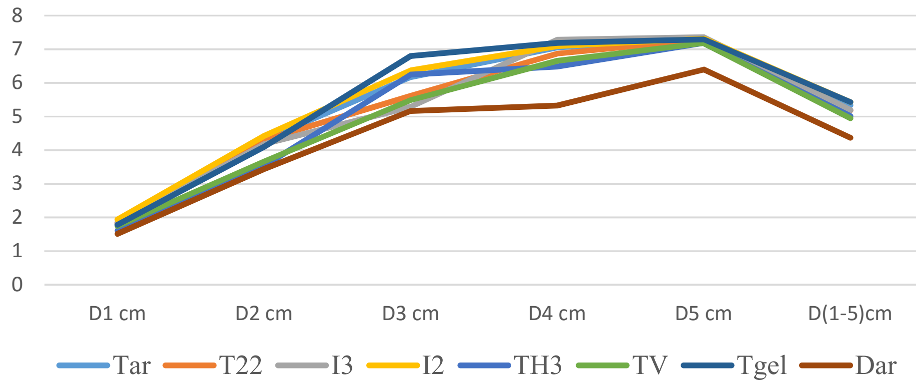


Figure 16: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Acremonium* spp.

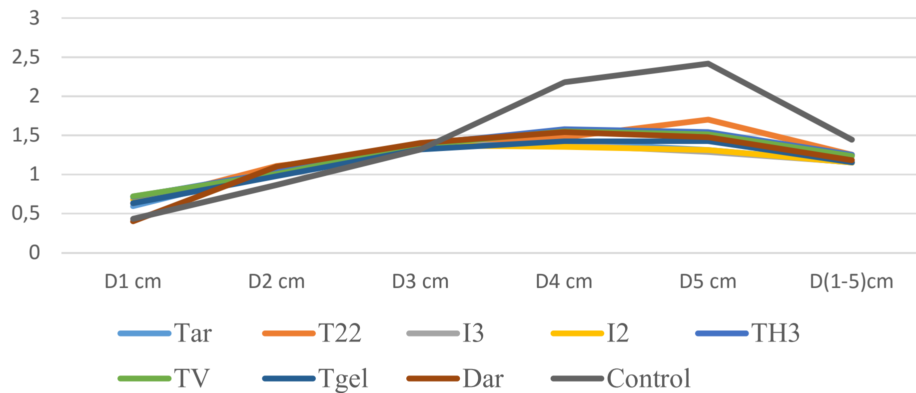


Figure 17: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Acremonium* spp.

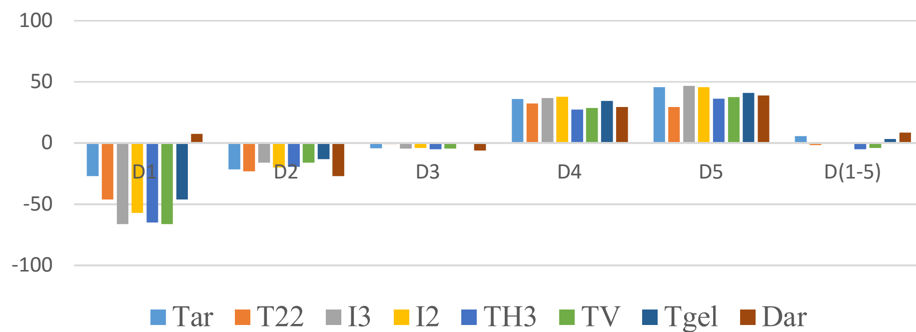


Figure 18: Growth inhibition percentage of *Trichoderma* isolates vs *Acremonium* spp.

G) Comparative study of T-isolates vs *Fusarium oxyporum* f. sp. *zingiberi*

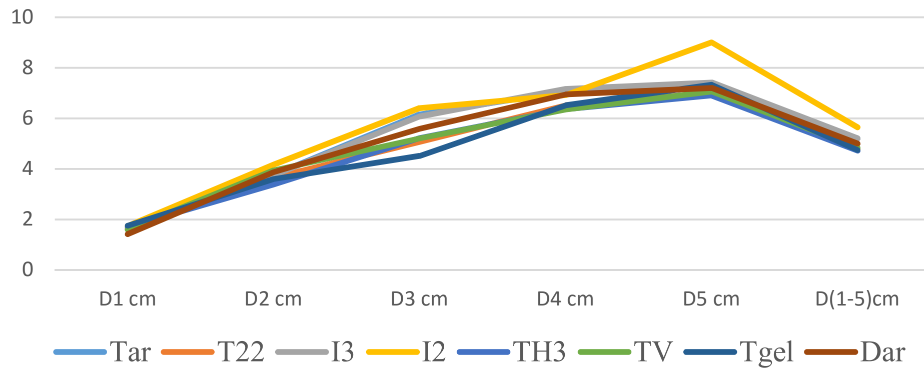


Figure 19: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *zingiberi*

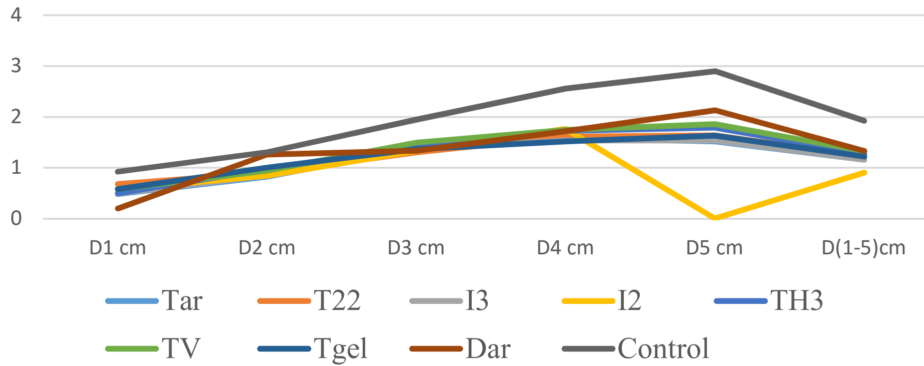


Figure 20: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *zingiberi*

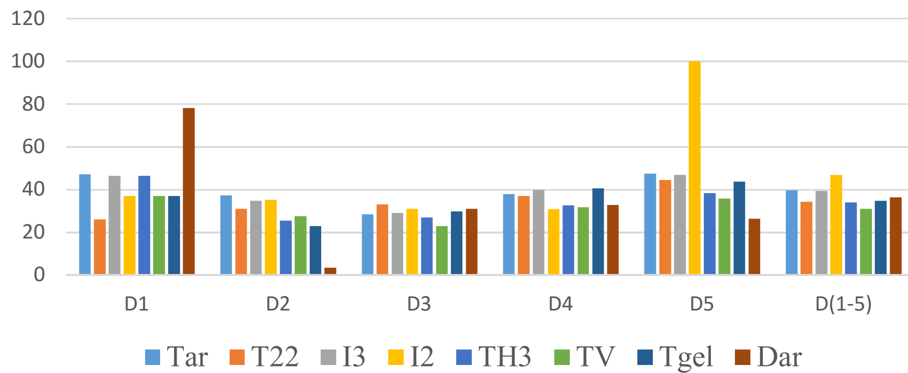


Figure 21: Growth inhibition of *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *zingiberi*

On this study, it is found that I2 got rank 1 due to 100% GI by day 5, Tar and I3 has approximately same potency thus getting same position of rank 2nd. The order of sequence in decreasing order of bio-control potential should be as "I2>Tar≥I3>T22>T-gel>TH3>TV>Dar". In this study Dar showed best lag stage performer indicated by day 1 growth measurement nevertheless, on further days maybe due to its gene and physiological activities, its potency remain lag behind than others thus positioned last in ranking though it is also local isolates.

H) Comparative study of T-isolates vs *Exsirohilum turcicum*

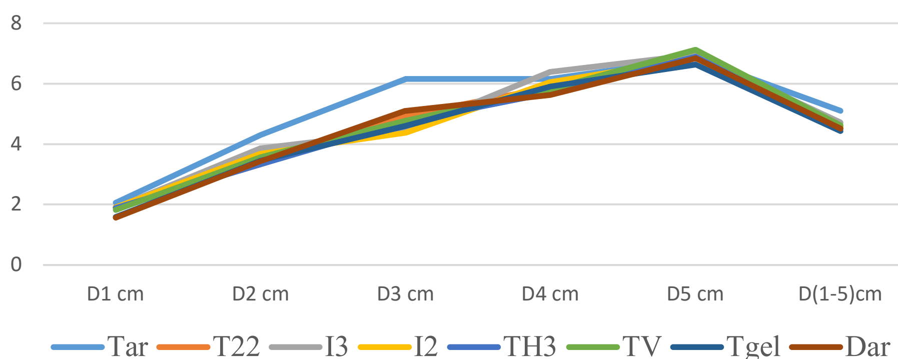


Figure 22: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Exserohilum turcicum*

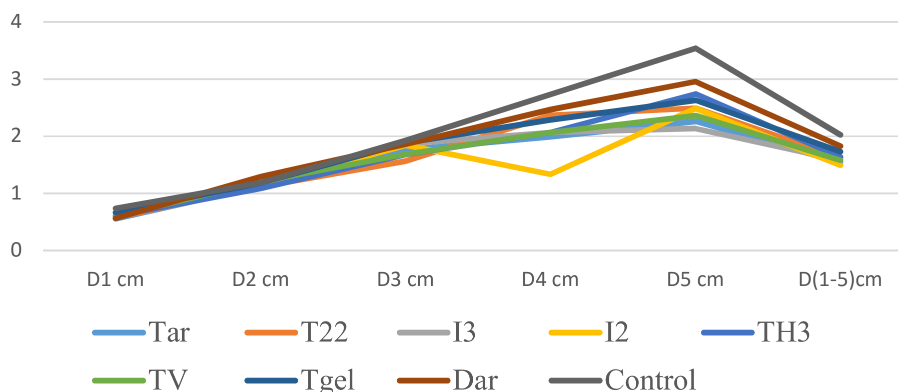


Figure 23: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Exserohilum turcicum*

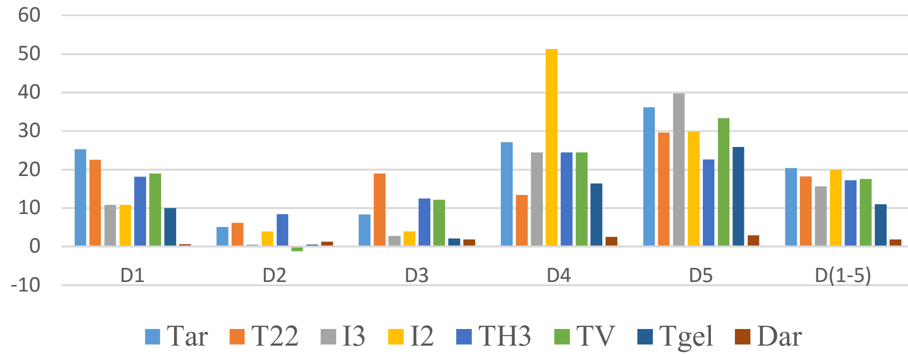


Figure 24: Growth inhibition percentage of *Trichoderma* isolates vs *Exserohilum turcicum*

From obtained result the mixed result of antagonistic potency of each isolates had been found. According to day 5 the order is I3>Tar>TV>I2>T22>T-gel>TH3>Dar. Similarly for day 4, order is I3>Tar>I2≥TH3≥TV>T-gel>T22>Dar and for D(1-5) order is Tar>I2>T22>TV>TH3>I3>T-gel>Dar. Thus final order should be in Tar≥I2≥I3>TV≥T22>TH3>T-gel>Dar.

I) Comparative study of T-isolates vs *Fusarium sambucinum.*

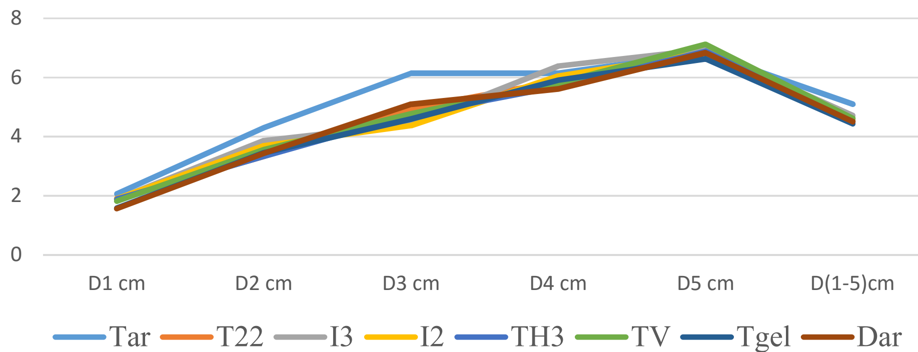


Figure 25: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Fusarium sambucinum*

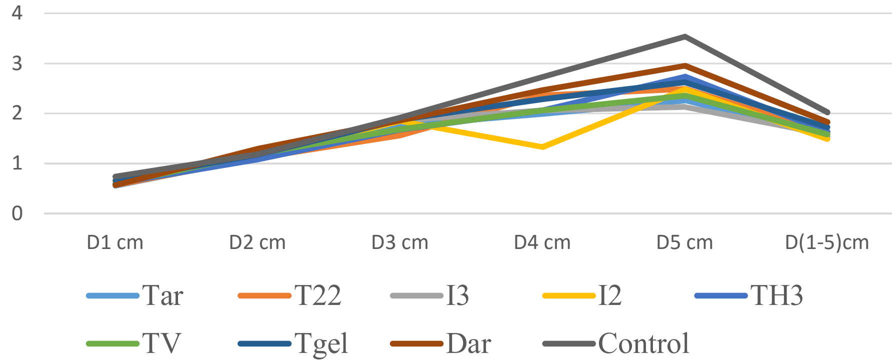


Figure 26: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Fusarium sambucinum*

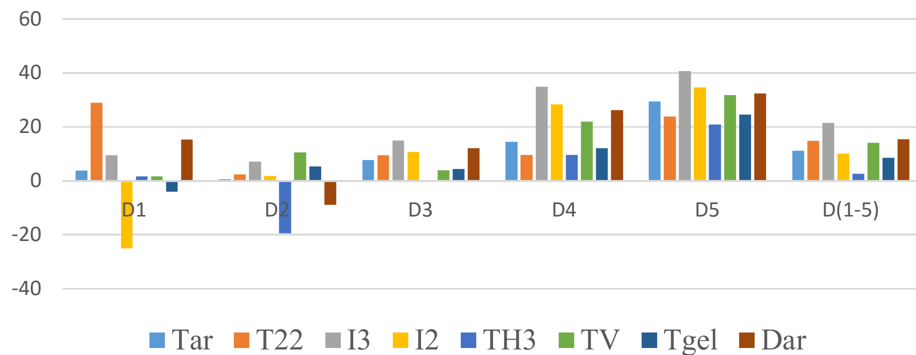


Figure 27: Growth inhibition percentage of T-isolates vs PDT.

On DC's experiment, it is found that I3 is most potent isolate and has consistency result. The order of sequence is I3>I2>Dar>TV>Tar>T22>T-gel>TH3.

J) Comparative study of T-isolates vs *Rhizoctonia solani* Kuhn

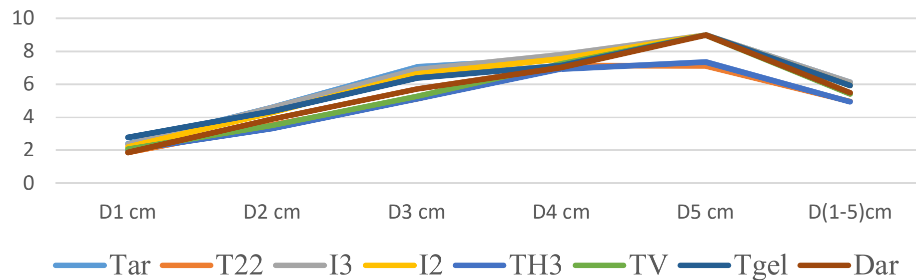


Figure 28: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Rhizoctonia solani* Kuhn

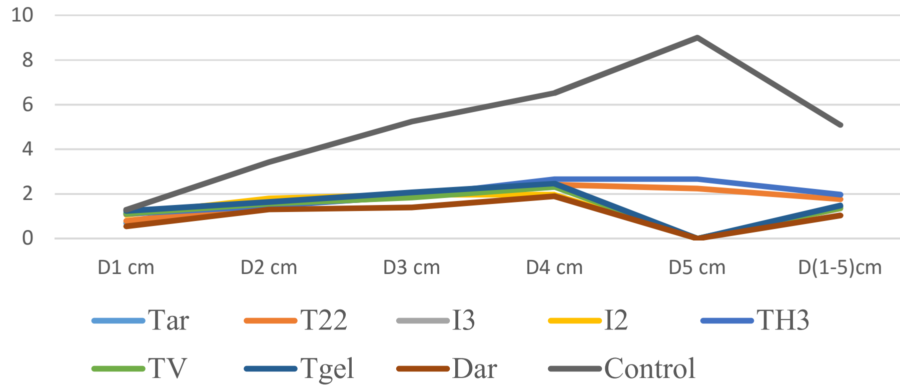


Figure 29: Growth chart of phytopathogen on dual culture for T-isolates vs *Rhizoctonia solani* Kuhn

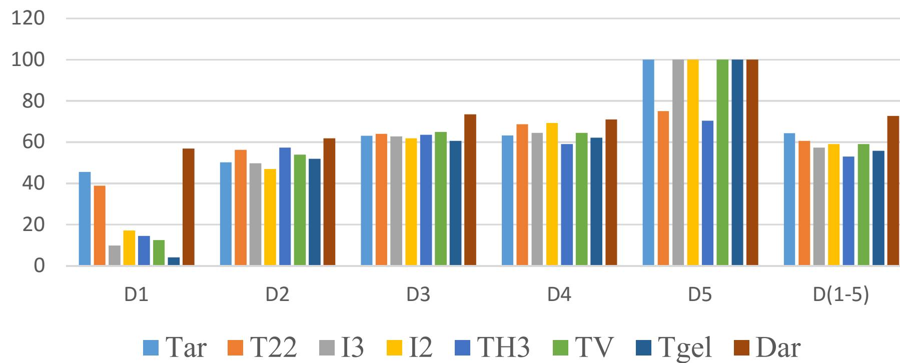


Figure 30: Growth inhibition percentage of *Trichoderma* isolates vs *Rhizoctonia solani* Kuhn

For T-isolates vs RS, DC's results show mycoparasitism following order of strength in decreasing order Dar>Tar>i2>i3>T-gel>TV>TH3>T22.From this study it is showed that most of the T-isolates are very useful in suppressing the growth of RS by day 5. It is found that T-isolates are very efficient in mycoparacitized RS than other phytopathogen.

K) Comparative study of T-isolates vs *Rhizoctonia solani*

The DC's method showed T=isolates were highly potent in competition and mycoparasitism as all selected T-isolates overgrow on nutrient medium and not let the growth of RSR freely. Chosen isolates were briefly analysed and are

divided into two range leading that Tar, I2 and T-gel were rank 1st and rest are 2nd.

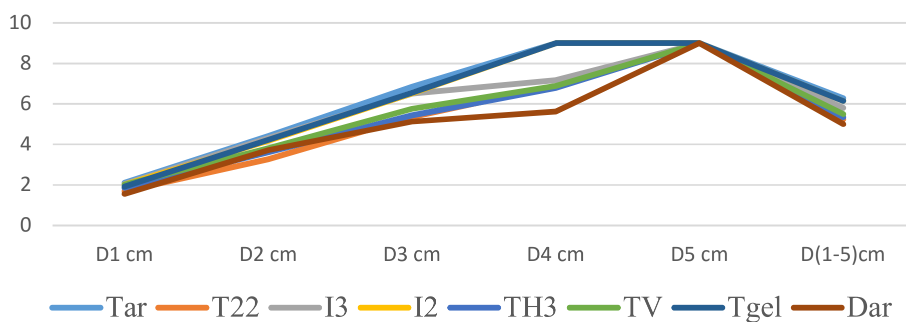


Figure 31: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Rhizoctonia solani* (Mustard green)

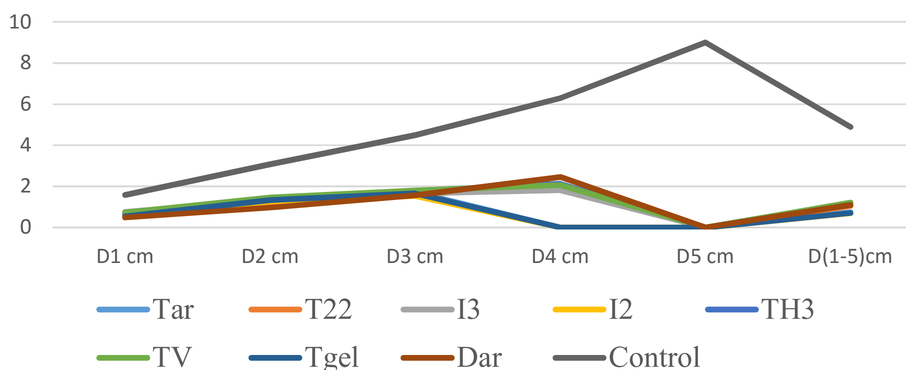


Figure 32: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Rhizoctonia solani* (Mustard green)

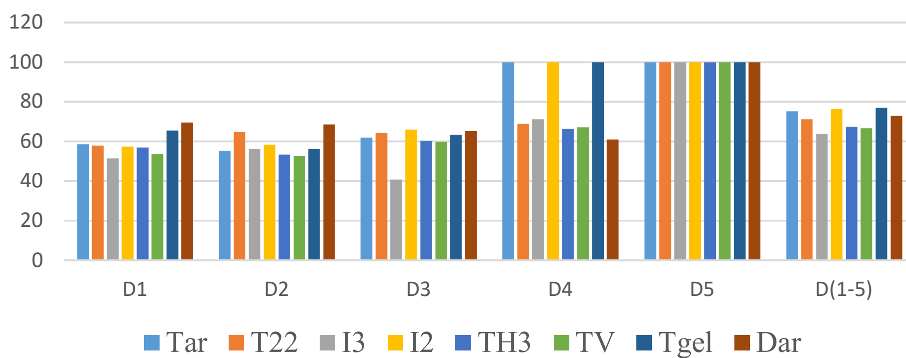


Figure 33: Growth inhibition percentage of *Trichoderma* isolates vs *Rhizoctonia solani* (Mustard green)

L) Comparative study of T-isolates and *Sclerotium rolfsii*

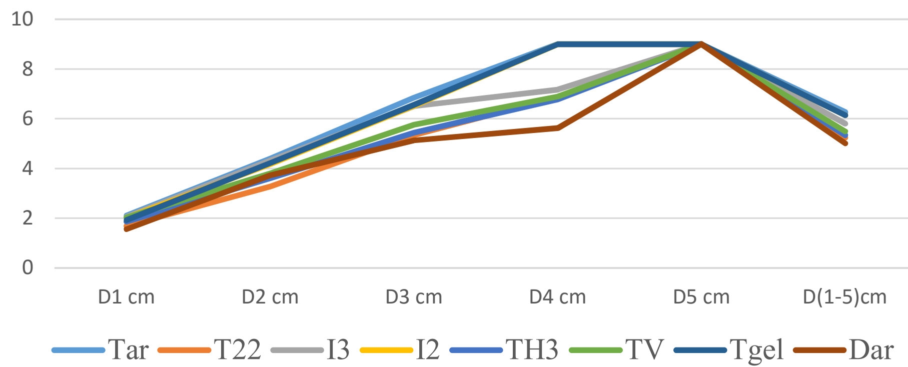


Figure 34: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Sclerotium rolfsii*

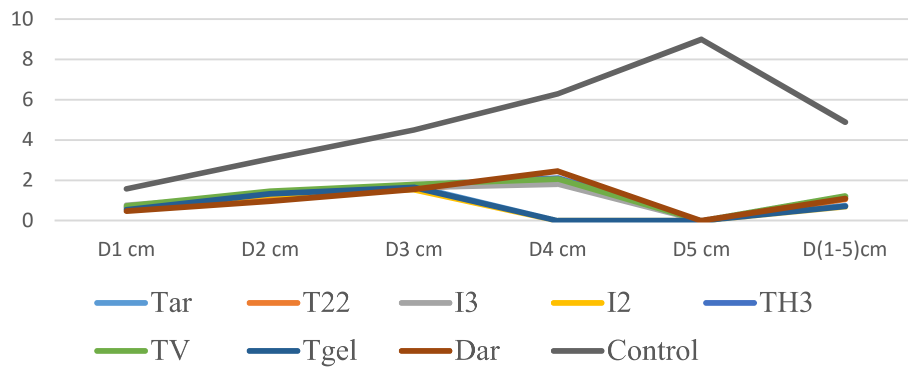


Figure 35: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Sclerotium rolfsii*

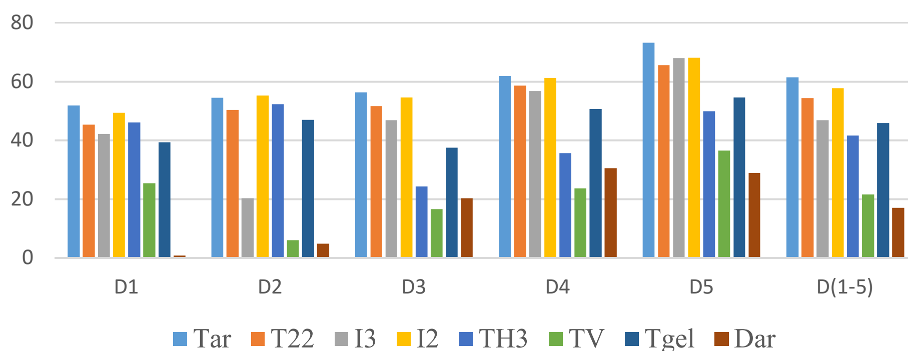


Figure 36: Growth inhibition percentage of *Trichoderma* isolates vs *Sclerotium rolfsii*

From DC's experiment, it is found that local isolates Tar is most potent isolates on suppressing the growth of SR on PDA medium. Only 4 (Tar, I3, i2 and T22) isolates were found to be significant in controlling seedling blight of rice. The effectiveness of T-isolates on suppressing the growth of SR in decreasing order were: Tar>I2≥I3>T22>T-gel>TH3>TV>Dar.

M) Comparative study of T-isolates vs *Macrophomina phaseolina.*

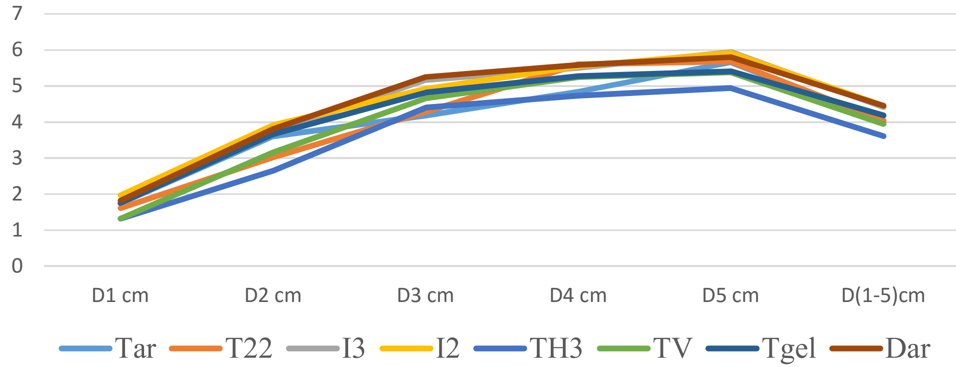


Figure 37: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Macrophomina phaseolina*

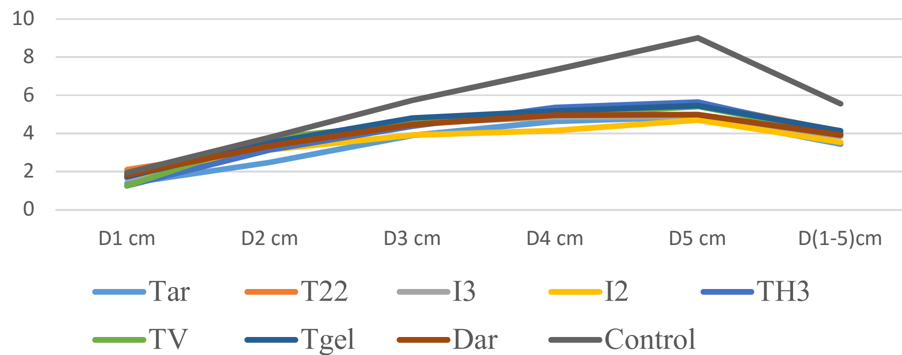


Figure 38: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Macrophomina phaseolina*

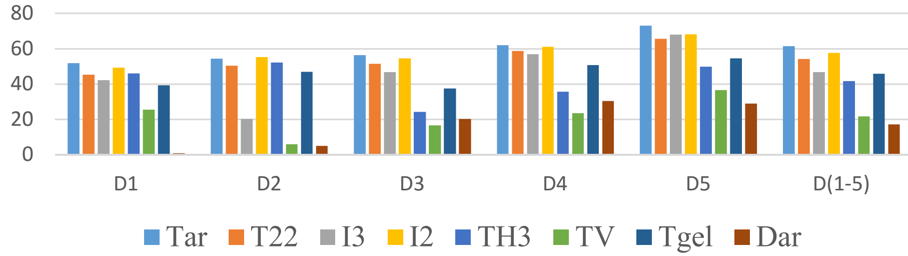


Figure 39: Growth inhibition percentage of *Trichoderma* isolates vs *Macrophomina phaseolina*; Investigation reveals that potency of local isolates Tar positioned 1st among all isolates and ranking should in order of Tar>I2>I3>Dar>TV>T-gel>T22>TH3.

N) Comparative study of T-isolates vs *Fusarium oxysporum* f. sp. *lycopersici*.

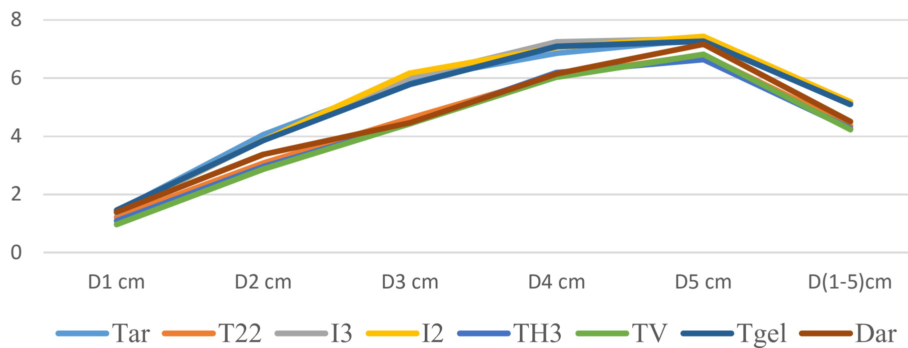


Figure 40: Growth chart of *Trichoderma* isolates on dual culture for *Trichoderma* isolates vs *Fusarim oxyporum* f. sp. *lycopersici*

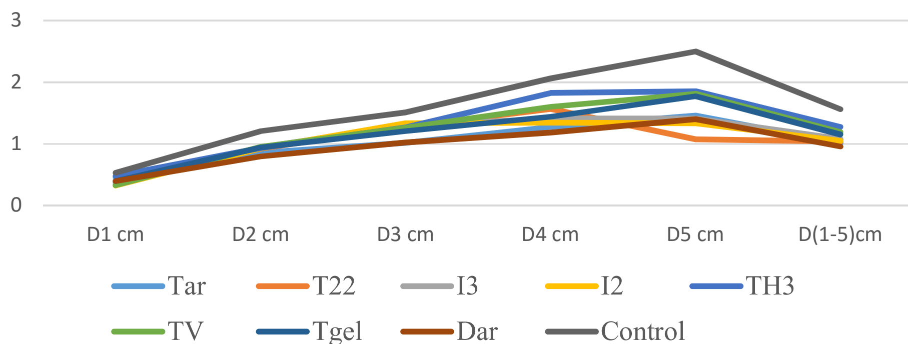


Figure 41: Growth chart of phytopathogen on dual culture for *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *lycopersici*

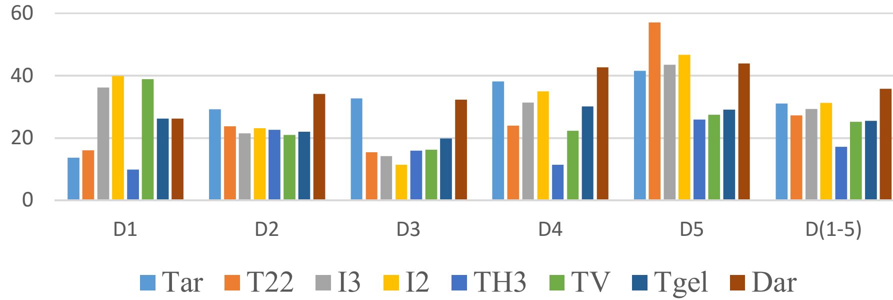


Figure 42: Growth inhibition percentage of *Trichoderma* isolates vs *Fusarium oxyporum* f. sp. *lycopersici*

DC's study reveals the potency of T-isolates in following order T22>I2>Dar>Tar>I3>T-gel>TV>TH3.

4.1.2 Antagonistic Efficacy of T-isolates against Phytopathogen in NVOC's Method

Table 2: Study of NVOC's effects of T-isolates on *Fusarium oxysporum* f. sp. *cubense*

E. on BDD by	NVOC'S effect of T-isolates on radial growth measurement BDD											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D (3-5-7)cm	D3 cm	D5 cm	D7 cm	D (3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	.533	1.788	1.186	1.169	.677	1.225	1.844	1.249	.733	1.877	1.988	1.533
Tar GI%	46.91	21.48	47.85	38.747	36.49	46.20	32.80	38.497	31.24	17.566	27.55	25.452
Dar	.511	1.665	1.222	1.133	.688	1.344	1.933	1.322	.766	1.91	1.944	1.54
Dar GI%	52.06	26.88	55.95	44.963	35.46	40.98	29.55	35.33	28.15	16.12	29.16	24.47
Control	1.066	2.277	2.774	2.039	1.066	2.277	2.744	2.029	1.066	2.277	2.744	2.029

Where E. = Effect; ln this study, GI% of day3 were recorded and they became shrinks by day 5 and raised again on reaching day 7. At 100% concentration Dar were more effective than Tar but in 50% concentration it became reversed and in 25% concentration Day 3 and day 5 shows Tar had more potency while day7 shows Dar.

Table 3: NVOC's effects of T-isolates on *Penicillium* spp.

Effect on CDL by	NVOC'S effect of T-isolates on radial growth measurement CDL											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	.377	.877	1.977	1.077	.621	1.099	2.177	1.299	.755	1.222	2.288	1.422
Tar GI%	66.07	53.55	29.67	49.763	44.11	41.80	22.56	36.157	32.04	35.28	18.61	28.643
Dar	.355	1.133	1.988	1.158	.544	1.166	1.799	1.17	.755	1.044	2.099	1.299
Dar GI%	68.05	39.99	29.28	45.773	51.04	38.24	36	41.76	32.04	44.70	25.33	34.023
C	1.111	1.888	2.811	1.936	1.111	1.888	2.811	1.937	1.111	1.888	2.811	1.937

Here, C = Control; the study clearly shows Tar has had slightly higher antibiosis potency than Dar at 100% concentration and 50% concentration though the result became zigzag by day wise and at 25% concentration Dar show more potency.

Table 4: Study of NVOC’s of T-isolates on *Alternaria brassicicola*

E.	NVOC’S effect of T-isolates on radial growth measurement CDT											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	.244	.811	.833	0.629	.4	.977	Ꞙ	0.792	.422	1.1	1.255	0.926
Tar GI%	71.83	46.29	48.64	55.587	53.81	35.3	Ꝏ	42.507	51.27	27.15	22.63	33.683
Dar	.299	.833	.999	0.710	4.433	.955	Ꞙ	2.129	.377	1.11	1.221	0.903
Dar GI%	65.47	44.84	38.41	49.573	50	36.76	Ꝏ	41.723	56.47	26.49	24.72	35.893
C	.866	1.51	1.622	1.333	.866	1.510	ҵ	1.333	.866	1.510	1.622	1.333

Where, E. = Effect on CDT by; Ꞙ = .999, Ꝏ = 38.41, ҵ = 1.622, C = Control; here, Tar>Dar at 100% concentration, Tar≥Dar at 50% concentration and Dar>Tar at 25% concentration. At day 3 GI% is high and falls down rapidly to nearly (40-50) % range of original value by day 5 and little raised in GI% is observed on reaching day 7.

Table 5: Study of NVOC’s of T-isolates on *Fusarium oxysporum* f. sp. *melongenae*

E.	NVOC’S effect of T-isolates on radial growth measurement FO											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D*
Tar	.3	.966	1.199	0.822	.366	1.222	1.3	0.963	.4	1.311	1.422	1.0443
Tar GI %	58.45	31.54	41.02	43.67	49.31	13.4	36.06	32.923	44.6	7.09	30.06	27.25
Dar	.2776	1.022	1.066	0.789	.355	1.233	1.311	0.966	.533	1.2886	1.4886	1.104
Dar GI %	58.45	31.54	47.57	45.853	50.83	12.62	35.52	32.99	26.12	8.68	26.78	20.527
Control	.722	1.411	2.033	1.389	.722	1.411	2.033	1.389	.722	1.411	2.033	1.389

Where, E. = Effect on FO by; D* = D(3-5-7)cm; from this study, following facts were found for 100% concentration Dar>Tar though both had equal performance on day 3 and 5. For 50% concentration Tar had barely better performance than Dar. For 25% concentration Tar shows better antibiosis than Dar.

Table 6: Study of NVOC's of T-isolates on *Fusarium solani* f. sp. *melongenae*

E.	NVOC'S effect of T-isolates on radial growth measurement FS											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*
Tar	.566	.733	.9	0.733	.663	1.4773	1.4773	1.206	.665	1.886	1.933	1.495
Tar GI %	43.32	66.33	59.5	56.383	33.61	32.14	33.52	33.09	33.41	13.37	13.01	19.93
Dar	.522	.7	.8886	0.704	.663	1.3886	1.3886	1.147	.9773	1.697	1.722	1.466
Dar GI %	47.73	67.85	60.01	58.53	33.61	36.22	37.51	35.78	2.13	21.93	22.50	15.52
C	.9986	2.177	2.222	1.799	.9986	2.177	2.222	1.799	.9986	2.177	2.222	1.799

Where D* = D(3-5-7)cm, E. = Effect on FS by, C = Control; result shows Dar≥Tar at 100% concentration and Dar>Tar at 50% concentration and 25% concentration.

Table 7: Study of NVOC's of T-isolates on *Acremonium* spp.

E.	NVOC'S effect of T-isolates on radial growth measurement IK1											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*
Tar	.333	1.111	1.133	0.859	.3773	1.1663	1.2106	0.918	.411	1.222	2.0886	1.241
Tar GI %	45.46	37.5	69.92	50.96	38.21	34.39	67.86	46.82	32.69	31.26	44.54	36.163
Dar	.333	1.122	1.222	0.892	.422	1.133	1.1663	0.907	.4773	1.177	2.0886	1.248
Dar GI %	45.46	36.88	67.56	49.967	30.89	36.23	69.03	45.383	21.83	33.77	44.54	33.38
C	.6106	1.778	3.766	2.052	.6106	1.7776	3.766	2.052	.6106	1.778	3.766	2.052

Where C = Control, E. = Effect on IK1 by, D* = D(3-5-7)cm; NVOC's effect on IK1 were nearly same in all concentration and days. Antibiosis effect on growth of mycelial growth of Ik1 found to be 100% concentration>50% concentration>25% concentration Overall GI pattern is found as such the certain amount of inhibition (30-50%) were exhibit on day 3 then it reduces little bit by day 5 and raised again even greater than original performance (day 3). The antibiosis potential of both Tar and Dar shows approximately same and had similar pattern of actions.

Table 8: Study of NVOC's of T-isolates on *Fusarium oxyporium* f. sp. *zingiberi*

E.	**NVOC'S effect of T-isolates on radial growth measurement IK2**											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	.233	.366	.5773	0.392	.244	.633	.711	0.529	.3	.666	1.022	0.663
Tar GI%	38.3	59.33	57.74	51.79	35.38	29.67	47.95	37.667	20.55	26	25.18	23.91
Dar	.2663	.6	.611	0.493	.2886	.644	.733	0.555	.3	.666	1.033	0.666
Dar GI%	29.48	33.33	55.27	39.36	23.57	28.45	46.34	32.787	20.55	26	24.38	23.643
C	.3776	.9	1.366	0.881	.3776	.9	1.366	0.881	.3776	.9	1.366	0.881

Where E. =Effect on IK2 by, C = Control; moderate growth inhibition was observed.

Table 9: Study of NVOC's of T-isolates vs *Exsirohilum turcicum*

Effect on IK2 by	NVOC'S effect of T-isolates on radial growth measurement MB											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7) cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	.233	.366	.5773	0.392	.244	.633	.711	0.529	.3	.666	1.022	0.663
Tar GI%	38.3	59.33	57.74	51.79	35.38	29.67	47.95	37.667	20.55	26	25.18	23.91
Dar	.2663	.6	.611	0.493	.2886	.644	.733	0.555	.3	.666	1.033	0.666
Dar GI%	29.48	33.33	55.27	39.36	23.57	28.45	46.34	32.787	20.55	26	24.38	23.643
Control	.3776	.9	1.366	0.881	.3776	.9	1.366	0.881	.3776	.9	1.366	0.881

Where E. = Effect on IK2 by; Tar≥Dar, moderate effect was observed on MB. Antibiosis pattern were raised-down-raised form.

Table 10: Study of NVOC's of T-isolates vs *Fusarium sambucinum*

E.	NVOC'S effect of T-isolates on radial growth measurement PDT											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*
Tar	.322	.422	.666	0.47	.3216	.8553	.9776	0.718	.855	1.7886	2.155	1.6
Tar GI %	71.33	77.39	74.71	74.4667	71.34	54.17	62.87	62.793	23.8	4.16	18.15	15.37
Dar	.3553	.666	.7553	0.592	.3773	1.0886	1.883	1.1163	.533	1.422	1.7	1.218
Dar GI %	68.33	64.32	71.32	67.99	66.37	42.74	28.48	45.863	52.5	23.81	35.44	37.25
C	1.122	1.866	2.633	1.874	1.122	1.866	2.633	1.874	1.122	1.866	2.633	1.874

Where C = Control, E. = Effect on PDT by, D* = D(3-5-7)cm; from obtained data, Tar>Dar at 100 and 50% concentration. But reversed at 25% concentration. Overall performance was ranged as moderate effect on growth inhibition of mycelium of *Fusarium sambucinum.*

Table 11: Study of NVOC's of T-isolates on *Rhizoctonia solani* Kuhn

ж	NVOC'S effect of T-isolates on radial growth measurement RS											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	NGM	NGM	NGM	NGM	NGM	π	π	NGM	.3886	.5553	.6776	.541
Tar GI %	100	100	100	100	100	100	100	100	91.03	87.66	84.94	.877
Dar	NGM	NGM	NGM	NGM	NGM	€	Ω	NGM	.433	.466	.633	.511
Dar GI %	100	100	100	100	100	100	100	100	90	89.65	85.93	88.45
C	4.33	4.5	4.5	4.5	4.33	4.5	4.5	4.5	4.33	4.5	4.5	4.443

Where, ж = Effect on RS by, C = Control, π = NGM/.266(DP), € = NGM/.2(DP), Ω= NGM/.2665(DP); NGM= No growth measurement, DP= Dual plate replication, SP= Single plate replication; Antibiosis of NVOC's against RS were seen to be highly potent as almost no growth had been recorded in vitro at 100 and 50% concentration. Very little mycelial growth was observed at 25% concentration.

Table 12: Study of NVOC's of T-isolates on *Rhizoctonia solani*

E.	**NVOC'S effect of T-isolates on radial growth measurement RSR**											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm	D3 cm	D5 cm	D7 cm	D(3-5-7)cm
Tar	NGM	NGM	NGM	NGM	NGM	NGM	NGM	NGM	.2886	.2886	.4773	0.352
Tar GI%	100	100	100	100	100	100	100	100	93.59	93.59	89.39	92.19
Dar	NGM	NGM	NGM	NGM	NGM	NGM	NGM	NGM	.2443	.3443	.4443	0.344
Dar GI%	100	100	100	100	100	100	100	100	94.57	92.35	90.13	92.35
C	4.5	¥	4.5	4.5	4.5	¥	4.5	4.5	4.5	¥	4.5	4.5

Where, E. = Effect on RS by, ¥ = 4.5/SP(4.226), SP = Single Plate replication, C= Control; Antibiosis of NVOC's against RSR were seen to be highly potent as almost no growth had been recorded in vitro at 100 and 50% concentration. Very little mycelial growth were observed at 25% concentration.

Table 13: Study of NVOC's of T-isolates on *Sclerotium rolfsii*

E.	NVOC'S effect of T-isolates on radial growth measurement SR											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*
Tar	.2	.4773	1.244	0.641	.444	.9886	1.9663	1.133	.4	1.	2.322	1.455
Tar GI %	81.81	85.77	72.36	79.98	59.62	70.53	56.31	62.153	63.62	50.98	48.4	54.333
Dar	.222	.266	1	0.496	.8553	Β	2.633	1.533	.444	1.333	α	2.092
Dar GI %	79.81	92.07	77.78	83.22	22.17	66.89	41.49	43.517	г	60.26	0	39.96
C	1.099	3.354	4.5	2.985	1.097	3.354	4.5	2.985	ǂ	3.354	4.5	2.985

Where, β = 1.1106, α = 4.5/SP(1.666), ǂ = 1.0996, г = 59.621, C = Control, E. = Effect on SR by D* = D(3-5-7)cm;

High level of growth inhibition of *Scelerotium rolfsii* mycelium was observed in vitro laboratory experiment. This result shows higher the concentrate ions of *Trichodermic* metabolites, more the inhibition phenomena on mycelium of SR. Growth of mycelium of SR was slowed down in comparison to control plate and 3, 5 and 7 DAI results shows mycelium growth either completely inhibited on higher concentration or little growth was observed whereas in lower concentration of *Trichodermic* metabolites solution, mycelium growth were observed fast growth than higher concentration but little lower than control plates.

Table 14: Study of NVOC’s of T-isolates on *Macrophomina phaseolina*

E.	NVOC’S effect of T-isolates on radial growth measurement TCD											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*
Tar	1.622	2.311	2.9773	2.303	2.433	3.777	4.344	3.518	2.7773	4.122	4.5	3.799
Tar GI %	63.07	48.65	33.84	4.5	45.93	16.06	3.466	ɥ	38.28	8.4	0	15.56
Dar	1.0106	1.966	2.7553	1.943	2.644	3.944	ıє	3.677	2.8443	4.177	4.5	3.840
Dar GI %	77.54	56.31	38.77	1.910	41.24	12.36	1.24	18.28	36.79	7.17	0	Ʊ
C	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5

Where, Ʊ = 14.653, ıє = 4.4443, ɥ= 21.819, C = Control, E = Effect m on TCD by, D =D(3-5-7)cm;

This result shows Dar>Tar at 100% concentration. But reversed in 50 and 25% concentration. On regarding result data, NVOC’s antibiosis of T-isolates on TCD moderately weak than other phytopathogen. GI pattern were found to be day3>day5>day7.

Table 15: Study of NVOC's of T-isolates vs *Fusarium oxysporum* f. sp. *lycopersici*

E.	NVOC'S effect of T-isolates on radial growth measurement TVD											
	100%				50%				25%			
	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*	D3 cm	D5 cm	D7 cm	D*
Tar	.433	µ	µ	1.018	.4996	1.522	1.522	1.181	.7443	1.633	2.444	1.607
Tar GI %	69.06	35.53	67.23	57.273	64.29	25.13	61.95	ʞ	46.82	19.68	38.89	35.13
Dar	.411	ɸ	ȸ	0.933	.522		Ꞧ	1.292	.7996	1.7553	2.2996	1.619
Dar GI %	70.64	41.54	70.01	60.73	62.70	17.51	58.06	46.09	42.87	13.66	42.51	33.013
C	ȹ	2.033	Ʈ	2.4774	ȹ	2.033	Ʈ	2.475	ȹ	2.033	Ʈ	2.4774

Where, C = Control, E. = Effect on TVD by, D* = D(3-5-7)cm; from result data, Dar≥Tar at 100% concentration But reversed order at 50 and 25% concentration GI pattern were found to be day 3>day 7>day.

4.1.3 Antagonism of T-isolates against Phytopathogen in VOC's Method

Table 16: Study of T-isolates of VOC's on BDD

Days and Organisms		**D5 cm**	**D7 cm**	**D (5-7) cm**
	Tar BDD	2.1773	2.7773	2.4773
T1	GI%	14.79	19.88	17.335
	Dar BDD	1.6883	2.5996	2.14395
T2	GI%	33.93	25	29.465
T3	Control-BDD	2.5553	3.4663	3.0108

VOC's study shows Dar>Tar and has very little influence on inhibiting the mycelial growth of BDD.

Table 17: Study of T-isolates of VOC's on CDL

Days and Organisms			D5 cm	D7 cm	D(5-7) cm
T1	Tar	CDL	2.744	3.1106	2.927
	GI%		.3993	7.154	3.777
T2	Dar	CDL	2.661	3.0663	2.864
	GI%		5.227	9.511	7.369
T3	Control-CDL		2.755	3.3886	3.072

VOC's investigation reveals Dar≥Tar and has almost no GI was observed.

Table 18: The effect of VOC's by T-isolates on CDT

Days and Organism			D5 Cm	D7 cm	D(5-7) cm
T1	Tar	CDT	1.223	1.7773	1.5
	GI%		41.75	24.18	32.965
T2	Dar	CDT	1.0883	1.5443	1.316
	GI%		48.17	34.12	41.145
T3	Control-CDT		2.0996	2.344	2.2222

VOC's trail found to be Dar>Tar.

Table 19: Study of T-isolates of VOC's on FO

Days and Organism			D5 cm	D7 cm	D(5-7) cm
T1	Tar	FO	2.0553	2.1773	2.116
	GI%		13.14	19.68	16.41
T2	Dar	FO	1.822	1.944	1.883
	GI%		23	28.28	25.64
T3	Control-FO		2.3663	2.7106	2.539

Here, Dar>Tar and has little influence on mycelial GI of FO.

Table 20: Study of T-isolates VOC's on FS

Days and Organism		**D5 cm**	**D7 cm**	**D(5-7) cm**
T1	Tar FS	1.744	2.4443	2.094
	GI%	22.66	17.90	2.028
T2	Dar FS	1.7886	2.5773	2.183
	GI%	20.68	13.44	17.06
T3	Control-FS	2.255	2.9773	2.616

Antibiosis of VOC's were Tar>Dar with small effect of GI.

Table 21: Study of T-isolates on VOC's on IK1

Days and Organism		D5 cm	D7 cm	D(5-7) cm
T1	Tar IK1	1.422	2.1663	2.094
	GI%	30.6	14.86	22.73
T2	Dar IK1	1.3553	2.2773	1.186
	GI%	33.33	10.5	21.915
T3	Control-IK1	2.033	2.5443	2.289

Tar≥Dar were observed with small effect on GI.

Table 22: Study of T-isolates on VOC's on IK2

Days and Organisms		**D5 cm**	**D7 cm**	**D(5-7) cm**
T1	Tar IK2	1.433	1.533	1.483
	GI%	38.29	49.46	43.875
T2	Dar IK2	1.733	2.5	2.1165
	GI%	25.37	17.57	21.47
T3	Control-IK2	2.322	3.033	2.6775

Tar>Dar were observed and moderate effect of VOC's metabolites was seen.

Table 23: Study of T-isolates on VOC's on MB

Days and Organism		D5 cm	D7 cm	D(5-7) cm
T1	Tar MB	2.633	3.021	2.287
	GI%	4.44	31.16	17.8
T2	Dar MB	2.555	3.544	3.05
	GI%	7.27	19.25	13.26
T3	Control-MB	2.7553	4.3886	3.572

On day 5 Dar>Tar and became reversed on reaching day 7.

Table 24: Study of T-isolates on VOC's on PDT

Days and Organisms		D5 cm	D7 cm	D(5-7) cm
T1	Tar PDT	2.166	3.1886	2.677
	GI%	9.74	15.33	12.535
T2	Dar PDT	2.133	2.8773	2.505
	GI%	11.11	23.6	17.355
T3	Control-PDT	2.3996	3.766	3.083

Here, Dar>Tar and has little influence on mycelial growth inhibition of MB.

Table 25: Study of T-isolates on VOC's on RS

Days and Organism		D5 cm	D7 cm	D(5-7) cm
T1	Tar RS	1.922	2.32	2.121
	GI%	57.29	48.45	52.87
T2	Dar RS	1.3773	3.7443	2.561
	GI%	69.39	16.79	43.09
T3	Control-RS	4.5	4.5	4.5

Until day 5 Dar>Tar and became opposite on reaching day 7. Overall effect of VOC's on RS were moderate GI of mycelial growth.

Table 26: Study of T-isolates on VOC's on RSR

Days and Organisms			**D5 cm**	**D7 cm**	**D(5-7) cm**
T1	Tar	RSR	2.044	2.996	2.52
	GI%		39.66	33.42	36.54
T2	Dar	RSR	1.933	2.844	2.389
	GI%		42.95	36.8	39.875
T3	Control-SR		3.3883	4.5	3.944

Result were Dar>Tar. Overall effect of VOC's on RS were moderate GI of mycelial growth.

Table 27: Study of T-isolates on VOC's on SR

Days and Organism			**D5 cm**	**D5 cm**	**D(5-7) cm**
T1	Tar	SR	1.4996	1.9	1.699
	GI%		23.29	41.01	32.15
T2	Dar	SR	1.333	1.966	1.649
	GI%		31.82	38.96	35.39
T3	Control-SR		1.955	3.221	2.588

Until day 5 Dar>Tar and became opposite on reaching day 7. Overall effect of VOC's on SR were moderate GI of mycelial growth.

Table 28: Study of T-isolates on VOC's on TCD

Days and Organism		D5 cm	D7 cm	D(5-7) cm
T1	Tar TCD	4.5	4.5	4.5
	GI%	0	0	0
T2	Dar TCD	4.5	4.5	4.5
	GI%	0	0	0
T3	Control-TCD	4.5	4.5	4.5

No effect were seen on GI of TCD mycelium by VOC's of T-isolates.

Table 29: Study of T-isolates on VOC's on TVD

Days and Organism		D5 cm	D7 cm	D(5-7) cm
T1	Tar TVD	1.933	2.4996	2.216
	GI%	6.44	14.13	10.285
T2	Dar TVD	1.944	2.344	2.144
	GI%	5.91	19.48	12.695
T3	Control-TVD	2.066	2.911	2.489

Very small effect of VOC's on TVD were observed. Tar and Dar has had nearly equal potency.

Table 30: Thermostability of T-isolates against SR and RS at high rpm

S.N.	Culture Media Undiluted 100%	Effect of fractionated culture filtrate 15000 rpm for 10 min							
		% inhibition against SR				% inhibition against RS			
		Top portion		Bottom portion		Top portion		Bottom portion	
		Tar	Dar	Tar	Dar	Tar	Dar	Tar	Dar
1	CDB	92.33	92.63	85.26	81.49	87.2	86.7	92.33	96.23
2	PDB	96.67	96.43	100	100	90.52	95.8	100	100

Table 31: Thermostability of T-isolates against SR and RS at low rpm

S.N.	Culture Media Undiluted 100%	Effect of fractionated culture filtrate 5000 rpm for 10 min							
		% inhibition against SR				% inhibition against RS			
		Top portion		Bottom portion		Top portion		Bottom portion	
		Tar	Dar	Tar	Dar	Tar	Dar	Tar	Dar
1	CDB	2.33	0	3.44	5.49	7.26	6.67	5.4	6.5
2	PDB	5.23	3.49	4.83	7.23	10.52	9.58	8.77	9.76

Results from Table 30 and 31 clearly shows *Trichoderma* produced secondary metabolites and are effective against soil bore phytopathogenic fungi which form sclerotial bodies in agriculture field. These table shows thermostability of T-isolates for high rpm than low rpm and are discussed in chapter v.

4.2 In Vivo Antagonism

4.2.1 Study of Seedling Blight of Rice

Table 32: Study of T-isolates vs Chaite-2 rice

T. N.	GI %	*Trichoderma* treated bio-primed Chaite-2 rice											
		DI of SR						DSI of SR					
		D_{9r}	D_{18r}	D_{27r}	D_{36r}	D_{45r}	$D_{\#}$	Ᵽ	D_{18r}	D_{27r}	D_{36r}	D_{45r}	$D_{\#}$
T_1	99	16	25	33.5	38	39.25	30.35	5	10.15	23.55	28.56	33.33	20.118
T_2	90	16	25	40	Δ	65.32	38.374	θ	13.33	21.23	34.43	37.73	22.99
T_3	99	13.33	28	35	43	38.33	31.532	5	12.25	20.63	34.55	43.33	23.152
T_4	99	15.34	26.89	37.56	Δ	63.33	37.734	Ω	15.43	26.43	35.56	44.56	25.64
T_5	95	17.65	27.75	38	ψ	65.76	41.698	ρ	17.89	34.44	39.76	45.32	29.168
T_6	93	17.33	28.73	43.33	σ	60.54	40.832	ϕ	16.75	33.33	41.11	40.76	29.542
T_7	95	25	32	40.43	55	57.75	42.036	ϕ	16.75	40.43	38.75	38.93	30.124
T_8	96	18.98	33.33	44.12	Δ	50.12	38.42	ԋ	23.43	32.13	35.55	44.54	30.464
T_9	91	21.11	36.32	45.55	ϵ	67.89	44.952	У	22.22	36.78	47.89	56.54	36.444

Where, $D_{\#} = D_{(9\text{-}45)r}$, Ᵽ = D_{9r}, Δ = 45.55, θ = 8.23, ω = 6.22, ψ = 59.33, ρ = 8.43, σ = 54.23, ϕ = 15.76, ԋ = 16.67, ϵ = 53.89, У = 18.79; PDIC values for T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8 and T_9 are 44.79, 36.91, 36.47, 29.64, 19.96, 18.93, 17.33 and16.4 percent respectively.

Table 33: Study of T-isolates vs Hardinath rice

T. N.	GI %	*Trichoderma* treated bio-primed Hardinath rice												
		DI% of SR						DSI% of SR						
		D_{9r}	D_{18r}	D_{27r}	D_{36r}	D_{45r}	$D_{\#}$	D	D_{18r}	D_{27r}	D_{36r}	D_{45r}	$D_{\#}$	PDIC
T_1	99	8	10	22.23	22.23	32.23	18.938	3	3	11	22.23	21	12.046	40.38
T_2	93	8	10	24.53	27.56	37.85	21.588	4	5	14.55	27.56	26	15.422	23.68
T_3	99	7.6	10	21.11	21.11	34.43	18.85	0	0	13.44	21.11	21.11	11.132	44.91
T_4	99	12	13.23	26.78	30.54	33.33	23.176	3	7	16.45	18.67	22.22	13.468	33.35
T_5	97	5	13.45	26.66	29.11	38.89	22.622	5	7	16.67	19.11	26.76	14.908	26.23
T_6	95	5	13.33	24.67	26.67	35.56	21.046	5	5.67	13.33	17.53	25.54	13.414	33.62
T_7	90	5	12.54	23.33	26.67	37.76	21.06	5	6.5	16.53	17.53	24.43	13.998	30.73
T_8	90	5	14.32	22.22	27.56	35.76	20.972	5	11.34	14.43	19.32	24.67	14.952	26
T_9	85	8.5	15.32	26.66	33.33	37.33	24.228	5.5	13.33	23.33	27.77	31.11	20.208	

Where D = D_{9r}, $D_{\#} = D_{(9-45)r}$; DI and DSI were found more on control than bioprimed rice. For Hardinath rice the order of potency of T-isolates was found to be Tar≥I2>Dar>TV>T-gel>I3>TH3>Concentration Similarly the order of potential of T-isolates for Chaite-2 rice were Tar>I2>I3>T22≥Dar>TV>TH3>T-gel>Concentration G% of Hardinath rice has following order: Tar=l2=I3>TH3>TV>T22>T-gel=Dar>Control while Chaite-2 had following

order: Tar=I2=Dar>TH3=T-gel>TV>Control>T22. Result shows Hardinath is more resistant to seedling blight of rice than Chaite-2.

4.2.2 Study of Sheath Blight of Rice

Table 34: Study of T-isolates vs Hardinath rice

T. N.	***Trichoderma* treated bio-primed Haedinath rice**												
	GI %	DI% of RS_{rice}						DSI% of $Rice_{rice}$					
		D_{9r}	D_{18r}	D_{27r}	D_{36r}	D_{45r}	$D_{(9-45)r}$	D_{9r}	D_{18r}	D_{27r}	D_{36r}	D_{45r}	D*	PDIC
T1	99	0	0	0	0	16	3.2	0	0	0	0	16	3.2	90.42
T2	96	0	0	0	16	30	9.2	0	0	0	0	20	4	88.02
T3	99	0	0	0	0	16	3.2	0	0	0	0	20	4	88.02
T4	99	0	0	0	22.22	40	12.444	0	0	0	20	20	8	76.05
T5	99	0	0	0	0	16	3.2	0	0	0	0	20	4	88.02
T6	97	0	0	16	0	16	6.4	0	0	0	0	20	4	88.02
T7	95	0	0	20.45	16	70	21.29	0	0	0	23	70	18.6	44.31
T8	97	0	0	0	16	70	17.2	0	0	0	16	23	7.8	76.64
T9	95	0	18	30.5	0	90	27.7	0	0	32	45	90	33.4	

G% of Hardinath rice had following order Tar=I2=I3>TH3=Dar>T22>T-gel=Control. Higher DI and DSI were observed on control than bioprimed rice. Biocontrol potential of T-isolates for DI and DSI of sheath blight of rice were in following order: Tar=I2=TH3>TV>I3>Dar>T-gel>Control, Tar=T22=I2=TH3=TV>Dar>I3>T-gel>Control. Result shows Hardinath rice is

generally resistant to sheath blight of rice. *Rhizoctonia* infection to rice was not observed on the early days of seedling and gradually shows infection as the time passes.

4.2.3 *Fusarium* wilt of Brinjal

Table 35: Study of FO disease in vivo

T.N.	FO G%$_{D9}$	PDIC %	FO*	FO$_{ar(1....5)}$ SL				FO$_{D45}$	
			D45 cm	D15 cm	D25 cm	D35 cm	D45 cm	DSI%	N
T_1	92.6	43.47	7.6	3	7.2	11.4	17	13	8
T_2	91.8	34.78	5.9	2.2	5.6	9.6	15	15	7
T_3	93	34.78	7.4	2.8	6.4	11.6	16.2	15	7
T_4	86.8	30.44	5.6	2.8	5.6	9.2	15	16	5
T_5	86	17.39	6.2	3.6	7	11	13.8	19	6
T_6	86	-8.7	6.6	2.4	5.4	9.6	13.6	25	6
T_7	83.8	43.48	5.4	3.4	6.4	10.4	11.8	13	5
T_8	90.8	21.74	6	2.6	5.8	9.4	12.8	18	8
T_9	76.8		5.6	2.4	5.4	9.2	12	23	7.4

Where N = NOL, FO* = FO$_{ar(1....5)}$ RL; G% of brinjal in case of FO organism were in following order: I2>Tar>T22>Dar>I3>TH3=TV>T-gel>Control. Biocontrol potential for reduction of PDI were found to be Tar=T gel>l3=l2>Dar>TH3>Control>TV. RL, SL and NOL of bioprimed seed with T-isolates generally higher than control.

Table 36: Study of FS disease in vivo

T.N.	FS G%$_{D9}$	PDIC %	FS$_{ar(1....5)}$ RL	FS$_{ar(1....5)}$ SL				FS$_{D45}$	
			D45 cm	D15 cm	D25 cm	D35 cm	D45 cm	DSI%	N
T$_1$	94.8	57.14	7.6	2.6	6.2	10.6	15.3	9	8
T$_2$	87.8	42.86	5.9	2.8	5.6	9.8	15.2	12	6
T$_3$	91	52.38	7.4	3.8	6.8	11	15.4	10	7
T$_4$	89	42.86	5.6	2.4	5	8.8	14.8	12	6
T$_5$	87.8	28.57	6.2	2.3	2.3	5.8	14.2	15	7
T$_6$	88.8	-14.29	6.2	1.9	5.4	6.2	14	24	6
T$_7$	88.8	42.86	5.4	2.4	5	8.8	13.2	12	6
T$_8$	93	14.29	7.2	2.2	5.6	9.4	12.2	18	7
T$_9$	79		5.6	2	5	8.8	11.6	21	5

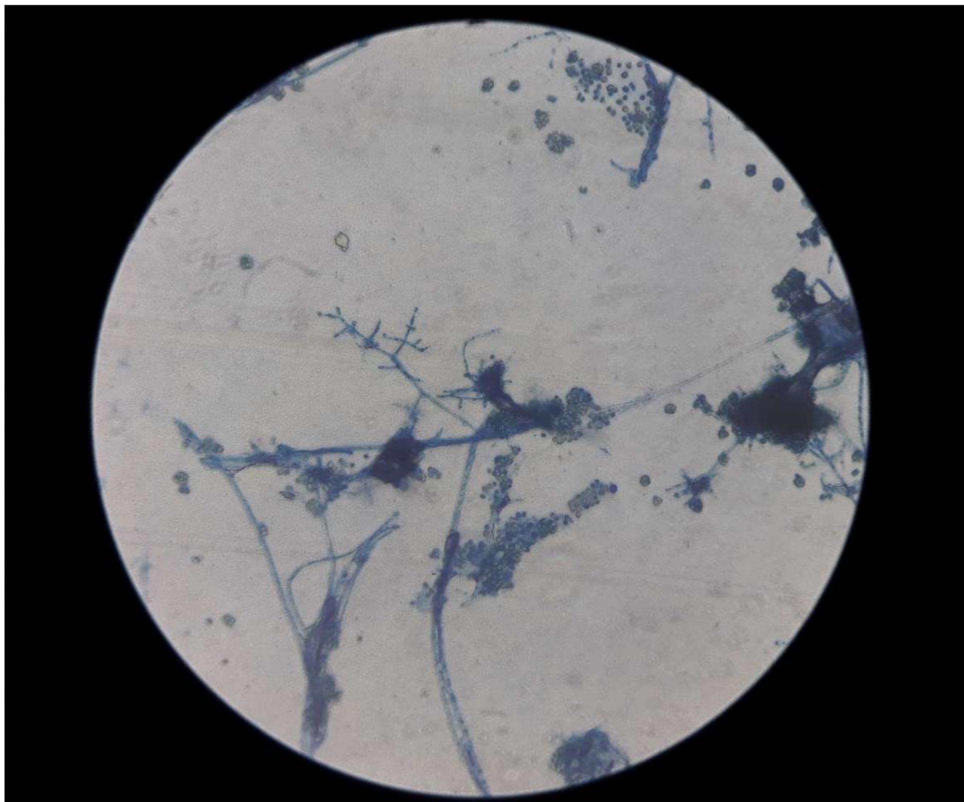
Where N = NOL, G% of brinjal in case of FS organism were in following order: Tar>Dar>I2>TV=T-gel>I3>T22>Control. Biocontrol potential for reduction of PDI were found to be Tar>I2>T22=I3>T-gel>TH>Dar>TV. RL, SL and NOL of bioprimed seed with T-isolates generally higher than control.

4.2.4 Damping off/Root rot of Mustard Green

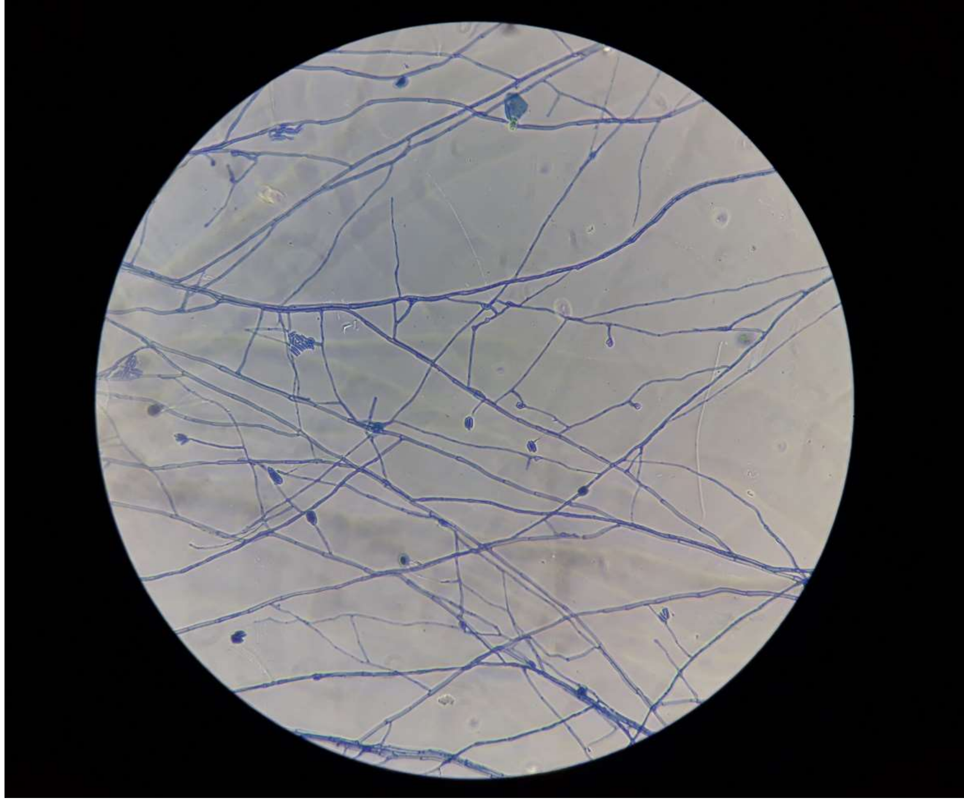
Table 37: Study of damping off disease of mustard green in vivo

T.N.	***Trichoderma* treated bio-primed mustard green**													
	GI %	DI% of Mustard green						DSI% of Mustard green						
		5r	10r	15r	20r	25r	D*	5r	10r	15r	20r	25r	D*	PDIC
T_1	97	0	0	5	13	15	6.6	0	0	3	4	7	2.8	65.85
T_2	96	0	0	7	16	19	8.4	0	0	4	5	9	3.6	56.09
T_3	98	0	0	6	14	16	7.2	0	0	4	4	7	3	63.41
T_4	96	0	0	8	21	23	10.4	0	0	4	7	12	4.6	43.902
T_5	95	0	0	9	25	25	11.8	0	0	5	9	13	5.4	34.14
T_6	96	0	0	9	27	26	12.4	0	0	4	11	13	5.6	31.70
T_7	95	0	0	8	26	27	12.2	0	0	3	10	14	5.4	34.14
T_8	97	0	0	7	16	27	10	0	0	5	12	16	6.6	19.51
T_9	95	0	0	12	34	39	17	0	0	7	15	19	8.2	

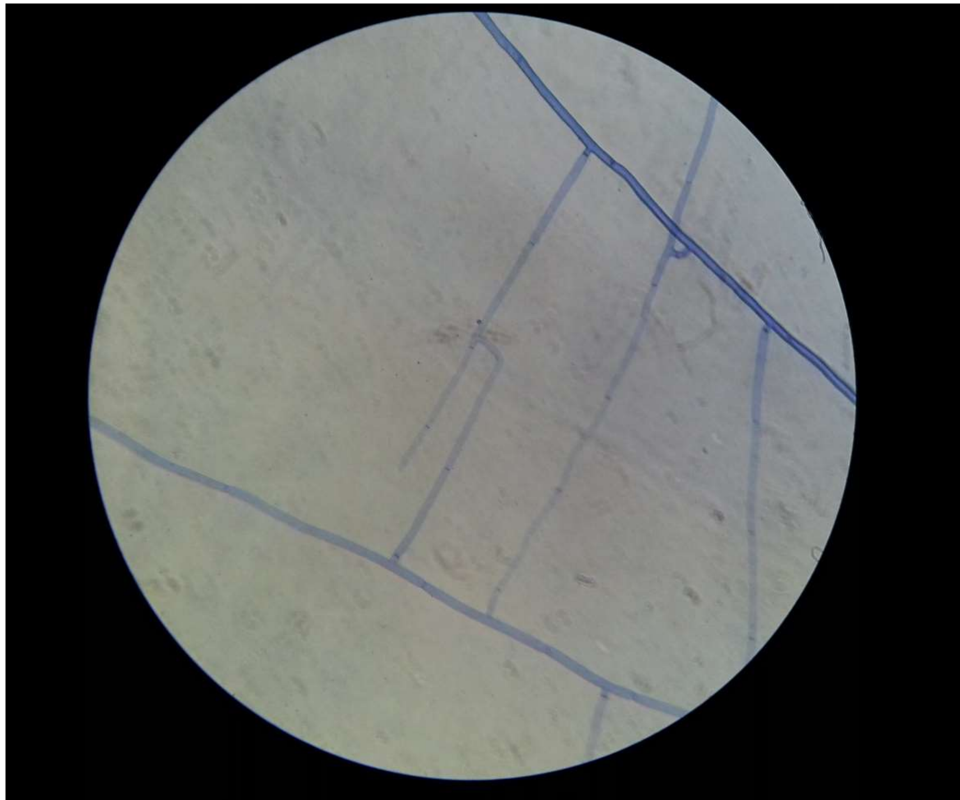
Where 5r = D_{5r}, 10r = D_{10r}, 15r = D_{15r}, 25r = D_{25r}, D* = $D_{(5\text{-}25)r}$; here BOP = Tar>I2>T22>I3>TH3=T-gel>TV>Dar. Results are shown in table 37 and possible interpretation was mentioned in chapter v. For chapter iv, abbreviation were designed as T.N.=Treatment Number, T_1 = Tar, T_2 = T22, T_3 = I2, T_4 = I3, T_5 = TH3, T_6 = TV, T_7 = T-gel, T_8 = Dar, T_9 = Control; D = Day; D(5-25)r = Average data for days 5 to 25, PDI= Percent Diseases Index. NOL=Number of Leaves, RL= Root Length, SL=Shoot Length, DI= Diseases Incidence, DSI/DII= Diseases Severity Index/Diseases Infection Index, $FS_{ar(1....5)}$ RL= FS average root length at day 45, $FS_{ar(1....5)}$ SL= FS average shoot length, NOL= Number of leaves, $D_{(9\text{-}45)r}$ = Average data for days 9 to 45.



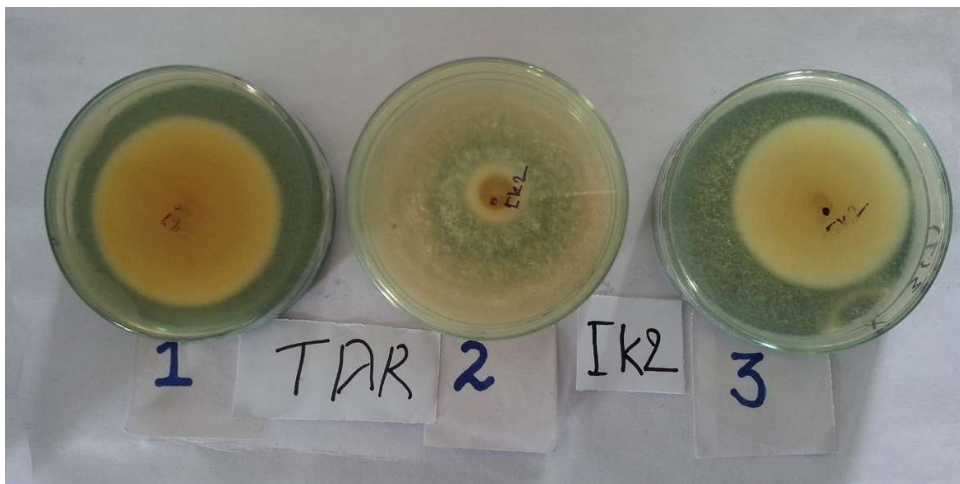
Photograph 1: Microscopy of *Trichoderma harzanium*



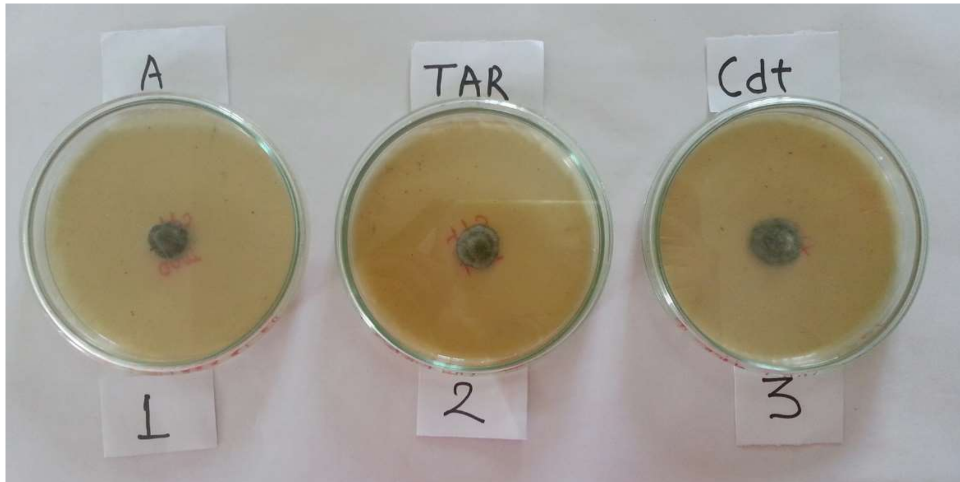
Photograph 2: Microscopy of *Acremonium* spp.



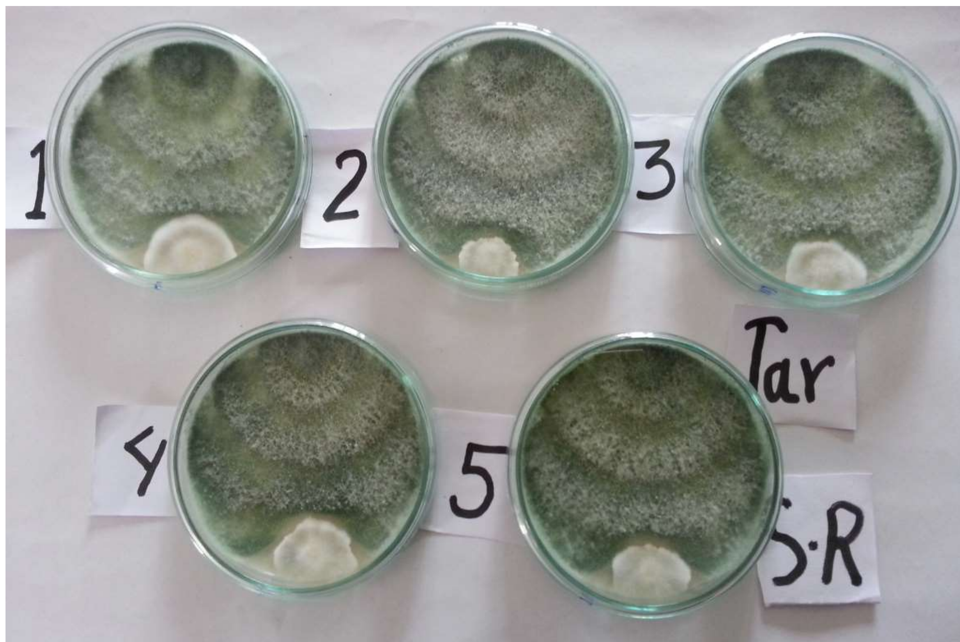
Photograph 3: Microscopy of *Rhizoctonia solani*



Photograph 4: VOC's method, Tar vs IK2 at 7DAI



Photograph 5: NVOC's method, Tar vs CDT on 7 DAI at 100% concentration



Photograph 6: Dual culture, Tar vs S.R. at 5 DAI

Photograph 7: Seedling blight diseases of rice

Photograph 8: In vitro laboratory perform

CHAPTER V

DISCUSSION

Result obtained from the investigation of "Evaluation of antagonistic potential of *Trichoderma* isolates on selected phytopathogenic fungi" stated in chapter iv have been examined critically and discussed here with appropriate interpretations, facts and comparison with previous works are mentioned in this chapter.

In this investigation, 8 *Trichoderma* isolates (T-isolates) were subjected to dual culture's (DC's) method against 14 isolated soil borne phytopathogen. Results reveals the following facts:

Biocontrol order of potential (BOP) for BDD (Banana *Fusarium* wilt) = I2>Tar≥Dar≥TH3>TV>T22>Dar>T-gel. While control plates of BDD showed regular radial growth, a diversity of interactions ranging from high growth inhibition to little influence was observed in the DC's treatments with *Trichoderma.* Antagonistic effects on the pathogen were seen after 3 Days After Inoculation (DAI), but the influence on growth and inhibition were more pronounced by 4 DAI. Mycelia of some of the *Trichoderma* covered a significant portion of plate surface and invaded major parts of the pathogen mycelium. Highest growth inhibition (GI) percentage of 100% on day 5 was observed by I2 i.e. overgrowth of I2 on BDD while T-gel has GI of 54.34%. The present study indicated the potential of 8 T-isolates in inhibiting the mycelial growth of the pathogen. Although in vitro assays do not always provide reliable data on the potential of biocontrol agents, they are a useful and rapid approach for identifying microorganisms that may be effective against pathogens by producing antibiotics or competing directly (Chaves et al 1991). This result were supported by earlier worker Bernal et al (2001) and observed the effective antagonism between *Trichoderma* spp. (*T.* spp.) and *Fusarium oxysporum* f. sp. *cubense*.

BOP for CDL (*Penicillium* rot) = Tar>I2>I3>T22>TV>TH3>T-gel>Dar. In dual cultures, *T.* spp. and *Penicillium* spp. were categorized as effective, based on their ability to over grow and inhibit the growth of the pathogens by giving

them a score as per modified Bell's scale. Where R1 = 100% over growth, R2 = 75% over growth, R3 = 50% over growth, R4 = locked at the point of contact (Ramaraju et al 2016). The hyphal interaction between the mycelia of opposite colonies was observed from 4 DAI. In this study at day 5, Tar got R4 (highest GI=52.74%) and Dar got lowest GI of 20.01%.

BOP for CDT (*Alternaria* Leaf Spot) = Dar>T22>I2>I3>TH3>TV>T-gel>Tar. Microscopic examination of dual culture plates revealed that all the eight isolates of *T.* spp. inhibited the growth of CDT. After 4 DAI, *Trichoderma* hyphae coiled around the test pathogen around zone of interaction (ZOI). Later it caused wrinkling of hyphae and Dar isolates completely overgrowth upon CDT. *T.* spp. developed with their sporulation on the *Alternaria* mycelium. *Trichoderma* conidia penetrated into the *Alternaria* mycelium and finally induced lysis of the mycelium of CDT (Mohan Venkata; Siva Prasad et al 2018). In such a way, Adarsh Pandey (2010) reported that *T. viride* and *T. harzianum* had controlled the pathogen *A. alternata* by mechanism known as mycoparasitism. Mokhtar and Dehimat (2012) reported that antagonistic fungi *T. harzianum* prevented the spore formation in *Alternaria* spp.

BOP for FO (*Fusarium* wilt) = TH3>T22>T-gel>Dar>I2>I3>Tar>TV. All T-isolates caused little to moderate inhibition of mycelial growth of *F. solani* f. sp. *Melongenae*. Taking day 5 reference, GI of *F. solani* f. sp. *melongenae* varied from 25.90(TV)-46.75(TH3)%. Present findings are supported the results of earlier workers. Abou-Zeid et al (2007) found that biological agents (*T. viride* and *T. hamatum*) significantly inhibited the radial mycelial growth of the pathogenic fungi- *Fusarium oxysporum*. Similarly, Altinok et al (2015) found that T16 and T23 (*T. harzanium*) significantly inhibited the radial mycelial growth of the *Fusarium oxysporum* f. sp. *melongenae* 70 and 72% respectively (Altinok; Handan and Erdogan; Oktay 2015).

BOP for FS (*Fusarium* wilt) = Tar>I2>T-gel>I3>T22>TV>TH3>Dar. All T-isolates caused little to moderate inhibition of mycelial growth of *F. solani* f. sp. *Melongenae*. Taking day 5 reference, GI of *F. solani* f. sp. *melongenae* varied from very little 12.89%(Dar)-50.84%(Tar). Present findings are supported the results of earlier workers. Sikder et al (2017) reported that the

inhibition of mycelial growth of T-isolates on *F. solani* f. sp. *melongenae* varied from 69.2-86.7%. Chakraborty et al (2009) reported that *T. harzianum* and *T. viride* were specifically found to have reduced the disease incidence of *Fusarium* wilt of eggplant caused by *Fusarium solani* up to 86% and 83%, respectively.

BOP for IK1 (Rhizome rot) = Tar>I2>I3>T-gel>Dar>TV>TH3>T22. Control plates of IK1 showed regular radial growth, from day 3, ZOI were observed in some isolates while some takes 4 days. These direct confrontation of T-isolates against the IK1 in vitro on PDA medium showed that when the mycelium of both cultures came in contact with each other the hyphal growth of the pathogenic fungus were found to be inhibited by the hyphae of T-isolates. A clear zone of interaction was formed in all *Trichoderma*-pathogen combination (Moqdad and Sajid Salahuddin 2014). Taking day 5 reference, mycelial GI varied from 29.62%(T22)-29.62%(Tar). Similar finding were observed by Moqdad and Sajid Salahuddin (2014) that *T. harzianum* 1 and *T. harzianum* 2 had mycelial GI of 21.05 and 28.95% respectively against *Acremonium* spp.

BOP for IK2 (Rhizome rot disease) = I2>Tar≥I3>T22>T-gel>TH3>TV>Dar. In dual culture, all eight T-isolates inhibited the growth of IK2 (*Fusarium oxyporium* f. sp. *zingiberi*), showing considerable increase in their biocontrol potency with time. It was observed that the different T-isolates exerted varied degree of stress on IK2 in dual culture. Among the antagonists, usually GI% increases with time and at day 5, I2 show maximum overgrowth plus colony degradation on plate (100% GI) while Dar has had as little as 26.45% GI of mycelium. Microscopic examination also showed that the hyphae of T-isolates coiled around the hyphae of IK2 followed by cell wall degradation and cellular coagulation of the pathogen (Kevimeo and Tiameren 2013). Mycoparasitic behaviours involving envelopment and coiling around of the hyphae of *F. oxysporum* by *Trichoderma* species has been reported by Otadoh et al (2011). Similar results were also observed by Gangrade (2006) showing *T. harzianum* plus IK2 and *T. viride* plus IK2 had average colony of IK2 diameter after 7 days (in mm) of 14.5 and 21.33 respectively in comparison to control 44.5.

BOP for MB (Northern corn leaf blight) = I3>Tar>TV>I2>T22>T-gel>TH3>Dar. All the *Trichoderma* isolates tested exhibited inhibition of growth of *Helminthosporium*. Percentage of GI of MB on 5 DAI by Tar, T22, I3, I2, TH3, TV, T-gel, Dar were 36.16, 29.58, 39.75, 29.75, 22.6, 33.33, 25.82, 16.59 respectively. In dual culture technique the mycelium of both the cultures came in contact with each other on 3 DAI. Four days after inoculations the hyphal growth of *Helminthosporium* was found to be inhibited by the hyphae of T-isolates. Similar results were observed by Jegathambigai et al (2009) shows the T-isolates tested against growth of *Helminthosporium* and growth inhibition of *Helminthosporium* by *T. harzianum* 1, *T. harzianum* 2*, T. viride* 1*, T. viride* 2*, T. viride* 3 found to be 79.18%, 69.03%, 83.75%, 82.99%, 74.11% respectively.

BOP for PDT (Dry Rot of Potatoes) = I3(40.76%)>I2>Dar>TV>Tar>T22>T-gel>TH3(20.84%). In dual culture, all eight T-isolates inhibited the growth of PDT at minimum to moderate amount. Similar results were reported by Riad et al (2012). They stated all tested isolates of *F. sambucinum* shows different degrees of growth inhibition. The highest reduction in the linear growth was found after 8 days of incubation with *F. sambucinum* No.1 (88%), where the least reduction was recorded with *F. sambucinum* No.3 (70%). *T. harzianum* inhibited the linear growth of all tested isolates of *F. sambucinum* by overcoming their growth in petri dishes. Since our investigation only last for five days in contrast to Riad et al (2012) plus ZOI were start to form only after 4 DAI, T-isolates overgrowth on PDT were not observed. Above statements proved that mycoparasitism of T-isolates on PDT takes more time than many phytopathogen and could be seen only after 8 DAI as mentioned in Riad et al (2012).

For RS (Sheath blight of rice), the order of sequence of BOP were: Dar>Tar>I2>I3>T-gel>TV>TH3>T22. T-isolates shows ZOI clearly at 3 DAI and took 5 DAI to overgrowth RS except T22 and TH3. For RSR, (*R. solani* disease for mustard green) the order of sequence of biocontrol potential were: Tar=I2=T-gel>Rest isolates. T-isolates shows ZOI clearly at 3 DAI and Tar, I2, T-gel got full overgrowth just at 4 DAI and rest of other isolates got full overgrowth at 5 DAI. This shows T-isolates has had very high antagonistic

properties against RS. In both cases, absence of zone of inhibition between the two cultures were found. Patibanda and Sen (2004) and Swathi (2010) stated that zone of inhibition is an indication of antibiosis by *Trichoderma* against *R. solani*. However, absence of such zone of inhibition in the present investigation indicated *Trichoderma* did not require antibiotic production to colonise or overgrow and antagonise *R. solani.* When both the interacting fungi joined together initially aerial mycelium was produced by *R. solani.* Such formation of aerial mycelium by *R. solani* was more in case of Tar and Dar. This indicated that *R. solani* had no chance to grow beyond T-isolates periphery. The formation of sclerotial bodies by *R. solani* was observed at two points. In case of Tar and Dar, sclerotial bodies were formed only at the point of inoculation and not at interaction zone. In case of other T-isolates, sclerotial bodies were not formed during the same period of incubation, i.e., 5 DAI. The resistant sclerotial bodies are formed under unfavourable conditions (Lee and Rush 1983; Mostapha 2004 and Vijay et al 2009). In the present investigation formation on sclerotial bodies in dual cultured plate signified unfavourable conditions within five days of incubation. Formation of sclerotial bodies at the point of inoculation by *R. solani* and not at interaction zone indicated that *R. solani* succumbed to Dar (for RS) and Tar (for RSR) at a faster pace giving no time to *R. solani* to react to invading them and form sclerotial bodies.

For SR, (Seedling blight diseases) BOP = Tar(73.12%)>I2≥I3>T22>T-gel>TH3>TV>Dar(29%). ZOI appears clearly at 3 DAI. Mycelial growth of pathogen was checked to a little extent with the hyphae of these biocontrol agents which continued its fast growth. Yellow band was noticed at the point of contact between mycelium of the pathogen and biocontrol agent which was clearly visible from underside of the petri plates. In case of T-isolates, at the beginning, it grew together on the same medium. After colonies met, the mycelium of T-isolates grew into areas that have already been occupied by *Sclerotium rolfsii*. The pathogen was checked and its colony was overrun by T-isolates which covers entire plate very quickly. Re-isolation of the same area where pathogen has been growing resulted in the recovery of T-isolates alone. It shows that T-isolates. is capable attacking and killing of *S. rolfsii* in dual culture. Similar result of T-isolates vs SR had been reported by Yaqub and

Shahzad (2005) and Puri et al (1998). Control of *Sclerotium rolfsii* using *Trichoderma harzianum* was reported by several researchers (Muthamilan and Jeyarajan 1996; Ganesan et al 2003; Ganesan 2004; Ganesan and Sekar 2004a). Upadhyay and Mukhopadhyay (1986); Muthamilan and Jeyarajan (1992); Biswasand Sen (2000) and Pant and Mukhopadhyay (2001) reported screening of antagonists against *S. rolfsii* using dual culture method originally described by Morton and Stroube (1955).

For TCD (Gummosis disease), BOP = Tar(46%)>I2>I3>Dar>TV>T-gel>T22>TH3(37.27%). While control plates of TCD (*Macrophomina phaseolina*) showed regular plus very fast radial growth (nearly same efficiency as T-isolates) resulting the first apparent physical contact between *T-isolates* and its host, TCD, occurred on 2 DAI followed by a diversity of interactions plus moderate growth inhibition. Similar results were reported by previous workers, Gajera et al (2012) stated that seven species of *Trichoderma* were evaluated against phytopathogen *Macrophomina phaseolina* by dual culture techniques and observed GI of antagonist *T. koningi* MTCC 796 (T4) (74.3%) followed by *T. harzianum* NABII Th 1 (T1) (61.4%) at 7 DAI plus mycoparasitism of antagonists were observed till 14 DAI (Gajera et al 2012). Khaledi et al (2016) stated that biological control capability of 11 *T. spp.* isolates against *M. phaseolina* (charcoal rot) in dual culture tests was varied from 20.22 to 58.67% (Nima and Parissa 2016).

BOP for TVD (Tomato *Fusarium* Wilt) = T22(57.12%)>I2>Dar>Tar>I3>T-gel>TV>TH3(25.88%) (Table no.15). While control plates of TVD showed regular radial growth resulting the first apparent physical contact between T-isolates and its host, TVD, occurred on 4 DAI followed by a diversity of interactions plus moderate growth inhibition. *Fusarium* spp. is a slow growing species which is also in agreement with this study. In general, the *Fusarium* spp. is characterized as creamish white to creamy, light pink and light purple to violet. Further, the isolate identification was confirmed by macro and micro-conidia characteristics which were thin walled 3-5 septate, fusoid falcate macro conidia with somewhat hooked apex and pedicillate base (Joshi et al 2013). *Trichoderma* colonies grew hastily and readily developed their typical yellow-green color, which aided in their identification from other soil-borne fungi

(Sundaramoorthy and Balabaskar 2013). Identical result were observed by previous workers, Goswami and Islam (2002) observed that biocontrol agent such as *T.* spp. exhibited greater mycelial inhibition of tomato wilt pathogen *F. oxysporum* f. sp. *Lycopersici* (Sikder et al 2013). Sundaramoorthy et al (2013) studied fifteen native isolates of *T.* spp. and screened for their in vitro antagonism against the *F. oxysporum* f. sp. *lycopersici* by dual cultural technique. The results found were ANR-1 inhibited the mycelial growth of *F. oxysporum* f. sp. *lycopersici* to an extent of 53.00 percent followed by KGI-3 (38.12 %), RTM-5 6 (31.11%) and KPI-9 (27.22 %) over control.

For DC's method some common thing were noticed i.e. Joining of both cultures in dual cultured plate 2/3/4 DAI ascertained beginning of interaction between the test fungi. Incubation beyond 3 days resulted in joining of both the test fungi in dual cultured plates with a "gap" of 0-0.8 cm between them. This zone was considered as zone of inhibition by Campbell (1989) who opined that such an inhibition zone can be taken as a clue for the production of antibiotics, thereby screening and selecting antagonists (Parkale 2017).

Two T-isolates were subjected to NVOC's method against 14 isolated soil borne phytopathogen. On day 3, day 5 and day 7 results reveals the following facts:

a) Generally more the concentration of NVOC's, higher the mycelial GI of phytopathogen by T-isolates (Dubey et al 2011; Anita et al 2012).

b) The rate of consumption and effect of NVOC's on phytopathogen varies with time, T-isolates and phytopathogen itself (Moqdad and Sajid 2014) e.g., T-isolates shows certain percent of GI on BDD at day 3 and it GI% decreases on reaching day 5 but again raises up on reaching day 7.

c) Only two local isolates are subjected to these trail resulting in some cases Tar shows higher GI% than Dar and vice-versa (Bell et al 1980).

d) In some cases, T-isolates NVOC's shows maximum GI e.g., at 3 and 5 DAI T-isolates completely inhibit the growth of RS and RSR mycelium while in some cases it doesn't show good effectiveness of GI e.g., at day 7 growth of TCD in tests equals that of control plates (Moqdad and Sajid 2014). In some

cases, NVOC's GI of T-isolates against phytopathogen depends on both time and isolates and shows different degrees of GI potency at respective time e.g., T-isolates vs TVD at 100% concentration shows Tar>Dar at 3 DAI and 5 DAI but reversed on reaching day7 i.e. Dar>Tar (Moqdad and Sajid 2014).

Above mentioned facts were supported by previous workers, (Nagamani et al 2017) reported that, all the T-isolates significantly inhibited the test pathogens by production of non-volatile inhibitors at 10%, 15% and 20% against *R. bataticola* (dry root rot of cheakpea) and shows GI ranged from 82.2% - 91.1% {NVOC's of *T. longibrachiatum* (ATPPE 6)} and *T. harzianum* (KNO 9)} at 20% concentration. Further, they found, *T. asperellum* (ATPU 6) had GI upto 93.3% against SR.

Dubey et al (2011) assessed and characterized the secondary metabolites and enzymes produced *Trichoderma* species and their efficacy against plant pathogenic fungi. They found the secondary metabolites extracted from culture as well as mycelial mass of *Trichoderma* species caused variable inhibition to the plant pathogenic fungi *R. bataticola* and *F. oxysporum* f. sp. *ciceris*.

Dubey et al (2011) identified compounds from the metabolites obtained from culture filtrate and mycelial extract of *Trichoderma* species as in Raut et al (2012) study confirms the potentialiaty of volatile and non-volatile metabolites from antagonistic *T.* spp. against phytopathogenic fungi. The biocontrol activity was tested versus the pathogens responsible for important losses in agriculture, such as: *Fusarium graminearum, Rhizoctonia solani* and *Pythium ultimum*. They had demonstrated that the volatile and non-volatile metabolites produced by the strains *Trichoderma* T36 and T50 displayed inhibitory effects on pathogens growth. The volatile metabolites assay revealed that *Trichoderma* T36 produced a higher inhibitory effect on pathogens growth as comparative with T50. Maximum inhibition (100%) by the volatile compounds occurred in *R. solani* - *Trichoderma* T36 interaction. Mycelial growth of target fungi was retarded by non-volatile metabolites from antagonistic strains. The pathogens growth was significantly restricted at higher concentrations of culture filtrate and a total inhibition of *R. solani* and *P. ultimum* was obtained with 50% (v/v) concentration of filtrate from *Trichoderma* T36. The strain of *Trichoderma*

T36 used in current study was capable to inhibit the growth of fungal pathogens and may be used as an efficient agent to control a broad spectrum of plant pathogens.

Similarly, two T-isolates were subjected to VOC's method against 14 isolated soil borne phytopathogen. On day 5 and day 7 results reveals the similar finding as mentioned in NVOC's method. Those mentioned facts were supported by previous workers investigation.

Amin et al (2010) investigated six isolates of *T.* spp. and tested for their ability to produce volatile metabolites against seven fungal plant pathogens viz., *Fusarium oxysporum* (causing chilli wilt), *Rhizoctonia solani* (causing sheath blight of rice), *Sclerotium rolfsii* (causing collar rot of tomato), *Sclerotinia sclerotiorum* (causing web blight of beans), *Colletotrichum capsici* (causing anthracnose of chilli fruit), *Helminthosporium oryzae* (causing brown spot of rice), *Alternaria brassicicola* (causing *Alternaria* blight of cabbage). Their studies shows that *T. viride* (Tv-1) was most effective in reducing the mycelial growth of *F. oxysporum* (41.88%), whereas, in case of *R. solani, T. viride* (Tv-2) accounted for maximum reduction in mycelial growth (30.58%) and sclerotial production (65.65%). Volatile metabolites from *T. viride* (Tv-1) caused maximum reduction in mycelial growth and sclerotial production in *S. rolfsii* and *S. sclerotiorum*. Maximum inhibition of mycelial growth of *C. capsici* and *A. brassicicola* was recorded with *T. viride* (Tv-1), whereas, in *H. oryzae, T. harzianum* (Th-1) accounted for maximum reduction in mycelial growth (37.16%) (Faheem 2010).

Acetaldehyde was identified tentatively as one of the metabolites of *Trichoderma viride* inhibitory to other fungi. Volatile secondary metabolites have been demonstrated to play a key role in the mycoparasitism of *Trichoderma* and its interaction with plants (Vinale et al 2008). The potential of *T.* spp. to produce many volatile (e.g. pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g., peptaibols) has been reviewed by Reino et al (2008). Microbial volatile organic compounds (MVOCs) appear as intermediate and end products of various metabolic pathways and belong to

numerous structure classes such as mono- and sesquiterpenes, alcohols, ketones, lactones, esters or C8 compounds (Korpi et al 2009).

Moya et al (2017) performed evaluation of the antagonistic effects of VOC's released by eight strains of two *Trichoderma* species against *Pyrenophora teres* Drechsler, the causal agent of barley net blotch and found that all *Trichoderma* strains inhibited mycelial growth of the pathogen in a range of 3 to 32%, showing weak and unpigmented mycelia with vacuolization. They performed VOCs extraction and identification using gas chromatography and mass spectrometry and through different methodologies. Their major findings about VOC's of T-isolates were given by Raut et al (2014) study also confirms present investigation as mentioned in NVOC's methods.

G% of brinjal in case of FO organism were in following order: I2>Tar>T22>Dar>I3>TH3=TV>T-gel>Control. Biocontrol potential for reduction of PDI were found to be Tar=T-gel>l3=l2>Dar>TH3>Control>TV. RL, SL and NOL of bioprimed seed with T-isolates generally higher than control. G% of brinjal in case of FS organism were in following order: Tar>Dar>I2>TV=T-gel>I3>T22>Control. Biocontrol potential for reduction of PDI were found to be Tar>I2>T22=I3>T-gel>TH>Dar>TV. RL, SL and NOL of bioprimed seed with T-isolates generally higher than control.

The results are in conformity with the results of several workers as described below:

Field experiments carried out by Faruq et al (2014) test the efficacy of *Trichoderma harzianum* T22 and some selected soil amendments viz. poultry waste, coco-dust, vermi-compost, ash, saw-dust, khudepana (Azolla pinnata), cowdung and solarized sand against *Fusarium* wilt disease of eggplant. They applied treatments in the soil at 15-30 days before transplanting. They recorded wilt incidence from 55 DAT to 95 DAT. They found that all the treatments appreciably reduced wilt incidence at different days after transplanting. In general, the wilt incidence was higher on control plot where no treatments were used. The most effective treatment was T22 followed by poultry waste in terms of suppressing wilt incidence, increasing plant growth and fruit yield. Poultry waste and vermi-compost also showed promising performance against the

disease. The applied treatments enhanced plant growth and increased fruit yield over untreated control treated plot (Abbas et al 2017).

Kareem et al (2017) performed greenhouse experiment shows biocontrol efficiency of *T. harzianum* (TF) against *F. oxysporum* resulting significant reduction in eggplant *Fusarium* wilt incidence compared with pathogens control treatments. They found pathogen *F. oxysporum* treatments with TF showed that all the treatments (TF) achieved high germination rate for eggplant seeds compared with control treatment, while the treatment TF13 was significantly superior than all other treatments in number of leaflets, stem height, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and root size which recorded 8.67, 19.00cm, 20.33gm, 4.73gm, 12.00gm, 1.53gm, 17.67cm^3 respectively. They mentioned *Trichoderma harzianum* significantly inhibited the growth of *F. oxysporum* and enhanced eggplant plant growth; this may be attributed to fact that *Trichoderma* species produce extracellular cellulase and pectinase enzymes that are capable of hydrolyzing the cell walls of pathogenic fungi (Marco et al 2003). According to Leiter et al (2004) and Kawabe et al (2011) this fungus has the enzyme Glucose-methanol choline oxidoreductases which play significant roles in the antibiosis against plant pathogenic fungi and biological control of plant diseases. The enzyme biosynthesis conception as a mechanism of biocontrol has been expanded to include synergism between enzymes and antibiotics (Schirmbock et al 1994). Moreover, the suppression of *F. oxysporum* growth by *T. harzianum* may be due to direct interaction between them, as in mycoparasitism, which involves physical contact supported with its enzymes and antibiotics (Benitez et al 2004).

Rice is cultivated under water logged conditions. S.R. and R.S. survives in soil as sclerotial bodies during off season which float on water and cause infection during next season. Being aerobic they cannot survive under submerged conditions. Further, they also infect the crops from the field bunds where grasseous hosts serve as collateral hosts. *Trichoderma* is also aerobic and cannot survive under submerged conditions. In the present investigation efforts to get the sheath/Seedling blight disease on rice plants through soil inoculation of sclerotonia on soil directly to give consistent disease development. Present

work investigates the biocontrol potency of T-isolates against them (Soujanya 2011).

DI and DSI were found more on control than bioprimed rice. For Hardinath rice BOP of T-isolates was found to be Tar≥I2>Dar>TV>T-gel>I3>TH3>Control. Similarly the order of BOP of T-isolates for Chaite-2 rice were Tar>I2>I3>T22≥Dar>TV>TH3>T-gel>Control. G% of Hardinath rice has following order: Tar=l2=I3>TH3>TV>T22>T-gel=Dar>Control while Chaite-2 had following order: Tar=I2=Dar>TH3=T-gel>TV>Control>T22. Result shows Hardinath is more resistant to seedling blight of rice than Chaite-2.

The results are in conformity with the results of several workers as described below:

Vishwanathet al (2008) isolated 17 *T.* spp. from 64 soil samples collected from rhizosphere of infected acid lime trees. Results of the experiment to evaluate the antagonistic activity of the 34 *Trichoderma* isolates revealed that the isolates SP2, WP5, GW5, GP3, CPK4 and RP4 were highly effective against *Sclerotium rolfsii* as they recorded 78.79 to 82.96 percent GI against the pathogen.

In present study, the effect of *T*. spp. on *S. rolfsii* was studied. The above findings are in agreement with the results obtained by Barakatet al (2006) in which they studied biological control of *S. rolfsii* by using indigenous *Trichoderma* isolates from Palestine. Similarly, Jegathambigai et al (2010) studied the effect *of T.* spp. on *S. rolfsii,* the causative agent of collar roton Zamioculcas (Parkle 2017).

Pacheco et al (2016) demonstrated efficacy of *Trichoderma* isolates to control *sclerotium* wilt (*Sclerotium rolfsii*) of common-bean (*Phaseolus vulgaris*) and used two isolates of *S. rolfsii* UB 193 and UB 228. Sixty-five *T*. spp. isolates were tested and the following ones were selected in vitro and in vivo tests: 5, 11, 12, 15, 102, 103, 127, 136, 137, 1525 (*T. longibrachiatum*), 1637 (*T. reesei*), 1642, 1643 (*T. harzianum*), 1649 (*T. harzianum*), 1700 (*T. asperellum*) and EST 5. The most promising isolates were identified by Sequencing of the

internal transcribed spacer regions ITS1, ITS2, and the 5.8s rRNA genomic region, using the ITS5 and ITS4 primers and compared with sequences in the National Center for Biotechnology Information (NCBI) database. These selected isolates 1649 (*T. harzianum*), 1525 (*T. longibrachiatum*) and 1637 (*T. reesei*) were tested for evaluation of sclerotial germination inhibition under laboratory conditions, and greenhouse conditions. The *Trichoderma* isolates 1649, 1525 and 1637 were more efficient in reducing *sclerotial* germination. In addition to 1649, 1525 and 1637, the isolates 5, 12, 102 and 1525 (*T. longibrachiatum*) significantly reduced de amount of diseased bean plants under greenhouse conditions.

Most notable things was Mohiddin et al (2010) reported *Trichoderma* is Considered Super Hero (Super Fungus) Against the Evil Parasites as per their review findings.

Germination (G) % of Hardinath rice had following order Tar=I2=I3>TH3=Dar>T22>T-gel=Control. Higher DI and DSI were observed on control than bioprimed rice. Biocontrol potential of T-isolates for DI and DSI had following order: Tar=I2=TH3>TV>I3>Dar>T-gel>Control and Tar=T22=I2=TH3=TV>Dar>I3>T-gel>Control respectively. Result shows Hardinath rice is generally resistant to sheath blight of rice. *Rhizoctonia* infection to rice was not observed on the early days of seedling and gradually shows infection as the time passes.

Singh et al (2010) investigated the combined application of fungal and bacterial antagonists *(Trichoderma harzianum & Pseudomonas fluorescens-27)* applied as seedling root dip and foliar spray against the sheath blight of rice caused by *R. solani* under glass house conditions. They found seedling root dip with *T. harzianum* + *P. fluorescens-27* and foliar spray with *T. harzianium* was the most effective in reducing the disease severity (45.8-48.0 %) and disease incidence (60.1-61.5 %) followed by seedling root dip with *T. harzianum* + *P. fluorescens-27* and foliar spray with *P. fluorescens-27* which resulted in the reduction of disease severity (43.0-43.4%) and disease incidence (56.7-56.9%), respectively (Kumar et al 2010).

Crop damage caused by sheath blight can decrease yield by upto 45%. Successful biological control of sheath blight by the bioagent *T.* spp. has been recorded and studied an experiment to evaluate the potential of indigenous *T.* spp. against *R. solani* in vitro as well as in the glass house (Abbas 2017).

Silva et al (2012) studied the T-isolates from Amazon forest soil samples, identify their potential for sheath blight (*R. solani*) suppression in rice, and promote plant growth. They found four out of the 13 isolates (T.06, T.09, T.12, T.52) which showed in vitro potential were evaluated through assays under greenhouse conditions. These four isolates also showed reduced *R. solani* mycelial growth and *sclerotial* viability (>50%) and were positive for phosphate solubilization and cellulose degradation. They significantly reduced sheath blight severity when applied as seed treatment, substrate incorporation or foliar spray. However, the preventive and curative sprays were the most efficient method, reducing sheath blight severity by 43% and the area under the disease progress curve by 45%. Isolates T.12 and T.52 applied in substrate treatment increased aerial and root dry weight by 61.2 and 32.9%, respectively. These two isolates showed potential as growth stimulants and can be used as novel biological products and bioinoculants in agriculture for increasing grain yield. Daghman et al 2006 reported the use of *Trichoderma harzianum* (UPM40) with compost (OPTCD) as the food-base carrier to control of *Rhizoctonia* damping-off in leaf mustard (Brassica rapa L). They found seeds sown in *Rhizoctonia*-infested soil treated with UPM40 dry preparation and fungicide (Brassicol®) gave significantly higher seedling emergence. The losses in emergence were only 2.83 and 3.71%, respectively, as compared to 21.99% in the untreated soil. This result were comparable to our study and shows BOP of T-isolates against *R. solani* damping off diseases of mustard green.

The major advantage of antibiosis observed in the current investigations with the metabolites (volatile or non-volatile) are the inhibitory substance produced by antagonists and it is not necessary for them to be in physical contact with pathogen for inhibition of mycelial growth. Several workers reported the inhibitory effect of volatile and non-volatile substances produced by *T.* spp. on several soil borne plant pathogens (Dennis and Webster 1971a; 1971b;

Hutchinson and Cowan 1972; Upadhyay and Mukhopadhyay 1983 and Mathur and Bhatnager 1994). The inhibitory activity of *T.* spp. might be due to diffusible metabolites secreted by them against the pathogen for inhibition of mycilial growth. Reduction in radial growth of *R. solani* and *S. rolfsii* was observed to be significant when PDA amended with the culture filtrates of *T.* spp. However, *R. solani* and *S. rolfsii* in control gave thick and profuse mycelial growth compared to other treatments. The culture filtrates obtained after growing the *T.* spp. on Potato dextrose broth (PDB) and Czapek's (dox) broth (CDB) *viz,* high-speed (15000 rpm for 10 min) in the centrifuger. Reduction of the mycelial growth of *R. solani* and *S. rolfsii* was maximum in both undiluted top and bottom fractions of the high speed centrifugation. The top and bottom fractions of the low speed centrifugation were least effective. Among the broths tested for production of anti *S. rolfsii* and *R. solani* metabolites in filtrate by *Trichoderma* spp, potato dextrose broth recorded high production of inhibitory substances i.e. PDB>Czapek dox agar. In high speed centrifugation both undiluted top and bottom fractions were equally effective on the test pathogen. This may be because of the separation of certain metabolites with different molecular densities at the time of centrifugation that are specifically toxic to the growth of the pathogen. Where as in case of low speed centrifugation the undiluted top and bottom fractions were not so effective as compared to the high speed centrifuged fraction. Further low centrifugation will not separate out the effective component to the pathogen. As above results also suggested that the PDB was good to produce metabolites by *T.* spp. The inhibitory activity of the undiluted culture filtrate was not affected by the temperatures. Whereas in control *R. solani* and *S. rolfsii* produced thick and profuse mycelium. This clearly indicated that antifungal substances produced in the filtrate ware thermostable (Siddanagoudar 2005).

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Eco-friendly disease/pest resistance strategies are modern concern in modern and sustainable agriculture. Bio-control agents have emerged as a new strategies of managing plant disease by inducing systamatic resistence plus growth promotion in plants against diseases. Biocontrol is an alternative, eco-friendly means for managing plant diseases/pest. Restriction on pesticides use and widespread emergence of pathogen resistence has increased global demand of biopesticides. It can be concluded from the present studies that *Tricoderma* isolates can serve as good option for biocontrol against phytopathogenic fungi based on its inhibition percentage in vitro experiment supported by positive PDIC values in vivo. These facts are concluded on the basis of following results found on this investigations:

- Isolates Tar and I2 should be preferred over all other tested isolates for treating the seed along with the other agrochemicals. All other tested isolates shows inconsistent and lower performance in vivo and vitro than them.
- The present study provides preliminary information on the antagonistics properties of *Trichoderma* and correctly identifying the fungal antagonists. Out of the eight *Trichoderma* isolates, two (Tar, I2) were highly producer secondary metabolites and showed strong antagonistic activity against fourteen different fungal pathogens of diseased plants.
- *Trichoderma* is a potent BCA's and used extensively for pre/post-harvest disease control; used successfully against various pathogenic fungi belonging to various genera, viz. *Fusarium, Phytopthora* etc. These facts are supported by present investigation where result sections shows positive values of percent diseases index increase or decrease over control (PDIC) due to various mechanisms involving *Trichoderma*-phytopathogen interaction described in literature review section.

6.2 Recommendations

Based on the results finding of the experiment, the recommendations are made as follows: -

- *Trichoderma*-phytopathogen shows various degrees of interaction and reveals different degrees of growth inhibition of phytopathogen. Some phytopathogen shows more resistant to biopesticides. In such cases, we recommend the local farmers to use biopesticides (e.g. *Trichoderma*) with lower doses of fungi/pesticides because their synergistic effect resulting the suppression of competitive soil microflora to overcome resistant phytopathogenic problems in field keeping no harm for eniviromental condition.
- Taking similar objective other antagonist (eg., *Glocladium*) can also taken into consideration for their antagonistic properties against other phytopathogen in future.
- In some cases, we recommend the farmers to use the transgenic plants (e.g. introduction of endochitinase gene from *Trichoderma* in tobacco, potato etc) on their fields because it shows increased resistance to fungal phytopathogenic diseases. Selected transgenic lines are highly tolerant to foliar pathogens such as *Alternaria alternata, A. solani*, and *Botrytis cinerea* as well as to the soil-borne pathogen, *Rhizoctonia* spp., *Sclerotium* spp. Transgenic plants has benefits over normal one as they are pesticides resistant (cancels cost on pesticides), drought resistant (in case of low water availability) and very competitive to adsorption of soil nutrient.
- We recommended biopesticides industry for mass production of only those BCA's (e.g. *Trichoderma*) isolates with expanded host range, temperature, moisture parameters and strains with better storage qualities.
- Still today, popularization of bio-pesticides is very slow as compared to chemicals and only 0.035% bio-pesticides are available in pesticides market share of Nepal as of 2011/12 (Dhital et al 2015). Therefore, government should make public awareness program from ground level as well as law enforcements regarding exessesive uses of agrochemicals to

enhance quality and healthy crop productions ensuring the elimination of side effects of synthetic pesticides/fertilizers/agrochemicals making healthy human heath plus eco-friendly enviroments.

- We strongly recommend Nepal Government to invest required budget on agriculture microbiologist and necessary facility needed to them to minimized crop losses and increase agriculture yield. Since, these man powers are directly related to research and development sector, Nepal government should facilitate research project on them to minimize cost of importing foreign agriculture technology plus keeping Nepalese agriculture up to date as in 21th century standard. Most important one is Agriculture microbiologist can work and find solution in extreme hazardous conditions during plaques of epi/endemic diseases that's why Nepal government should open separate agriculture divisions regarding Agriculture microbiologists.
- Nowadays investigator confirm his/her findings with the help of genetic tool i.e. 16s RNA. But developing countries like Nepal could not afford the costing price of it mostly, unless very necessary to do so. Thus, it is recommended to Ministry of Agriculture and Livestock Department of Nepal for detailed study of this dissertations findings to find out mechanism of antagonist action over phytopathogen as well as appropriates method of application of them by farmers on ground level directly.

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APPENDIX: A

Scientific classification of *Trichoderma* spp. and Soil Borne Phytopathogen Used as Model Organism

Position	**Asexual stage (Conidia)**	**Sexual stage (Ascospore)**
Phylum	Dueteromcota	Ascomycota
Sub-Division	Dueteromycotina	Ascomycotina
Class	Hyphomycetes	Pyrenomycetes
Order	Monilliales	Sphariales
Family	Monilliaceae	Hypocreaceae
Genus	*Trichoderma*	*Hypocrea*

Position	***Alternaria***	***Penicillium***	***Fusarium***
Kingdom	Fungi	Fungi	Fungi
Division	Ascomycota	Ascomycota	Ascomycota
Class	Dothideomycetes	Eurotiomycetes	Sordariomycetes
Order	Pleosporales	Eurotiales	Hypocreales
Family	Pleosporaceae	Trichocomaceae	Nectriaceae
Genus	*Alternaria*	*Penicillium*	*Fusarium*

Position	***Acromonium***	***Exserohilum***	***Rhizoctonia***
Kingdom	Fungi	Fungi	Fungi
Division	Ascomycota	Ascomycota	Basidiomycota
Class	Euascomycetes	Dothideomycetes	Agaricomycetes
Order	Hypocreales	Pleosporales	Cantharellales
Family	Hypocreaceae	Pleosporaceae	Ceratobasidiaceae
Genus	*Acromonium*	*Exserohilum*	*Rhizoctonia*

Position	***Sclerotium***	***Macrophomina***
Kingdom	Fungi	Fungi
Division	Basidiomycota	Ascomycota
Class	Agaricomycetes	Dothideomycetes
Order	Atheliales	Botryosphaeriales
Family	Atheliaceae	Botryosphaeriaceae
Genus	*Athelia* (sexual stage)	*Macrophomina*

APPENDIX: B

Habit and Habitat of *Trichoderma*

2.4 Biodiversity of *Trichoderma*

The genus *Trichoderma/Hypocrea* can be characterized with high adaptability to temperate and tropical soils, commonly found in variety of soil types such as agriculture, forest, prairie, saline and desert soils in all climatic zones. Besides this, it is also found colonizing roots, litter, decaying/decorticated wood, decaying bark and various plant materials at all climatic zones/latitudes (Danielson and Davey 1973).

2.4.1 Natural soils, Decaying wood and Plant Material

Danielson and Davey (1973) surveyed the *Trichoderma* propagules in a variety of the forest soils in the southeastern U.S and Washington State and identified the isolates as *T. hamatum, T. harzianum, T. koningii, T. polysporum, T. pseudokoningii* and *T. viride. T. hamatum* and *T. koningii* were reported as the most widely distributed species aggregates while *T. harzianum* was reported to be characteristic of warm climates. *T. polysporum* and *T. viride* were found to be largely restricted to cool temperate regions; while *T. hamatum* and *T. pseudokoningii* were the dominants forms under excessive moisture conditions. Vajna (1983) survey *Trichoderma* spp. from dead wood of apple twigs, oak and cork wood samples collected from Hungary, however the isolates as *T. aueroviride*, *T. koningii*, *T. harzianum*, *T. longibrachiatum*, and *T. viride* were identified based on morphological and cultural characteristics only. In a recent study, Vasanthakumari and Shivanna (2011) reported the isolation, morphological and cultural characteristics based identification of *Trichoderma asperellum, T. koningii, T. harzianum* and *T. viride* from the rhizospheric plane of grasses of the subfamily *Panicoideae* in the Lakavalli Region of Karnataka, India. ITS barcoding based identification of 12 taxa (*T. asperellum, T. atroviride, T. brevicompactum, T. citrinoviride, T. erinaceum, T. hamatum, T. koningiopsis, T. harzianum, T. reesei, T. spirale, T. stromaticum, T. vermipilum* and *T. virens*) from Chinese forest soil explored by Sun et al (2012). *Trichoderma* spp. may also be sensitive to environmental pollution as indicated by low rate of recovery

of *T. viride* from coniferous forests that had been subjected to alkaline dust. The presence of CO_2 has been reported to favour growth of *Trichoderma*. Apart from these factors, the iron content of the soil, HCO_3^-, salt and organic matter content and presence and absence of other microbes in soil are also important determinants of microsite preference by *Trichoderma* spp. (Papavizas 1985).

2.4.2 Agricultural Habitats

In agricultural ecosystem *Trichoderma* spp. can be isolated and showing positive effects on cultivated plants such as biological control of phytopathogens, increasing nutrient availability and uptake, inducing systemic resistance, promotion of plant growth, improving crop yields and degrading xenobiotic pesticides (Harman 2006). Diversity of *Trichoderma* spp. was very high in wheat fields of China (Liang et al 2004). In another study, 11 *Trichoderma* spp. were identified by ITS barcoding from wheat rhizhospheric soil of winter season in Hungary comprising *T. atroviride, T. brevicompactum, T. gamsii, T. harzianum, T. koningiopsis, T. longibrachiatum, T. pleuroticola, T. rossicum, T. spirale, T. tomentosum* and *T. virens* (Kredics et al 2012).

2.4.3 Endophytes

Trichoderma species have the ability to utilize a wide range of compounds as sole carbon and nitrogen sources and can utilize mono/di/poly-saccharides etc. for carbon with ammonia being the most preferred source of nitrogen. The members of *Trichoderma* are generally considered to be aggressive competitors although this trait has also been found to be species dependent (Parkle 2017). Certain strains of *Trichoderma* can also colonize the plant roots and take part in symbiotic relationship with several plants such as cocoa (*Theobroma cacao*), rubber tree (*Hevea* spp.), Maire's Yew (*Taxus mairei*) and banana (*Musa acuminata*). Zhang et al (2007) described *Trichoderma taxi* from *Taxus mairei* tree in China, Chaverri et al (2011) demonstrate *T. amazonicum* as a novel isolate from rubber tree (*Hevea* spp.). Six different banana endophytic *Trichoderma* spp. reported by *TrichO*KEY identification (Xia et al 2011), among which four species: *T. asperellum, T. brevicompactum, T. harzianum* and *T. virens* found inside the roots while two species: *T. atroviride* and *T. koningiopsis* were

detected on root surface, while Samuels et al (2012) described *T. solani* as an endophyte in tubers of *Solanum hintonii* in Mexico.

2.4.4 Mushroom-Related substrata

The green mould disease caused by *Trichoderma* species showed a negative economic impact in the commercial production of *Agaricus bisporus* (button mushroom) and *Pleurotus ostreatus* (oyster mushrooms). Hatvani et al (2007) demonstrate green mould affected oyster mushroom in Hungary were *T. asperellum, T. atroviride, T. longibrachiatum* and one undescribed species *Trichoderma* sp. DAOM 175924, which represented 90% of the isolates, Szczech *et al*. (2008) revealed the dominance of *T. harzianum* was found to cause deleterious infections of green mold on Polish mushroom farms. In Poland, seven mushroom associated *Trichoderma* species (*T.aggressivum, T. atroviride, T. citrinoviride, T. harzianum, T. longibrachiatum, T. virens,* and *T. viride*) were identified with *T. aggressivum* as most abundant species (60% of the isolates) (Błaszczyk et al 2011) while Kim et al (2012) *described Trichoderma mienum as a new* species of the Semiorbis clade isolated from *Pleurotus ostreatus* and shiitake bed logs in Japan.

APPENDIX: C

Culture Media Used in Research

A) Potato Dextrose Agar

Ingredients	Amounts
Peeled and Sliced Potato	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 ml
Ph	6.5 ± 0.2

Required amount of peeled potato was cut into small pieces and boiled in 500 ml of distilled water for 15 minutes and filtered through muslin cloth to have desired extract. Thereafter, 20 g of dextrose and 20 g of Agar-agar were dissolved in 500 ml boiling water, then Potato extract was added and mixed thoroughly by stirring with glass rod to have homogenized mixture. After few minutes of boiling 200 ml was transferred to 500 ml capacity flasks. The pH of the medium was adjusted to 7.0 ± 0.2 and plugged with non-absorbent cotton and autoclaved at 15 lbs p.s.i. at 120°C for 20 minutes

B) Potato Dextrose Broth

Ingredients	Amount
potato	200 g/l
Dextrose	20 g
Distilled water	1000 ml
ph	6.5 ± 0.2

C) Water agar

Ingredients	Amount
Agar	20 g
Distilled water	1000 ml

D) *Trichoderma* Selective Medium (TSM)

Ingredients	Amount (g/l)
Glucose	3.0
MgSO4.7H2O	0.20
K2HPO4	0.9
NH4NO3	1.0
KCl	0.15
FeSO4.7H20	1.0
MnSO4.4H2O	0.65
ZnSO4.7H2O	0.90
Agar	20.0
ph	5.5 ± 0.2

Autoclave at 15 lbs for 15 min.

Before pouring the TSM a final ingredient was added: 0.5 mL of 5 mg mL^{-1} chloramphenicol solution (0.25 g of chloramphenicol dissolved in 50 mL 99.99% Ethanol). The nutrient ingredients such as MgSO4 (0.2 g), K2HPO4 (0.9 g), KCl (0.15 g), NH4NO3 (1 g) and glucose (3 g) were added to 1000 ml of boiling water and then Agar (20 g) was melted directly in this boiling nutrient solution. This was filled into 250 ml conical flasks (150 ml per each), which were plugged with non- absorbant cotton and autoclaved at 15 psi for 15 min. before plating the medium remaining ingredients like Chloramphenicol (0.25 g), captan (0.02 g), metalaxyl (0.3 g), PCNB (0.2 g) and Rose Bengal (0.15 g) were added to the medium, mixed thoroughly and then poured into Petriplates.

E) Czapek Dox Broth (liquid medium)

Ingredients	Amounts
Sodium nitrate ($NaNO_3$)	2 g
Dipotassium hydrogen phosphate (K_2HPO_4)	1 g
Magnesium sulphate($MgSO_4$)	0.5 g
Potassium Chloride (KCl)	0.5 g
Ferrous sulphate ($FeSO_4$)	0.01 g
Sucrose	30 g
Distilled water	1000 ml

The chemicals listed above except sucrose were heated in a water bath (1000 ml distilled water) for 15 min. after cooling sucrose (30 g) was added to the nutrient solution. Culture medium was filled in 250 ml Erlen Meyer flasks @ 50 ml per flask, plugged with non-absorbant cotton and autoclaved at 15 psi pressure for 15 min.

F) Czapek- Dox Agar (solid medium)

Composition: To the Czapek- Dox broth 20 g / L agar was added. The final volume was made upto 1000 ml filled in 250 ml. Erlen Meyer flasks and was autoclaved.