EVALUATION OF *Trichoderma harzianum* AS A BIOCONTROL AGENT ON *Fusarium* WILT OF TOMATO GROWN IN EASTERN NEPAL

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:
Romika Shrestha
T.U. Reg. No.: 5-2-459-0006-2011
Roll No.: MB 440/072
Dharan
2019

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE & TECHNOLOGY
Central Campus of Technology
Department of .....
Phone No. : 025-520228
025-526530
Post Box No. 4
DHARAN-14, HATTISAR
SUNSARI, NEPAL

Ref. .....

Date : .....

RECOMMENDATION

This is to certify that **Mrs. Romika Shrestha** has completed this dissertation work entitled **"Evaluation of *Trichoderma harzianum* as a biocontrol agent on *Fusarium* wilt of tomato grown in Eastern Nepal"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (**Agriculture**) under my supervision. To my knowledge, this work has not been submitted for any other degree.

.....

Shiv Nandan Sah
Assistant Professor
Department of Microbiology
Central Campus of Technology
Hattisar, Dharan, Nepal

Date: ...../...../.....

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE & TECHNOLOGY
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Phone No. : 025-520228
025-526530
Post Box No. 4
DHARAN-14, HATTISAR
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Date : .....

CERTIFICATE OF APPROVAL

On the recommendation of Mr. Shiv Nandan Sah this dissertation work of **Mrs. Romika Shrestha** entitled **"Evaluation of *Trichoderma harzianum* as a biocontrol agent on *Fusarium* wilt of tomato grown in Eastern Nepal"** has been approved for the examination and is submitted for the Tribhuvan University in partial fulfillment of the requirements for M.Sc. degree in Microbiology (Agriculture).

.....
Dr. Kamana Sahani
Programme Coordinator
M.Sc. Microbiology
Department of Microbiology
Central Campus of Technology
Tribhuvan University

Date: ...../...../.....

Board of Examiner

Recommended by:

……..
(Supervisor)
Shiv Nandan Sah
Assistant Professor

Approved by:

…….
(Program Co-Ordinator)
Dr. Kamana Sahani
Teaching Assistant
Department of MSc Microbiology
Central Campus of Technology,
Hattisar, Dharan, Sunsari

Examined by:

……..
(Internal Examiner)

……..
(External Examiner)

Date: ……. / …….. / ……

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Romika Shrestha

Date: ..... / ..... /.....

ABSTRACT

Fusarium wilt acts as a limiting factor for the yield of tomato for which *Trichoderma* spp. has been evidently used as a biological control agent against the wilt. However, the efficiency of fungi is not well understood. The main purpose of this study was to investigate *Trichoderma harzianum* isolates towards their contribution to growth of tomato in Eastern Nepal to counteract the effect caused by *Fusarium* wilt. Investigation of *T. harzianum* was performed under in vitro and in vivo conditions against the pathogen (*Fusarium oxysporum*). The most dominant species and causative agent of *Fusarium* wilt was identified as *Fusarium oxysporum.* Three native *Trichoderma* antagonists were isolated from fifteen soil samples of different geographical regions of Eastern Nepal. Under in vitro conditions, the results revealed that *Trichoderma harzianum*, isolate Th-TJ, was found to inhibit effectively the radial mycelial growth of the pathogen by (57%). Under greenhouse conditions, the application of *T. harzianum*, Th-TJ exhibited the least disease incidence. Also, tomato plants treated with *T. harzianum*, Th-TJ isolate showed a significant stimulatory effect on plant height by (78.33 cm) and the dry weight by (3.33 g) of tomato plants, in comparison to untreated control (1.4 g). Therefore, the antagonist *T. harzianum*, Th-TJ is chosen to be the most promising bio-control agent for *F. oxysporum* f.sp. *lycopersici*. On the base of this study, the biocontrol agents of plant diseases might be exploited for sustainable disease management programs to save environmental risk.

Keywords: Biological control; Fungi antagonist; *Fusarium* wilt; *Lycopersicon esculentum*

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BCA	-	Biological Control Agent
CMA	-	Corn Meal Agar
CRD	-	Completely Randomized block Design
DAI	-	Days After Inoculation
DAT	-	Days After Transplantation
DI	-	Disease Incidence
DN	-	Dharan
FO	-	*Fusarium oxysporum*
LPCB	-	Lacto Phenol Cotton Blue
PDA	-	Potato Dextrose Agar
PN	-	Panwari
RB	-	Rose Bengal
T-isolates	-	*Trichoderma* isolates
TJ	-	Tahrara Jungle
TL	-	Tomato Leaves
TSM	-	*Trichoderma* Selective Media

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CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Tomato (*Solanum lycopersicum*) is one of the most important crops in Southeast Asia and Nepal. It is globally cultivated and produced because of is high nutrients value Vitamins (A and C), beta-carotene pigment and minerals like calcium, iron, magnesium, phosphorous etc. (Abdullah et al 2013). It is also rich in medicinal values (Chavan et al 2011) and is very well reported to have antiseptic properties against intestinal infections. Being rich source of lycopene, tomato is used in the treatment of cancer; especially the prostate cancer (Giovannucci, 1999). Tomato belongs to the family *Solanaceae* and is an important nursery-based vegetable crop cultivated for its fleshy fruits. This fruit can be eaten raw or cooked with various ingredients. Tomato is known as productive as well as protective food. It is considered crucial in large production of processed products such as ketchup, pasta sauce, tomato juice, soup, and many ready-to-eat products (Subramanian, 2016). Tomato is also known as the poor man's apple in Nepal with an average national consumption of 11.97 kg/person/year (Ghimire et al 2017). It is cultivated in about 20,000 hectares (ha) in Nepal and around 0.3 million metric tons (MT) tomato is produced annually in the country (MoAD, 2014). The major tomato growing countries are China, USA, Italy, Turkey, India and Egypt. Tomato is affected by many pathogens and one of them is *Fusarium* wilt.

Most *Fusarium* species are soil fungi and have a worldwide distribution. Some are plant pathogens, causing root and stem rot, vascular wilt or fruit rot. Currently the genus *Fusarium* comprises at least 300 phylogenetically distinct species, 20 species complexes and 9 monotypic lineages (Balajee et al 2009, O'Donnell et al 2015). Most of the identified opportunistic *Fusarium* pathogens belong to the *F. solani complex*, *F. oxysporum complex* and *F. fujikuroi* complex. Less frequently encountered are members of the *F. incarnatum-equiseti*, *F. dimerum*and, *F. chlamydosporum* complexes, or

species such as *F. sporotrichioides* (O'Donnell et al 2015, Van Diepeningen et al 2015).

Fusarium wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* is a devastating disease in major tomato growing regions worldwide and has been reported in at least 32 countries (Jones et al 1991). First symptoms are yellowing of the foliage, beginning with the lower leaves and working upward. Yellowing often begins on one side of the vine. Infected leaves later show downward curling, followed by browning and drying. The top of the vine wilts during the day and recovers at night, but wilting becomes progressively worse until the entire vine is permanently wilted. Vascular browning can be seen in infected stems and large leaf petioles. Affected plants and their root systems are stunted. The degree of stunting depends upon time of root infection. Plants infected when they are young will be more severely stunted than plants infected at a later stage (Pawan Kumar et al 2018).

The pathogen is soil borne and persists for many years in the soil without a host. *Fusarium* fungi survive in the soil or associated with plant debris for up to ten years (Baker & Snyder 1970). Most infections originate from the fungus associated with infected tomato debris. The fungi enter the plants through their roots and are then spread throughout the plant by the plant's water-conducting vessels. Disease development is favored by warm temperatures (for example, 27–28°C), dry weather, and acidic soil (pH 5–5.6). Rapidly growing, highly succulent tomato plants exposed to fertilization with ammonium nitrate are especially susceptible to the disease. The fungus can be disseminated by infected seed or by transplants grown in infested soil. The fungus can be introduced into a field on contaminated equipment, training stakes, packing crates or shoes. Soil particles from infested fields may be blown into disease-free fields (Biosecurity SA, 2017).

Although use of clean equipment to avoid infesting new fields or preventing the introduction of infested soil into production fields through contaminated tools, hands, clothing, shoes etc. can be used to prevent the wilt disease, there are other alternatives of controlling them. Utilization of modern pesticides and chemical compounds has been done by farmers to control such plant pathogen.

However, these chemicals do not degrade completely leaving behind toxic residue in soil. As a biological alternative, scientists have long considered to use fungus as the controlling agent against wilt. *Trichoderma* species is one of such fungus showing inhibition of plant pathogen. Genus *Trichoderma* has gained immense importance since last few decades due to its biological control ability against several deadly plant pathogens (De Medeiros et al 2017). *Trichoderma* spp. are found in almost all soil types viz. cultivated soil, garden soil, fallow and pasture land, forest soil etc. (Harman et al 2004b). The impact of *Trichoderma harzianum* is tested on the growth of tomato against *Fusarium* wilt caused by the strain of *Fusarium oxysporum* isolated from the roots of tomato. The co-inoculation of tomato plants with *T. harzianum* provides a basis for the comparison of various agronomic parameters such as number of flowers, fruits number, leaves number, aerial part length, root length, fresh weight of the aerial part and fresh weight of root part.

Trichoderma harzianum is a common soil, litter, and wood fungus. It possesses highly cellulolytic activity and is main agents of decomposition. Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants. Most biocontrol agents are from the species *T. harzianum, T. viride* and *T. hamatum* (Wikipedia Contributors, 2019). *Trichoderma* species are an efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi.

Chemical treatments against soil-borne root pathogens are very dangerous; they cause technical, environmental and economic problems. These limits of chemical control and the high concern for the preservation of the environment (Alabouvette et al 2006) are the major reasons for the increased interest in the use of biological control through its ability to provide an effective protection in the long term and without negative impact on the environment or on human health (Demir et al 2015). As plant growth promoter (Kasa et al 2015) and antagonist against plant pathogens, *Trichoderma* strains are appealing alternatives to hazardous fumigants and fungicides.

Therefore, the objectives of the present study were to assess the ability of *Trichoderma* spp. in decreasing the disease severity of *Fusarium* spp. in tomato under *in vitro* and *in vivo* conditions.

1.2 Objectives

1.2.1 General Objectives:

- To isolate *Trichoderma harzianum* from different soil samples and *Fusarium* sp. from the root and leaves of infection suspected plants.

1.2.2 Specific Objectives:

- To use different isolates of *T. harzianum* for the control of tomato wilt disease caused by *Fusarium oxysporum*.
- To isolate and study the impact of *Fusarium oxysporum* causing wilt on the plant selected.
- To compare various agronomic parameters of inoculated and un-inoculated plant.

CHAPTER II

LITERTURE REVIEW

2.1 *Fusarium* wilts disease of Tomato

Tomato (*Solanum lysopersicon*) is one of the important crops used as fresh vegetable as well as in a variety of processed product such as ketchup, sauce, juice, puree, tomato-based powder, etc. (Kumari and Singh, 2018). It is most important vegetable crop having high market potentialities. In Nepal, production peaks in summer in hills (from May to September) when it is off-season in Terai region. On the other hand, it can be produced in the Terai in winter (from November to March) when it is too cold in the hills. Tomatoes can make people healthier and decrease the risk of conditions such as cancer, osteoporosis and cardiovascular disease (Freeman and Reimers, 2010). Tomato is also good for liver health. Tomato has detoxification effect in the body. However, the production trend of tomato has been on the decline over the years mostly as a result of infectious diseases from fungal pathogens (Paul and Simon, 2017). One such pathogen is *Fusarium oxysporum* f.sp. *lycopersici* which causes *Fusarium* wilt of tomatoes (Srinivas et al 2019). It has adverse effect on tomato production with severely infected fields recording loses of up to 50% (Orzolek et al 2010).

Aydi Ben Abdallah et al (2016) found that *fusarium* wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* is one of the economically most important disease in major tomato growing regions worldwide. It is a highly destructive pathogen, causing 10 to 50% yield loss in many tomato production areas (Ghazalibiglar et al 2016). Brammall and Lynch, (1990) tested the tomato wilt isolates for pathogenicity in tomato and identified it as a *Fusarium oxysporum* f.sp. *lycopersici.* Snyder and Hans, (2003) studied the introduction, host range and distribution, isolation identification, symptoms, ecology and life cycle of *Fusarium oxysporum* f.sp. *lycopersici* (Sacc). Reis et al (2005) first reported *Fusarium oxysporum* f.sp. *lycopersici* race 3 on tomato from Brazil.

2.2 Fusarium oxysporum f. sp. *lycopersici*

Reis et al (2005) reported that three races of *Fusarium oxysporum* f.sp. *lycopersici,* are the most important races of tomato (*Lycopersicon esculentum*). Races 1 and 2 are distributed worldwide whereas race 3 has a more limited geographic distribution with no report thus far in Brazil. Virulence assays were performed using a set of the race differential cultivars: 'Ponderosa' (susceptible to all races), 'IPA-5' (resistant to race 1), 'Floradade' (resistant to races 1 and 2) and 'BHRS-2, 3' (resistant to race 3). All isolates were highly virulent to 'Ponderosa', 'IPA-5' and 'Floradade' and were able to infect only a few plants of 'BHRS- 2, 3'. An additional virulence test was conducted including the same set of cultivars plus *Lycopersicon pennellii* 'LA 716'. Identical results were obtained with *L. pennellii* displaying an extreme (immune-like) resistant response.

Anitha and Rabeeth, (2009) reported that the production of tomato and pepper fruit is of worldwide agricultural importance. Many diseases and disorders can affect tomatoes during the growing season. *Fusarium oxysporum* f.sp. *lycopersici* (FOL) is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and minimal or absent crop yield. There may be a 30 to 40% yield losses due to this disease. Nirmaladevi and Srinivas, (2012) isolated *Fusarium oxysporum* f.sp. *lycopersici* strains from wilted tomato plants and soil samples collected from different tomato fields in and around Karnataka. A total of 114 isolates named as (MB1-MB114) were subjected to cultural, morphological and pathogenicity studies. Significant variations existed among the isolates with respect to rate and type of growth, colony color, mycelial growth pattern, sporulation, septation of the conidia, number and pattern of chlamydospore formation and their pathogenic variability by inoculating to five different tomato varieties by standard root dip method. The pathogenicity of the isolates could be categorized based on their virulence. The invasive growth of the isolates was assayed by injecting the conidial suspension of the isolates to tomato fruits.

Raithak and Gachande, (2013) isolated pathogenic fungi, *Fusarium oxysporum* f.sp. *lycopersici* and *Alternaria solani* (Elis and Mart) Sorauer from infected

tomato roots and fruits respectively from different varieties of tomato. The effect of culture filtrate of these fungi was observed on seed germination, root, and shoot length and vigour index. Culture filtrate obtained from *F. oxysporum* f.sp. *lycopersici* isolated from tomato varieties. Laxmi (NP-5005) increased seed germination up to 80 % vigour index 904.0, root length 6.14 cm and shoot length 5.16 cm as compared to other varieties. *A. solani* isolated from var. Priya (BSS-908) increased seed germination up to 70%, vigour Index 784.7, root length 5.93 cm and shoot length 5.28 cm as compared to other varieties.

2.3 Isolation, pathogenicity and symptomatology

Matta and Dimond, (1963) reported the symptoms of *Fusarium* wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* and observed that, all leaves were completely wilted after 20 days from inoculation, showing stem drying from the apex to downward as the growing point died. The first browning of the vascular bundles appeared 14 days after inoculation and then wilted rapidly in advanced stage. Patel and Prasad (1963) described the symptoms of *Fusarium* wilt of cumin caused by *Fusarium oxysporum* f.sp. *cumini.* The disease was characterized by wilting of leaves and the tips of the stem fallowed by prompt death of the plants. Mycelium was confined mostly to vascular tissue. Malathrakis (1985) isolated and identified *Fusarium oxysporum* f.sp. *lycopersici* from affected root systems of tomato. Tello et al (1988) isolated monosporic structure of the fungus from the vascular system of a tomato cultivar resistant to race 1 of *Fusarium oxysporum* f.sp. *lycopersici.*

Agrios (1988) reported the symptoms produced due to *Fusarium oxysporum* as slight vein clearing on the outer leaves, epinasty on older leaves, followed by stunting, yellowing of the lower leaves, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant. Narnavar and Kalekar, (1997) reported the symptoms of wilt caused by *Fusarium oxysporum* was dropping of the leaves of tomato ground level was girdled and the infected plants were stunted and in advanced stage, completely wilted and collapse on the ground, blackening of the vascular element was observed. Patil et al (2011) studied the

pathogenicity of *Fusarium* spp. isolated from rhizosphere soils of tomato from Karnataka, Tamil Nadu and Gujarat states. Among these isolates, six found non-pathogenic to tomato and these non-pathogenic *Fusarium* isolates can be exploited for the biocontrol of wilt disease.

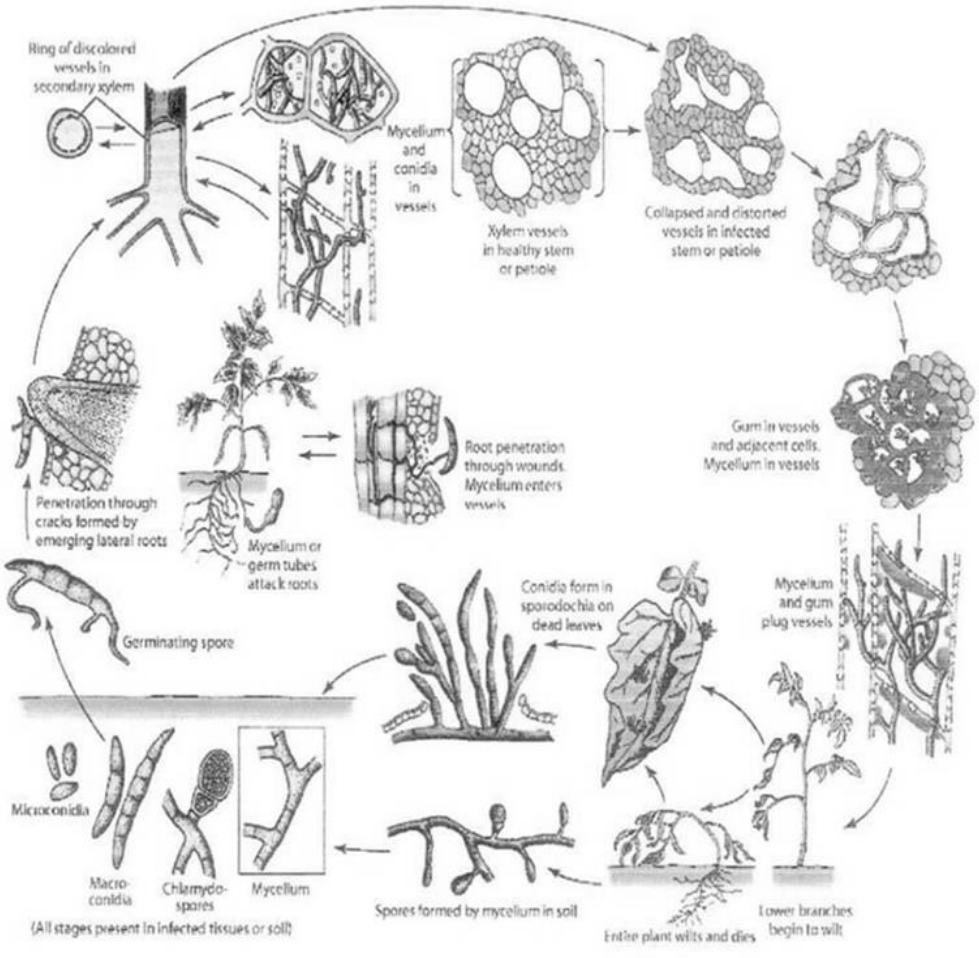


Figure 2.1 *Fusarium* wilt of *Solanum lysopersicum* caused by *Fusarium oxysporum* f.sp. *lycopersici*

2.4 Morphology of pathogen

Chattopadhyay and Gupta, (1967) studied the morphology of *Fusarium* spp. of 20 days old culture and reported that when grown on potato dextrose agar the size of conidia measured $9.3 - 29.7 \times 2.7 - 6.0$ µm. Godage (1979) reported the morphological characters of *Fusarium oxysporum* f.sp. *lycopersici* and further stated that, the conidia were measured 3.48 (2.28–5.32 µm) in width and 10.76 µm (7.60–19.76 µm) in length. Microconidia were usually oval, mostly 0-1 septate and hyaline, whereas macrocondia were 1-3 Septate fusiform and slightly curved. Agrios (1988) reported that *F. oxysporum* produces three types of asexual spores microconidia, macroconidia and chlamydospores. Smith et al (1988) reported that the *Fusarium oxysporum* has varying appearances like the aerial mycelium first appears white and they may change to variety of colors from violet to dark purple in potato dextrose agar medium.

Narnavar and Kalekar, (1997) reported the morphological characters of *Fusarium oxysporum* f.sp. *lycopersici* and reported that, microconidia were 15.37 to 2.4 µm, macroconidia – 24.31 to 40.95 µm in length and 3.32 to 5.04 µm in width. Chlamydospores are hyaline pale brown in color intercalary either single or in chains 8-10 µm in diameter. Caroll (2003) reported that the colonies of *Fusarium oxysporum* are pigmented with a reddish-purple color and surrounded by a pinkish white aerial mycelium.

2.5 Biological Control

Gnanamanickam et al (2002) reported that successful biological control systems commonly employ and naturally occurring, antagonistic microorganisms that can reduce the activities of plant pathogens. Such antagonists can compete with pathogens for nutrients, inhibit pathogen growth by secreting antibiotics, or reduce pathogen populations through parasitism. In addition, some of these microorganisms induce resistance in host plants, which enhances the plant's ability to defend itself from pathogen attack.

Montealegre et al (2005) evaluated *Trichoderma harzianum* 650 (Th650) and *Paenebacillus lentimorbus* 629 (Pl629) against root damage caused by *F. oxysporum* f.sp. lycopersici, *Pyrenochaeta lycopersici* and for *R. solani* in the

summer assay. Ethyl bromide (MeBr) decreased tomato root damage caused by the complex from 88.7% to 21.2% and from 78.4% to 35.7% in the summer and in the winter assay, respectively. None of the biocontrollers could replace MeBr in the winter assay, but *Trichoderma harzianum* 650 (Th650) and *Paenebacillus lentimorbus* 629 (Pl629) reduced root damage caused by this complex in the summer assay. Treatments with biocontrollers were improved by their combination with solarization in this season. Independent evaluations showed that the positive control of Th650 towards *R. solani* and the lack of effect on *P. lycopersic*i correlate well with the endochitinase pattern expressed by Th650 in response to these phytopathogens. Root damage caused by *R. solani* can be controlled at a similar level as it does MeBr in summer assays, thus representing an alternative to the use of this chemical fungicide for the control of this phytopathogen.

Morsy et al (2009) reported that *Trichoderma* and *Bacillus* genera are most feasible bio control microorganisms which suppress several pathogens like *Fusarium solani.* The efficiency of these antagonistic' treated plant by strains was evaluated using an in vitro assay. In pot experiment, the *T. viride* and *B. subtilis* suppressed *F. solani* as indexed by survival rate. Field experiment was carried out at El-Fayoum Farm Research Station during 2007 and 2008 seasons. Their results showed that, these treatments favored greater proliferation of rhizosphere microflora and higher dehydrogenase activity in the rhizosphere. The dual treatment by *T. viride + B. subtilis* decreased the percentage of infection and increased survival rate than individual one. Moreover, the dual inoculation gave the highest records of growth parameters, fruit yields and plant nutrient content than individual one. Thus, it is recommended to use these strains as a common biocontrol practice in agriculture.

Mishra et al (2009) tested biocontrol efficacy of five species of *Aspergillus* and five species of *Trichoderma* in vitro against *Fusarium oxysporum* f.sp. *lycopersici.* In both the experiments (dual culture and culture filtrates) *T. harzianum* was found to be highly effective against the isolates of *Fusarium oxysporum* f.sp. *lycopersici* (Fol). Followed by *A. niger* biocontrol potential of *A. terreus* is least among all the isolates tested. Culture filtrates obtained from

A. luchuensisn exerted least inhibition of *Fusarium oxysporum* f.sp. *lycopersici* (Fol). The most sensitive isolate of Fol. against all the antagonists tested was identified as IIVR-2 (Fol. 9). Inherent diversity among Fol. isolates, from different tomato growing regions in India, was determined using RAPD primers. The genetic similarity coefficients ranged from 0.20 to 0.96, indicating that no any two or more isolates were 100% similar. RAPD profiles revealed up to 20% genetic diversity among ten isolates of *Fusarium oxysporum* f.sp. *lycopersici*.

2.6 Introduction of *Trichoderma* spp.

Trichoderma spp. are among the most frequently isolated soil fungi and present in plant root systems (Harman et al 2004a). The discoveries of the antifungal abilities of these beneficial micro-organisms date back to the 1930s (Chet et al 2006; Schubert et al 2008). There is still considerable interest in finding more efficient mycoparasitic fungi especially within *Trichoderma* spp., which differ considerably with respect to their biocontrol effectiveness. It is important to isolate *Trichoderma* spp. having potentially higher antagonistic efficiency by the selection of isolates with high potential to secret extra cellular lytic enzymes chitinase and β-1, 3-glucanase. Numerous studies have confirmed that *Trichoderma* spp. can directly affect mycelium or survival propagules of other fungi through production of toxic secondary metabolites, formation of specialized structures and secretion of cell wall degrading enzymes. To date many *Trichoderma* strains have been identified as potential bio-control agents of plant pathogenic fungi on crops such as beans (*Phaseoli vulgaris*), strawberries (*Fragaria vesca*), peas *(Pisum sativum*), cucumber (*Cucumis sativus*), tomatoes (*solanum lycopersicon*), radish (*Raphanus sativum*), sugar beets (*Beta vulgaris*) and cotton (*Gossypium hirsutum*) (Nusret and Steven, 2004). *Trichoderma* affects most soil borne and foliar plant pathogens (Agosin et al 1997; Kucuk and Kivan, 2002; Nusret and Steven, 2004) which are responsible for major crop losses in agricultural production, some of which include: *Phytophthora, Pythium, Botritis, Armillaria, Fusarium, Rhizoctonia, Sclerotinia, Verticillium, Phoma* (Monte, 2001).

2.7 Biodiversity of *Trichoderma*

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

2.8 Overview of *Trichoderma* spp. effect on wilt

The work conducted by Sghir et al (2016) showed that inoculation of tomato plants and eggplant with *T. harzianum* isolated from compost strongly decreased the effect of *F. oxysporum* on both *Solanaceae* judging by the decrease of the dwarfing indices and leaf alteration indices. Such a biological control agent is very effective against seed line burns diseases of eggplant and promotes seeds germination (Meah et al 2004). After the study regarding the potentiality of *Trichoderma* species by Wells, it has been reported as one of the most efficient biocontrol agents against various plant diseases, including tomato wilt., the plant suffers tremendously from a wilt pathogen, *Fusarium oxysporum* (Khatun and Chatterjee, 2011) that result in tremendous economic loss to our country. Another study conducted by Zehra et al (2017) on synergistic effects of *Trichoderma harzianum* in tomato against *Fusarium* wilt showed that pathogen caused strong reduction in the dry weight of roots and shoots. They conducted 2 tests in which they inoculated tomato with just *T. harzianum* in the first test and pretreated the tomato with *T. harzianum* and chemical inducers in the second. While all the treatment protected tomato seedlings against *Fusarium* wilt disease, the pathogen still challenged the plant which was only pretreated with *T. harzianum.*

Singh et al (2016) tested *Trichoderma* isolate (Th Azad) against lentil wilt pathogen *Fusarium oxysporum* f.sp. *lentis* (Fol) for its abiotic stress tolerance and its antagonistic potential both *in vitro* and *in vivo* conditions. Th. Azad effectively inhibited the growth of Fol (69.23%) under *in vitro* and field conditions against the seven seed quality attributes viz. germination, shoot length, root length, seedling length, seedling dry weight. Siameto et al (2010) recorded sixteen selected isolates of *T. harzianum* were selected for antagonism against five soil-borne phytopathogenic fungi *(Rhizoctonia solani, Pythium* sp., *Fusarium graminearum, F. oxysporum,* f.sp. *phaseoli and F. oxysporum* f.sp. *lycopersici)* using dual culture assay. All *T. harzianum* isolates had considerable antagonistic effect on mycelial growth of the

pathogen in dual cultures compared to the controls. Maximum inhibitions occurred in *Pythium* spp. compared to other pathogens. Since all *T. harzianum* isolates evaluated were effective in controlling colony growth of the soil borne pathogens both in dual cultures and in culture filtrates they could be tried as a broad-spectrum biological control agent in the green house and under field conditions.

Kumar et al (2007) tested three *Trichoderma* spp. *i.e. T. virens, T. viride* and *T. harzianum* against var. subglutinans and found them effective. Isolates of *Trichoderma* sp. grew considerably faster than pathogenic *Fusarium* under the same conditions. The rapid growth gives *Trichoderma* an added advantage in competition for the space and nutrient with plant pathogenic fungi, even before it develops its arsenal of mycotoxins. *Trichoderma* was also found to control many 22 crop diseases. Our studies clearly indicated that *Trichoderma* spp. is a suitable antagonistic agent against *F. oxysporum* f.sp. *psidii* and *F. solani* isolates.

However, the study conducted by Trabelsi et al (2017) demonstrated that 104 of isolates recovered from 150 samples collected from olive trees showed die back and wilting symptoms were identified as *Fusarium* spp. Many *Fusarium* isolates were considered as weak or opportunistic as they can attack only the plants already weakened by other abiotic stress such as drought, wind, and insect pests (Palmer and Kommedahl, 1960). Trabelsi et al (2017) further concluded that the pathogenicity test performed on young olive trees (cv. Chemlali), demonstrated only 23 out of 104 *Fusarium* spp. isolates collected to be pathogenic. One of their interesting finding was the many of these crops (cotton, potato, tomato, alfalfa, or even olive itself) increase the pathogen population in soil in a very efficient way. This information will help farmers to rotate the crop to minimize the susceptibility of *Fusarium*.

2.9 Modes of actions of *Trichoderma*

Trichoderma can work as biocontrol agents in several ways:

- A biocontrol agent may excrete a compound that slows down or completely inhibit the growth of pathogens in the surrounding area of such a compound called antibiosis.
- It may promote a plant to produce a chemical that protects it from the pathogen, which is induced resistance.
- It may grow faster or use its food source more efficiently than the pathogen, thereby crowding out the pathogen and taking over, known as nutrient competition.
- They can grow in an endophytic way in other species and supports plant growth.
- It may feed on or in a pathogenic species directly known as parasitism.

2.9.1 Antibiosis

The mechanism of antibiosis is commonly reported among many species including microorganisms and plants. In case of *Trichoderma*, small size diffusible compounds or antibiotics produced by these species inhibit the growth of other microorganisms (Benitez et al 2004). These secondary metabolites are important natural products used to inhibit microbial growth and are produced during microbial development and sporulation (Vinale et al 2008; Vinale et al 2009). The spectrum of secondary metabolites secreted by Trichoderma is species and strain-dependent and includes volatile and nonvolatile antifungal substances (Vinale et al 2009). In tobacco plants, exogenous application of peptaibols activated defense responsive genes and showed reduced susceptibility to Tobacco mosaic virus (Wiest et al 2002). Coconut smell is typical of *T. viride* isolates suggesting the presence of volatile compounds that are inhibitory to pathogen growth. These metabolites include harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid (Vey et al 2001; Raaijmakers et al 2009).

2.9.2 Induced Resistance

This is caused in plants where the *Trichoderma* in association with the plant roots activates the plant's own defense mechanism against any potential attack from phytopathogens including fungi, bacteria and viruses (Vinale et al 2006). The first clear demonstration of induced resistance with *T. harzianum* strain T-39 showed that treated soil made leaves of bean plants resistant to diseases caused by the fungal pathogens such as *B. cinerea* and *C. lindemuthianum*, even though T-39 was applied only on the roots and without any on the foliage (Bigirimana et al 1997). Induced resistance was found to be beneficial in more than 10 different dicots and monocots, to infection by fungi (*B. cinerea, R. solani*, *Colletotrichum* spp., *Phytophthora* spp., *Alternaria* spp., *Magnaporthegrisea*, etc.), bacteria (*Xanthomonas spp., Pseudomonas syringae*, etc.), and even some viruses like CMVT.

2.9.3 Nutrient competition

(Harman et al 2004) found *Trichoderma* grow very fast, quickly occupying free space and colonize substrates in order to use free nutrients and this is important in their suppressive activity against the growth of soil borne pathogens. Secondly the *Trichoderma* spp. possess the ability to grow alongside the developing root system of the plants, in addition it is rhizosphere competent, this further enhances its ability as a biocontrol agent against soil borne pathogens (Ahmad and baker, 1987). Biocontrol by competition has been demonstrated during the biocontrol of *Fusarium oxysporum* by *T. harzianum* through competition for Rhizosphere colonization and nutrients and the best control is achieved with diminishing nutrient concentration (Lorito, 1994). Carbon and iron are two essential elements in most of the filamentous fungi, required for viability. Competition for carbon is effective mode not only in *Trichoderma* but also some other fungi such as strains of *F. oxysporum* (Sarrocco et al 2009; Alabouvette et al 2009). Under iron starving conditions; most fungi produce small size ferric-iron specific chelators to mobilize iron from surrounding environment. *T. harzianum* T35 also controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients (Tjamos et al 1922).

2.9.4 Endophytes

Endophytic activity of many microorganisms (growth inside plant tissue without any harm) may useful to host plant by stimulating of plant growth, a postponement to the beginning of drought stress and the obstruction to pathogens (Piotrowski and Volmer, 2006). Endosymbiotic species can establish colonies in plant roots and triggers the expression of many plant genes affecting stress responses. Recently, there are reports showing *Trichoderma* isolates acting as endophytic plant symbionts in some woody plants (Gazis and Chaverri, 2010; Chaverri and Gazis, 2011). Phylogenetic analysis classifies all known endophytic species as a separate taxon with the exception of *T. koningiopsis, T. stilbohypoxyli* and *T. stromaticum* within their clades at terminal position suggesting endophytism is not an old trait but recently evolved in Trichoderma species (Chaverri et al 2011; Samuels et al 2006; Samuels and Ismiel, 2009; Druzhinina et al 2011).

2.9.5 Parasitism

Mycoparasitism is one of the main mechanisms involved in the antagonisms of *Trichoderma* as a biocontrol agent. The process apparently includes, chemotropic growth of *Trichoderma*, recognition of the host by the mycoparasites, secretion of extra cellular enzymes, penetrations of the hyphae and lysis of the host (Zeilinger et al 1999). Once *Trichoderma* recognizes signals from a fungal target organism, it grows towards it by chemostatic hyphal branching; it then either grows alongside the hyphae of the pathogen or coils around it (Harman et al 2004). The result of the interaction is loss of turgor and collapse of cells. This comes about due to the different lytic enzymes such as chitinase, glucanase and pectinase secreted by *Trichoderma* and are of key importance in this process (Tahia et al 2004).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipment, media and reagents used in this study are listed in Appendix A.

3.2 Methods

3.2.1 Study design

The study was conducted from December 2018 to May 2019. This study was a laboratory based cross-sectional study. All the work concerning this research was carried out in microbiology laboratory of Central Campus of Technology, Dharan and at garden.

3.2.2 Soil sampling

In this study, soil samples (50 g each) were taken from different ecological habitat (forest area and agricultural area) of Eastern Nepal for the isolation of *Trichoderma harzianum*. The samples were collected from top 2-5 cm depth of rhizospheric soil. The soil samples were collected from a field in the polythene bag labeled separately. The samples were stored at 4^{o}C in the laboratory.

3.2.3 Laboratory set up

Laboratory setting was done in microbiology laboratory, Central Campus of Technology, Dharan.

3.2.4 Cleaning and sterilization of glass wares

The Petri plates, pipettes, conical flasks, test tubes, beakers etc. used in the experiments were thoroughly washed and dried. The Petri-plates and pipettes were wrapped in a silver foil and sterilized in hot air oven at 160^{o}C for 2 hours (Aneja 2004).

3.2.5 Isolation of *Trichoderma harzianum*

1 g of soil sample was taken and added to 1 ml of sterilized distilled water to make dilution of 10^{-1}. Six-fold serial dilution of each soil samples were prepared in sterilized distill water and 0.5 ml of each dilution i.e. 10^{-4} and 10^{-6} dilutions were poured onto THSM contained in petriplates and spread uniformly by adopting spread plate method. The petriplates were incubated at $25 \pm 3℃$ for 168hrs. Morphologically different colonies appearing on the plates were purified in the Potato Dextrose Agar (PDA). The purified isolates were preserved at 4^{o}C.

3.2.6 Phenotype characters of the *Trichoderma* isolates

After the isolation of all isolates, growths observed on those plates were taken for studying colony characteristics, morphology and microscopic examination of each *Trichoderma* isolates. It was examined under a microscope for the identification and confirmatory of *Trichoderma harzianum*. Microscopic observation of specimens was done by preparing slide culture method.

3.2.7 Isolation and purification of plant pathogenic fungi

Infected vascular tissues from root and leaf regions of tomato showing wilt symptoms were collected separately from field. Tissue bits were surface sterilized with 10% sodium hypochlorite for 5-10 min. and subsequently three washings with sterile distilled water. Then, they were placed on potato dextrose agar (PDA) medium separately and incubated at the laboratory conditions at $25 \pm 3℃$ for five days.

The fungi were purified separately by transferring the tip of the mycelia into PDA slants and maintained as stock cultures for further studies. Isolated fungus was identified according to their morphological characters based on T suneo Watanabe (2010) and stored at 4^{o}C until use.

3.3 *In vitro* effect of *Trichoderma* antagonists against FO-TL pathogen

3.3.1 Slide culture preparation

Pertiplates and glass slides were sterilized for slide culture. A thin layer of CMA 1×1 cm was placed on glass slide. From actively growing plate cultures of each *Trichoderma* isolates and *Fusarium oxysporum* thin mycelium was taken with the help of sterile inoculating loops and inoculated at the edge of CMA on opposite sides; a cover slip was then placed on the slide cultures; and incubated for 5 days at 25ºC. Microscopic observations were performed by transferring the cover slip to another microscope slide, LPCB was used to stain the fungi.

3.3.2 Dual plate culture

Dual plate culture technique (Dennis and Webster, 1971c) was followed to determine the antagonistic activity of *Trichoderma harzianum* isolates against plant pathogen *Fusarium oxysporum*. 4mm disc of fifteen days old antagonistic fungi and pathogen cultures were placed on PDA medium one cm away from the edge of the plate, separately. Five replicated plates for each treatment was maintained and incubated at 25 ± 3℃ control plates were inoculated with phytopathogen only. Growth of each phytopathogen isolates in dual culture and in control (without antagonist) was measured after different intervals from the 5th DAI i.e., 120hrs, 144hrs, 168hrs, 192hrs and 216hrs and Per cent inhibition over control was calculated as per the formulae:

$$\mathrm{PI} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100\%$$

Where, PI = Per cent inhibition over control

C = Growth of test pathogen with absence of *Trichoderma harzianum* (cm)

T = Growth of test pathogen with *Trichoderma harzianum* (antagonist) (cm)

3.4 Development of *Trichoderma harzianum*

Hadar et al (1979) investigated that *Trichoderma harzianum* in the form of wheat bran culture as soil treatment can effectively control the wilting of tomato caused by *Fusarium oxysporum*. Wheat bran: water 1:1 (w\v) was autoclaved in 500 ml flask for one and half hour at 121℃ at 1.5 kg\cm^2 for two successive days. 5 mm bit of *Trichoderma harzianum* was inoculated after cooling of the medium and incubated at 23 ± 2℃ for three weeks until all substrates were covered with *T. harzianum* mycelium.

3.5 Development of phytopathogen

For this purpose, FO–TL was grown on PDA for 15 days. Inoculum of *F. oxysporum* was multiplied by transferring the 5cm diameter culture to Erlenmeyer flasks containing Maize meal sand medium (100 g sand, 5 g maize meal and 20 ml of sterile distilled water). Then, the inoculated substrates were incubated at room temperature for three weeks until all the substrates were covered by pathogen mycelium (Frommel et al 1991).

3.6 *In vivo* experiment of *F. oxysporum* and *Trichoderma harzianum* on tomato

A pot culture study was conducted to test the antagonistic potential of selected antagonists (*Trichoderma harzianum*-DN, *Trichoderma harzianum*-PN and *Trichoderma harzianum*-TJ) against *F. oxysporum*-TL. The soil was sterilized by autoclaving it for 1hr for two consecutive days and filled in plastic pots (25 cm diameter) of 5 kg capacity. Tomato (F1 hybrid) seeds were sown in autoclaved soil in plastic pot. After 25 days, the seedlings were transplanted in the pots at the rate of three seedlings per pot. Both multiplied inoculums were of the pathogen and antagonists were incorporated into the pots at 5% (w/w) (FO–TL was used one day before transplanting and *Trichoderma* isolates were applied just the day of seeding). The observation on the percent disease incidence was recorded at the time of harvest.

$$\mathrm{DI} = \frac{\text{Total No. of infected plant}}{\text{Total No. of plant assessed}} \times 100\%$$

Where, DI = Disease Incidence (%)

Each treatment was replicated thrice in Completely Randomized Block Design (CRD). Treatments were:

T0: Uninoculated control (healthy)

T1: Inoculated control with *F. oxysporum*-TL

T2*: F. oxysporum*-TL + *T. harzianum*-PN

T3: *F. oxysporum*-TL + *T. harzianum*-TJ

T4: *F. oxysporum*-TL + *T. harzianum*-DN

Plants were maintained in normal conditions at garden by watering daily and equal moisture was maintained in each pot. In all the treatments, plant height and number of leaves were measured at 10, 20, 30, 40 and 45 days after transplantation (DAT). While the fresh root and dry roots were measured after 45 DAT.

3.6.1 The measured parameters

a) The height of the plant (cm):

It was measured from the rhizospheric region to the upper end of the plants.

b) Number of leaves

Total number of leaves per plant was counted and recorded.

c) The fresh weight of the roots (g)

The underground root biomasses of the plants were weighed fresh after harvesting.

d) The dry weight of the roots (g)

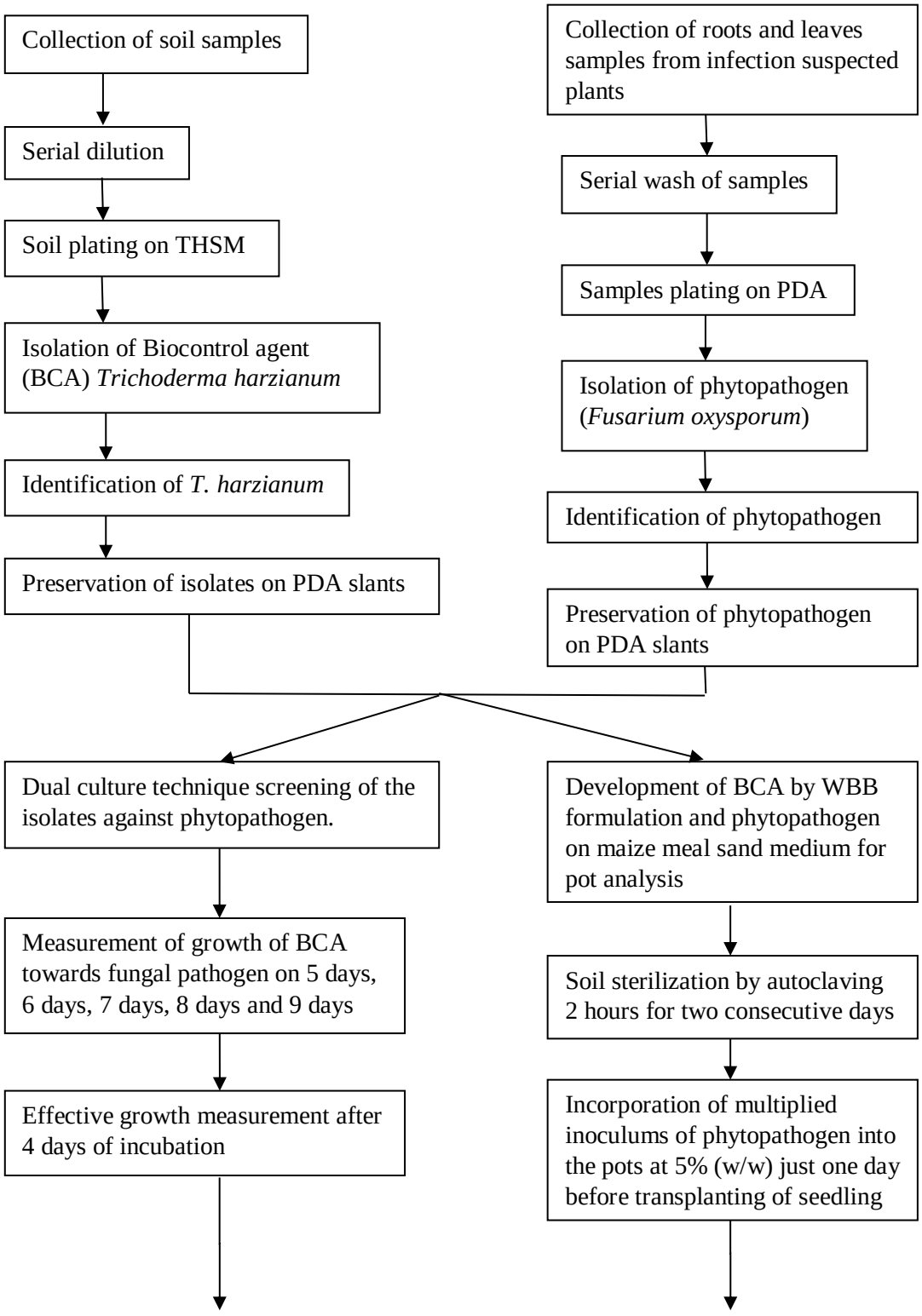
The underground root biomass of the plants were estimated after they were carefully rinsed with water, dried with filter paper then placed at oven at 75 °C until the weight was stabilized.

3.7 Data Analysis

The data recorded from dual culture and pot culture were documented and tabulated. The data were statistically analyzed using SPSS version 16. One way ANOVA test was used to determine the association of plant growth parameters with different treatments. The test was statistically significant if $P<0.05$ with 95% confidence interval.

3.8 Flow Chart

Flow chart of the study



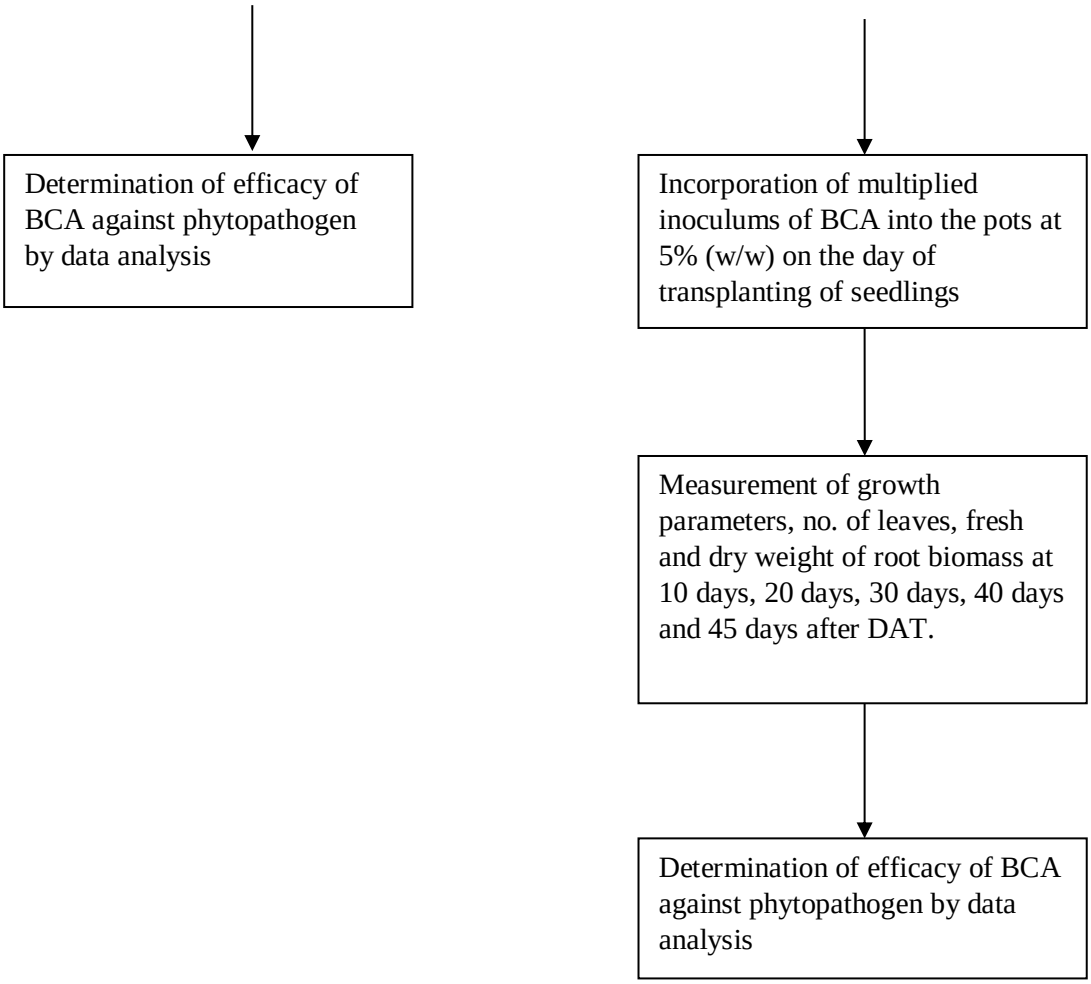


Figure 3.0: An overview of overall methodology

CHAPTER IV

RESULTS

4.1 Isolation of *Fusarium oxysporum*

In this investigation, sixty samples were collected from infection suspected tomato plants. The samples were collected from infected vascular tissues of roots and leaf regions of tomato showing wilt symptoms. Out of sixty samples, four samples gave the positive signal. Sample was identified on the basis of morphological and colonial characterization under microscope. And among four isolates, single isolate was further subjected for study.

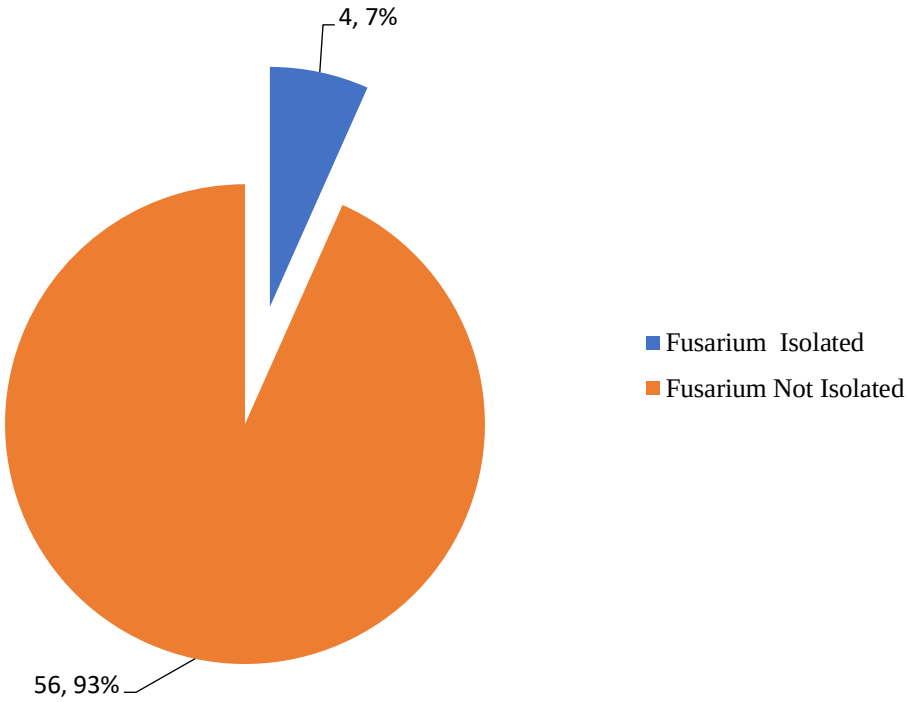


Figure 4.1 Isolation of *Fusarium* from infection suspected tomato plant

4.2 Isolation of *Trichoderma harzianum*

Similarly, phytopathogen antagonist *Trichoderma harzianum* was isolated from different soil of Eastern Nepal. Out of fifteen samples, three samples gave the positive result and it was further subjected for microscopic confirmation. The isolates were named based on their isolated areas. These selected isolates were further subjected for dual culture assay and *in vivo* trial.

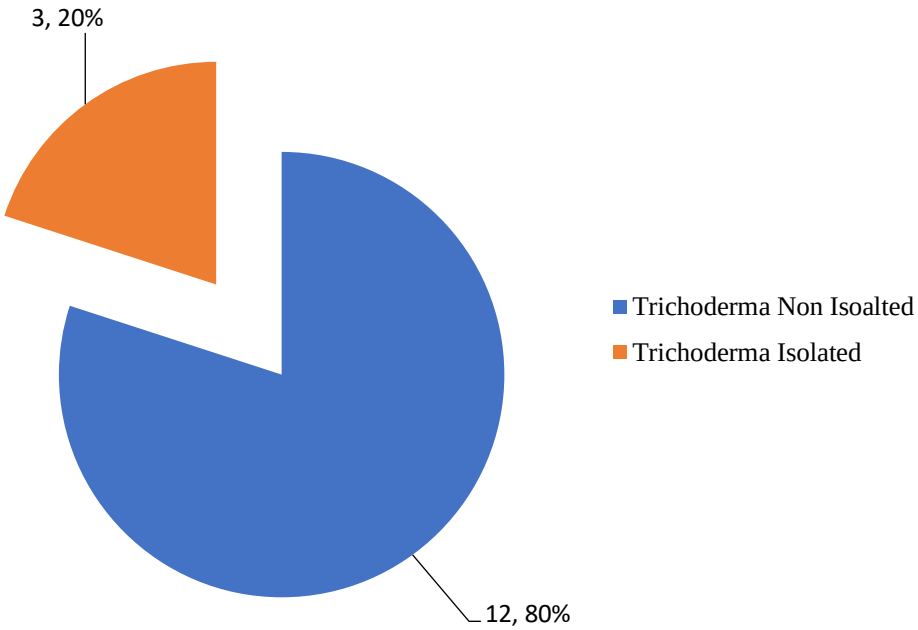


Figure 4.2 *Trichoderma harzianum* isolation form soil sample

4.3 Morphology of the *Fusarium oxysporum* isolate

Four distinct *Fusarium oxysporum* isolates were obtained from the leaves of infected tomato plant. The isolates exhibited a slow growth on PDA plates reaching between 5.1 and 5.7 cm in diameter after 5 days of incubation at 25^{o}C. Out of four isolates, one isolate was used in the investigation. The microscopic features on CMA plates, presented by the isolates were; macroconidia which were elongate, curved, septate and slightly foot shaped, the microconidia produced were cylindrical, non-septate and abundant.

Table 4.1: Characteristics of the *Fusarium* isolates by morphological features.

Characteristics	***Fusarium oxysporum***
Color of Colony on PDA	White/pinkish aerial mycelium, purple Undersurface
Diameter length on PDA (cm) after 7 days	4.0 cm
Type of macroconidia µm (CMA)	Elongate, curved, three septate and with a slight foot shaped end cell
Type of microconidia µm (CMA)	Cylindrical, non-septate, Abundant
Type of Chlamydospores	Both terminal and intercalary chlamydospores, smooth walled
Phialides	Short and non-septate

4.4 Morphological characteristics of *Trichoderma harzianum* isolates

Out of 15 soil samples, three isolates of *T. harzianum* was obtained. Cultural characteristics comprising growth rate, color and colony appearance were examined. These characteristics were regarded as taxonomically useful characteristics for *Trichoderma harzianum* (Samuels et al 2002).

On PDA, *T. harzianum* formed 1-2 concentric rings with green conidial production. The conidia production was denser in center then towards the margins. Some white pustules were also found growing on the green mat of conidia. *T. harzianum* form cottony white mycelium with dark green conidiation towards the margins, while on the reverse the color was pale, tan or yellowish. After then, examination of the shape, size, arrangement and development of conidiophores or phialides or conidia provided a tentative identification of *Trichoderma harzianum*.

Table 4.2 Characteristics of the *Trichoderma harzianum* isolates by morphological features.

Characteristics	***Trichoderma harzianum***
Color of Colony on PDA	Greenish to white cottony mycelium
Diameter length on PDA (cm) after 5 days	7.3 cm
Phialides	Flask shaped
Chlamydospores	Subglobose, short hyphae
Type of conidia µm (CMA)	Ellipsoidal, smooth, dry

4.5 Effect on dual plate culture

Growth inhibition of the pathogen by all the *Trichoderma harzianum* isolates was evident from the fifth day of incubation. The mycelial growth of the pathogen was daily evaluated by measuring the diameter of the petri dish in which the radius of the pathogen was found next to the antagonist. This evaluation was realized every 24 hours for 5 days. The reduction of the mycelial growth of pathogen (*Fusarium oxysporum*) was significantly higher in the dual culture compared to the pathogen control. It was probably due to the competition for available nutrient and space. The three well known mechanisms associated with pathogen control by *Trichoderma* are; competition for nutrients, antibiosis and myco-parasitism (Sharma 2011; Hermosa et al 2012). *Trichoderma harzianum*-TJ showed significantly higher inhibition of the mycelial growth of the pathogen (56%) than *Trichoderma harzianum*-PN (55%) and *Trichoderma harzianum*-DN (36%) (Table 4.3). The differences in the mycelial inhibition may be due to the diversity in the *Trichoderma* isolates.

In this test, after a maximum of 8 days, it was observed that the colonies of *Trichoderma* isolates recovered those of the fungi thus, revealing their inhibitory power. This situation has also been obtained by the different confrontations (Pathogen -Antagonist).

Table 4.3 Effect of *Trichoderma harzianum* isolates on mycelium Per cent (%) inhibition on growth of *Fusarium oxysporum*

	% Inhibition on growth of *Fusarium oxysporum*					
Treatments	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Average**
Th–PN	41%	45%	59%	*62%*	68%	55%
Th–DN	2%	21%	45%	*53%*	59%	36%
Th–TJ	39%	47%	57%	*65%*	69%	56%

4.6 Pot assay experiment

All the treatments of *Trichoderma harzianum* compared to the control and *Fusarium* resulted in better plant height of tomato. The plant height of tomato at 45 DAT was even lower in *Fusarium* treated soil compared to the control. All the parameters related to the plant growth and yields were also maximum in *Trichoderma* treated soil; while most of them were the lowest in *Fusarium* treated soil. These results are in agreement with previous studies (Cotxarrera et al 2002; Ghazalibiglar et al 2016). The treatment of plants by *Trichoderma* isolates has had a beneficial effect on its growth by promoting the development of it in the presence of pathogen and delaying the onset of symptoms. The observation of the state of the plants was inoculated by the pathogen and the antagonist, compared to that of the inoculated control, and showed that the plants treated with *Trichoderma* isolates exhibited a greater vegetative development. The treatment with *Trichoderma* isolates resulted an increase in the height of the plant, root length, fresh weight of the aerial part, the fresh weight of the roots, the dry weight of the aerial part, the dry weight root and fineness of the root system.

The ability of *Trichoderma* to increase tomato biomass in the absence of the pathogen suggests that these isolates are likely to be able to influence the production of phyto-stimulators or phytohormones (Martínez-Medina et al 2014; Lee et al 2016). Previous research has also shown that Trichoderma promotes plant root development and solubilize nutrients (Contreras-Cornejo et al 2009).

Table 4.4 Efficacy of *Trichoderma harzianum* on *Fusarium* wilt control in tomato at 45 DAT

Treatments	Fungal Native Antagonist	FO-TL	Total no. of Plant examined	Infected plants with Wilt	% Disease Incidence
T0	Healthy Control	-	3	2	67%
T1	Inoculated Control	+	3	3	100%
T2	FO-TL + Th-PN	+	3	2	67%
T3	FO-TL + Th-TJ	+	3	1	33%
T4	FO-TL + Th - DN	+	3	2	67%

4.6.1 Effect on shoot length of tomato

After 25 DAT of seedlings to the pot, plant height (cm) was recorded at the interval of 10, 20, 30 40 and 45 days. All the treatments of *Trichoderma* compared to the control and *Fusarium* resulted in better plant height of tomato (Table 4.5). The plant height of tomato at 45 DAT was even lower in *Fusarium* treated soil compared to the control. All the parameters related to the plant growth and yields were also maximum in *Trichoderma* treated soil; while most of them were the lowest in control and *Fusarium* treated soil. As seen in table below, the shoot length showed progressive growth with maximum length of 78.33 cm in 45 DAT. This fungus may produce growth promoting phytohormones like indole acetic acid (IAA), or auxin analogues and vitamins that supports plant growth.

Table 4.5 Effect of *Trichoderma* and *Fusarium* on shoot length (cm) of tomato

Treatments	10 DAT	20 DAT	30 DAT	40 DAT	45 DAT
T0	13.67^{c}	24^{c}	31.5^{d}	42.17^{d}	56.33^{c}
T1	8.67^{d}	19^{d}	27.5^{e}	34.5^{e}	42.5^{d}
T2	15.33^{b}	28^{b}	38.67^{c}	50.83^{c}	65.67^{b}
T3	16.17^{a}	32.67^{a}	45.5^{a}	66.33^{a}	78.33^{a}
T4	13.5^{c}	30^{b}	40.67^{b}	55.67^{b}	67^{b}

Different letter in each column denote significant differences ($P<0.05$) among the treatments according to Duncan's multiple range test, DAT- Days after transplantation.

4.6.2 Effect on number of leaves of tomato

After 25 DAT of seedlings to the pot, the number of leaves of tomato plant was recorded at the interval of 10, 20, 30 40 and 45 days. As seen in table below, the *Trichoderma* treated plant showed greater number of leaves as compared to the control and *Fusarium* inoculated plant. The highest number of leaves were found in tomato treated with T3 (*F. oxysporum*-TL + *T. harzianum*-TJ) at 90 leaves in 45 DAT. However, the *Fusarium* inoculated plant had only 33 leaves in same duration of time.

Table 4.6: Effect of *Trichoderma* and *Fusarium* on number of leaves of tomato

Treatment	10 DAT	20 DAT	30 DAT	40 DAT	45 DAT
T0	34^{c}	44.33^{c}	48.67^{d}	48.33^{c}	51.67^{d}
T1	18^{d}	28.67^{d}	34.33^{e}	35^{d}	33^{e}
T2	39.33^{b}	51.33^{b}	60.33^{b}	64.67^{b}	74.67^{b}
T3	45.33^{a}	61.33^{a}	71.67^{a}	82.67^{a}	90.67^{a}
T4	39^{b}	51.6^{b}	56.67^{c}	64.33^{b}	66.33^{c}

Different letter in each column denote significant differences ($P<0.05$) among the treatments according to Duncan's multiple range test, DAT- Days after transplantation.

4.6.3 Effect on fresh root weight of *Trichoderma* inoculated plants

During the experiment, the weight of fresh root of tomato plant was also measured at 45 DAT and it was evident from the root mean weight that the *Trichoderma* increase the overall biomass of root system due to promotion of root development. The maximum weight was measured for treatment T3 and the minimum was for T1 (control).

Table 4.7 Mean fresh root weight (g) of control and *Trichoderma* treated plants

Root Weight Mean (g)	**T0**	**T1**	**T2**	**T3**	**T4**
Root fresh wt.	2.9	2.13	3.47	4.4	3.87

4.6.4 Effect on dry root weight of *Trichoderma* inoculated plants

After the analysis of fresh roots, the roots were washed properly, air dried on filter paper and then placed at oven at 75 °C until the weight was stabilized. Dried roots were measured (g) and tabulated. The maximum weight was measured for treatment T3 and the minimum was for T1 (control).

Table 4.8 Mean dry roots of control and treated tomato

Root Weight Mean (g)	**T0**	**T1**	**T2**	**T3**	**T4**
Root dry wt.	2.13	1.4	2.6	3.33	2.95

CHAPTER V
DISCUSSION

Fusarium wilt caused by the *Fusarium oxysporum* f.sp. *lycopersici* is a destructive disease of tomato crop worldwide. This disease is caused by a soil borne fungal pathogen that infects plants through root at all stages of plant growth and is a major problem in tomato crop in the warm, moist and tropical regions of the world, causing damping off in nursery seedlings as well as stem rot, wilting and blight in adult plants with consistent loss of production (Flores-Moctezuma et al 2006). The pathogen enters through the roots of the plants and proliferates in the vascular tissue leading to breakdown of the water economy of the infected plants (Singh et al 2017). The disease causes great losses, on the susceptible varieties of tomatoes especially when soil and air temperature are rather high during the warm season. Typical symptoms of the disease are yellowing and wilting of leaves progressing upward from the base of the stem. Initially, only one side of the leaf, one branch or one side of the plant is affected. The symptoms soon spread to the plant and finally kill it (Roy et al 1997).

Three *Trichoderma harzianum* species were obtained from the area of study. This result is in concurrence to earlier research that have reported that *Trichoderma* species are cosmopolitan fungi, that occur widely worldwide and are frequently present in all types of soil, manure and decaying plant tissues (Rahman et al 2011). This *Trichoderma harzianum* isolates showed potential in controlling of *Fusarium oxysporum* f.sp. *lycopersicon* both in vitro and in vivo but with varying degrees of inhibition amongst the isolates. Competition, mycoparasitism and antibiosis resulting from production of secondary metabolites were the main mechanisms observed in the effect of *Trichoderma harzianum* against the pathogen. This finding is similar to other research that has reported that *Trichoderma* uses several mechanisms in controlling soil pathogens (Vinale et al 2008). This study also agrees with reports that have suggested that different strains of *Trichoderma* control every pathogenic fungus. However, most *Trichoderma* strains are more efficient for control of

some pathogens than others and may be largely ineffective against some fungi (Shelton 2012).

In this study the inoculated plant showed wilting symptoms and percentage of diseases incidence was (100%). The result is supported by the findings of Begum (2007) who observed that *F. oxysporum* f.sp. *lycopersici* was able to produce wilting symptoms in tomato plants. Among all the microbial antagonists studied the highest (75.75%) inhibition of *F. oxysporum* f.sp. *lycopersici* was observed in case of *Trichoderma harzianum* (Sarker et al 2014). Sundar et al (1995) and Deshmukh and Raut, (1992) reported that *Trichoderma harzianum* grew over the colonies of *Fusarium oxysporum*. Chabbi and Matrod, (2002) achieved 77% growth inhibition of *Fusarium oxysporum* with *Trichoderma harzianum*.

It was revealed from the results, *Trichoderma* isolates varied in their effect on tomato plants and ability to reduce the effect of *F. oxysporum* when subsequently applied in the pot experiment. Reduction of *Fusarium* wilt disease was observed by 100% and 67% in comparison to the control. Best disease control was achieved in treatment (T3) demonstrating only 33% of disease incidence followed by treatment (T2) and treatment (T4) with 67%. These results agree with previous studies of several workers. Two *Trichoderma* isolates significantly ($P<0.05$) reduced tomato *Fusarium* wilt incidence, as shown by 69% fewer plants with vascular discoloration (Ghazalibiglar et al 2016). In several studies, *T. harzianum* has also been reported to be effective against the *Fusarium* wilt pathogen (Datnoff et al 1995; Sivan et al 1987; Srivastava et al 2010). They reported that isolates of *Trichoderma* showed promise for controlling *Fusarium* wilt and improving the growth and yield of tomato.

The results of *Trichoderma* treatments showed statistically significant effects of *Trichoderma* treatments on plant growth parameters (Table, 4.5, 4.6, 4.7 and 4.8). Root length, fresh weight and dry weight and number of leaves per plant found that the maximum was recorded in the treatment. These results agree with Ozbay and Newman, (2004) who reported that seedling treated with *T. harzianum* increases number of true leaves, fresh and dry weights of root of tomato plants. Sundaramoorthy and Balaskar, (2013) also observed that

tomato plants treated with *T. harzianum* (ANR-1) stimulate plant height by 73.62 cm and increased the dry weight by 288.38 g in comparison to untreated control. Harman (2000) established that *Trichoderma* spp., are opportunistic plant colonizers that affect plant growth by promoting abundant and healthy plant roots, possibly via the production or control of plant hormones. The increased growth response caused by *Trichoderma* isolates may be through modification of the rooting system as Yedida et al. (1999) reported for *T. harzianum* inoculation which improved uptake of nutrients by the plants at a very early growth stage.

The increase in bio matter production may be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake and by producing antibiotics to protect plants from deleterious rhizosphere organisms. Therefore, the antagonist *T. harzianum* is chosen to be the most promising bio-control agent for *F. oxysporum* f.sp. *lycopersici*. On the base of present study, the bio agents of fungi might be exploited for sustainable disease management programs to save environmental risk.

In this study the results from the dual plate cultures showed that different isolates of *Trichoderma harzianum* have differing abilities in the bio-control of *Fusarium oxysporum* f.sp. *lycopersicon*. Among the 3 *Trichoderma* isolates collected from Eastern Nepal, Dharan (Th - DN), Panwari (Th - PN) and Tahrara Jungle (Th - TJ), Th - PN showed the highest inhibition (41%) at day 5 in comparison to Th - DN (2% only). However, the data recorded day 6 to day 9 showed increased inhibitions for Th-TJ from 47% to 69%. This outcome is similar to earlier experiments that have demonstrated that *Trichoderma spp*. mycoparasitize the hyphae and resting structures of plant pathogens in vitro and also in natural soil (Papavizas 1985). However, most *Trichoderma* strains are more efficient for control of some pathogens than others and may be largely ineffective against some fungi (Shelton 2012).

Use of fungicides is quite uneconomical and it's often leads to atmospheric pollution, development of resistance in the pathogen, and the risk of health hazards to human being. Use of alternative methods like BCA against wilt disease is cheaper and eco-friendly. One of the most promising means to achieve this goal is using new tools based on BCAs for pest and disease

control alone or to integrate with reduced doses of chemicals in the control of plant pathogens resulting in minimal impact of the chemicals on the environment (Vinale et al 2009). Biological control of plant diseases has been considered a viable alternative method to manage plant diseases (Heydari and Pessarakli, 2010). The use of PGP/BCA offers an attractive way to replace chemical fertilizer, pesticides, and supplements; most of the isolates result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants (Sharma 2003). *Trichoderma* spp. is active rhizosphere colonizers and act as BCA and PGP. So, *Trichoderma* application for disease management is one of the cheapest, ecofriendly and reliable measure for controlling *Fusarium* wilt that helps in enhancing and promoting plant growth (Sevugapperumal et al 2016). *Trichoderma* spp. has antifungal properties due to the production of secondary metabolites such as: trichodermine, dermadina, sequisterpeno, suzukacillina, alamethicina, trichotoxina, acetaldehyde, as well as extracellular enzymes such as β-1,3 glucanase, chitinase and cellulase that degrade the host cell walls and allow the penetration of the antagonist's hyphae, reducing its propagation in the root (Elad et al 1982; Chet et al 1967; Cherif and Benhamou, 1990). Fungi belonging to the genus *Trichoderma* are the most promising bio-control agent against a range of plant pathogens under a variety of environmental conditions (Chen et al. 2007). *T. harzianum* stimulates the growth of plants by producing metabolites that promote developmental processes, which allow greater root development and absorbent hairs, which favors the mobilization of nutrients in the soil, thus improving nutrition and water absorption; also accelerates the decomposition of organic matter and minerals (Schirmbuck et al 1994).

Thus, the application of *Trichoderma harzianum* on *Fusarium* infected plant enhanced the plant growth along with its biomass and it proves to be a potent BCA.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The results in this study are highly promising. They support the potential of *Trichoderma* as suppressor of pathogen growth in in-vitro condition and as growth promoter in in-vitro and in-vivo condition. *Trichoderma* sp. decreased disease incidence of *Fusarium oxysporum* f.sp. *lycopersici* by as less as 33%, and increased plant growth parameters such as shoot length, number of leaves and root weight in tomato. Moreover, the inoculation of *Trichoderma* spp. proved better than control without it. Thus, the finding of present investigation holds a good promise in tomato wilt management. However, further studies on the effect of these treatments in field conditions need to be undertaken so that *Trichoderma* could be recommended as a biocontrol agent.

T. harzianum isolated from Tarhara Jungle (Th-TJ) and Panmara (Th-PN), increased per cent inhibition on growth of *F. oxysporum*. The growth inhibition showed increasing trend with time.

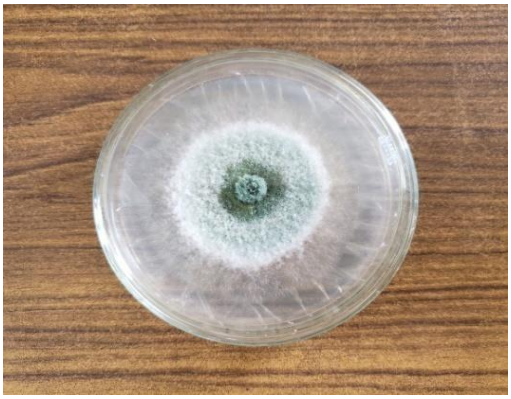
Therefore, the antagonist *T. harzianum* is chosen to be the most promising bio-control agent for *F. oxysporum* f.sp. *lycopersici*. On the base of this study, the biocontrol agents of plant diseases might be exploited for sustainable disease management programs to save environmental risk.

6.2 Recommendations

Fusarium oxysporum f.sp. *lycopersici* is a major tomato rot pathogen which causes *Fusarium* wilt, an economically important disease in tomato production causing damage to tomatoes of between 10-100%. Destruction by this pathogen results into overwhelming losses to the farmers due to reduced crop productivity hence reduced market prices. The search for and development of effective methods for control of this pathogen will contribute a great deal to the objective of attainment of food security among the small-scale farmers. The strategy of integrated disease management (IDM) that incorporate use of bio-control agents which are safe to use, easy to adopt and environmentally friendly is one such system that can contribute greatly to management of this disease.

While in vitro experiments are important in the initial stages of selection of *Trichoderma* species that can act as bio-control agents through different synergistic mechanisms against the *Fusarium oxysporum* f.sp. *lycopersici*; it is always difficult to extrapolate the bio-control activity of a given strain from the laboratory to natural environments. *Trichoderma* ecology and biological control activity are greatly influenced by soil properties. In agricultural systems the soil properties are affected by farming practices such as ploughing, irrigation, liming and fertilizer and pesticide applications. Future work should focus on; further evaluation of the local isolates *T. koningiopsis* and *T. viride* under field conditions against *Fusarium oxysporum* f.sp. *lycopersici* to determine their effectiveness. It is also important to determine the farming practices that would inhibit or enhance the antagonistic activity of the *Trichoderma* species to realize their optimum bio-control ability.

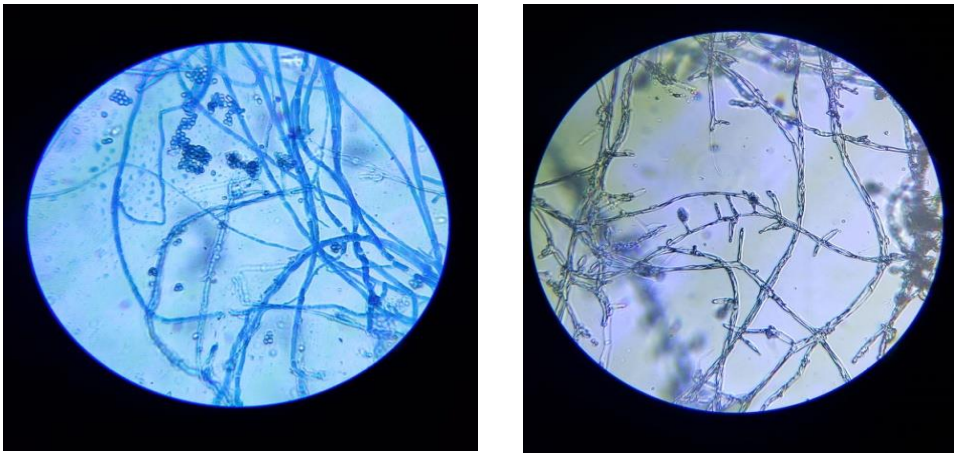
The results from this study could be used to develop bio-control strategy against *Fusarium oxysporum* f.sp. *lycopersici*. This could have a significant beneficial impact on the management of *Fusarium* wilt disease in tomatoes. Availability of local *Trichoderma* isolates that are effective against this pathogen would provide a source of readily available bio-control agents for use against *Fusarium* wilt of tomatoes and as a result the small-scale farmers will realize improved productivity of tomatoes.



Trichoderma harzianum ***Fusarium oxysporum***

Photograph 1: Morphological view of antagonist and fungal pathogen

Photograph 2: Researcher working on lab



Trichoderma harzianum ***Fusarium oxysporum***

Photograph 3: Microscopic view of antagonist and fungal pathogen

Photograph 4: Dual culture, *Trichoderma* vs *Fusarium* at 8 DAI

Photograph 5: *In-vivo* trial (pot culture)

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APPENDIX-A

Materials and equipment

1. Equipment

Microscope, Incubator, Refrigerator, Digital balance, Autoclave, Hot air oven, Laminar air flow.

2. Materials

Measuring cylinder, Glass slides, Cover slips, Petri plates, Inoculating loops, Beakers, Spatula, Wire gauges, Teat tubes, Pipettes, Glass rods, Conical flasks, Ruler, Plastic pots, Plastic bags, Price tags, Cello tapes, Markers, etc.

3. Chemicals

LPCB, $MgSO_4.7H_2O$, Glucose, Rose Bengal, Dextrose, Agar, KCL, NH_4NO_3, K_2HPO_4, etc.

4. Media

Trichoderma harzianum Selective Media (THSM), Potato Dextrose Agar (PDA), Corn Meal Agar (CMA), Maize meal Sand Medium, Wheat bran Formulation, etc.

5. Antibiotics

Chloramphenicol, Streptomycin in powder form

APPENDIX-B

Scientific classification of *Trichoderma, Trichoderma harzianum* and *Fusarium oxysporum*

a. *Trichoderma*

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

b. *Trichoderma harzianum*

Position	Trichoderma
Kingdom:	Fungi
Division:	Ascomycota
Class:	Sordariomycetes
Order:	Hypocreales
Family:	Hypocreaceae
Genus:	*Trichoderma*
Species:	*T. harzianum*

c. *Fusarium oxysporum*

Position	Fusarium
Kingdom:	Fungi
Division:	Ascomycota
Class:	Sordariomycetes
Order:	Hypocreales
Family:	Nectriaceae
Genu:	*Fusarium*
Species:	*F. oxysporum*
Subspecies:	*F.* o. f.sp. *lycopersici*

APPENDIX-C

Habit and habitat of *Trichoderma*

2.6 Biodiversity of *Trichoderma*

Trichoderma species frequently are predominant over wide geographic regions in all climatic zones, where they are significant decomposers of woody and herbaceous materials. *Trichoderma* can be characterized with high adaptability to temperate and tropical soils, commonly found in variety of soil type such as agriculture, forest, saline and desert soils in all climatic zones. *Trichoderma* isolates are also frequently isolated from water logged soils including mangrove swamps, alkaline mudflats and estuarine sediments. Many species of *Trichoderma* are closely associated with plant root and specific strains may form endophytic associations with their plant host (Bailey et al 2006; Evans et al 2003, Hoyos-Carvajal et al 2009b; Manesh et al 2006, Sette et al 2006, Viterbo & Chet, 2006; Yedidia et al 2000).

Besides this, it is also found colonizing roots, litter, decaying wood, decaying bark and various plant materials at all climatic zones. The largest species diversity study of *Trichoderma* based on sexual morph specimens collected predominantly from dead wood and bark was carried out by Jaklitsch (2009) and Jaklitsch (2011) who reported 75 species among 620 Hypocrea specimens in Central and Northern Europe. Danielson and Davey (1973) surveyed the *Trichoderma* propagules in a variety of the forest soil in the southeastern U.S and Washington State and identified the isolates as *T. hamatum, T. harzianum, T. koningii, T. polysporum, T. pseudokoningiians T. viride.* 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 rhizospheric soil of winter season in Hungary comprising *T. atroviride, T. brecvicompactum, T. gamsii, T. harzianum, T. koningiopsis, T. longibrachiatum, T. rossicum, T. spirale, T. tomentosum* and *T. virens*(Kredics et al 2012). *Trichoderma* spp. May also be sensitive to environmental pollution as indicated by low rate of recovery of *T. viride* from coniferous forest that had been subjected to alkaline dust. The presence of CO_2 has been reported to favor growth of *Trichoderma*. Certain strains of *Trichoderma* can

also colonize the plant roots and take part in symbiotic relationship with several plants such as cocoa, rubber tree and banana.

APPENDIX-D

Culture Media Used in Research

A. *Trichoderma harzianum* Selective Media (THSM)

Ingredients	Amount (g/l)
Magnesium sulphate heptahydrate	0.20
Dipotassium hydrogen phosphate	0.90
Ammonium nitrate	1.00
Potassium chloride	0.15
Glucose	3.0
Rose Bengal	0.15
Agar	20.0
pH	5.5 ± 0.2

Autoclaved at 15 lbs for 15 min

After autoclaving of the mixture, a final ingredient solution was added to the medium when the mixture was cooled around 40-50^{o}C: Chloramphenicol (25.0 mg), Streptomycin (0.90 mg) and Metalaxyl (9.26 mg) dissolved in 40ml of 99% ethanol was mixed thoroughly and then poured into the sterilized petriplates.

B. Potato Dextrose Agar (PDA)

Ingredients	Amount (g/l)
Potatoes, infusion form	200.00
Dextrose	20.00
Agar	15.00
pH	5.6 ± 0.2

Himedia containing this entire ingredient was used. 39 g of PDA base was taken in a conical flask and suspended in 1000 ml distilled water. The mixture was mixed thoroughly by stirring with glass rod to have homogenized mixture.

After few minutes of boiling, the pH of the medium was adjusted to 7.0 ± 0.2 and autoclaved at 15 lbs at 120^{o}C for 20 mins. To enhance the media more potent 25.0 mg of chloramphenicol was added after autoclaving when the media was cooled to temperature 40-45^{o}C.

C. Corn Meal Agar (CMA)

Ingredients	Amount (g/l)
Corn meal, infusion form	50.00
Agar	15.00
pH	6.0 ± 0.2

17 g of the constituents were dissolved in 1000 ml distilled water. The constituents were heated to boiling to dissolve the medium completely and were autoclaved at 15 lbs pressure for 15 mins. After autoclaving the mixture was cooled to 40-50^{o}C and streptomycin (0.90 g) was added and the final pH was adjusted.

APPENDIX-E

Statistical Analysis Output

ANOVA

1. Plant Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2152.733	4	538.183	41.240	.000
Within Groups	130.500	10	13.050		
Total	2283.233	14			

H_0= There is no statistical difference in height of plants with different treatment Groups

H_1=There is statistical difference in height of plants with different treatment Groups

Result: P< 0.05, so result is statistically significant

Conclusion: T3 Treatment exhibited better growth of plant than control and other treatment groups.

ANOVA

2. Plants leaves yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5822.267	4	1455.567	21.965	.000
Within Groups	662.667	10	66.267		
Total	6484.933	14			

H_0= There is no statistical difference in leaves yield of plants with different treatment Groups

H_1=There is statistical difference in leaves of plants with different treatment Groups

Result: P< 0.05, so result is statistically significant

Conclusion: T3 Treatment exhibited better growth of plant leaves than control and other treatment groups.

ANOVA

3. Fresh root weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.109	4	2.277	18.267	.000
Within Groups	1.247	10	.125		
Total	10.356	14			

H_0= There is no statistical difference in fresh root weight of plants with different treatment Groups

H_1=There is statistical difference in fresh root weight of plants with different treatment Groups

Result: P< 0.05, so result is statistically significant

Conclusion: T3 Treatment exhibited better root growth of plants than control and other treatment groups.

ANOVA

4. Dry root weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.823	4	1.706	18.917	.000
Within Groups	.902	10	.090		
Total	7.724	14			

H_0= There is no statistical difference in dry root weight of plants with different treatment Groups

H_1=There is statistical difference in dry root weight of plants with different treatment Groups

Result: P< 0.05, so result is statistically significant

Conclusion: T3 Treatment exhibited better root growth of plants than control and other treatment groups.

ANOVA

5. Dual Culture of Th-TJ / FO-TL

inhibition(cm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	27.889	1	27.889	6.506	.034
Within Groups	34.292	8	4.287		
Total	62.181	9			

H_0= There is no statistical difference in dual culture inhibition of *Trichoderma* (Th-TJ) against *Fusarium*.

H_1=There is statistical difference in dual culture inhibition of *Trichoderma* (Th-TJ) against *Fusarium*.

Result: P< 0.05, so result is statistically significant

Conclusion: *Trichoderma* (Th-TJ) exhibited better inhibition against *Fusarium* culture.

ANOVA

6. Dual Culture of Th-DN / FO-TL

inhibition(cm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.964	1	24.964	6.010	.040
Within Groups	33.232	8	4.154		
Total	58.196	9			

H_0= There is no statistical difference in dual culture inhibition of *Trichoderma* (Th-DN) against *Fusarium*.

H_1=There is statistical difference in dual culture inhibition of *Trichoderma* (Th-DN) against *Fusarium*.

Result: P< 0.05, so result is statistically significant

Conclusion: *Trichoderma* (Th-DN) exhibited better inhibition against *Fusarium* culture.

ANOVA

7. Dual Culture of Th-PN / FO-TL

Inhibition (cm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.964	1	24.964	5.675	.044
Within Groups	35.192	8	4.399		
Total	60.156	9			

H_0= There is no statistical difference in dual culture inhibition of *Trichoderma* (Th-PN) against *Fusarium*.

H_1=There is statistical difference in dual culture inhibition of *Trichoderma* (Th-PN) against *Fusarium*.

Result: P< 0.05, so result is statistically significant

Conclusion: *Trichoderma* (Th-PN) exhibited better inhibition against *Fusarium* culture.

ANOVA

8. T0 Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.733	2	4.867	.018	.982
Within Groups	3266.000	12	272.167		
Total	3275.733	14			

ANOVA

9. T1 Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.933	2	.467	.003	.997
Within Groups	2090.800	12	174.233		
Total	2091.733	14			

ANOVA

10. T2 Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.133	2	4.067	.011	.989
Within Groups	4617.200	12	384.767		
Total	4625.333	14			

ANOVA

11. T3 Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43.200	2	21.600	.034	.966
Within Groups	7541.200	12	628.433		
Total	7584.400	14			

ANOVA

12. T4 Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	31.600	2	15.800	.036	.965
Within Groups	5296.000	12	441.333		
Total	5327.600	14			

Result: $p>0.05$. There was no significant difference in plant height among plants within each treatment groups.

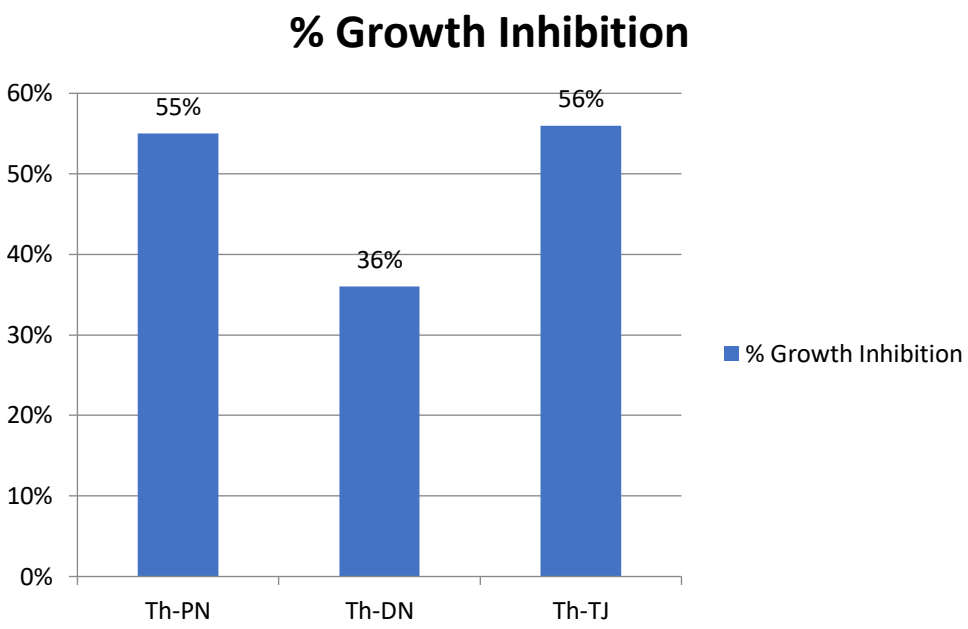

Figure 4.3: Per cent Growth inhibition in dual culture

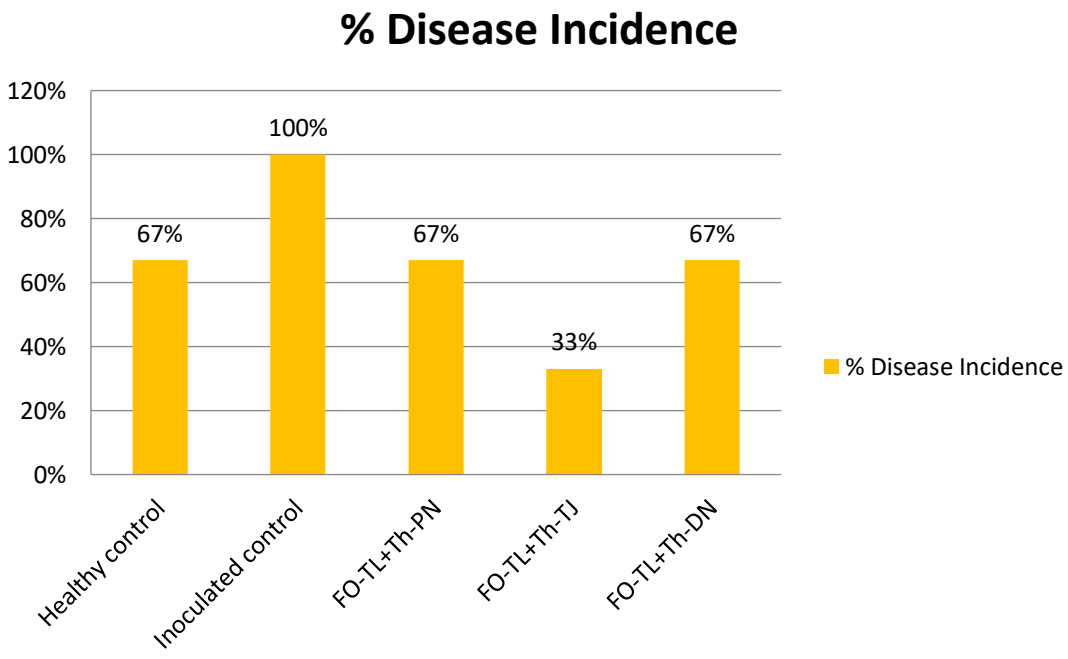

Figure 4.4: Per cent Disease Incidence