ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

INSTITUTE OF BIOTECHNOLOGY

In vitro and *In vivo* Test of Antagonistic *Trichoderma* Isolates against the Causative Agent of Tomato Wilt Disease (*Fusarium oxysporum f. sp. lycopersici*), in the Central Rift Valley of Ethiopia

By

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements of the Degree of Master of Science in Biotechnology

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Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in this or any other universities and that all sources of materials used for the thesis have been fully acknowledged.

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ABSTRACT

The study was initiated with the objectives of controlling tomato wilt diseases caused by Fusarium oxysporum species *and using Trichoderma species as a biocontrol agent against the test pathogen. Fusarium oxysporum the causative agent of tomato wilt disease was isolated from diseased tomato plants grown in five selected kebeles of Dugda Bora and Adami Tulu Jido-Kombolcha woredas of the Central Rift Valle (CRV) region of Ethiopia. Three Trichoderma species were isolated from the rhizosphere soil of healthy tomato plants. Trichoderma isolates were grown in different culture media namely; PDA, MEA and CDA to determine their mycelial growth. The pathogencity of isolate was determined on three different tomato varieties namely Cochoro, Miya and Fetane were grown in 20 cm plastic pots containing 3 kg of autoclaved soil in the greenhouse. The antagonistic effect of Trichoderma isolates against the test pathogen was tested both in vitro and under greenhouse conditions. Fusarium oxysporum isolate produced three types of spores on PDA medium namely microconidia, macroconidia and chlamydospores. The antagonistic effect of Trichoderma isolates against the test pathogen was tested by infesting 20 ml of Fusarium oxysporum isolate spores at a concentration of 1.5 $x10^6$ conidia/ml and 20 ml Trichoderma isolate spores with the same concentration after 7 days of inoculation of Fusarium oxysporum isolate spore. All the Trichoderma isolates showed maximum mycelial growth on MEA and minimum mycelial growth on CDA. From the three tomato varieties, Miya variety was more susceptible to Fusarium oxysporum isolate infection than both Cochoro and Fetane varieties. The antagonistic effect of Trichoderma isolates culture filtrate on the test pathogen showed that Trichoderma isolate AUT9 exhibited the highest percent inhibition 66 % on the mycelial growth of test pathogen. Trichoderma isolates AUT8 and AUT10 showed percent inhibition of 61 and 58%, respectively on the mycelial growth of the test pathogen. The symptoms of wilt were observed after 60 days of sowing. All Trichoderma isolates achieved maximum mycelial growth at 25^0C and minimum mycelial growth at 15^0C. The effect of p^H on the mycelial growth of Trichoderma isolates in vitro showed that Trichoderma isolate AUT8 showed maximum mycelial growth at p^H 5.5 and Trichoderma isolate AUT9 showed maximum mycelial growth at p^H 6.5.*

Key words: *Pathogen, Resistance, Pesticide, Symptom, Antagonism, Antibiosis*

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Acronyms and Brief Description

ANOVA = Analysis of variance

ATP = Adenosine Triphosphate

AUT = Addis Ababa University *Trichoderma*

BCA = Biological Control Agent

bsl = below sea level

CDA = Czapeck Dox Agar

CRBD = Completely Randomized Block Design

CRV = Central Rift Valley

FAO = Food and Agricultural Organization

GDP = Gross Domestic Product

HR = Hypersensitive Response

ISR = Induced Systemic Resistance

LSD= Least Significance Difference

MARC = Malkassa Agricultural Research Center

masl = meters above sea level

MEA = Malt Extract Agar

MoARD = Ministry of Agriculture and Rural Development

P = Phosphate

PDA = Potato Dextrose Agar

PDB = Potato Dextrose Broth

PSM = Phosphate Solubilizing Microorganisms

RP = Rock Phosphate

SAR = Systemic Acquired Resistance

SD = Standard Deviation

SDW = Sterile Distilled Water

SFP = Sterile Filter Paper

SPSS = Statistical Program for Social Science

TWD = Tomato Wilt Disease

WFD = World Food Day

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.), belongs to the *Solanaceae* family and it is grown worldwide for its edible fruit (Kocchar, 1981). The fruit is consumed fresh, sliced and used in the preparation of salads, milled and made in to pastes and added to stews. Tomato (*Lycopersicon esculentum* Mill.) is one of the most important and widely grown vegetable in Ethiopia. Fresh, processed and cherry type are produced in the country by small scale farmers/ producers that contribute to the bulk of fresh market tomatoes whereas processed types are mainly produced by large scale horticultural farms (MoARD, 2009). It is an important source of vitamin A, B and C as well as minerals. The yellow type tomatoes that are high in β-carotene are also becoming dietary important in the market. Farmers are interested in tomato production more than any other vegetables for its potential production all year round, which increase in high profit per unit area. The processed products such as tomato paste, tomato juice, tomato ketchup and whole peel tomato are produced for local market and export. Recently tomato is recognized for treating various human diseases (Beutner *et al.,* 2001).

The production of tomato is of worldwide agricultural importance. They are rich in protein, mineral, vitamins and fiber. Tomato is one of horticultural crop and highly cultivated in the central rift valley (CRV) region of Ethiopia. It is the most profitable vegetables which give net income of about 11000 to 14000 Eth birr/ha (Lemma and Herath, 1994). It is the most prominent and known fruit vegetables cultivated in Ethiopia. It is widely accepted and commonly used in variety of dishes as raw, cooked or processed product more than any other vegetables. It is also a cash generating crop to small farmer and provides employment in the production and processing industries. Farmers are interested in tomato production than other vegetables for its multiple harvests, high profitability and its

potential to improve income and nutrition of household. Such diverse uses made it an important vegetable in irrigated agriculture (Lemma, 2003).

The economy of the country predominantly depends on agriculture which contributes about 50% to the total gross domestic product (GDP) and 90% of export items of which crops are leading component. The country has the favorable conditions for the production of a number of vegetable crops. The wide range of altitude, ranging from below sea level (bsl) to over 3000 meter above sea level (masl) gives it a wide range agro-ecological diversity ranging from humid tropics to alpine climates, where most types of vegetable crops can be successfully grown. Vegetable crops are also important for food security in times of drought, famine and food shortage. They provide a source of income for the farmers; create employment opportunity and contribution to the national economy as export commodities as cited by (Fekadu and Dandena, 2006).

Tomato requires a relatively cool, dry climate for high yield and premium quality. However, it is adapted to a wide range of climatic conditions from temperate to hot and humid tropical. The optimum temperature for most varieties lies between 21 and 24^0C. The plants can survive a range of temperatures, but the plant tissues are damaged below 10^0C and above 38^0C (Naika *et al.,* 2005).

The production and quality of tomatoes are affected by biotic and abiotic factors in tomato growing areas of the world. Various diseases and disorders can affect tomatoes during the growing season. Among these, *Fusarium oxysporum* f. sp. *lycopersici* 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 loss (Kirankumar *et al.,* 2008).

1.1. Statement of the Problem

Fusarium wilts of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* is one of the most economically important and widespread diseases of the cultivated tomato (*Lycopersicon esculentum* Mill.). It is one of the most important diseases which are highly destructive to tomatoes grown in greenhouse and in the field in many warm regions of the world, where it causes 10-50 % yield loss (Larkin and Fravel, 1998; Borrero *et al*., 2004). The management of *Fusarium* wilt pathogen is particularly complex because it lives in or near the dynamic environment of rhizosphere and can frequently survive long periods in soil through the formation of certain resistant structures of the pathogen (Blum and Rodriquez-Kabana, 2004). Tomato wilt disease becomes one of the most prevalent and damaging disease wherever tomatoes are grown intensively because the pathogen can persist indefinitely in infested soils (Agrios, 2005).

The current population of 80 million people in Ethiopia is expected to double within the next 30 years. Almost 80% of the populations live in the country side while the rest situated in urban area. An estimated five million people suffer from lack of vitamins and essential minerals of which 80% are children. Vegetables are the major sources of most micronutrients and the only practical and sustainable way to ensure their supply as cited by (Fekadu and Dandena, 2006)

1.2. Significance of the Study

This study will help to develop different biocontrol agents against the test pathogens especially *Fusarium oxysporum* that cause wilt disease in tomato plant and reduce the yield of tomato. Different approaches may be used to prevent, mitigate or control plant diseases. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years.

However, the environmental pollution caused by excessive use and misuse of agro-chemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in people's attitudes towards the use of pesticides in agriculture (Pal and Gardener, 2006).

Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove the most hazardous chemicals from the market. Additionally, the spread of plant diseases in natural ecosystems may preclude successful application of chemicals, because of the scale to which such applications might have to be applied. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. A variety of biological controls are available for use, but further development and effective adoption will require a greater understanding of the complex interactions among plants, people, and the environment (Pal and Gardener, 2006). In fact, the antifungal abilities of these beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then (Samuels, 1996).

Chemical compounds have been used to control plant disease, but using chemical fungicides abuse in their employment and favored the development of pathogens resistant to fungicides.

Finally, this study has been initiated to isolate, identify, characterize and evaluate the potential biological control agents such as; *Trichoderma* isolates from the rhizosphere soil and testing them against *Fusarium oxysporum* species from the Central Rift Valley region of Ethiopia.

1.3. Objectives of the Study

1.3.1. General Objective

- ✓ To isolate, identify and characterize *Fusarium oxysporum* species from diseased tomato plants and *Trichoderma* species from the rhizosphere soil of tomato plants from the Central Rift Valley (CRV) region of Ethiopia and *in vitro* and *in vivo* evaluation of the antagonistic activities of *Trichoderma* isolates against the test pathogen.

1.3.2. Specific Objectives

- ✓ To isolate, identify and characterize *Fusarium oxysporum* species from diseased tomato plants
- ✓ To isolate, identify and characterize *Trichoderma* species from the rhizosphere soil of healthy tomato plants
- ✓ To evaluate *in vitro* antagonistic activities of *Trichoderma* isolates against the test pathogen
- ✓ To study the effect of different growth factors such as temperature, pH and culture media on the growth of *Trichoderma* isolates
- ✓ To study *in vivo* antagonistic activity of *Trichoderma* isolates against the test pathogen under greenhouse conditions

2. Literature Review

2.1. Tomato Plant and Its Origin

Tomato has its origin in the South American Andes. The cultivated tomato was brought to Europe by the Spanish conquistadors in the sixteenth century and later introduced from Europe to southern and eastern Asia, Africa and the Middle East. More recently, wild tomato has been distributed into other parts of South America and Mexico (Naika *et al.,* 2005). In Ethiopia, no local cultivars of tomato have evolved or been developed and hence all varieties grown are introduced (Yayeh Zewdie, 1989).

2.2. Economic Importance of Tomato

The tomato (*Solanum lycopersici L.*), according to the FAO (2004), is the second most cultivated vegetable in the world, after potato, with an annual production of nearly 10^8 ton of fresh tomato in $3.7x10^6$ hectare (ha) worldwide, China, USA and Turkey are the leading tomato producers. In addition to its economic importance, tomato consumption has recently been demonstrated to be beneficial to human health, because of its content of phytochemicals such as lycopene, β-carotene, flavonoids, vitamin C and many essential nutrients (Beutner *et al.,* 2001). This composition explains the high antioxidant capacity in both fresh and processed tomatoes (Gahler *et al.,* 2003), associating the fruit with the lower rates of certain types of cancer and cardiovascular disease (Rao and Agarwall, 2000). Tomatoes are a major source of lycopene, a carotinoid with a notable capacity to eliminate active-oxygen species (Rao *et al.,* 1998; Toor and Savage, 2005).

2.3. Tomato Production in Ethiopia

In Ethiopia, it is produces between 700 and 2000 meters above sea level, which is characterized as warm and dry day and cooler night. These conditions are favorable for optimum growth and development of tomatoes. A temperature range between 21 to 27^0C day and 10 to 20^0C night is

favorable for plant development, and fruit set in the country. It grows better at a constant day and night temperature. A difference of 6^0C between day and night temperatures was found sufficient for good plant growth and development. Fruit setting is poor when the temperature is either high or low. Extreme temperatures cause flower drops and poor fruit set. At high temperatures such as above 35^0C day and 20^0C night temperatures from March to July cause a high blossom drop. The cultivars that are currently in production failed to set fruits and gave low yield when the day and night temperatures were above 32^0C and 21^0C, respectively. However, heat tolerant genotypes could be the potential ones for such growing conditions. It must be also noted that tomato flowers fail to set fruits as the result of poor nutrient imbalances and poor management.

Tomato can be grown in many types of soils. However, well drained friable sandy loam soil with pH of 6.7 is preferable for early and high fruit yield. Tomato is widely produced under irrigation. Production in the rainy season is also possible, but need intensive pest and disease management. The bulk of tomato production is concentrated in the Central Rift Valley (CRV) region. However; there are favorable growing pockets in the different parts of the country (MoARD, 2009).

2.4. Biology of *Fusarium oxysporum* Species

Fusarium oxysporum is an anamorphic species circumscribed by a suite of morphological criteria (Nelson *et al.*, 1983), principally the shape of the macroconidium, the structure of the microconidiophore, and the formation and disposition of chlamydospores. Notwithstanding these unifying criteria, considerable morphological and physiological variation within *Fusarium oxysporum* has long been recognized; many such variants were initially assigned specific status (Wollenweber and Reinking, 1935). Subsequently (Snyder & Hansen, 1940), proposed to consolidate all of these species into *Fusarium oxysporum*, a concept that has received wide acceptance. Nevertheless, it is clear that *Fusarium oxysporum* comprises a wide diversity of strains,

and the extent to which these differences are sufficient to justify recognition of separate species is by no means a settled issue.

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder and Hansen is one of the most common pathogen that causes wilt of tomato in areas of upland cultivation which can cause economic losses (Agrios, 2005). *Fusarium oxysporum* f. sp. *lycopersici* has become one of the most damaging and difficult to control wherever tomatoes are grown intensively, because it grows endophytically and persists in infested soils.

Table1: Some of the common fungal diseases of tomato and there causative agents

Common names of tomato diseases	**Causative agents (pathogens)**
Fusarium wilt	*Fusarium oxysporum f. sp. lycopersici*
Early blight	*Alternaria solani*
Septoria leaf spot	*Septoria lycopersici*
Anthracnose	*Colletotrichum coccodes*
Verticillium wilt	*Verticillium albo-atrum*
Late blight	*Phytophthora infestans*
Gray mold	*Botrytis cinerea*
Leaf mold	*Fulvia fulva*
Powdery mildew	*Oidium neolycopersici*
Southern blight	*Sclerotium rolfsii*

Sources: Mark and Brooke (2006)

2.5. Symptoms of Tomato Wilt Disease

Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum*, initially causes a yellowing and wilting of lower leaves on infected plants. Symptoms can be seen on a single branch, or on several branches on one side of the plant, or on all the lower branches. The yellowing and wilting progress up the plant as the fungus spreads within its host. Yellowed, wilted leaves often dry up and drop prematurely. Eventually the entire plant wilts and dies early, producing few or no fruits. Plants infected with *Fusarium* wilt will have brown discoloration of vascular system, which can be used as an aid in diagnosis. The discoloration can extend from the roots up the stem through the branches and into the petioles of the plant. *Fusarium* usually enters its host through feeder roots and subsequently multiplies and colonizes the food and water conducting vessels of the plant. The disease is more severe when air and soil temperatures are above 78^0F and 90^0F and is more likely to occur in poorly drained soil. Infection may occur at any time during the life time of the plant. The fungus can persist in most soils indefinitely, because of its ability to colonize the roots of a number of weeds and its ability to produce resistant spore structures (Edward and William, 2009).

Fusarium wilt is an important disease of tomato in many warm regions in the world, caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici*, which is highly destructive both in greenhouses and field grown tomatoes destroying up to 10 – 50 % of yield (Kallo, 1991; Mao *et al.*, 1998). *Fusarium oxysporum* f. sp. *lycopersici* is a known pathogen of tomato plant which is an economically important crop (Suárez-Estrella *et al.*, 2007). Tomato yield is significantly reduced by *Fusarium oxysporum* f. sp. *lycopersici* because it can destroy roots of tomatoes at growth stages. Numerous strategies have been proposed to control this fungal pathogen (Ahmed, 2011). Currently, the most effective method in preventing tomato from *Fusarium* wilt is mixing of tomato seeds with chemical

fungicides. However, the use of chemical fungicides can be harmful to other living organisms besides reduction of soil microorganisms (Lewis *et al.,* 1996).

Fusarium wilt diseases, caused by pathogenic *formae speciales* of the soil inhabiting fungus *Fusarium oxysporum* can cause severe losses in a wide variety of crop plants. On tomato, two symptomlogically distinct forms of the pathogen can cause either a vascular wilt (*Fusarium oxysporum* f. sp. *Lycopersici,* Snyder & Hansen, 1940) or a crown and root rot (*Fusarium oxysporum* f. sp. *radicis-lycopersici*, Jarvis, 1988). Both of these pathogens occur throughout most tomato growing areas and either can devastate a crop. Although the use of *Fusarium* -resistant tomato cultivars can provide some degree of control of these diseases, the occurrence and development of new pathogenic races is a continuing problem, and currently there are no commercially acceptable cultivars with adequate resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* or race 3 of *Fusarium oxysporum* f. sp. *lycopersici*.

According to (Ozbay and Newman, 2004; El-Khallal, 2007), *Fusarium oxysporum* is a soil borne fungal pathogen that infects plants through roots at all stages of plant growth, causes major economic losses by inducing necrosis and wilting symptoms in many crop plants. Tomato wilt, caused by *Fusarium oxysporum f. sp. lycopersici* is a serious disease in greenhouses and open fields in the temperate regions of the world.

Fusarium oxysporum is an abundant saprophyte in soil and organic matter and occurs worldwide in the rhizosphere of many plant species. The fungus has numerous specialized forms of known as *formae speciales* (f. sp.) that infect a range of host plants causing diseases such as vascular wilt, corm rot, root rot or damping-off (Armstrong and Armstrong, 1981). Two *formae speciales* of this fungus are known to affect tomato, a crop plant of great economic importance. *Fusarium oxysporum*

f. sp. lycopersici is the cause of a severe wilt disease; where as *Fusarium oxysporum f. sp. radicis* causes crown and root rot (Jones, 1991).

The yield tomato is significantly reduced by fungal diseases. *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is a serious problem for tomato production in many areas which cause yield reduction of up to 25% (Fravel *et al.*, 2005). This pathogen enters tomato through the roots and causes yellowing of the oldest leaves, often on only one side of the plant. The yellowed leaves gradually wilt and die and the infected plants can be severely stunted. Agrochemicals are commonly used to control this pathogen, but are relatively expensive and subjected to various environmental constraints.

Apart from their role as plant pathogens several strains of *Fusarium oxysporum* are known to control *Fusarium* wilt diseases (Fravel *et al*., 2003) and are involved in the suppressiveness of soils (Alabouvette, 1990). Non-pathogenic strains are present in the rhizosphere soil and colonize plant roots, but seen not to invade the vascular system (Olivain and Alabouvette, 1999). The mechanisms by which non-pathogenic *Fusarium oxysporum* isolates antagonize pathogenic isolates and the biochemical responses of the plant are only partially understood (Olivain and Alabouvette, 1997).

Tomato wilt disease (TWD) caused by *Fusarium oxysporum f. sp. lycopersici* is one of the limiting factors in the production of tomato. It has become one of the most prevalent and damaging disease wherever tomatoes are grown intensively because the pathogen persists indefinitely in infested soils. Controlling such diseases mainly depend on fungicides treatments (Rauf, 2000; El-Mougy *et al*., 2004). However, fungicidal applications cause hazards to human and animal health and increase environmental pollution. Therefore, alternative eco-friendly approach treatments for control of plant diseases are needed (Abd-El-Kareem, 2007; Rojo *et al*., 2007; Mandal *et al*., 2009).

2.1.1. Chemical Control

Disease problems and control are by no means peculiar to modern agriculture. Farmers are in general familiar with chemical pesticides because of their quick, effective actions. However, chemical control poses risks to human lives and environments hence the necessity of changing to biological control (Denis and Webster, 1971). The use of chemical pesticides increases the cost of production and also makes the products more expensive (FAO, 1989). Another negative feature of chemical pesticides is the capacity of the pests and disease species to quickly evolve genetic resistance to the plethora of chemical agents used against them (Dean, 1991).

Soil erosion and underground water contamination are known to be caused by pesticide residues and fertilizer runoffs (Basim *et al.,* 1999). As environmental and health hazards mount as a result of heavy pesticide usage, , a new holistic perspective emerges in food production - sustainable agriculture and this is a dynamically evolving system in which widely divergent agricultural practices and conditions are evaluated, modified and verified in order to create a productive and continuing sustainable agriculture (Salami *et al.,* 2005).

Pesticides are essentially organic compounds widely used to control plant pathogens in many countries. However, the non-degradable components of their compounds have accumulated over the years and entered the food chain causing higher toxicity in animals (Cigdem and Merih, 2003; Chet, 1987; Lynch, 1990). Fungicides and soil fumigants are used to control the disease. The toxicity of these compounds has led to a general trend to reduce their application in the soil. An estimated 37 percent crop loss is due to pests, of which 12 percent is due to pathogens. The extensive use of fungicides in various parts of the world for years has increased the pollution level in soil and water, and adverse effect on food quality and human health. Apart from this, the chemicals tend to become less efficient due to the development of resistance among the pathogens over time.

The excessive misuse of a wide range of fungicides has led to it being harmful to the environment and increases the resistant pathogen populations (Ozgonen *et al*., 2001). *Fusarium oxysporum* f. sp. *lycopersici* becomes resistant to those chemical fungicides. For this reason, alternative methods with emphasis on biological control using fungi or bacteria of the controlling the disease have been studied by several researchers to reduce fungicide application and decrease cost of plant production. Chemical control of soil borne phytopathogenic fungi is costly and causes great damage to beneficial resident microorganisms and human being. Moreover, it represents an environmental risk leads to series of hazardous events of which the development of chemical-resistant pathogens is exuberant. All these facts along with the phase out of methyl bromide by the year 2015 in the globe (Ishikawa *et al*., 2005) raised the need to study non chemical alternatives. Hence, it is necessary to look for alternative disease management practices, which include the use of eco-friendly biological control agents (BCAs) and pathogen-resistant crop cultivars.

2.2.1. Biological Control

Biological control of plant pathogens has been increasingly interesting for plant pathologists and researchers. It can serve as long-term protection of the disease as well as maintain environmental conditions for natural balance (Soytong and Quimio, 1989). There have been many reports about using bioactive compounds from biological control agents which were extracted from different fungi that inhibit many plant pathogenic fungi, including *Fusarium* wilt of tomato (Soytong, 1992).

2.2.2. *Trichoderma* species

Trichoderma species are imperfect filamentous fungi with teleomorphs belonging to the Hypocreales order of the Ascomycota division. The ecological role of this genus is that *Trichoderma* strains take part in the decomposition of plant residues in the soil (Harper and Lynch, 1985).

Some *Trichoderma* species are very good cellulase producers and therefore they are important for the biotechnological industry (Reczey *et al.,* 1996).

Trichoderma are common fungi in soil and rhizosphere ecosystem of that have been known not only for potential to control several plant pathogens that caused soil-borne (Koch, 1999; Spiegel and Chet, 1989; Barker and Paulitz, 1996; Harman and Hadar, 1983), air-borne (Elda, 2000) and postharvest (Freeman *et al*., 2004) and diseases in a wide range of crops by different mechanisms (Howell, 2003), but also for their ability to promote plant growth (Hoyos-Carvajal *et al*., 2009; Shanmugaiah *et al*., 2009; Harman *et al*., 2004;Ouseley *et al*., 1994; Barker, 1989; Barker, 1988) and improve nutrient uptake (Chet, 2001; Yedidia *et al*., 2001) as well as improve plant defense level against biotic and or a biotic stress (Mastouri *et al*., 2010; Hoitink *et al*., 2006; Honson and Howell, 2004; Yedidia *et al*., 1999).

Trichoderma are, for the most part, classified as imperfect fungi, in that they have no known sexual stage. Rapid growth rate in culture and the production of numerous spores (conidia) that are varying shades of green characterize fungi in this genus. The reverse side of colonies is often uncolored, buff, yellow, amber, or yellow-green, and many species produce prodigious quantities of thick-walled spores (chlamydospores) in submerged mycelium (Gams and Bisset, 1998). The potential of *Trichoderma* species as biocontrol agents of plant diseases was first recognized in the early 1930s (Weindling, 1932).

Many *Trichoderma* spices have been identified as having potential applications in the biological control of plant pathogenic fungi on many crops including strawberries (*Fragaria vesca*), cucumbers (*Cucumis sativus*), tomatoes (*Lycopersicon esculentum M*.), radishes (*Raphanus sativus*), sugar beets (*Betavulgaris*), cotton (*Gossypium hirsutum L*.). *Trichoderma harzianum*, a number of the fungal genus *Trichoderma*, has been extensively studied as biological control agent (Lewis and Papavizas,

1991; Elda, 2000) due to its ability to successfully antagonize other fungi including plant pathogenic species.

Trichoderma species are generally considered to be aggressive competitors (Samuels, 1996), grow very fast and rapidly colonize substrates to exclude pathogens such as *Fusarium* species (Papavizas, 1985). Rhizosphere competence, following seed treatment is an important strategy to create a zone of protection against pathogens (Howell, 2003). *Trichoderma* species, either added to the soil or applied as seed treatments, grow readily along with the developing root system of the treated plants (Ahmad and Baker, 1987).

The mode of hyphal interaction and parasitism of *Trichoderma* species with several soil-borne pathogenic fungi has been documented (Chet *et al*., 1981; Dennis and Webster, 1971). *Trichoderma* grows tropically toward hyphae of other fungi, coil around them in a lectin-mediated reaction, and degrade cell walls of the target fungi by the secretion of different lytic enzymes. This process (mycoparasitism) limits growth and activity of plant pathogenic fungi. *Trichoderma* attaches to the host hyphae via coiling, hooks and appressorium like bodies, and penetrate the host cell wall by secreting lytic enzymes. The interaction is specific and not merely a contact response. *Trichoderma* recognizes signals from the host fungus, triggering coiling and host penetration (Dennis and Webster, 1971; Sivan and Chet, 1989). Chitinolytic enzymes have been suggested as the key enzymes in mycoparasitism (Elad *et al*., 1982; Cherif and Benhamou, 1990).

Among different biological control agents (BCAs), *Trichoderma* species has proved effective and selective enough against most of the fungal diseases. *Trichoderma* species have evolved numerous mechanisms that are involved in attacking other fungi.

These mechanisms include competition for space and nutrients (Elad *et al*., 1999), mycoparasitism (Haran *et al*., 1996; Lorito *et al*., 1996), production of inhibitory compounds (Sivasithamparam and

Ghisalberti, 1998), inactivation of the pathogen's enzymes (Roco and Perez, 2001) and induced resistance (Kapulnik and Chet, 2000).

Today, more than 50 different *Trichoderma*-based agriculture products can be found as registered in many countries in five continents, and are sold and applied to protect and improve yield of vegetables, ornamentals and fruit trees (Lorito, 2005).

Biological control of plant pathogens is an alternative approach to decrease dependence of modern agriculture on chemical fungicides, which cause environmental pollution and development of resistant strains (Harjono and Widyastuti, 2001). Biological control of plant disease is defined as the involvement of the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause (Lewis and Papavizas, 1991). Different biological control agents (BCAs) can be used for the control of diseases. These include bacteria, fungi and actinomycetes.

2.2.3. *Trichoderma* Species as Biocontrol Agents

Antagonistic *Trichoderma* species are considered as promising biological control agents against numerous phytopathogenic fungi including *Fusarium oxysporum* (Sarhan *et al.,* 1999). These filamentous fungi are very common in nature, with high population densities in soil and plant litters (Samuels, 1996). They are saprophytic, promptly growing and easy to culture besides producing huge amount of conidia with long life-time.

Biological control of root pathogenic fungi is eco-friendly and is a promising solution for many agriculture pest problems. It could be achieved directly through introducing antagonists able to suppress pathogens activity, indirectly by manipulation of microbial balance in the soil or through host resistance induced, acquired, or inherited (Lucas, 1998).

Trichoderma is a fungus that exists in almost all soils and a wide range of habitats. In soil, they are the most widespread and culturable fungi. They prefer locations with a large supply of plant roots, which they promptly colonize. Additionally, the *Trichoderma* species attack, parasitize or derive nutrition from other fungi. Because the species flourishes best when there are copious amounts of healthy roots, they have developed many mechanisms for attacking other fungi and for improving plant and root growth (Benitez *et al*., 2004).

Many species of *Trichoderma*, if given optimal conditions, establish stable and long-lasting colonization of root surfaces and even penetrate into the epidermis and a few cells below this level (Harman *et al.*, 2004). This intimate relationship between *Trichoderma* and the host root cells is what induces localized and systemic resistance responses to pathogen attack. *Trichoderma* also has the ability to dissolve the cell wall membranes of pathogenic fungi. Initially *Trichoderma* species were only thought to have suppressive effects on a small number of plant root pathogens, however as research into the methods of this suppressive effect were studied, it was found that *Trichoderma* had other beneficial properties on plant growth and development. These growth enhancement effects went further than just suppression of pathogen in the root zone allowing a return to normal healthy growth. It has been found that the species *Trichoderma* increase the uptake and concentration of a variety of nutrients (copper, phosphorus, iron, manganese and sodium) in the roots in hydroponic culture (Yedidia *et al.*, 2001).

Apart from pathogen suppression and growth promotion activities, *Trichoderma* has been selected for widespread use in horticulture for a number of other reasons. The spores of *Trichoderma* can be easily formulated into long shelf life products that, upon reactivation, rapidly colonize the growing media under suitable conditions.

Trichoderma is a strong competitor in the root zone and generally has a good chance of establishment even when there are pre-existing healthy populations of other microbial species. It has also proven to be compatible with a number of other bio control agents, including beneficial fungi in a number of different studies, so that mixtures of effective microbes can be safely applied. In general *Trichoderma* species have also been found to be highly resistant to a variety of toxins including chemical fungicides, heavy metals and antibiotics produced by other microbes. While *Trichoderma* is commonly applied to the root zone or nutrient solution, certain strains have also been applied to fruit, flowers and foliage to control certain plant pathogens and even used to prevent post harvest rot disease. One study found that application of *Trichoderma* species on greenhouse strawberries could control post harvest rotting, while several *Trichoderma* species have been used to protect fruit such as banana, apple, mango and tomato during post harvest storage (Verma *et al.*, 2007). There is evidence to suggest *Trichoderma* assists plant growth and development under stressful conditions and the growth promoting potential of *Trichoderma* appear to be stronger in crops growing under less than ideal conditions.

2.2.1.1. Mechanism of Inhibition of Fungi by *Trichoderma* Species

2.2.1.2. Antibiosis

Antibiotics are generally defined as low molecular weight organic compounds that are produces as secondary metabolites by certain groups of microorganisms, mostly soil inhibitors that are inhibitory to the growth or other metabolic activities of other microorganisms at low concentrations (George, 2002).

Antibiosis is considered as the most common mechanisms of action deployed by the microorganisms up to now investigated (Howell, 1998). It is defined as antagonism brought about

the metabolites of fungal antibiotics or antibiotic-like compounds, lytic enzymes, volatile compounds, siderophores or other toxic substances.

The potential use of the *Trichoderma* species as a biocontrol agent was suggested more than 70 years ago by Weindling (1932), who was first to demonstrate the parasitic activity of a member of this genus against soil-borne fungal or bacterial pathogens. Antibiosis is required as one of the most important attributes in deciding the competitive saprophytic ability of *Trichoderma* species. The first knowledge of toxic metabolite production by species of *Trichoderma* was largely due to Weindling (1934) who showed the production of an antifungal metabolite by *Trichoderma lingorum*.

2.2.1.3. Hydrolytic Enzymes

The role of enzymes in biocontrol can often be assigned to mechanisms, parasitism and antibiosis. In particular cell wall degrading enzymes such as chitinases, 1,3- β- glucanases and cellulases, are not only important features of mycoparasites for colonization of their host fungi, but also may exhibit considerable antifungal activity on their own (Hermosa *et al.*, 2000). One of its main mechanisms, antibiosis, relies on the recognition, binding and enzymatic disruption of the host fungus cell wall (Haggag, 2002).

Trichoderma produces a variety of lytic enzymes that have a high diversity of structural and kinetic properties, thus increasing the probability of this fungus to counteract the inhibitory mechanisms used by neighboring microorganisms (Ham *et al.*, 1997). Further, *Trichoderma* hydrolytic enzymes have been demonstrated to be synergistic, showing an augmented antifungal activity when combined with themselves, other microbial enzymes, PR proteins of plants and some xenobiotics compounds (Lorito *et al.*, 1994b). *Trichoderma* strains may be employed in many different ways in order to obtain beneficial effects to the plant, such as biocontrol and plant growth promotion.

Recently, it has been demonstrated that hundreds of genes and gene products are involved in the multiple interaction processes of this biocontrol agent (BCA): mycoparasitism, antibiosis, competition (for nutrients or space), improvement of plant stress tolerance by enhancing the root and aerial development, solubilization and sequestration of inorganic nutrients, induced resistance, and inactivation of enzymes produced by pathogens (Monte, 2001). Some of these genes have been identified, characterized, patented and used transgenically to improve plant disease resistance against fungal pathogens (Lorito *et al.,* 1998). Volatile compounds from the biological control agents have an important part of the inhibitory mechanism, especially under closed conditions. Production of volatile ammonia (NH_3) has been implicated as possible mechanism to control soil borne pathogens (Paultiz *et al.,* 2000).

2.2.1.4. Competition

The nature of competition is fungistatics (inhibitory). Good antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites that are produced by other species including plants and no survive under extreme competitive conditions. *Trichoderma* strains grow rapidly and resistant to many toxic compounds including herbicides, fungicides and pesticides such as DDT and phenolic compounds (Chet *et al.,* 1997).

Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Chet *et al.,* 1997). One of the most sensitive stages for nutrient competition in the life cycle of *Fusarium* is chlamydospore germination (Barker, 1986). In soil the chlamydospores of *Fusarium oxysporum* need nutrition maintain germination rate of 20-30%. The germination may decrease due to sharing of nutrients by other microorganisms. Root exudates are major source of nutrients in soil which are excreted from the root

tips. Thus, colonization in the rhizosphere of root tip by an antagonist might reduce infection by *Fusarium*-like pathogens (Cook and Baker, 1983). In addition, *Trichoderma harzianum* controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients with biocontrol becoming more effective as the nutrient concentration decreases (Alabouvette and Couteadier, 1992). Competition for carbon has also been involved in the determination of the antagonism expressed by different strains of *Trichoderma* species against several plant pathogens, especially *Fusarium oxysporum* (Siva and Chet, 1989). *Trichoderma* has a superior capacity to mobilize and take up nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide spread in fungal environments; cellulose, glucan and chitin among others, all of them rendering glucose (Chet *et al.*, 1997).

2.2.1.5. Mycoparasitism

Mycoparasitism, the direct attack of one fungus on another is a very complex that involves sequential events including recognition, attack and subsequent penetration and killing of the host. Trichoderma species may exert direct biocontrol by parasitizing arrange of fungi detecting other fungi and growing toward them. The remote sensing is particularly due to sequential expression of cell wall degrading enzymes, mostly chitinases, glucanases and proteases (Harman *et al.*, 2004). The production of cell wall degrading enzymes and peptaibols (Howell, 2003), which facilitates both the entry of *Trichoderma* hyphae into the lumen of the parasitized fungus and the assimilation of the cell wall content.

2.2.1.6. Stimulation of Host Defense Response

The ability of *Trichoderma* strains to protect plants against root pathogens has long been attributed to an antagonistic effect against the invasive pathogen (Chet *et al.,* 1997). However, these root fungi association also stimulate plant defensive mechanisms (Howell *et al.,* 2000; Hanson and Howell, 2004). Strains of *Trichoderma* added to the rhizosphere protect plants against numerous classes of pathogens. Example those that produce aerial infections, including viral, bacterial and fungal pathogens which point to the induction of resistance mechanisms similar to the Hypersensitive Response (HR), Systematic Acquired Resistance (SAR) and Induced Systematic Resistance (ISR) in plants (Harman, 2004).

2.2.1.7. Plant Growth Promotion by *Trichoderma* Species

Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stress and the uptake and use of nutrients (Arora *et al*., 1992). *Trichoderma* produces growth factors that enhance the rate of seed germination, plant growth and yield (Benitez *et al.,* 1998). Activity of biocontrol agents could also reduce the concentration of substances in the soil that are inhibitory to plant growth (Windham *et al.,* 1986). Thus, the plant growth promotion may be due to production of plant hormones or increased uptake of nutrients by the plant (Chet *et al.,* 1993); control of one or more sub potential pathogens (Barker, 1986) and /or strengthen plant's own defense mechanism (Zimand *et al.*, 1996).

3. Materials and Methods

3.1. Description of the Study Area

The samples were collected from the Central Rift Valley (CRV) region of Ethiopia especially Dugda Bora and Adami Tulu-Jido Kombolcha Woredas, which are part of the East Showa Zone of the Oromia Regional State. Geographically the study areas are located 134 km and 160 km from Addis Ababa and between $8^0$01'-$8^0$25'N latitude and $38^0$32'- $39^0$04'E longitude and $7^0$37'-$8^0$04'N latitude and $38^0$32'-$39^0$04' E longitude, respectively.

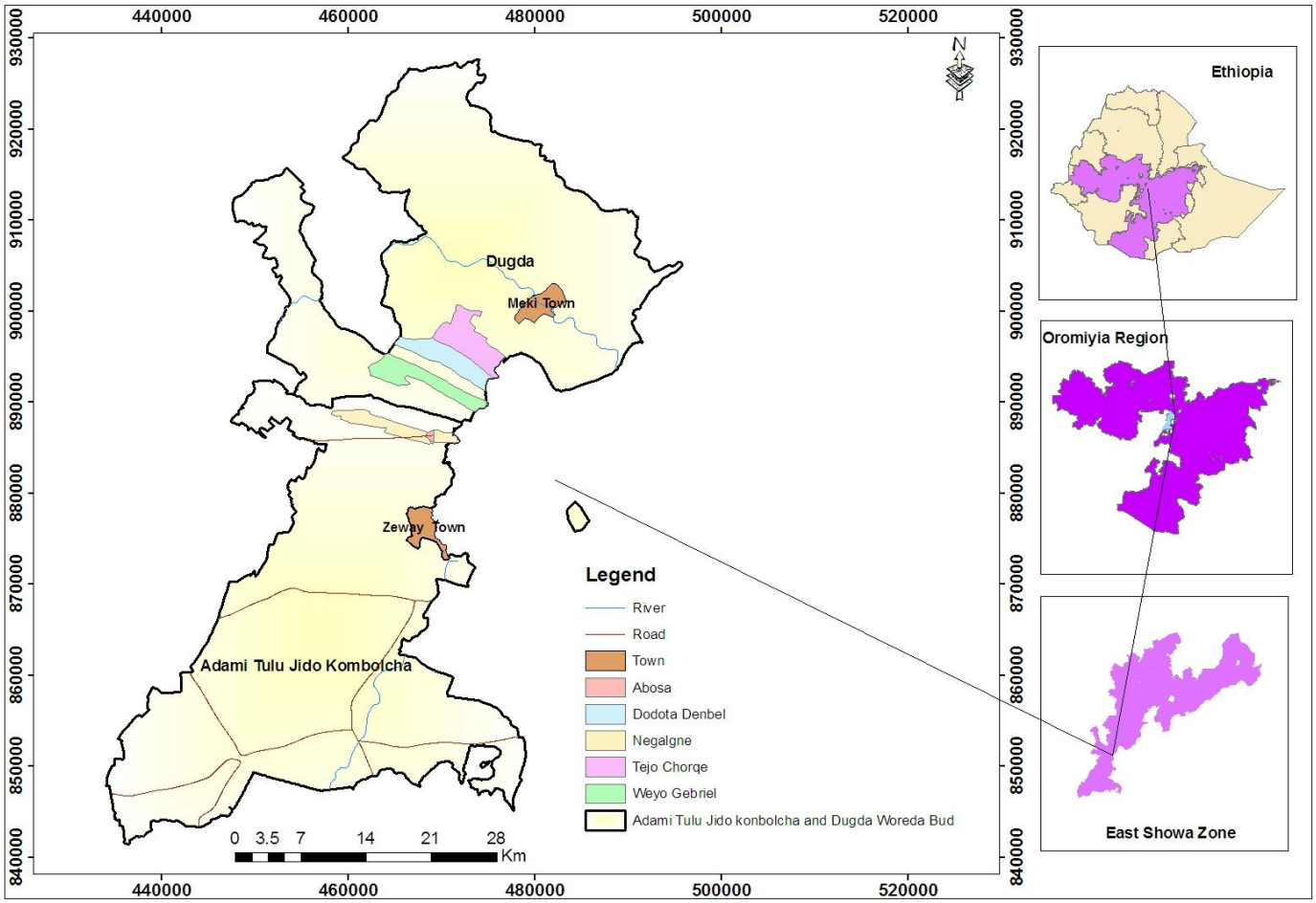


Figure 1. Map of Study Area

3.2. Experimental Sites

All experiments were carried out in the mycological research laboratory and greenhouse, Department of Microbial, Cellular and Molecular Biology, College of Natural Sciences, Addis Ababa University. It is located $9^{o}01'N$ and $038^{o}45'$ E. The minimum and maximum temperatures were $9.9^{0}C$ and $24.6^{0}C$, respectively.

3.2.1. Sample Collection Sites

Diseased tomato plants and rhizoshere soil samples were collected from Dugda Bora and Adami Tulu-Jido Kombolcha Woredas in the Central Rift Valley (CRV) region. These two woredas were selected due to the fact that the Central Rift Valley (CRV) region of Ethiopia, especially Dugda Bora and Adami Tulu-Jido Kombolcha Woredas are known in the country for having the highest potential for horticultural crops production (WFD, 2010). The soil samples and diseased tomato plant parts were collected from five different kebeles namely Dodota dembel, Weyo gebriel and Tajo choroqe are found in Dugda Bora woreda and Negalegne and Abossa are found in Adami Tulu- Jido Kombolcha woreda.

3.2.2. Isolation of *Trichoderma* Species from the Soil Samples

Collected soil samples were homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using 2.0 mm sieve. *Trichoderma* species were isolated from the soil samples according to (Kader *et al.,* 1999). Soil suspensions were prepared by adding 1.0 g homogeneously mixed soil to 9 ml sterile distilled water (SDW) and mixed thoroughly for 15 minutes on rotary shake. Immediately, each suspension was serially diluted to 10^{-5}. From the 10^{-5}dilution, 1 ml was spread on potato dextrose agar (PDA) with an antibiotic Neomycinsulfate at a concentration of 0.1 g per liter to suppress bacterial contamination and incubated at $25^{0}C$ for 7 days. Based on the appearance, *Trichoderma* colonies were repeatedly transferred to PDA plates to get pure cultures.

The *Trichoderma* species were isolated, identified and maintained on PDA slants. The inoculums were prepared by adding 5 ml of SDW to 7 days old slant culture and scrapped with sterile inoculation needle. The suspension was transferred into 25ml molten PDA, mixed thoroughly and poured into sterile Petri plates and incubated at 25^0C for 7 days. The mycelia discs of 5 mm diameter was cut randomly from 7 days old culture with sterile cork borer and used throughout the investigation. Pure colonies were confirmed as *Trichoderma* species according to the identification key based on the branching of conidiophores, shape of the phialides, emergence of phialospores (Rifai, 1969). All the confirmed *Trichoderma* isolates were further maintained on PDA slants at 4^0C and used for further studies.

3.2.3. Isolation of *Fusarium oxysporum* Species

Fusarium oxysporum species was isolated from diseased tomato plant that showing typical symptoms of *Fusarium* wilt. Plant tissues were surface disinfected with 2% Sodium hypochlorite (NaOCl) solution for 2 minutes to remove surface contamination and then rinsed three times with sterile distilled water (SDW) and the piece was placing on Potato Dextrose Agar (PDA) with an antibiotic Neomycinsulfate at a concentration of 0.1g per liter to suppress bacterial contamination and incubated at 25^0C for 7 days (Katan *et al.*, 1991). The identification of *F. oxysporum* isolate was made on the basis of their morphological characters (Henni *et al.*, 1994). Colonies with colony morphology of *F. oxysporum* isolate was sub cultured for 7days on PDA at 25^0C. An agar block of *F. oxysporum* isolate was preserved on PDA slants at 4^0C and used for further studies.

3.2.4. Sterilization and Maintaining of Cultures

The sterilization of media and glassware were done by autoclaving them at 121^0C temperature and 15 lb pressure for 15 minutes. Autoclaved glassware was dried in hot air oven at 80^0C for 45 minutes. The maintenance of cultures of *Fusarium oxysporum* isolate and *Trichoderma* isolates were

maintained on PDA slants in the plugged test tubes. The slants were stored in the refrigerator at 4^0C for further studies.

3.3.5. Slide Culture Methods

Antagonistic activity of *Trichoderma* isolates was evaluated against the test pathogen by slide culture method (Silvakumar *et al.,* 2000). A clear slide, cover slip and filter paper were placed on a V-shaped glass rod in 90 mm Petri dish and autoclaved. Then small amount of molten potato dextrose agar (PDA) medium was poured and evenly spread over the slide to make a thin agar film. A disc of 5 mm diameter of 7 days old culture of both and the test pathogen were placed 1 cm apart from each other in aseptic condition. A few ml of sterile distilled water was added to Petri dish to prevent drying of the slide and incubated at 25^0C for 7 days. At the end of incubation period, region where hyphae of *Trichoderma* isolate met the hyphae of the test pathogen was observed under light microscope.

A.  **B.**  **C.**  **D.** 

Figure 2. Slide Culture of *Trichoderma* isolates and *Fusarium oxysporum* isolate

A. *Trichoderma* isolate AUT8 B. *Trichoderma* isolate AUT9 C. *Trichoderma* isolate AUT10 and D. *Fusarium oxysporum* isolate

3.2.6. Single Spore Isolation of *Trichoderma* Isolates

The method developed by Gregory (1983) the single spore isolation was employed for isolation and purification of the *Trichoderma* isolates. A very dilute spore suspension was made on a sterile slide in water by dipping flamed, moisture loop into the sporulating culture. This spore suspension was streaked across a marked line on a very thin plate agar (1.5%) and was incubated at 25^0C for 12 hours. The germination of spore was observed using light microscope. The selected spore was examined under low power microscope in order to insure that only one spore was involved, and then after spore was transferred to another agar plate. The pure cultures were maintained on PDA at 4^0C for further study.

3.2.7. Preparation of Inoculum of *Trichoderma* Isolates

Trichoderma inoculum was prepared by the method proposed by (Smith *et al*., 1990). The conidial inoculum for each *Trichoderma* isolate was prepared by adding sterile distilled water (SDW) to 7 days old cultures under aseptic conditions and centrifuged at 5000 rpm for 10 minutes. The conidial spore suspension was adjusted to $1.5x10^6$ conidia/ml by using haemocytometer and aseptically transferred into 250 ml flasks containing 100 ml Potato Dextrose Broth (PDB). The flasks were incubated for 15 days on a rotary shaker at 121 revolution per million (rpm). The young germinated mycelia were harvested by filtration using sterilized cloth and aseptically washed with sterile distilled water (SDW).

3.3. In vitro Assay of *Trichoderma* Isolates against the Test Pathogen

Two agar discs 5 mm in diameter one with the mycelium of *F. oxysporum* isolate and another with mycelium of *Trichoderma* isolates were cut with the help of sterilized cork borer from the edge of 7 days old culture and placed on the PDA plates 3cm apart from each other and assessed for about 7

days whether any inhibition zone were appeared or not. An agar disc of the *F. oxysporum* isolate was placed at the center of the plate and *Trichoderma* isolate was placed at the periphery 3 cm apart from the test pathogen. The *Trichoderma* isolate was placed after three days because the growth rate of the *F. oxysporum* isolate was slower than *Trichoderma* isolate. Three replicates with each fungus were prepared. The plates were incubated for 7 days at 25^0C. The growth of pathogen in both the test and control experiment was recorded. Measurement of the percentage of inhibition and inner colony distance was taken daily for 7 days (Srinon *et al*., 2006). Radial growth of pathogen in the control and radial growth of pathogen in dual culture with antagonist and the width of the zone of inhibition (ZI) were measured as the smallest distance between the colonies in the dual culture plate (Royse and Ries, 1978; Garrett, 1956).

$$I = \frac{(R1-R2)}{R1} \times 100$$

Where, I= Percent inhibition in mycelial growth

R1= Radial growth of *Fusarium oxysporum* in the control

R2= Radial growth of *Fusarium oxysporum* in dual culture

3.3.1. Assay of Culture Filtrates of *Trichoderma* Isolates on the Mycelial Growth of the Test Pathogen

Secondary metabolites were isolated from the *Trichoderma* culture filtrates as described in Vinale *et al*. (2006). To test the production of inhibitory substance by the *Trichoderma* isolates, Potato Dextrose Broth (PDB) was used to grow them and for testing their potential to produce such substances. 250 ml flasks containing 100 ml of PDB were used to culture the *Trichoderma* isolates. Agar disc 5 mm diameter of *Trichoderma* isolates were inoculated in 250 ml flasks containing 100

ml of Potato Dextrose Agar (PDB). For each experiment, flasks in triplicate were incubated at 25^0C for 7 days. The filtrate culture of *Trichoderma* isolates were removed by centrifuging the culture at 9000 rpm for 30 minutes. The supernatant was used immediately (Agarry *et al.,* 2005). The cell free culture filtrates of *Trichoderma* isolates were examined to determine their influence on the mycelial growth of *F. oxysporum* isolate on Potato Dextrose Agar (PDA). About 5 ml of culture filtrate of *Trichoderma* isolates was added in sterilized and cooled PDA (45^0C), then 5 mm disc taken from the periphery of a 7 days old culture of *F. oxysporum* isolate and placed in the center of the Petri plate containing PDA and culture filtrate. Plates were incubated for 7 days at 25^0C and fungal growth was measured and compared to the control growth where the filtrate was replaced with equal amount of sterile distilled water (SDW).

3.3.2. The Effect of Culture Media on the Mycelial growth of *Trichoderma* Isolates

Potato Dextrose Agar (PDA) is commonly used for the isolation and growth of wide range of fungi in laboratories and its compositions are well defined (Sharma and Pandey, 2010). The growth requirements for fungi may vary from strain to strain, although cultures of the same species and genera tend to grow best on similar media (Dhingra and Sinclair, 1993; Aneja, 2005). The effect of different culture media on the growth of *Trichoderma* isolates were investigated by growing them in three different culture media namely Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapeck Dox Agar (CDA). An agar disc 5mm diameter of inoculums was cut from 7 days old culture of *Trichoderma* isolates and inoculated into the freshly prepared culture media. Each medium was prepared in triplicate and incubated at a temperature of 25^0C. After 7 days of incubation, the diameter of the mycelium was measured and recorded.

3.3.3. The Effect of Temperature on the Mycelial Growth of *Trichoderma* Isolates

The majority of filamentous fungi are mesophilic, growing at temperatures within the range of 10-35^{0}C, of which most grow with temperatures between 15 and 30^{0}C (Kapoor and Kar, 1989).

To evaluate the effect of temperature on the mycelial growth of *Trichoderma* isolates, three *Trichoderma* isolates were grown on Potato Dextrose Agar (PDA) for determining their comparative growth rate. In each of Petri plate 25 ml of molten PDA medium was aseptically poured. The mycelial disc of each *Trichoderma* isolate, 5 mm in diameter was taken from the edge of 7 days old culture which grown on PDA by using sterile cork borer and placed at the center of 90 ml Petri plate containing freshly prepared PDA. The plates were incubated at five different temperature ranges (15^{0}C, 20^{0}C, 25^{0}C, 30^{0}C and 35^{0}C). Mycelial diameter of each *Trichoderma* isolate in a plate was measured in millimeter at right angle by using plastic ruler. Three replicate plates were used for each *Trichoderma* isolates.

3.3.4. The Effect of pH on the Mycelial Growth of *Trichoderma* Isolates

Biocontrol *Trichoderma* strains are applied in agricultural soils with certain pH-characteristics. Therefore, it is important to collect information about the effects of pH on mycelial growth and on the *in vitro* activities of extracellular enzymes involved in nutrient competition and mycoparasitism of *Trichoderma* strains with biocontrol potential. pH can also play a role in the regulation of extracellular enzyme production, as it was demonstrated by Delgado-Jarana *et al*. (2000) for β-1, 6-glucanase of *Trichoderma harzianum*.

The Potato Dextrose Broth (PDB) (200 g potato, 20 g dextrose per liter of distilled water) was prepared and adjusted to different pH levels (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) in order to obtain the optimum and suitable pH value for the growth of the *Trichoderma* isolates. These pH ranges were adjusted by using 1N HCl and 1N NaOH. The media was autoclaved and a disc of 5

mm diameter inoculum was taken from the margin of 7 days old culture grown on PDA and inoculated into the 250 ml flasks containing 100 ml PDB and incubated at 25^0C for 15 days. After 15 days of growth in the potato dextrose broth (PDB) mycelial dry weight was measured by using electronic balance.

3.4. In vivo Evaluation of *Trichoderma* Isolates against *Fusarium oxysporum*

A pot experiments were conducted in the greenhouse of Addis Ababa University using a complete randomized block design (CRBD) and three replicates for each experiment were used to evaluate the performance of *Trichoderma* isolates as a biocontrol agent against tomato wilt disease caused by *F. oxysporum* isolate. Seeds of three tomato varieties were obtained from Melkassa Agricultural Research Center (MARC). They were Cochoro, Miya and Fetane that have high potential yield per a given area. Six seeds of tomatoes were sown in each 20 cm diameter plastic pot which was surface-sterilized by using 2% Sodium hypochlorite (NaOCl) and 70% ethanol and filled with 3kg of autoclaved soil. The inoculum of both test pathogen and *Trichoderma* isolates was adjusted to $1.5x10^6$ conidia /ml by using Haemocytometer before infesting into the soil. Efficacy of *Trichoderma* isolates to control the wilt disease was tested after application and sowing of tomato seeds into artificially made *Fusarium oxysporum* isolate infested soil described by Bell *et al.* (1982) with suitable modification. About 20 ml of spore suspension of the test pathogen at a concentration of $1.5x10^6$ conidia /ml were inoculated in to the soil by drenching/flooding method before 7 days of inoculation of *Trichoderma* isolates spore. After 7 days of the inoculation of the test pathogen 20 ml of *Trichoderma* isolates spore at a concentration of $1.5x10^6$conidia/ml were inoculated into the soil by the same method. The control seeds were inoculated with equivalent amount of sterile distilled water (SDW). The experiment included the following treatments: 1) Non- infested soil (control); 2) Soil treated with *F. oxysporum* isolate only, 3) *F. oxysporum* isolate + *Trichoderma* isolates.

Six seeds were sown per pot and three pots were used for each test as replicates. Percentage of disease incidence (% DI) was recorded (Ishikawa *et al.,* 2005). Pots were kept under greenhouse conditions till the end of the experiment.

$$\text{Percent Disease Incidence} = \frac{\text{Number of Diseased plants}}{\text{Total Number of Plants}} \times 100$$

3.5. Statistical Analysis

All experiments were performed in triplicate. Statistical analysis of growth characteristics of isolates at different media, temperature, and pH and mean comparisons of isolates based on different parameters were analyzed by least significant difference (L.S.D.) test at probability of 0.05 to identify significant effect of a treatment. Duncan Multiple Range Test was used to evaluate the significant differences between treatments ($P \leq 0.05$). ANOVA analysis was done with the SPSS statistics software.

4. Results

4.1. Morphological Characterization of *Fusarium oxysporum* Isolate

The characterization of isolate was made based on their cultural characteristics and conidial features. Snyder and Hansen (1940), delicate that the mycelia of *Fusarium oxysporum* f. sp. *lycopersici* were white to pink, often with purple tinge, and are sparse to abundant. The fungus produces three types of spores: microconidia, macroconidia, and chlamydospores in Figure 3. Microconidia are borne on simple phialides arising laterally and are abundant, oval-ellipsoid, straight to curved, and non-septate. Macroconidia, sparse to abundant, were borne on branched conidiophores or on the surface of sporodochia and were thin walled, fusoid-subulate and pointed at both ends, have pedicellate base. Three septate spores were more common. Chlamydospores, both smooth and rough walled, were abundant and form terminally or on an intercalary basis. They were generally solitary, but occasionally formed in pairs or chains. After the appearance of the mycelial growth, the single spore isolation technique was employed to purify the fungal spores to obtain the pure culture of *Fusarium oxysporum* isolate. Simultaneously, the spores were transferred to PDA and continuous observation was made on the sporulation, pigmentation, cultural characteristics and conidial structures to confirm the test pathogen.

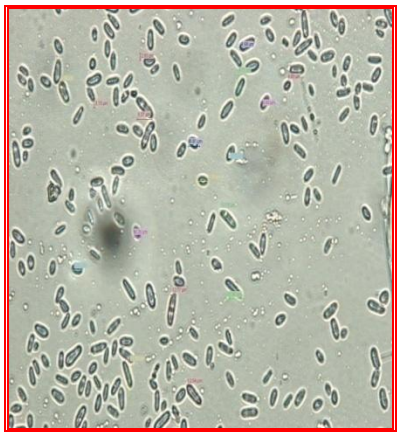
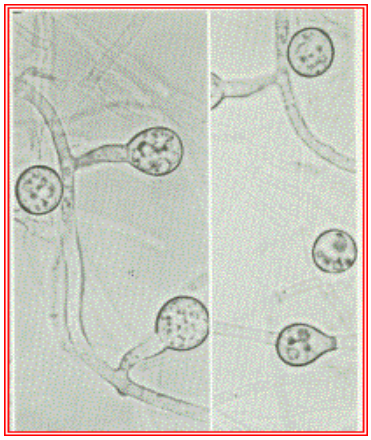
A.  B.  C. 

Figure 3. Colony morphology and microscopic observation of *Fusarium oxysporum* isolate Spore (magnification power 40x),

A. Colony morphology of *Fusarium oxysporum* isolate on PDA,

B. Both microconidia and macroconidia and C. chlamydospore

Table 2: Average length of conidia (µm) of *Fusarium oxysporum* isolate after 7 days of growth on PDA medium

Isolate	**Microconidia (µm)**	**Macroconidia (µm)**
***Fusarium oxysporum* isolate**	3.76	9.37
	2.80	10.92
	5.79	12.77
	4.01	12.54
	4.26	11.59
	5.45	12.06
	4.98	13.04
Mean	4.43	11.76
Range	2.80 -5.79	9.37 - 13.04

As the result shown in Table 2, the mean length of microconidia was 4.43 µm and 11.76 µm for macroconidia. Macroconidia had 3-5 septated parts and microconidia were not septated.

4.2. Morphological Characterization of *Trichoderma* Isolates

The mycelia of *Trichoderma* isolates on potato dextrose agar (PDA) cultures was typically recognized by the presence of fast growing colonies producing white, green, or yellow cushions of sporulating filaments or conidiophores produce side branches bearing spherical to ovoid green colored spores. *Trichoderma* isolates were grown fast with the optimal temperatures between 25-30^{0}C. The hyphae were initially transparent or whitish, and depending upon the species, the mycelium became greenish, yellowish or less frequently white within one week. Conidiophores are highly branched and thus difficult to define or measure shown in Figure 4.

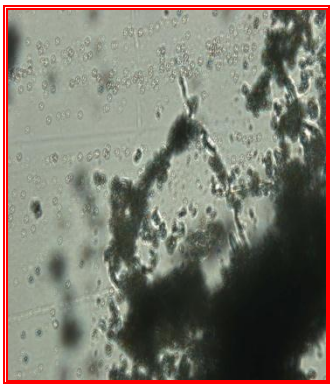
A.  B.  C. 

Figure 4. A. Colony Morphology of *Trichoderma* isolate on PDA Medium

B. Microscopic Observation of *Trichoderma* isolate AUT9 spores and

C. Microscopic Observation of *Trichoderma* isolate AUT10 spores

4.3. In vitro Assay of *Trichoderma* Isolates against the Test Pathogen

The antagonistic effect of *Trichoderma* isolates against *Fusarium oxysporum* isolate in the dual culture technique was shown in Table 3. *Trichoderma* isolates were showed differences to inhibit the mycelial growth of the test pathogen. Mycelial growth inhibition of the test pathogen by *Trichoderma* isolates was also shown in Figure 5. *Trichoderma* isolate AUT9 gave significantly higher percent inhibition of the mycelial growth of the test pathogen with inhibition of 50.83 % compared to isolates AUT10 and AUT8. *Trichoderma* isolates AUT10 and AUT8 gave percent inhibition of 41.99 % and 28.24 %, respectively on the mycelial growth of the test pathogen.

Table 3: The antagonistic effect of *Trichoderma* isolates on the mycelial growth of the test pathogen in the dual culture on PDA medium after 7 days of incubation at 25^0C

Isolates	**Mycelial growth of pathogen in the dual culture (mm)**	**Average % inhibition of the mycelial growth of the test pathogen**
AUT8	20.30	28.24
AUT9	13.91	50.83
AUT10	16.41	41.99
Control	28.29	0.00

[a] Each value is an average of three replicates

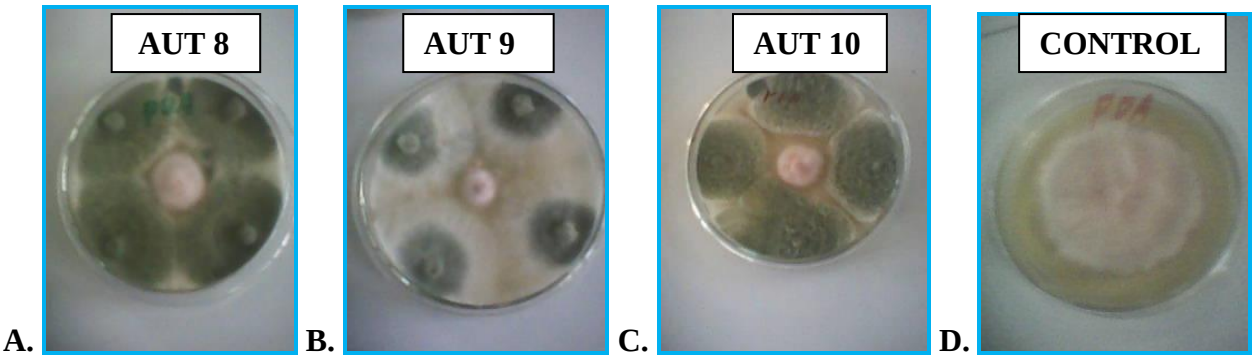


Figure 5. The antagonistic effect of *Trichoderma* isolates on the mycelial growth of *Fusarium oxysporum* isolate in the dual culture technique A. *Trichoderma* isolate (AUT8) B. *Trichoderma* isolate (AUT9) C. *Trichoderma* isolate (AUT10) and D. Control

4.4. Assay of Culture Filtrates of *Trichoderma* Isolates on the Mycelial Growth of the Test Pathogen

The effect of culture filtrates of *Trichoderma* isolates on the mycelial growth of the test pathogen was shown in Table 4. *Trichoderma* isolate AUT9 culture filtrate showed maximum mycelial growth inhibition of the test pathogen with percent inhibition of 66 % whereas isolate AUT10 showed minimum mycelial growth inhibition of 58 % on the test pathogen. The maximum and minimum mycelial growth inhibition of the test pathogen by *Trichoderma* isolates culture filtrate was also shown in Figure 6.

Table 4: The effect of culture filtrates of *Trichoderma* isolates on the mycelial growth of the test pathogen after 4 days of incubation on PDA

Isolates	Mycelial growth of pathogen in the culture filtrates of *Trichoderma* isolates (Diameter in mm)	Average % inhibition of the mycelial growth of the test pathogen
AUT8	10.44	61
AUT9	9.10	66
AUT10	11.24	58
Control	26.78	0.00

[a] Each value is an average of three replicates

4.5. The Effect of Culture Media on the Mycelial growth of *Trichoderma* Isolates

All the *Trichoderma* isolates got maximum mycelial growth on Malt Extract agar (MEA) and minimum mycelial growth on Czapeck Dox Agar (CDA). Isolate AUT8 was the only one that got maximum mycelial growth on all types of culture media and isolate AUT10 got minimum mycelial growth on all types of culture media. The maximum mycelial growth of 60.89, 58.44 and 57.11 mm was displayed on Malt Extract Agar (MEA) by isolates AUT8, AUT9 and AUT10, respectively (Table 5). Likewise, the minimum mycelial growth of 39.33, 36.67 and 34.33mm was recorded on Czapeck Dox Agar (CDA) by the same isolates respectively.

Table 5: The effect of different culture media on the mycelial growth of *Trichoderma* isolates

Culture Media	*Trichoderma* isolates (Mycelial Diameter in mm) (Mean ± SD)		
	AUT8	**AUT9**	**AUT10**
PDA	57.67 ± 28.40[b]	56.33 ± 28.50[b]	52.89 ± 28.00[b]
MEA	60.89 ± 28.70[a]	58.44 ± 28.60[a]	57.11 ± 28.70[a]
CDA	39.33 ± 25.24[c]	36.67 ± 24.64[c]	34.33 ± 24.17[c]

[a]Each value is an average of three replicates ± Standard deviation. Data followed by the same letter are not significantly different (P≤ 0.05), according to Duncan's multiple range test.

4.6. The Effect of Temperature on the Mycelial Growth of *Trichoderma* Isolates

The effect of different temperature range (15, 20, 25, 30, 35^{0}C) on the mycelial growth of *Trichoderma* isolates was evaluated by measuring mycelial growth on PDA (Table 6). The best mycelial growth for all *Trichoderma* isolates was obtained at 25^{0}C whereas slow mycelial growth for all *Trichoderma* isolates was obtained at 15^{0}C. The maximum mycelial growth of 66.11, 61.44 and 59.68 mm was displayed at 25^{0}C by isolates AUT8, AUT9 and AUT10, respectively. Whereas, the minimum mycelial growth of 36.44, 34.44 and 31.78 mm was recorded at 15^{0}C by the same isolates respectively.

Table 6: The effect of temperature on the mycelial growth of *Trichoderma* isolates on PDA medium after 5 days of incubation

Temperature (^{0}C)	*Trichoderma* isolates (Mycelial Diameter in mm) (Mean ± SD)		
	AUT8	**AUT9**	**AUT10**
15^{0}C	36.44 ± 24.77^e	34.44 ± 23.19^e	31.78 ± 20.87^e
20^{0}C	45.56 ± 22.40^d	43.44 ± 21.60^c	40.56 ± 22.40^c
25^{0}C	66.11± 28.00^a	61.44 ± 26.40^a	59.68 ± 27.50^a
30^{0}C	62.00 ± 29.00^b	55.00 ± 32.20^b	50.78 ± 33.90^b
35^{0}C	51.56 ± 22.16^c	48.56 ± 21.50^{bc}	46.22 ± 19.22^{bc}

[a]Each value is an average of three replicates ± Standard deviation. Data followed by the same letter are not significantly different ($P \leq 0.05$), according to Duncan's multiple range test.

4.7. The Effect of pH the Mycelial Growth of *Trichoderma* Isolates

The effect of p^H on the mycelial growth of *Trichoderma* isolates was shown in Table 7. *Trichoderma* isolates were grown on potato dextrose broth (PDB) medium adjusted to different P^H values ranging from 3.0 to 8.0. The best mycelial growth was recorded from isolate AUT8 at pH 5.5 with a mycelial biomass of 1228.80 mg followed by mycelial biomass of 1133.20 mg at pH 3.5 by the same isolate. The least mycelial growth of 552 mg was also measured from isolate AUT8 at a pH of 7.0. In general good mycelial growth for isolate AUT8 was recorded at pH between 5.5- 6.5.

With regard to isolate AUT9, the best mycelial grwth was detected at pH 6.5 with mycelial biomass of 722.70 mg. The least mycelial biomass 441.60 mg was recorded at pH 3.0 by the same isolate.

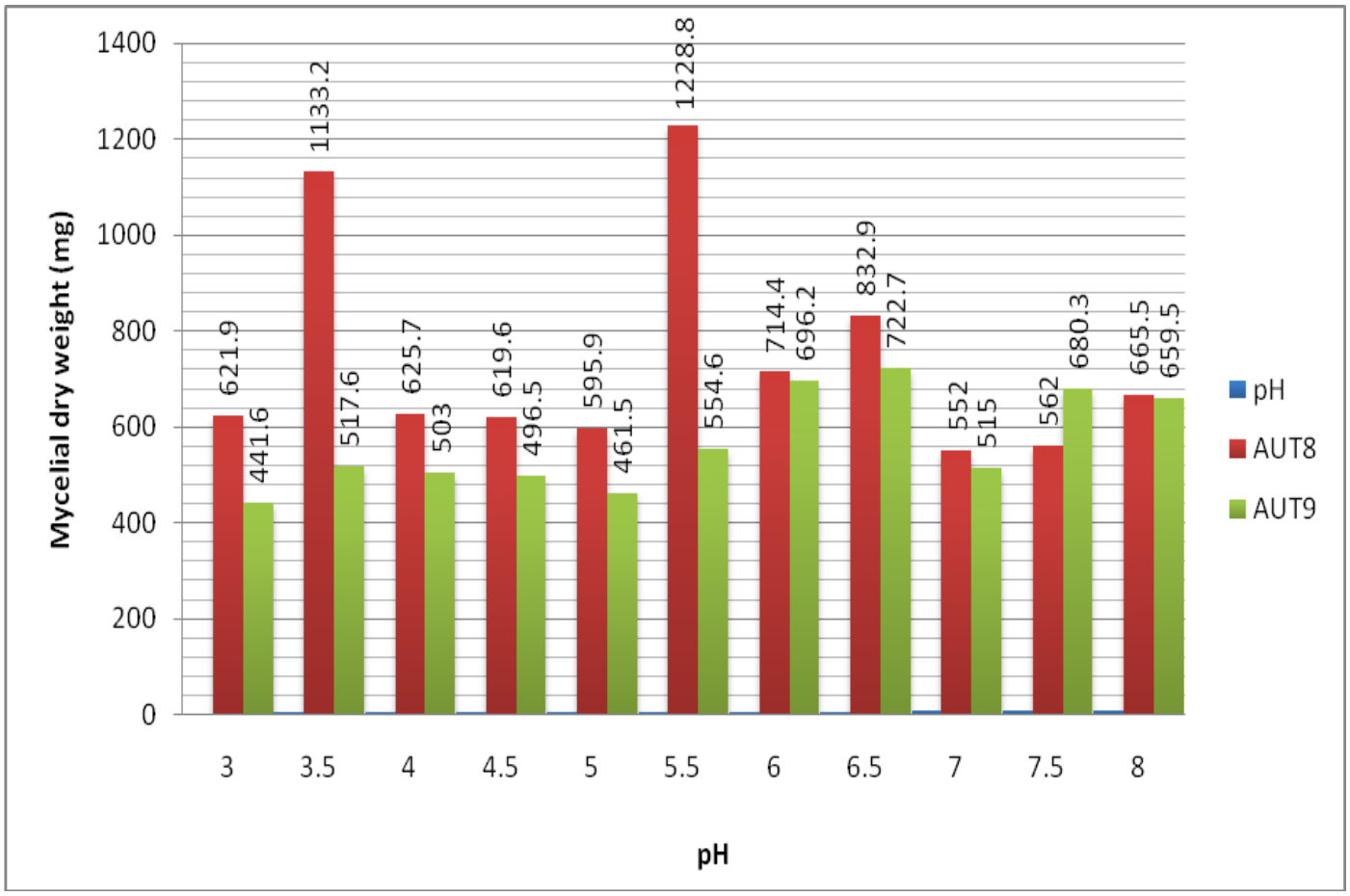


Figure 6: The effect of pH on the mycelial growth of *Trichoderma* isolates

4.8. Antagonism Test in the Greenhouse Conditions

Wilting symptoms were seen in all the three tomato varieties after 60 days of sowing, but Miya variety was more susceptible to *Fusarium* wilt compared to the Cochoro and Fetane varieties (Figure 7). The symptoms that observed were yellowing of lower leaves and discoloration of stem appeared. The re-isolated *F. oxysporum* isolate from the diseased tomato plant parts especially from stem which were grown in the greenhouse revealed that the isolated fungal pathogen was the same as the original isolate.

As the result indicated in Table 7, Miya variety showed minimum shoot length, shoot fresh weight, root fresh weight and minimum shoot and root dry weight in all treatments. Whereas, Fetane variety showed maximum shoot length, shoot fresh weight, root fresh weight and maximum shoot and root dry weight in all treatments. All tomato varieties showed maximum shoot length, shoot fresh weight and root fresh weight when treated with *Trichoderma* isolate AUT9. Whereas, they showed minimum shoot length, shoot fresh weight and root fresh weight with isolate AUT8. All tomato varieties showed significant differences between the treatments and negative controls. The disease incidence for Miya, Cochoro and Fetane varieties was19.44, 16.67 and 11.11 %, respectively when treated with isolate AUT9 and 27.78, 22.22 and 21.22 %, respectively with isolate AUT8. The highest disease incidence was 83.33, 77.78 and 72.22 %, respectively with *F. oxysporum* isolate (negative control) in Table 8. In general Miya variety was more susceptible to *Fusarium* wilt than Cochoro and Fetane varieties.

Table 7: The effect of *Trichoderma* isolates on tomato plant shoot length, shoot and root fresh weight and dry weight under greenhouse conditions

Tomato varieties	Treatments	Plant fresh weight (g) (Mean ± SD)		Plant dry weight (g) (Mean ± SD)		Shoot length (cm) (Mean ±SD)
		Shoot	Root	Shoot	Root	
1. Cochoro	AUT8 + *Fol*	61.83 ± 18.81^{c}	15.18 ± 3.79^{c}	11.67 ± 2.53^{c}	1.70 ± 0.79^{c}	39.00 ± 8.66^{d}
	AUT9 + *Fol*	136.33 ± 30.14^{b}	35.67 ± 10.01^{a}	20.33 ± 5.50^{b}	4.93 ± 0.90^{a}	52.33 ± 3.51^{b}
	AUT10+*Fol*	69.18 ± 30.72^{c}	16.18 ± 6.66^{c}	12.40 ± 6.73^{c}	2.33 ± 1.17^{b}	41.33 ± 12.50^{c}
	Positive Control (Seed Only)	175.00 ± 44.93^{a}	32.33 ± 6.79^{b}	30.17 ± 3.75^{a}	5.50 ± 1.32^{a}	57. 67 ± 6.51^{a}
	Negative Control	41.67 ± 29.88^{d}	10.50 ± 4.92^{d}	9.67 ± 3.21^{c}	1.33 ± 0.50^{d}	36.67 ± 6.43^{d}
2. Miya	AUT8 + *Fol*	40.33 ± 7.02^{c}	11.67 ± 1.53^{c}	7.00 ± 2.29^{d}	1.97 ± 0.40^{d}	29.00 ± 4.36^{a}
	AUT9 + *Fol*	77.50 ± 44.33^{b}	24.33± 22.23^{b}	17.33 ±13.32^{b}	4.50 ± 1.32^{b}	43.67 ± 14.43^{a}
	AUT10+*Fol*	59.83 ± 47.79^{c}	22.68± 18.56^{b}	10.43 ± 7.54^{c}	2.87 ± 2.15^{c}	41.33 ± 13.65^{a}
	Positive Control (Seed Only)	161.00 ± 74.98^{a}	49.33 ± 15.22^{a}	31.67 ± 20.60^{a}	6.90 ± 2.65^{a}	58.33 ± 7.64^{a}
	Negative Control	37.33 ± 21.13^{d}	8.00 ± 1.80^{d}	6.50 ± 1.00^{d}	1.17 ± 0.35^{e}	28.67 ± 5.03^{a}
3. Fetane	AUT8 + *Fol*	70.00 ± 50.57^{b}	21.00 ± 7.94^{b}	12.10 ± 5.37^{c}	2.10 ± 0.36^{c}	48.33 ± 9.71^{b}
	AUT9 + *Fol*	119.83 ± 58.87^{a}	47.00 ± 19.94^{a}	14.67 ± 6.43 ^{b}	6.43 ± 3.39^{b}	56.33 ± 3.51^{a}

	AUT10+*Fol*	115.67 ± 67.45[a]	24.67 ± 4.16[b]	13.17 ± 8.91[b]	3.90 ± 0.78[b]	54.40 ± 7.21[a]
	Positive Control (Seed Only)	133.33 ± 32.32[a]	47.50 ± 22.79[a]	24.00± 14. 93[a]	7.40 ± 6.60[a]	59.00 ± 4.58[a]
	Negative Control	64.00 ± 32.75[b]	13.33 ± 5.51[c]	10.33 ± 4.51[d]	1.70 ± 0.61[d]	44.00 ± 2.00[b]

[a]Each value is an average of three replicates. Data followed by the same letters are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 8: The effect of *Fusarium oxysporum* isolate on disease incidence under greenhouse conditions

Tomato Varieties	**Treatments**	**Disease incidence (%)**
1. Cochoro	AUT8 + *Fol*	22.22
	AUT9 + *Fol*	16.67
	AUT10 + *Fol*	18.32
	Positive Control (Seed Only)	5.56
	Negative Control (*Fol* Only)	77.78
2. Miya	AUT8 + *Fol*	27.78
	AUT9 + *Fol*	19.44
	AUT10 + *Fol*	25.00
	Positive Control (Seed Only)	8.33
	Negative Control (*Fol* Only)	83.33
3. Fetane	AUT8 + *Fol*	21.22
	AUT9 + *Fol*	11.11
	AUT10 + *Fol*	16.67
	Positive Control (Seed Only)	2.78
	Negative Control (*Fol* Only)	72.22

5. Discussion

The *Trichoderma* isolates showed mycelial growth inhibition of the test pathogen as compared to the control. *Trichoderma* isolate AUT9 gave the highest mycelial growth inhibition 58.83% of the test pathogen and isolate AUT8 gave the least mycelial growth inhibition 28.24% of the test pathogen. Similarly, the antagonistic activity of different *Trichoderma* isolates against different phytopathogenic fungi such as *Razoctonia solani*, and *Sclerotium rolfii* was reported by (Deshmukh and Raut, 1992).

All *Trichoderma* isolates showed mycelial growth inhibition of the test pathogen as compared to the control. *Trichoderma* isolate AUT9 showed maximum mycelial growth inhibition and AUT10 showed minimum mycelial growth inhibition against the test pathogen. Different mechanisms such as competition, production of antibiotics inhibiting fungal growth by producing volatile and non-volatile compounds were reported by (Michrina *et al.,* 1995; Calistru *et al.,* 1997). Similarly mycoparasitism of fungal hyphae, endoconidia and chlamydospores and finally lyses of protoplasm was observed in case of *Trichoderma harziaum* and *Trichoderma viride* against pathogenic fungi (Ramanujiam *et al.,* 2002). Culture filtrates of *Trichoderma* isolates had an inhibitory effect on the radial growth of the test pathogen and mycelial accumulation suggesting action of non-volatile antibiotics in the filtrates.

Similarly, the presence of inhibition zones in dual cultures between *Fusarium oxysporum f. sp. lycopersici* and *Trichoderma harzianum* suggested secretion of diffusible non-volatile inhibitory substance by the *Trichoderma harzianum* isolates was reported by (Grodona *et al.,* 1997; Behzad *et al.,* 2008).

All *Trichoderma* isolates showed maximum mycelial growth on Malt Extract Agar (MEA) and minimum mycelial growth on Czapek Dox Agar (CDA). *Trichoderma* isolate AUT8 showed

maximum mycelial growth in all the three types of culture media and isolate AUT10 showed minimum mycelial growth in all the three types of culture media. Different *Trichoderma* isolates showed different mycelial growth in the same culture media. The difference in growth and population was observed in the three *Trichoderma* isolates. This was due to inherent ability of the *Trichoderma* species to utilize the different culture media and difference in their composition and to their natural ecological behavior and the fast growth rate of *Trichoderma* species was described by (Wang *et al.,* 1999). Similarly, the growth and morphological features of *Trichoderma* species was studied on different culture media as reported by (Elad *et al*., 1981; Shalini *et al.* 2006; Harman *et al.* 1991). These results have implications for the use of such in-vitro test as a part of general screen for biological efficiency of different biocontrol agents.

Different *Trichoderma* isolates responded differently to various temperatures ranges. Mean mycelial growth for all *Trichoderma* isolates was maximum at 25^0C and minimum mycelial growth was observed at 15^0C. Similarly, (Lilly and Barnett, 1951) reported that temperature affect almost every function of the fungi. Pavavizas (1985) also stated that different species of *Trichoderma* have their own ecological preferences. Similarly, the effect of temperature on the mycelial growth of *Trichoderma* species was reported by (Samuels, 1996).

Maximum and minimum mycelial growth for *Trichoderma* isolate AUT8 was obtained at pH 5.5 and 7.0, respectively whereas for isolate AUT9 maximum and minimum mycelial growth was observed at pH 6.5 and 3.0, respectively. Similarly, (Jackson *et al.,* 1991) have found that optimum biomass production of *Trichoderma* isolates occurred at pH ranges between 4.6 and 6.8. Similarly, (Seyis *et al.,* 2005) observed that activity of *Trichoderma harzanium* was maximum around pH 5. It has been demonstrated that *Trichoderma* strains are active under a wide range of pH (Kredics *et al.,* 2003).

As the results indicated Miya variety was the most susceptible to *Fusarium oxysporum* wilt and Fetane variety was resistant one. Similarly, (De Cal *et al.,* 1997) reported that *Fusarium* wilt of tomato is an intractable problem because management strategies such as cultivar resistance and chemical control are not always appropriate.

All *Trichoderma* isolates increased the tomato plants shoot length, shoot fresh weight and root fresh weight compared to pathogen inoculated (negative control). As the result indicated *Trichoderma* isolate AUT9 showed maximum shoot length, shoot fresh weight and root fresh weight in all tomato varieties and *Trichoderma* isolate AUT8 showed minimum shoot length, shoot fresh weight and root fresh weight in all tomato varieties. Similarly, (Ozbay and Newman, 2004), agreed that *Trichoderma harzianum* strains were significantly increased the height, shoot and root dry weight in tomato seedlings transplanted into pots in the greenhouse. Similarly, (Altomare *et al.*, 1999) reported that *Trichoderma* species increases the solubility of phosphates and micronutrients with low solubility and this enhances growth of the roots and the above ground parts of the plant.

6. Conclusion and Recommendation

6.1. Conclusion

- In this study three *Trichoderma* isolates were selected based on their *in vitro* antagonistic activity against the test pathogen
- *Trichoderma* isolates AUT9 was the most effective against the test pathogen both in vitro and under greenhouse conditions. Whereas isolate AUT8 was the least effective.
- All *Trichoderma* isolates showed maximum mycelial growth at 25^0C and minimum at 15^0C
- All isolates showed maximum mycelial growth on Malt Extract Agar (MEA) and minimum on Czapeck Dox Agar (CDA)
- All tomato varieties showed their maximum shoot length, shoot fresh weight and root fresh weight with isolate AUT9 and minimum with isolate AUT8
- *Trichoderma* isolates AUT8 showed maximum mycelial growth at pH 5.5 whereas, isolate AUT9 showed maximum maximum mycelial growth at pH 6.5
- Miya variety was the most susceptible to the *Fusarium* wilt than Fetane and Cochoro varieties.
- Fetane variety was the most resistant one to *Fusarium* wilt under the greenhouse condtions
- As the result indicated there was significant difference between treatment and positive and negative controls

6.2. Recommendations

Based on the results of the study, the following recommendations are forwarded:

- ✓ More research on tomato wilt disease caused by *Fusarium oxysporum* species should be done in the near future
- ✓ *Trichoderma* species as a biocontrol agents should be tested in the field
- ✓ *Trichoderma* species as a biocontrol agents should be produced in a large commercial scale
- ✓ Further study on the area of biocontrol agents should be done in the near future
- ✓ Resistant tomato varieties should be released to the farmers and farmer association such as Fetane and Cochoro varieties

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Appendices

Appendix I. Composition of medium for phosphate solubilizers' microorganisms

(Pikovskays's medium) is general medium for the selection of phosphate solublizer

1. Components	**Amounts (g/l)**
Glucose	10.0
$Ca_3 (PO_4)_2$	5.0
$(NH4)_2SO_4$	0.5
NaCl	0.2
$MgSO_4.H_2O$	0.1
KCl	0.2
Yeast extract	0.5
$MnSO_4.H_2O$	0.002
$FeSO_4.7H_2O$	0.002
pH	7.0

Appendix II. Composition and preparation of different culture media.

2. Malt Extract Agar (MEA)

Malt extract----------30.0 g

Dipotassium hydrogen phosphate----------2.0 g

Ammonium chloride----------1.0 g

Citric acid 1 N----------15.0 ml

Agar----------12.0 g

Distilled water----------1000.0 ml

3. Potato Dextrose Agar (PDA)

Potato peeled----------200.0 g

Dextrose----------20.0 g

Agar----------15.0 g

Distilled water----------1000.0 ml

4. Potato Dextrose Broth (PDB)

Potato peeled----------200.0 g

Dextrose----------20.0 g

Distilled water.....1000.0 ml

5. Czapeck Dox Agar (CDA)

Sodium nitrate..... 2.0 g

Potassium chloride…….……0.5 g

Magnesium glycerol phosphate……..0.5 g

Ferrous sulphate……0.01 g

Potassium sulphate……0.35 g

Sucrose…….30.0 g

Agar…….…….12.0 g

Distilled water……1000.0 ml

6. Nutrient Agar (NA)

Peptone …….5.0 g

Sodium chloride…….5.0 g

Yeast extract…….……2.0 g

Beef extract……....1.0 g

Agar ……..15.0 g

Distilled water……...1000.0 ml

Appendix III. Pathogencity Test on Tomato Plants under Greenhouse Conditions

Figure 7. Cochoro Variety (A-E), Miya Variety (E-J) and Fetane Variety (K-O)