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Addis Ababa University

College of Natural and Computational Sciences
Department of Zoological Sciences

Isolation and characterization of Endophytic *Trichoderma* Isolates for Antagonistic Activity against Coffee wilt disease (*Gibberella xylarioides*) in Keffa zone, Southwestern Ethiopia

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A Thesis Submitted to Addis Ababa University in Partial Fulfillment for the requirements of the Degree of Masters of Sciences (MSc) in Biology

September, 2018

Addis Ababa, Ethiopia

ACKNOWLEDGMENTS

First of all, I want to express my deepest thanks to my advisor Tesfaye Alemu (PhD), Department of Microbial, Cellular and Molecular Biology, Addis Ababa University (AAU) for his great support, valuable suggestion, keen supervision and constructive comments to finalize this thesis work. His kindness and being available whenever I need his advice are highly appreciated.

I would like to thank Summer Post Graduate Coordinator Dr.Tilaye Wube and Department Head of Zoological Sciences Prof. Abebe Getahun who are stand by my side and encouraging me.

I am grateful to Mr. Afrasa Mulatu (PhD candidate) and Mr. Ayelign Melesse (PhD candidate) for their guidance, support and facilitation of the research progress from the start to the end. I appreciate their willingness for sharing their knowledge and experiences. They gave me the very first step into the depth of laboratory work. I would also like to thank W/ro Nigatuwa Mokenen, mycology laboratory technicians, for her encouragement, support and making laboratory materials available.

Finally, I thank the Department of Microbial, Cellular and Molecular Biology and Department of Zoological Sciences, Addis Ababa University for all round assistance and for allowing me to carry out the research in the Mycology laboratory, and the School of Graduate Studies of AAU for financial support.

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LIST OF ABBREVIATIONS AND ACRONYMS

BCAs	Biological Control Agents
CBD	Coffee Berry Disease
CLR	Coffee Leaf Rust
CWD	Coffee Wilt Disease
CWDEs	Cell Wall Degrading Enzymes
CZA	czapeck –Doxis agar
CZI	Clear Zone of Inhibition
SD	Standard devision
DRC	Democratic Republic Congo
GDP	Gross domestic product
ha	hectare
ISR	Inheritance systematic inhibition
MCMB	Department of Microbial, Cellular and Molecular Biology
IPM	Integrated Pest Management
JARC	Jimma Agricultural Research Center
MEA	Malt Extract Agar
PAP	6-Penthyl-alpha-pyrone
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGPR	Plant Growth Promoting Rhizobacteria
PIRG	Percentage Inhibition of Radial Growth
PR-protein	Pathogenesis related protein
OD	optical density
TSM	Trichoderma specific media
VOCs	Volatile organic compound

ABSTRACT

The present study aimed to evaluate, test and characterize potential biocontrol of endophytic *Trichoderma* isolates against coffee wilt pathogen (*Gibberella xylarioides*). In this study we examined the effect of pH and temperature on the mycelia growth and spore yield of *Trichoderma* isolates in batch culture. Coffee root samples were collected from different ecological habitat of Kaffa zone for the isolation of endophytic *Trichoderma* isolates. The ability of *Trichoderma* isolates to grow at pH 4.5, 6.5 and 7.5 were tested in liquid potato dextrose broth (PDB) medium. The effect of volatile and non-volatile compounds produced by *Trichoderma* isolates on radial growth of pathogenic fungi, *F. xylarioides* was also determined. *In vitro* antagonistic bioassays were performed to evaluate and determine the potentiality of *Trichoderma* isolates as biocontrol agents against *F. xylarioides.* The pH and temperature had significant effect on the growth and sporulation of *Trichoderma* isolates. It was observed that the optimum pH for maximum mycelial growth and spore yield produced by *Trichoderma* isolates in batch cultures was pH 7.5 while the optimum temperature for mycelial growth and the maximum spore yield was produced at optimum temperature was 25^{O}C. This work has revealed the important role that environmental conditions plays in the mycelia growth and spore yield of *Trichoderma* isolates, a biocontrol agent. In *in-vitro* bioassay the experimental results showed that all isolates of *Trichoderma* were able to inhibit the growth of *F. xylarioides* under *in vitro* experiment at rates ranging from 42.2 % to 69 % after 10 days of incubation. The highest mean inhibitory effect on the growth of the test pathogen was achieved by Gimbo *Trichoderma* isolate 05 (Gim-T05) (69%) followed by Gim-T10 (67.6%) and Gim-05 (66.7%), while Gowata *Trichoderma* isolate 01 (Gow-01) isolate showed the lowest (42.2%) mean inhibitory effect restricting it almost completely in plates as compared to the control consisting of *F. xylarioides* growing alone. Gimbo *Trichoderma* isolate 05 (Gim-T05) produced zones of inhibition which is an indicative of the production of secondary metabolite(s) inhibiting the mycelial growth of *F. xylarioides*. Thus, the use of novel isolates of *Trichoderma* with efficient antagonistic capacity against *F. xylarioides* is a promising alternative strategy to pesticides for coffee wilt disease management.

Keywords/phrases: Antagonism, *Coffee arabica*, endophytes, pH, temperature

1. INTRODUCTION

1.1 Background

Coffee (*Coffea arabica* L.*)* is one of the most important commodities in the international agricultural trade, representing a significant source of income in many tropical countries (Girma Adugna *et al.,* 2008). Coffee cultivation is confined to the tropical areas of the world consisting of over 80 developing countries, including Ethiopia. One of the most important gifts of Ethiopia is coffee, which has tremendous economic, social and spiritual impacts on many people of different geographical regions at cultural backgrounds (Tefestewold Biratu, 1995). Coffee is not only one of the highly preferred international beverages, but also one of the most important trade commodities in the world next to petroleum (Eshetu Derso, 2000).

Coffee represents, for most coffee-growing countries, the major source of revenue for foreign exchange. The contributions of coffee in Ethiopian economy is more than 60% of the country's foreign exchange earnings, over 5% of the GDP, 12% of the agricultural output, and 10% of the government revenues (Petit, 2007). In Ethiopia, coffee provides employment for over 25 million people, who are involved in production, processing, marketing, and related services. It also employs 25% of the domestic labour force. About 55% of the production is exported and the rest is consumed locally. It is mainly cultivated in western, southern and eastern parts of Oromia, SNNP, Gambella, Benishangul Gumuz and Amhara in Ethiopia (Tesfaye Alemu, 2012; Girma Adugna *et al.*, 2008; Phiri and Baker, 2009).

According to Brimner and Boland (2003), plant diseases every year pose 10-20% decrease in the total world food production which leads to loss of billions of dollars. Among them, fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants, especially in the tropical and subtropical regions. Various coffee diseases are reported on *Coffea arabica*, such as fungal, bacterial, nematodes and viral. The major coffee diseases in Ethiopia are coffee berry disease (CBD), coffee wilt disease (CWD) and coffee leaf rust (CLR). One of the most important and limiting factor of coffee production in the coffee growing African countries is the coffee wilt disease caused by a fungus, *Fusarium xylarioides*, a conidial (imperfect, anamorph) stage which also has a sexual stage (teleomorph; *Gibberella xylarioides* (Rutherford, 2006).

Coffee wilt disease was first observed in 1927 in a plantation of *Coffea excelsa*, in the Central African Republic (Musoli *et al.*, 2008). Subsequently, many hectares of *C. excelsa* throughout west and central Africa were destroyed by coffee wilt disease. It attacks all parts of coffee trees at all stages of development (Fernández; Flood, 2010; Gashaw *et al.*, 2014; Geiser *et al.*, 2005). The pathogen was first described in Republic of Democratic Congo ex- Zaire in 1948 (Steyaert, 1948). The perithecia represent the sexual stage of the fungus called *F. xylarioides* (Heim and Saccas). *Coffea arabica, C. canephora, C. excelsa* and wild *Coffea* species are all susceptible. Pieters and Van der Graaff (1980) reported that the disease was endemic in all coffee growing areas of Ethiopia and reached epidemic proportions in some areas.

CWD is currently having a devastating effect on coffee production in parts of eastern and central Africa and continues to spread at an alarming rate. Unlike many other diseases of coffee, CWD rapidly kill infected mature trees, often within as little as 6 months following appearance of the first external symptoms, and thus ultimately result in total yield loss (Rutherford, 2006). There are no curative control methods and recommendations for control are limited to phytosanitary measures against the source and spread of diseased material from infected trees (Haarer, 1963; Hocking, 1965; Sihen Getachew *et al.*, 2012). To control the pathogen with fungicide is impossible. However, to overcome the existing problem of this pathogen, there will be one possible control that is biological control.

Biocontrol agents such as soil borne microbes showing antagonism towards disease-causing plant pathogens that cause severe economic losses in commercial crop production were getting more attention. Antifungal metabolites isolated from them appear to be promising as viable supplements or alternatives to plant disease control, compared to synthetic chemicals (Stewart *et al.*, 2010; Ab Rahman *et al.*, 2017). Biological control agents would help in preventing the increase of pathogen population and also to check health hazards caused due to excessive use of chemicals (Butt and Copping, 2000). Now days, there are initiatives emerging worldwide to control plant diseases using useful fungi and bacteria by using as a bio control in order to mitigate the associated environmental impacts of using chemical control.

Endophytic fungi play an important role in enhancing plant health, and have been recognized as an important resource of biocontrol agents to suppress plant pests including both insects and pathogens (Backman and Sikora, 2008; Kumar and Kaushik, 2013; Zhang *et al.*, 2014).

Endophytes are microbes that colonize living internal tissues of plants without causing any immediate overt negative effect (Bacon and White, 2000). They are known to affect the interactions of plants with their environment, and to alter the course of their interactions with plant pathogens. Considerable evidence has been documented in recent years to support and identify the benefits associated with the use of endophytes in crop protection (Afzal *et al.*, 2013; Tariq *et al.,* 2014).

According to Temesgen Belayneh *et al.* (2010), *Trichoderma* species are reported as bio-control agents against coffee wilt diseases. The interaction effect of *Trichoderma* species against coffee wilt diseases is of special interest in biological control because of the ability of the species to proliferate in the treated environment as well as demonstrated ability to produce antibiotics, involve various mode of action, compete for nutrients and space, act as a mycoparasite and secretion of lytic- enzymes (Butt and Copping, 2000; Cannell, 1985). Among the major endophytes used as biocontrol agents (BCA), members of the fungus *Trichoderma* genus have so far been mainly registered as effective antagonists of soil-borne pathogens such as *Sclerotinia sclerotiorum* (IbarraMedina *et al*., 2010), *Sclerotinia rolfsii* (Suraiya *et al.*, 2014), *Macrophomina phaseolina* (Larralde-Corona *et al*., 2008), *Rhizoctonia solani* (Maisuria and Patel, 2009), *Pythium* species (Maisuria and Patel, 2009), *Phytophthora spp*. (Mpika *et al.*, 2009) and *Fusarium* species. In addition to their effects against diseases, endophytes might contribute to the overall plant improvement.

Trichoderma species are ubiquitous soil-borne Ascomycete noted for their biocontrol capabilities against many economically important plant pathogens. In general, commercial preparations of *Trichoderma* sp. for biological control consist of bulk produced conidia, which are the asexual reproductive units of this fungus. Bulk production of conidia typically relies on manipulation of nutrients and substrates to promote conidiation, which has led to much research into the optimal growth conditions for *in vitro* conidiation in many species of *Trichoderma*. The ambient pH and temperature are the main environmental factors influencing conidiation in *Trichoderma. Trichoderma* isolates are of great importance as biocontrol strains should have better stress tolerance levels than the plant pathogens against which they are going to be used for biological control.

1.2 Statement of the Problem

Coffee is vital to the economy of Ethiopia providing a major source of foreign exchange earnings as a cash crop, supporting the livelihood of millions of people who are involved in cultivation, processing, marketing, and export (Paulos Dubale and Demil Tektay, 2000). However, the production of coffee is hampered by various biotic and abiotic factors. The major biotic factors are fungal, bacterial, viral, nematodes, insect pests and weeds. Among the coffee diseases, coffee wilt pathogen fungus is the major constraints to the production, processing and quality of coffee (Kimani *et al*., 2002; Tesfaye Alemu and Kapoor, 2004; Noah Phiri and Peter Baker, 2009; Phiri *et al*., 2010).

This Pathogen has a problem of limiting the production of coffee in Ethiopia and the disease attacks all commercial coffee species including *Coffea arabica* and *Coffea canephora* at any stage (Rutherford, 2006). The disease has contributed to a decline in revenue for several African nations not only Ethiopia due to reduced coffee production. However, to overcome the existing problem of the CWD biological control can be used as one of the strategies for reducing disease incidence and severity by direct or indirect manipulation of antagonists (Afrasa Mulatu *et al.*, 2013 and Tesfaye Alemu and Kapoor, 2004).

Therefore, the main purpose of this study was to evaluate the interaction between the antagonists *Trichoderma* isolates against the test pathogen of coffee wilt disease (*F. xylarioides*) under *in-vitro* condition.

1.3 Objectives

1.3.1 General objective

- To isolate, characterize, evaluate and determine the efficacy of endophytic *Trichoderma* isolates for the biocontrol of coffee wilt disease (*F. xylarioides)* under *in vitro* conditions.

1.3.2 Specific objectives

- To isolate the *Endophytic Trichoderma* isolates from Kaffa zone districts purposively.
- To identify, test and evaluate the biocontrol potential of endophytic *Trichoderma* isolates against *F. xylarioides* under *in vitro* conditions
- To determine the biomass production and spore yield of *Trichoderma* isolates at different growth parameters

- To examine the effect of pH and temperature on the mycelia growth and spore yield of *Trichoderma isolates* in batch culture

2. LITERATURE REVIEW

2.1. Fungal Diseases of Coffee Plants

Plant diseases have been the concern of mankind since agriculture began and played a crucial role in the destruction of natural resources contributing 13 to 20 per cent losses in crop production worldwide. Phytopathogenic fungi, such as *Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia* and *Fusarium* are widely distributed through infected material with healthy coffee plant during the last few years due to change in farming practices with their detrimental effects on crops of economic importance. Not only growing crops but also stored fruits were host to fungal infection (Chet *et al.*, 1997). Coffee diseases are caused by pathogenic fungi and occasionally by bacteria and some viruses; they affect different plant organs resulting in debility, deformity and sometimes the death of the whole plant. The fungal diseases of coffee are the major constraints for coffee production and quality in major coffee producing countries of Africa (Kimani *et al.*, 2002).

Appropriate measures are often necessary to prevent diseases developing to a level that would reduce the productivity and/or quality of the crop. The need to undertake disease control depends upon the effects of particular diseases and for this purpose they can conveniently be grouped according to the part of the coffee plant they attack (Dean *et al.*, 2012).

In Ethiopia it has been reported that there are more than 45 fungal pathogens of coffee (Tesfaye Alemu, 2012). The major coffee diseases in Ethiopia are the coffee berry disease (CBD), coffee wilt disease (CWD) and coffee leaf rust (CLR).Coffee berry disease (CBD) is the top among the diseases of coffee in Ethiopia, which attacks mainly the green berries of coffee. Next to coffee berry disease (CBD), the most limiting factors in terms of severity and wide distribution for coffee production in Central and East African countries is *Tracheomycosis. Tracheomycosis* is a vascular wilt disease of coffee caused by *F. xylarioides* Steyaert imperfect stage (*F. xylarioides* Heim and Saccas Perfect stage). The major differences between Tracheomycosis and many other coffee diseases is that, the former kills all affected trees at all stages of development (Kimani *et al.*, 2002). Some of the fungal diseases of coffee in Ethiopia are indicated below (Table 1).

Table 1. Economically important fungal diseases of coffee in Ethiopia (Hindolf and Omondi, 2011)

Some Common name of coffee diseases	**Some Scientific names of causative agent**
Coffee wilt disease (Tracheomycosis)	*Gibberella xylarioides*
Coffee berry disease	*Colletotrichum kahawae*
Coffee leaf rust	*Hemileia vastatirx*
Damping off	*Rhizoctonia salani, Pythium & Fusarium* spp
Armillaria root rot	*Armillaria mellea*
Black rot (Thread blight)	*Corticium koleroga*
Pink disease	*Corticium salmonicolor*
Collar rot /Bark diseases	*Fusarium latritium, Fusarium stilboides*
Stem blight dieback (Ascochyta blight)	*Ascochyta tarda*
Brown eye spot (Berry blotch or berry spot)	*Cercospora coffeicola*
Anthracnose (Twig dieback or stalk rot of berries)	*Colletotrichum gloeosporioides*
Post-harvest fungal disease (mould)	*Aspergillus sp, Penicillium sp, Fusarium, Botrytis, Alternaria*

2.2 Coffee Wilt Disease (CWD) *Gibberella xylarioides*

The coffee wilt disease (CWD) is caused by a fungus (*F. xylarioides*) that blocks water and nutrients from traveling to other parts of the coffee plant from the roots, in turn causing wilting and eventually death. *Tracheomycosis* or vascular wilt of coffee historically was first observed in 1927 on *Coffea excelsa* in Central Africa Republic and first reported on *C. excelsa* in the Central African Republic in 1946 and the causal agent was identified as *F. xylarioides* by Steyaert (Flood, 2010; Girma Adugna *et al.*, 2008).

The *Fusarium* wilt disease on *Coffea arabica* was first observed in Ethiopia (in Keffa province) (Beaert *et al.*, 1957). Stewart described the wilting symptom and identified the causal organism to be *Fusarium oxysporum.* Later on, the fungus inciting Tracheomycosis was authentically confirmed to be *F. xylarioides*, of which *F. xylarioides* is the conidial stage (imperfect stage). This was based on comparative studies of the isolates collected from dying arabica coffee trees of different origin (including isolates from *Coffee arabica* L., Ethiopia) and different *Coffea* spp (Mogk, 1975). This disease can attack almost all above ground parts of the plant, and is most common in young plants. Die back begins with the lower branches but may spread to the whole plant as the disease develops. Stem tissues around the collar of the plant are killed, and blue black streaks appear in the wood, under the bark. In severe attacks, trees wilt and collapse (Cannell, 1985; Arega Zeru, 2006; Hindorf and Omondi, 2011).

On berries, sunken brown lesions appear at the stalk end of the berry, which can cut off the flow of nutrients to the berries, causing them to die prematurely. Dark brown lesions may also appear elsewhere on the berries, especially where the flower was attached, which turn the infected berries red, so that they appear too ripened.

Figure 1. Coffee wilt disease in Ethiopia (Gera) (Picture by Dr. Tesfaye Alemu)

The fungus is soil dwelling and enters the plant through wounds either above or below ground. The fungus is apparently not able to survive long in the soil and survival from one season to the next is mainly through seed from infected berries, however, insects and rain splash may also contribute to the spread of the disease (Girma Adugna and Hindorf, 2001).

In recent years, the emergence of *Fusarium* wilt disease (*F. xylarioides*) across East Africa has affected 90% and 30% of farms in Uganda and Ethiopia, respectively (Hein and Gatzweiler, 2006; Waller *et al.*, 2007). According to Waller *et al.* (2007) it has been estimated that affected coffee households are facing a reduction by a third of their income due to coffee wilt disease. The level of infection by this pathogen has confirmed the presence of Tracheomycosis with an incidence of up to 40 % (Hein and Gatzweiler, 2006; Rutherford, 2006; Waller *et al.*, 2007).

2.3 Spread of CWD and Survival under field condition

The fungal pathogen, *F. xylarioides* survives in the soil in the form of microconidia, macroconidia, chlamydospores and perithicium with ascospores (Girma Adugna, 2004). The timing from first symptoms to death of the tree varies from days in young plants to eight months in trees more than ten years old. Once the fungus infects the coffee tree, all affected trees eventually die. Wrigley (1988) has observed that the lateral and feeder roots of coffee spread on the surface plate parallel to soil surface for a distance of 1.2 to 1.8 meters from the trunk, and *F. xylarioides* is abundantly recovered from root parts of symptomatic and asymptomatic trees (Girma Adugna, 2004; Flood, 2010). The pathogen spreads two meters up to four plants on either sides of the inoculated focus plant through the infection of the roots in greenhouse experiment (Lewis Ivey *et al.*, 2003; Rutherford, 2006).

Closely spaced trees are more liable to wounding and cross inoculation while slashing or hoeing coffee fields. Girma Adugna (2004) reported that almost all coffee trees have wounds at the crown level or few centimeters above, and on average healthy trees have 1-3 wounds per coffee stem. Weeds are slashed frequently, some times more than ten times a year, depending on the dominating weed flora in plantation coffee. Most of coffee trees are found with wound at least once at all locations, where slashing is employed to control coffee weeds. When seedlings with healthy roots are transplanted into either naturally or artificially infested soils, no wilting symptoms appeared.

Infection exhibits when the tap roots are injured and transplanted into naturally or artificially infested soils, and also only on those seedlings inoculated by stem wounding through ditching with *F. xylarioides* infested scalps or by injecting the conidial suspensions with needles (Lewis Ivey *et al.*, 2002). The stem nicking or root drenching inoculation methods also elaborate the roles of contaminated farm implements in cross inoculating coffee trees as well as disseminating the coffee wilt pathogen in the field (Tesfaye Alemu, 2012).

2.4 Coffee Wilt Distribution in Ethiopia

Semi-forest coffee is estimated to contribute 35% to Ethiopia's total coffee (Paulos Dubale and Demil Tektay, 2000), even though yields are low. Coffee, intercropped with a variety of other crops enhances spread of disease where more intensively managed by slashing and pruning, together with some mulching and other organic materials. Weeding by slashing is done once a year around the picking season (Workafes Woldetsadik and Kasu Kebede, 2000).

The coffee wilt disease (CWD) was found to occur in all of the production systems; forest, Semi-forest, garden and plantation coffee (Girma Adugna, 2001; Girma Adugna, 2004). Noah Phiri and Peter Baker (2009) have reported that CWD incidence in Ethiopia is greatly affected by the farming system, with relatively low rates of infection in forest and semi-forest coffee and much higher rates in garden and plantation coffee. This may be due to the greater level of intervention in the latter, which gives increased opportunity for the fungus to spread, and may also be related to the greater genetic homogeneity of the coffee planted. Pieters and Van der Graaff (1980) have reported that the disease was endemic in all coffee growing areas of Ethiopia and reached epidemic proportions in some areas (Tables 2 and 3). Although CWD is not the major constraint to coffee production until recently, it existed in Ethiopia for many years, and yet at present the disease is less noticed by farmers in semi- forest than garden and plantation coffee (Girma Adugna, 2004).

2.4.1 Coffee wilt disease in the forest and semi-forest coffee plantations

Coffee wilt disease was found in four forest coffee zones in south-west and south-east rainforests with incidences ranging between 5% at Sheko and 30% at Yayu. Arega Zeru, (2006) reported increasing occurrence of CWD in the forest areas of Harenna (Bale) and Bonga (Keffa). The mean incidence in semi-forest coffee ranged from 4% at Mettu to, 16% at Gera in the South-West coffee producing areas with severity between 19 and 25% in some parts of Yirga cheffe (Girma Adugna, 2004).

2.4.2 CWD in garden coffee

Garden coffee is mostly in the southern and south-eastern regions, with plots usually less than 0.5 ha, intercropped with a variety of other crops. The coffee often consists of many ancient landraces. This system is more intensively managed by slashing and pruning, together with some mulching and other organic materials (Workafes and Kassu, 2000). Coffee wild disease is prevalent in the three major quality coffee-producing districts of the southern region, i.e. Wonago, Kochore and Yirgacheffe of Sidama and Gedeo zones, with the highest incidence in Yirgacheffe. The severity of wilting seen in Yirgacheffe varied between 27 and 44% (Girma Adugna, 2004). Disease incidence varied widely across coffee growing areas of the Southern Nations and Nationalities and Peoples state (SNNP) region. It was especially high in Sidama and Gedeo zones, with an incidence rate above 90% and severity of 25%. The incidence of CWD was above 35% in garden coffee of West Gojam Zone of Amhara regional state but it was very low in Wolaita (SNNP) and West Harerghe (Oromiya) (Petit, 2007; Phiri and Baker, 2009) (Fig.2).

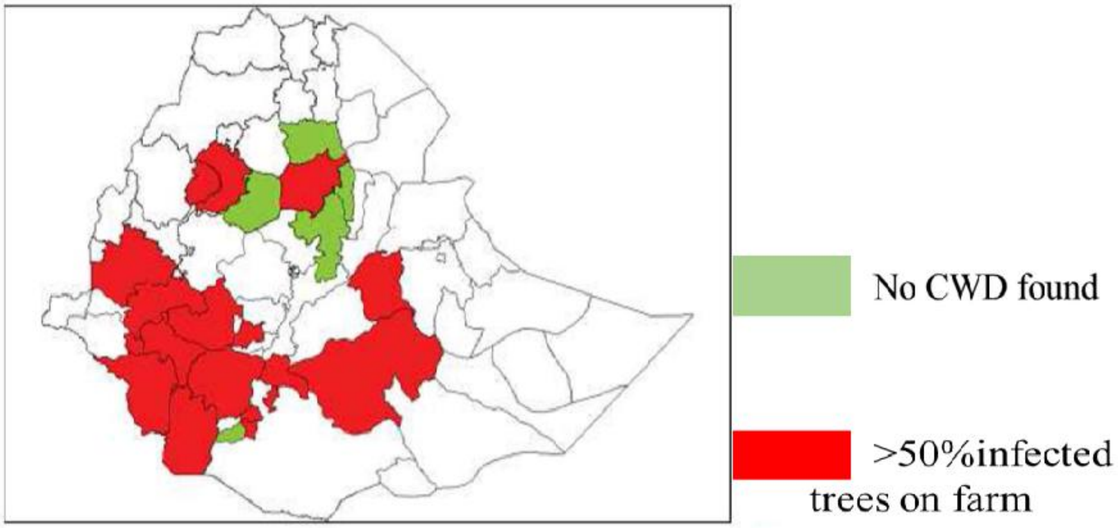


Figure 2. Distribution of coffee wilt disease (CWD) in Ethiopia (CABI, 2009)

2.4.3 CWD in plantation coffee

Incidence is severe in both plantation coffee and research center plots. On plantations in Gera, Chira and Gechi districts, mean incidence ranged respectively from 22 to 26%, 32 to 77% and 35 to 60% (Table 2). The overall tree loss in farmers' plantations was more than 30% and a small amount of coffee plantation had been abandoned completely. Girma Adugna (2001) confirmed that the disease was severe in coffee plantation at Bebeka, Teppi, Gera and Jimma.

Table 2. Incidence (%) of CWD under farmers' condition in South West Ethiopia (CABI, 2009).

Location	Field	Estimated area (ha)	Incidence (%)	
			Range	Mean
Gera	Gicho1	1.0	11.5-35.0	24.5
	Gicho2	1.5	8.7-38.0	21.7
	Sedi-loya	1.0	23.9-27.1	25.5
Chira	Gure-Genj	5.2	38.0-75.0	51.5
	Chira1	4.5	55.0-89.0	77.0
	Chira2	1.5	14.0-42.0	32.3
Tobba	Yachi	0.3	12.1-20.8	16.5
	Kilole	0.4	14.6-23.9	19.3
	Ageyu	0.2	8.3-27.0	16.1
Gomma	Shashamene	0.5	12.7-19.4	10.8
	Echemo	0.3	12.5-15.5	13.6
	Sombo	0.2	25.8-34.2	29.2
Gechi	Camp	0.5	25.0-70.0	48.9
	Mine-kobba	5.0	15.0-55.0	35.0
	Asendabo	5.0	37.7-78.6	59.7
Yayo	Jitto	1.0	11.0-34.0	22.5
Mettu	Sor	0.5	8.0-33.3	20.4
Mean of the ranges and mean	(Total 17)	(Total 28.6ha)	8.3-89.0	30.9±18.2

Coffee wilt disease was commonly encountered in the research plots at different rates. The lowest incidence of 8.7 ± 3.4 was recorded at Wenago and the higest incidence of 48.2 ± 23.1 was recorded at Jimma research plots respectively. Variations in the altitudes of the study area were also observed (Table 3).

Table 3**.** Incidence of CWD in experimental plots in Coffee Research Centers (Girma Adugna *et al.*, 2009).

Research Center	Number of fields (*n*)	Incidence (%)		Altitude (m)
		Range	Mean and SD	
Jimma	10	19.8-82.0	48.2 ± 23.1	1750
Agaro	3	5.2-12.1	8.7 ± 3.4	1650
Gera	15	21.0-61.1	42.5 ± 18.7	2000
Mettu	3	23.3-30-9	27.1 ± 5.4	1550
Teppi	3	6.5-13.4	10.0 ± 4.9	1200

Wenago	3	5.7-14.6	9.8 ± 4.5	1850
Mean of the range and means		5.2-82.0	24.4 ± 17.7	

According to Waller *et al.* (2007), most farmers observed the disease 40 years ago and since then the disease increased at lower rate. Its spread and control methods are not well known by farmers, extension workers and agricultural officers, although the observation by farmers coincided more or less with the first record of disease by Stewart (1958) who first discovered symptoms of wilting in Ethiopia. Kranz and Mogk (1973) observed the disease on a few single trees scattered in some plantations around Agaro, Jimma and Bonga. Girma Adugna (1997) has reported the disease outbreaks are observed on some trees at Bebeka and in the Baya at Tepi in 1992. Later the disease distributed to Chira, Gechi, Choora, Yayo districts and other coffee growing regions of Ethiopia.

2.5 Status of CWD in Ethiopia

The Arabica strain of the disease is present only in Ethiopia, and although it has been there since 1957, the incidence and severity of the disease is mostly less acute than Democratic Republic of Congo (DRC) or Uganda. Van der Graaff, (1981) reported that coffee lines of *C. arabica* in Ethiopia differed widely in their resistance to *F. xylarioides* and considered that these differences provided an excellent opportunity to control the disease using resistant varieties. It is extremely worrying that CWD is found in forest coffee, which must be considered a threat to the genetic base of Arabica, which is already under threat because of land-use change and climate change (Phiri and Baker, 2009).

2.6 Symptoms of Coffee Wilt Disease

The first signs of CWD are yellowing, folding and curling inward of leaves. The leaves to touch, then dry up and feel papery and then turn brown. Eventually, the leaves drop off leaving the infected trees completely bare. Affected branches may turn black brown or blackish and dry up (Lewis Ivey *et al.*, 2002; Phiri and Baker, 2009). These signs are known as dieback, often start on the branches on one side of the tree but rapidly spread to the whole tree (Figure 3).

Figure 2. Symptoms of an arabica coffee wilt in Ethiopia (Photo source: Dr. Tesfaye Alemu)

The bark on the trunk, especially near the base of the tree, may become swollen and have many vertical and spiral cranks. Underneath the bark the wood appears blue-black in color. Towards the end of the rainy season black structures resembling soil occur on the bark, usually at the base of the plant (Lewis Ivey *et al.*, 2002; Phiri and Baker, 2009). These structures are dark-violet perithecia; contain spores (ascospores) of the fungus that enable it to spread to other coffee trees and to survive in the soil or on plant material. In the roots, a moist black rot is observed.

Another important early sign of CWD is that berries on infected trees turn red prematurely and appear to ripen early. Most affected trees die within six months after the first external symptoms are observed. Although other symptoms are caused by other problems, only CWD causes the blue-black discoloration of the wood (Lewis Ivey *et al.*, 2002; Phiri and Baker, 2009).

2.7 Management of Coffee Wilt Disease

2.7.1 Quarantine measures

For races currently free from *Tracheomycosis* strict quarantine measures, which help to prevent its entry and spread must be followed. Movement of coffee materials (seedlings, husks, and other organs), soil and farm implements between affected and unaffected areas should be isolated properly (Girma Adugna *et al.*, 2010). These measures need to be backed up with dissemination of information about the disease to farmers, extension workers, scientists and the general public. Dissemination of information on the symptoms of the disease is essential to allow monitoring and early detection of the disease. (Girma Adugna, 2004; Rutherford, 2006; Flood, 2010). However, it is not easy as most areas were already affected.

2.7.2 Resistant varieties

These are undoubtedly the most feasible option for controlling CWD in all affected countries. Use of resistant cultivars was found to be highly effective when combined with other control measures during the previous outbreak of the disease. The combined use of selected cultivars and biocontrol agents can provide better disease control than the use of any of them alone (Derso, 1997; Girma Adugna, 2004; Arega Zeru, 2006). It has been reported that varietal differences in resistance to the pathogen and suggested the use of resistant varieties as a means of control. However, developing resistant varieties is long-term and requires considerable resources (human, facilities and financial). Megan *et al*. (2006) reported that Uganda has advanced further with its CWD breeding program, using single-tree selection, and some of the more promising selections are currently being evaluated on-farm.

2.7.3 Cultural practices

Systematic elimination of affected plants over vast areas combined with the development of breeding programs effectively reduced its impact. Affected trees and trees adjacent to affected trees should also be uprooted and burnt although appear healthy because while symptoms of the disease may not be visible, the fungus may be inside the plant (Rutherford, 2006).

Frequent inspection of the crop, along with uprooting and burning infected material at the spot where they were uprooted, minimizes disease spread. In addition, replanting should not be done for 2-3 years after uprooting infected bushes to allow the viability of the soil inoculum to decline (Wrigley, 1988) when symptoms are recognized quickly and uprooting and burning done efficiently, the farmers may save some of the crops. If the farmers delay, the infected trees act as source of inoculum to other trees and leads to whole crop losses. Trees cut down as control measure should not be used as fuel as affected trees drugged through healthy trees in the farm will aggravate the spread of the disease. Diseased trees must be burnt where they are uprooted (Girma Adugna *et al.*, 2010).

To prevent spread from one field to another in large plantation, it is recommended that a 300 m strip of land should be cleared of coffee (by uprooting and burning) ahead of the disease front. Any kind of wounding to the tree will allow the fungus to enter. Wounding may occur through weeding and pruning with machete or hoe, or even by livestock feeding on and around the tree.

Great care should be taken to minimize damage to the tree and all tools should be sterilized with fire or with disinfectant before moving to another tree (Girma Adugna and Hindorf, 2001; Girma Adugna *et al.*, 2008). Mulches and soil amendments including cow dung and urine have been claimed to control the disease, but bring only temporal improvement to infected trees by increasing plant vigor and stimulating new growth of roots, shoots, and leaves.

Mulches and soil amendments are therefore unlikely to control the disease in already infected trees, but may be useful in preparing the land for replanting after affected trees have been uprooted and burnt (Papavizas, 1985). Following destruction of the diseased trees and preparation of the land, replanting should not be carried out for at least two years to allow the inoculum of the fungus in the soil to decrease. Replanting should be done with plants raised from the disease free cuttings and seeds collected from areas that are free from the disease (Rutherford, 2006; Temesgen Belayneh *et al.*, 2010)

2.7.4 Chemical Control

There are several reports where fungicides have been used for the control of diseases caused by soil-borne pathogens. Captan and benomyl have been used successfully against several seed-borne fungi under laboratory and field condition. Commonly used fungicides which are effective against *Fusarium species* are: Carbendazim, Dithane M-45, Thiovit and Thiophanate-methyl significantly reduced the growth of *F. oxysporum (*Abdul *et al.*, 2006).

Aminoglycosides: amikacin, gentamicin, kanamycin A, kanamycin B, neomycin, and ribostamycin showed the best fungicidal activities against *F. graminearum* and suppressed fungal infection (Sharma *et al.*, 2016). The pathogen, *F. xylarioides* is thought to live in the soil and inside the plant, making it hard to target the fungus even with systemic fungicides (Tesfaye Alemu and Kapoor, 2004). Copper oxychloride (50%) sprayed on to the stem only, to be diluted at the rate of 40 g per 7.5 l of water and applied once a month during the rainy season, and once every 3 months during the dry season (Phiri and Baker, 2009).

2.7.5 Biological control

Biological control is the use of microbial antagonists to suppress host-specific pathogens, insect pests and weeds. The organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA). The biocontrol mechanism could be reduction of inoculum density or disease producing activities of a pathogen or a parasite in its active or dormant state using one or more organisms. The application of the biocontrol agents can be accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists (Sundaramoorthy and Balabaskar, 2013). More specifically, through the use of microbial inoculants to suppress a single type or class of plant diseases or managing soils conditions to promote the combined activities of native soil and plant associated organisms that contribute to general suppression (Junaid *et al.*, 2013).

The term biological control has also been applied to the use of the natural products extracted or fermented from various sources. These formulations may be very simple mixtures of natural ingredients with specific activities or complex mixtures with multiple effects on the host as well as the target pest or pathogen (Kimani *et al.*, 2002).

2.7.6 Characteristics of Biological Control Agents

The BCAs exhibit different modes of action and hence, a good testing program should elucidate all the mechanisms involved in the biocontrol activity of the BCA. Apart from bio control ability, the BCAs possess other traits such as rhizosphere competence, tolerance of fungicides, saprophytic competitive ability, ability to tolerate high and low temperatures, adaptability to different edaphic conditions, good searching ability, host specificity, high reproduction rate, short life cycle, adaptability, well adapted to different stages of life cycle of target host, able to maintain itself after reducing host population (Harman *et al.*, 2004). These traits are useful for good BCA as they help in the establishment of the BCA in a given agro-ecological region.

2.7.7 Antibiotic-mediated suppression

Antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. Most microbes produce and secrete one or more compounds with antibiotic activity. Antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens. Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens. *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. The potential of *Trichoderma* species as biocontrol agents of plant pathogens was first recognized in the early 1930s (Haas and Keel, 2003).

Trichoderma species can directly affect mycelia or survival propagules of other fungi through production of toxic secondary metabolites, formation of specialized structures, and secretion of cell wall-degrading enzymes. Mycoparasitic activity of *Trichoderma* spp. against phytopathogenic fungi and Oomycetes due to lytic activity of cell wall-degrading enzymes has been widely studied. In addition to mycoparasitism, other mechanisms have been proposed to account for biocontrol of plant disease by *Trichoderma* species including the induction of resistance in the host plant and competition for nutrients and potential infection sites (Harman *et al.,* 2004).

Trichoderma are widely used in agriculture, and some of the most useful strains demonstrate a property known as rhizosphere competence, the ability to colonize and grow in association with plant roots (Harman *et al.*, 1991). *Trichoderma harzianum* and *Trichoderma viride* are the most studied of all the *Trichoderma* species for biological control and the most effective in reducing diseases caused by soil borne plant pathogens. *Trichoderma* would be especially suitable for combating coffee wilt disease because many of its species are rhizosphere competent, and the coffee roots are the first target for the attack by pathogens Bowen and Rovira (Scala *et al.*, 2007)

Trichoderma species have been widely studied, and are presently marketed as biopesticides, biofertilizers and soil amendments, due to their ability to protect plants, enhance vegetative growth and contain pathogen population under numerous agricultural conditions. Many members of the genus *Trichoderma* are prolific producers of extracellular proteins, and best known for their ability to produce enzymes that degrade cellulose and chitin, although they are also capable of producing other useful enzymes for industry and agriculture (Ramanujam *et al.*, 2010)

2.8 The genus *Trichoderma* and their distribution

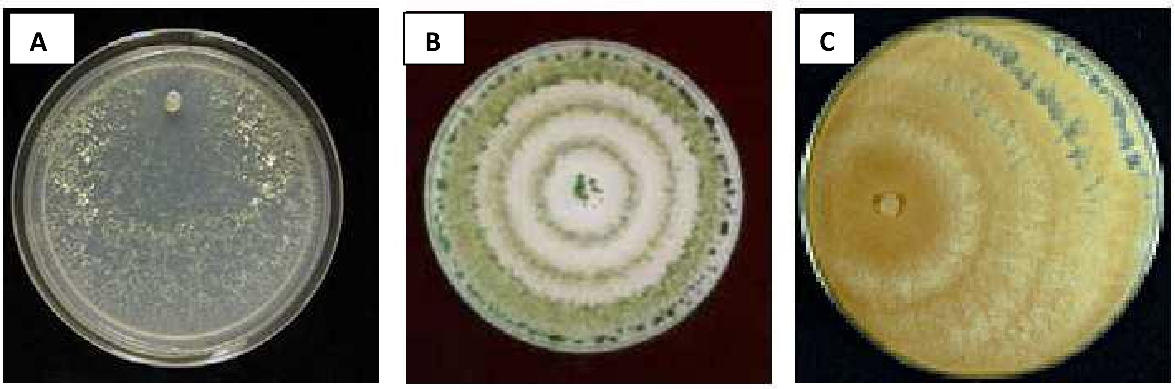
Trichoderma are free living, asexually reproducing and filamentous fungi. They are members of a genus belonging to a group of largely asexually reproducing fungi that includes a wide spectrum range from very effective soil colonizers with high biodegradation potential to facultative plant symbionts that colonize the rhizosphere. *Trichoderma* species are exceptionally good model of biocontrol agent as they are widely spread, easy to isolate and culture, multiply rapidly on many substrates, act as mycoparasite, strong opportunistic invaders, avirulent plant symbionts, competes for food and site, prolific producers of spores and powerful antibiotics, antifungal compounds, secondary metabolites and enzymes. These properties make these fungi ecologically very successful and are the reasons for their ubiquitousness (Harman *et al.*, 2004).

Trichoderma are present nearly in all types of temperate and tropical soils, commonly found in variety of soil types such as agriculture, forest, prairie, salt marsh and desert soils in all climatic zones. Besides this, they are also found colonizing roots, litter, decaying/decorticated wood, decaying bark and various plant materials at all climatic zones/latitudes. It is reported that *Trichoderma* constituted up to 3 per cent of the total fungal propagules in a wide range of forest soils and 1.5 per cent in pasture soils in a wide range of crops (Hagn *et al.*, 2003; Ha, 2010; Junaid *et al.*, 2013).

2.8.1 Morphology

*Trichoderm*a is usually recognized by the presence of fast-growing colonies producing white, green, or yellow cushions of sporulating filaments, the fertile filaments or conidiophores produce side branches bearing whorls of short phialides that support the spherical to ovoid green colored.

The mycelia of *Trichoderma* spp. on potato dextrose agar (PDA) plate cultures is typically fast growing, with the optimal temperatures between 25-30 ^{O}C. The hyphae are initially transparent or whitish, and depending upon the species, the mycelium become greenish, yellowish or less frequently white within one week (Fig. 4). Conidiophores are highly branched and thus difficult to define or measure. They may be loosely grouped or compactly tufted, and often develop in distinct concentric rings (Khalid, 2009).



Source: Khalid (2009)

Figure 3. *Trichoderma* spp grown in culture media (PDA) A. *T. atroviride*; B. *T. viride*; C. *T. harzianum*.

2.9 Mechanism of action of *Trichoderma* species as biocontrol

The mechanisms that *Trichoderma* spp uses to antagonize phytopathogenic fungi include competition, colonization, antibiosis and direct mycoparasitism (Harman *et al.*, 2004). This antagonistic potential serves as the basis for effective biological control applications of different *Trichoderma* strains as an alternative method to chemicals for the control of a wide spectrum of plant pathogens. *Trichoderma* species stimulates plant growth by producing substances that stimulate plant growth and development. These substances act as catalysts or accelerators in the primary meristem tissues in the young parts of plants, accelerating cell reproduction, so that the plants achieve faster growth than those which have not been treated with this microorganism (Dennis and Webster, 1971; Baker, 2013).

The benefits of using *Trichoderma* in agriculture are multiple, and depending upon the strain the advantages for the associated plant can include colonization of the rhizosphere by the BCA, allowing rapid establishment within the rhizosphere of a stable microbial community, control of phytopathogenic and competitive micro flora or fauna, overall improvement of the plant health, enhancing nutrient availability and uptake, and inducing systemic resistance (ISR) similar to that stimulated by beneficial rhizobacteria (Harman *et al.*, 2004).

Trichoderma biocontrol strains utilize numerous mechanisms for both attacking other soil organisms and enhancing plant and root growth. The colonization of the root system by rhizosphere competent strains of *Trichoderma* results in increased development of root and/or aerial systems and crop yields (Yedidia *et al.*, 2001). *Trichoderma* has also been described as being involved in other biological activities such as the induction of plant systemic resistance and antagonistic effects on plant pathogenic nematodes (Sharon *et al.*, 2001). Some strains of *Trichoderma* species have also been noted to be aggressive bio-degraders in their saprophytic phases, in addition to acting as competitors to fungal pathogens, particularly when nutrients are a limiting factor in the environment. These facts strongly suggest that in the plant root environment Trichoderma actively interacts with the components in the soil community, the plant, bacteria, fungi, other organisms, such as nematodes or insects that share the same ecological niche.

Trichoderma species is important participants in the nutrient cycle. They aid in the decomposition of organic matter and make available to the plant many elements normally inaccessible. Yedidia *et al.* (2001) noted that the presence of the fungus increased the uptake and concentration of a variety of nutrients (copper, phosphorous, iron, manganese and sodium) in the roots of plants grown in a liquid medium. These increased concentrations indicated an improvement in plant active-uptake mechanisms.

Corn that developed from seeds treated with *T. harzianum* strain produced higher yields, even when a fertilizer containing 40% less nitrogen was applied, than the plants developed from seed that was not treated (Harman *et al.*, 1991; Harman, 2006)). This ability to enhance production with less nitrate fertilizers provides the opportunity to potentially reduce nitrate pollution of ground and surface water, a serious adverse consequence of large-scale maize culture. In addition to effects on the increase of nutrient uptake and the efficiency of nitrogen use, the beneficial fungi can also solubilize various nutrients in the soil that would be otherwise unavailable for uptake by the plant.

The success of *Trichoderma* spp. as a biocontrol agent is believed to involve various modes of action, including antibiotic production, secretion of lytic-enzymes, mycoparasitism, competition for space and nutrients, and induction of systemic resistance. A given *Trichoderma*-host interaction may involve any of these mechanisms individually or encompass more than one of them acting simultaneously and in fact it seems advantageous for a biocontrol agent to suppress a plant pathogen using multiple mechanisms (Harman, 2006)

2.9.1 Antibiosis

Both volatile and non-volatile antibiotics are known to be produced from *Trichoderma* species. Peptaibols (trichorizianines, trichokindins, trichorzins, *Trichorozins* and *harzianins*), a class of antibiotics, are produced by most species and strains of *Trichoderma*. They generally exhibit antimicrobial activity against fungi and gram positive bacteria. Peptaibols are thought to act on the membrane of the target fungus to inhibit membrane associated enzymes involved in cell wall synthesis (Vinale *et al.,* 2008). The antibiotics *Trichodermin, Trichodermol, harzianins* A and *herzianolide* are also known to be produced from *T. viride* and other species of *Trichoderma* (Howell, 2003).

2.9.2 Lytic enzymes

Studies have shown that mycoparasitic strains of *Trichoderma* produce a complex set of extra cellular enzymes including β-(1,3)-glucanase, chitinases, lipases and proteases when grown on isolated cell walls of pathogenic fungi (Viterbo *et al.*, 2002). These lytic enzymes are probably responsible for hyphal lysis through the digestion of major cell wall components. It is believed that these enzymes act synergistically with the antibiotics to inhibit the growth of fungal pathogens (Schuster and Schmoll, 2010). It appears that the weakening of the host cell wall by the enzymes increases the rate of diffusion of the antibiotics through the cell wall. *Trichoderma* spp also produce both volatile and non-volatile compounds that inhibit the growth of the mycoparasites. Harman *et al.* (2004) identified five classes of volatile compounds, such as alcohols, esters, ketones, acids and lipids, produced by some fungi and bacteria.

2.9.3 Mycoparasitism

Mycoparasitism occurs when one fungus exists in intimate association with another from which it derives some or all of its nutrients while conferring no benefit in return. The best-known mycoparasite is the fungus *Trichoderma* species. This is because *Trichoderma* spp attacks a great variety of phytopathogenic fungi that are responsible for most important diseases of major economic importance worldwide. It appears that mycoparasitism is a complex process involving several steps. The mycoparasitic relationship between *Trichoderma* spp and its potential host might involve biochemical and physiological interactions that lead the microscopically visible phenomena of hyphal coiling, appressorium formation, penetration and cytoplasmic degradation (Howell, 2003).

2.9.4 Competition

Competition is an indirect effect whereby pathogens are excluded by depletion of food bases or by physical occupation of sites. The study of Barbosa *et al.* (2001) in the *in vitro* antagonism of *Trichoderma* species on *Cladosporium herbarum* revealed that the colonies of *Trichoderma* species grew always faster than *C. herbarum* in single or mixed culture. *T. viride* compete for the same niches with the pathogens. Thus, the rapid growth of *Trichoderma* species gives it an important advantage in the competition for space and nutrients with plant pathogenic fungi.

In the rhizosphere competition for space as well as nutrients is one of major importance of microbial interaction. Thus, an important attribute of a successful rhizosphere biocontrol agent would be the ability to remain at high population density on the root surface, providing protection of the whole root for the duration of its life (Vos *et al.*, 2015). In addition to their biocontrol effects, the ability of *Trichoderma* species to increase the rate of plant growth and development has been known for many years. It was found that a number of *Trichoderma* strains were simultaneously plant growth promoters in vegetables and various seedlings, and biocontrol agents. *Trichoderma* species may affect minor pathogens in the soil but it may also directly affect the plant by excreting a regulating hormone which may, in turn, increase the growth rate or the efficiency of nutrient uptake (Tapwal *et al.*, 2015).

2.9.5 Induction of host resistance

Plants actively respond to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability. Plants also respond to a variety of chemical stimuli produced by soil and plant associated microbes. Molecules produced by Trichoderma and/or its metabolic activity also have potential for promoting plant growth. Applications of *T. harzianum* to seed or the plant resulted in improved germination, increased plant size, augmented leaf area and weight, greater yields.

Metabolic changes occur in the root during colonization by *Trichoderma* species such as the activation of pathogenesis-related proteins (PR-proteins), which induce in the plant an increased resistance to subsequent attack by numerous microbial pathogens. Such stimuli can either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens (Hoitink *et al.*, 2006). Induction of host defenses can be local and/or systemic in nature, depending on the type, source, and amount of stimuli.

2.10 Isolation and testing of fungal biological control agents from soil samples

The method used to isolate microorganisms is a soil dilution technique. Dried, crushed and sieved soil samples (10 g) were shaken in 90 ml of sterile water for 10 min then left standing for a further 20 min. A dilution series was made up to 10-6. Aliquots (0.5 ml) are spread onto three Czapeck-Dox's agar (CZA) plates and incubated at 25°C for 2 weeks. Resulting colonies are purified on PDA plates and identified using standard mycological keys (Chaverri *et al.*, 2015). Testing must begin with the identification of the BCA and continue up to the commercial product. In *in vitro* tests are tests which have been designed for identification or selection of potential BCAs and elucidate biocontrol mechanisms of known BCAs.

Dual culture method is also known as bicultural, cross culture, or paired culture has been extensively used for preliminary screening of large populations of fungal, bacterial, and actinomycetes BCAs in a Petri dish under optimum conditions for both the pathogen and the BCA. The inhibition is recorded either in the form of the inhibition zone produced or the over growth of the pathogen by the BCA (Anees *et al.*, 2010).

3. MATERIALS AND METHODS

3.1 Coffee Root Sample Collection

Coffee root samples were collected from different ecological habitat of Kaffa zone for the isolation of endophytic *Trichoderma* isolates (Figure 5). At each sampling site, a visually healthy adult *C. arabica* plant was located and a root sample was taken within its rhizosphere area. Sample root were collected from three districts (Gimbo, Gowata and Chana districts) from garden coffee, forest coffee, coffee plantation and semi-forest coffee growing areas, at the depth of 5-7 cm of soil surface in March, 2018. A total of 30 root samples were randomly collected, placed in the paper envelope, labeled with locations (district, localities, coordinates and altitude) and collected date and brought to laboratory and refrigerated at 4^{O}C until isolation took place.

The isolation, evaluation, characterization and testing of biocontrol agent *Trichoderma* isolates were conducted under *in vitro* condition at Mycology laboratory, Department of Microbial, Cellular and Molecular Biology (MCMB), Addis Ababa University (AAU).

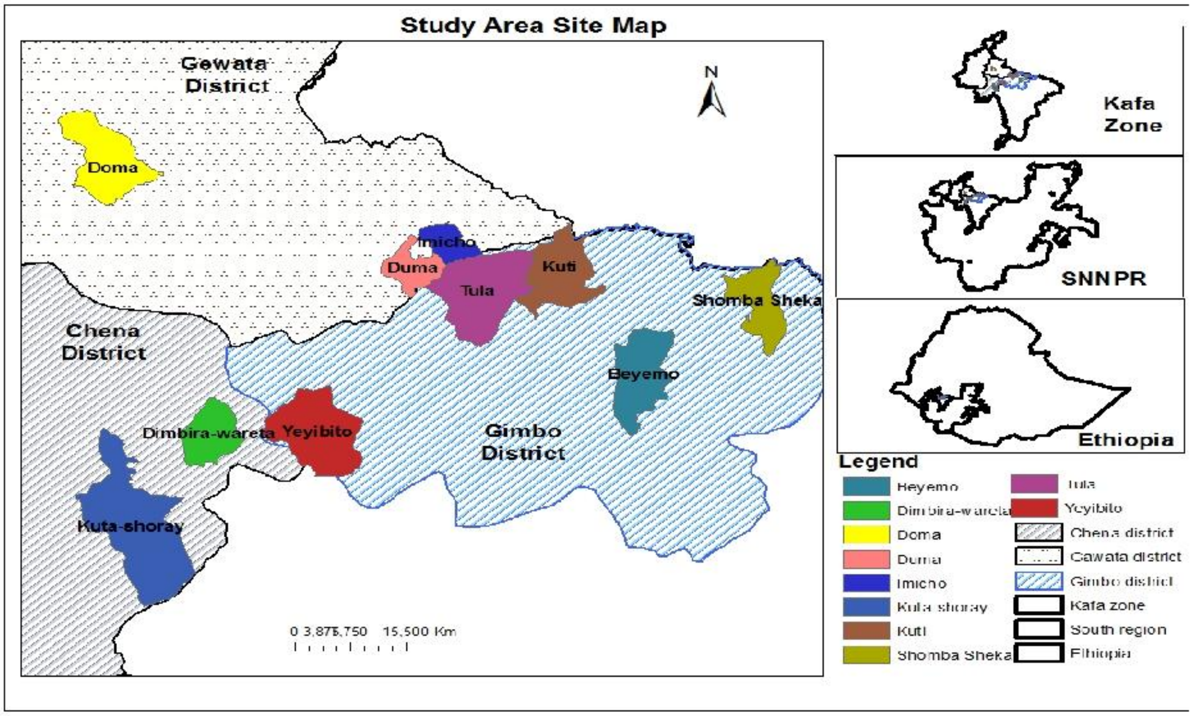


Figure 5. Study area Map of Kaffa zone and sample area Districts

3.2 Sterilization

The sterilization of media and glass wares (wrapped in brown papers/Kaki papers) were done by autoclaving at temperature of 121 ^{O}C for 15 minutes. Autoclaved glass wares were dried in hot air oven at 80 ^{O}C for 45-60 minutes.

3.3 Isolation of endophytic *Trichoderma* isolates and test Pathogen (*F. xylarioides*)

The root samples were washed with tap water to remove dust and dirt residues, and cut into pieces (1×1 cm). Then the root samples were immersed in 70% ethanol for 30 seconds and 1% sodium hypochlorite solution for 2 minutes. The root was then thoroughly rinsed twice in sterile distilled water. After this process, four root pieces were placed in separate Petri dishes (90 mm) containing Trichoderma specific medium (TSM) supplemented with chloramphenicol (250g/L) after the medium was autoclaved. Plates were incubated in at 25 ± 2 °C for 7 days. After endophyte mycelium emerged from plant tissues into the agar, mycelial fragments were transferred to fresh PDA plates (Sánchez Marquez et al., 2007). Endophytes were identified up to genus level based on colony characters, growth and structure of mycelium, conidiophores, phialides and conidia as described by Kubicek and Harman (2002).

The maintenance of cultures of *F. xylarioides* isolates and biological control agents (*Trichoderma* isolates) was done on Potato Dextrose Agar (PDA) slants in the plugged test tubes. The slants were stored in the refrigerator at 4 ^{O}C for further study. Continuous maintenance of endophytes was achieved by regular transfer on PDA slants under aseptic conditions to keep the culture fresh and viable. They were screened for biological activity and the most potent biological control agent against *F. xylarioides*, the causal agent of CWD. The coffee wilt pathogen (*F. xylarioides*) was also obtained from the above mentioned laboratory (MCMB) and it is used as a test pathogen.

Table 4. *Trichoderma* isolates studied and their identification

S/No	***Trichoderma* Isolates**	**Isolate Designation**	**Number of root Sample taken**	**Place of Collection**
1	Gowata Trichoderma 01	Gow-01	4	Gowata
2	Gimbo Trichoderma 02	Gim-02	3	Gimbo
3	Gimbo Trichoderma 03	Gim-03	3	Gimbo
4	Gimbo Trichoderma 05	Gim-05	4	Gimbo
5	Gimbo Trichoderma 06	Gim-06	4	Gimbo
6	Gimbo Trichoderma 08	Gim-08	4	Gimbo
7	Channa Trichoderma 10	Chan-10	4	Channa
8	Channa Trichoderma 11	Chan-11	4	Channa

3.4 Cultural and morphological characterization of *Trichoderma* isolates

The cultural characteristics of *Trichoderma* isolates were studied in PDA. The identification was performed using an interactive key for strain identification based on the growth characters on PDA along with microscopic observations of the isolates. Cultural characteristics such as colony appearances, mycelia textures and pigmentations on both obverse and reverse on PDA plates was observed after 3–7 days of incubation under the standard incubation conditions (Afrasa Mulatu *et al.*, 2013). Growth rate via colony diameter also on PDA was measured initially standardized at 5 mm using a cork borer and incubated for 3 days in a total darkness. Trials were repeated three times and in triplicates.

3.5 Physiological Characterization of Trichoderma Isolates

3.5.1 Growth at different Temperatures

The ability of *Trichoderma* isolates to grow at 4, 25 and 37^{O}C over 8 days was tested on PDA medium (Hermosa *et al.*, 2001).

3.5.2 Biomass production and spore yield determination of *Trichoderma* species at different pH values

The ability of *Trichoderma* isolates to grow at pH 4.5, 6.5 and 7.5 were tested in liquid Potato dextrose broth (PDB) medium. For biomass production Erlenmeyer flask (250ml) containing 50 ml of each PDB medium was inoculated with four mycelial plugs/disc (5mm in diameter) of the *Trichoderma* isolates taken from seven days old cultures on PDA. The flasks were plugged with cotton in aseptic conditions and placed in incubator at 25^{o}C for 10 days. The culture was harvested finally from each replicate. The fungal biomass yield was assessed by collecting fungal biomass on pre-weighed filter paper. The dry weight was determined after 24 hours of oven drying at $60^{O}C$. Number of conidia per mg of the biomass was determined by dilution method with the aid of Haemocytometer.

The spore yield was determined using the modified method of Waghunde *et al.,* (2010). Each flask was harvested by filtering the spore suspension through a sterilized double layered muslin cloth. The stock suspension was kept on rotary flask shaker for 5 minutes, after which 2 ml of the suspension was added into a cuvette. The equipment was calibrated with 2 ml of blank solution of potato dextrose broth (PDB). The spore yield was determined at a wavelength of 550 nm using Perkin Elmer Lambda 25 UV Spectrophotometer.

3.6 Evaluation of Antagonistic Activity of *Trichoderma* isolates on radial growth of *F. xylarioides*

3.6.1 Dual culture plate testing

The antagonistic effects of eight *Trichoderma* isolates were evaluated against *F. xylarioides* in *in vitro* condition using the direct confrontation method. Briefly, one disc (5 mm) obtained from one week old culture of *Trichoderma* isolate and *F. xylarioides* respectively were placed on PDA at opposite sides on the same diagonal line at 1.5 cm distance from the edge. The isolates were placed 6 cm apart on the same plate. Three plates were considered for each fungus-fungus interaction and the biological control potential of each isolate against the test pathogen was studied. One disc of test pathogen *(F. xylarioides*) and each isolate of *Trichoderma* alone were prepared as a control. Both the dual and individual cultures were incubated at $25 \pm 2\ ^{O}C$ and the radial mycelial growth of Trichoderma linear growth of the fungi was measured within two days interval for 10 consecutive days. Microscopic examinations was done on the area of intermingling contact (pathogen - antagonist) (Butt and Copping, 2000; Atanasova *et al.*, 2013; De la Cruz Quiroz *et al.*, 2015).

The interaction between *F. xylarioides* and *Trichoderma* isolates were assessed using the following predetermined criteria. 1= the hyphae of the two colonies intermingle but remain clearly distinguishable. 2= the growing margins of the two fungi meet; the phytopathogenic fungus is inhibited and overgrown by *Trichoderma*. 3= the hyphae of the two organisms approach one another and stop growing and 4= the growth of the phytopathogenic fungus is inhibited at a distance leaving a clear zone of inhibition between the two organisms. Interaction types scored as 2 and 4 were considered to be antagonistic (Steyaert *et al.*, 2016). The Percentage Inhibition of Radial Growth (PIRG) was calculated at the last day following incubation using the formula given below (Pakdaman's *et al.*, 2013):

$$PIRG = \frac{(Rc - Ra)}{Rc} \times 100$$

Where PIRG = Percent Inhibition of pathogen growth by antagonists; Ra =Radius against antagonist colony; Rc = control radius.

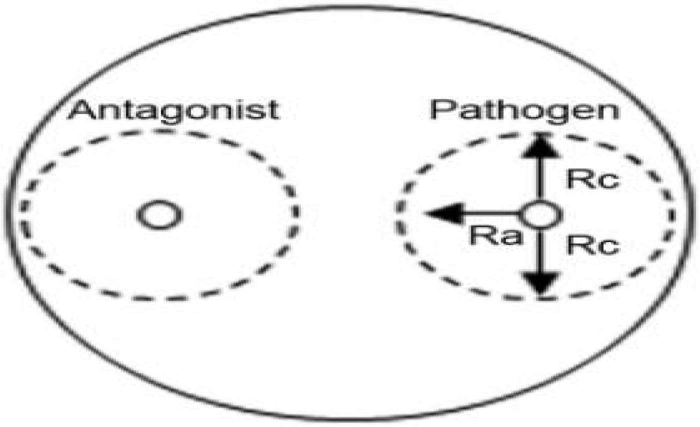


Figure 6. Scheme of inoculation of confrontation plates

Antagonism effect of *Trichoderma* isolates were assessed in semi-quantitative means according to Temesgen Belayneh *et al.*, (2010): >85 PIRG indicating very high antagonistic activity, 61–85 PIRG indicating high antagonistic activity, 51– 61 PIRG indicating moderate antagonistic activity, < 50 PIRG indicating low antagonistic activity, and 0 indicating no activity. Clear zone of inhibition (CZI) was also determined by measuring the clearance between the colony margins of the *F. xylarioides* and *Trichoderma* isolates.

3.6.2 Effect of volatile metabolites of *Trichoderma* isolates *on mycelial growth F. xylarioides*

The effect of volatile compounds produced by *Trichoderma* isolates on radial growth of pathogenic fungi, *F. xylarioides* was determined following the method described by Muthukumar *et al.* (2011). Five millimeter disc of *Trichoderma* colony was inoculated centrally in Petri plates containing PDA medium in triplicates and incubated for 48 h at 25 ^{O}C. Additional PDA plates with a mycelial plug (5 mm) from the colony margin of an actively growing pathogen (*F. xylarioides*) colony (typically 3-5 days old) was inoculated. The plate lid from the *Trichoderma* culture was replaced by the PDA plate inoculated with the test pathogen so that both fungi were facing each other. The test pathogen fungi was inverted over *Trichoderma* to avoid contamination by the mycoparasitic fungal conidia. The Petri dishes were sealed together with Parafilm and incubated for further 5 days at 25 ^{O}C. For the control, an uninoculated PDA plate was used in place of the *Trichoderma* culture. The colony radius of the test pathogen was measured on six day and the radial growth rate was calculated. The percentage inhibition of radial growth (PIRG) was determined after seven days of incubation by using the same formula as described in dual culture plate testing.

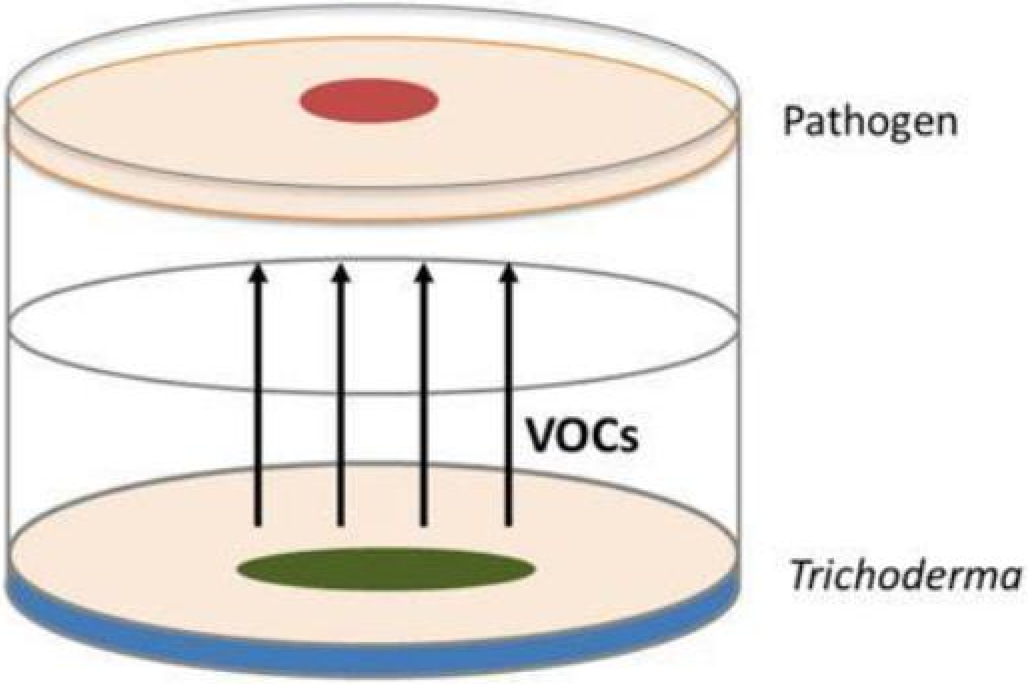


Figure 7. Volatile organic compounds emitted by *Trichoderma* and their effect on radial growth of *F. xylarioides*: A graphic representation of the bio-assay

3.6.3 Effect of culture filtrates of Trichoderma isolates on mycelial growth F. xylarioides

The production of non-volatile substances by the *Trichoderma* isolates against the test pathogen was studied using the method described by Dennis and Webster, (1971). *Trichoderma* isolates were inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 25 ± 2 ^{o}C on a rotatory shaker set at 100 rpm for 15 days. The control flasks were not inoculated with any of the culture. The liquid culture was filtered through Whatman no.1 filter paper for removing mycelia mats and then sterilized by passing through 0.45µm pore biological membrane filter (Aneja *et al.*, 2005). The filtrate was added to molten PDA medium (at 40 ± 3^{o}C) to obtain a final concentration of 10% (v/v).

Five milli liters was mixed with 100 ml PDA, poured in Petri plates and 5 mm diameter culture disc of test pathogen was inoculated at the center and incubated at 27± 2°C for 7 days. There were three replicates for each treatment. The observation was taken and mycelial growth inhibition percent was recorded and calculated in relation to the growth of the controls (Aneja *et al.*, 2005).

3.7 Statistical Data Analysis

The data collected were analyzed using SPSS (version 24.0). Descriptive statistics was used for data presentation. Comparative analysis of experimental results was also analyzed by using spread sheet software (Microsoft Excel 2013).The result was considered significant if $p<0.05$.

4. RESULTS AND DISCUSSION

4.1 Isolation and identification of Endophytic *Trichoderma* isolates

Eight different *Trichoderma* isolates have been identified from the collected samples. Use of morphological characteristics is one of the conventional methods to identify *Trichoderma* isolates and it remains as a potential method to identify *Trichoderma* up to genus level (Samuels *et al*., 2002). Pure *Trichoderma* colonies grown in the PDA plates at 25^{O}C showed different growth patterns with different colony characteristics. The color of the colonies varied from light green to dark green and deep yellow. Each *Trichoderma* isolate having similar colony morphology, may be, most probably, belong to the same species. But colony characteristics are not sufficient to identify them into species level. Therefore, the characteristics of sporulating structures and spores were used.

4.2 Cultural and morphological characterization of *Trichoderma* isolates

Isolates of *Trichoderma* isolates were examined macroscopically and microscopically. They were found to form colonies with white mycelia, becoming green when forming conidia and conidiophores. There were conidia formed densely over the center and undulating concentric rings toward the edge. Observation through the bottom of Petri dishes showed production of yellowish/cream-white pigmentations by some isolates at early age. These colours either remained with time or changed into purple or black.

4.3 Biomass production and spore yield determination of *Trichoderma* isolates at different pH growth

The effect of pH on the mycelia growth and spore yield by *Trichoderma isolates* after 10 days in batch culture were shown in Figure 8 and 9. Analysis revealed that pH 7.5 supported the maximum mycelial growth of 0.32, 0.28 and 0.27 g/ml produced by isolates Gim-T06, Gim-T03 and Gim-T08 in batch culture respectively while the lowest mycelial growth of 0.08g/ml was recorded at pH 4.5 (Fig.8). The optimum pH for the mycelial growth of *Trichoderma* isolates was 7.5 in batch culture. The biomass production after 10 days i.e., at the end of the experiment ranged from 0.08-0.32 g in all treatments. With increasing time all isolates showed a significant increase in biomass at all pH levels. A specific value of pH was required to note the maximum growth where these biocontrol agents can be multiplied and pathogen can be controlled.

The abiotic factors deteriorated the antagonistic properties were temperature and pH that influence the mycelial growth of test fungi as well as biocontrol agents, *Trichoderma* isolates. As in all microorganisms even in *Trichoderma*, the external factors modify its morphological characteristics as well as physiological functions. Among these factors, pH was the most important environmental parameter affecting the mycoparasitic activities of *Trichoderma* strains. The studies on the variation of pH by different workers revealed that *Trichoderma* isolates showed optimum growth and sporulation rate at different pH values ranging from 2 to 7 (Arega Zeru, 2006). In Ethiopia, there is great diversity in soil characteristics especially with respect to soil pH. Different *Trichoderma* species are able to grow in a wide range of pH from 2.0 to 8.0 with maximal growth rates at 4.0, the optimum range being 4.5 to 7.5 (Cannell, 1985). However, there is a need to have isolates specifically for saline soils and acidic soils.

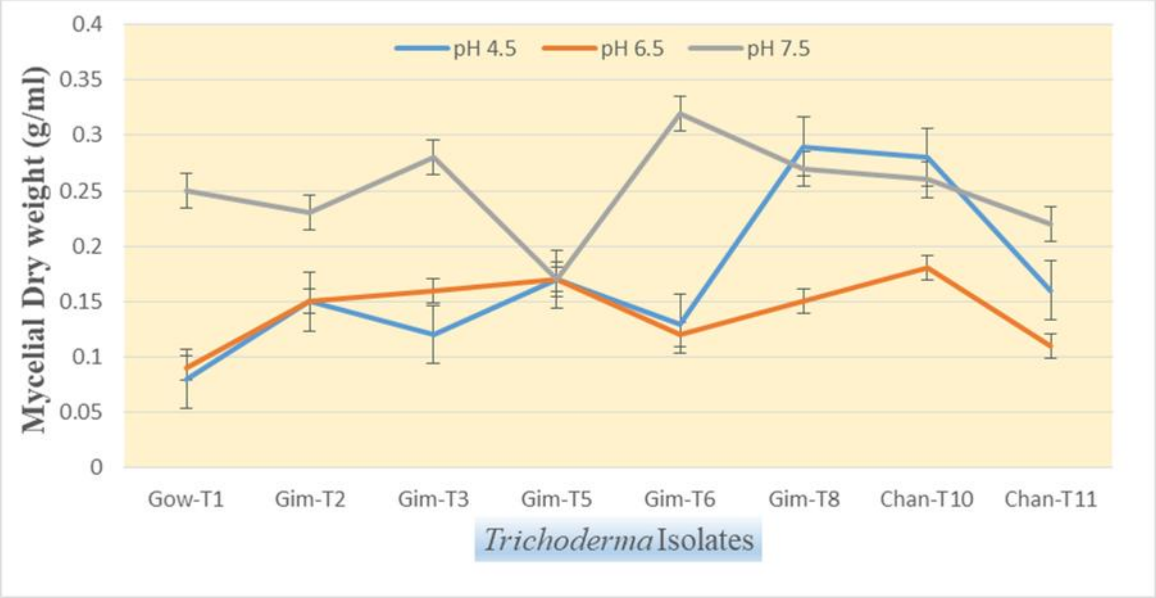


Figure 8. Influence of pH on the mycelial growth of *Trichoderma isolates* after 10 days in batch culture

On the other hand, the maximum spore yield (0.32 g/ml) of optical density (OD) was supported at pH 7.5 while the lowest spore yield with OD of 0.4 was produced at pH 4.5. The optimum pH range that favored high spore yield of *Trichoderma isolates* in fed-batch culture was pH 4.5-7.5 (Fig.9).

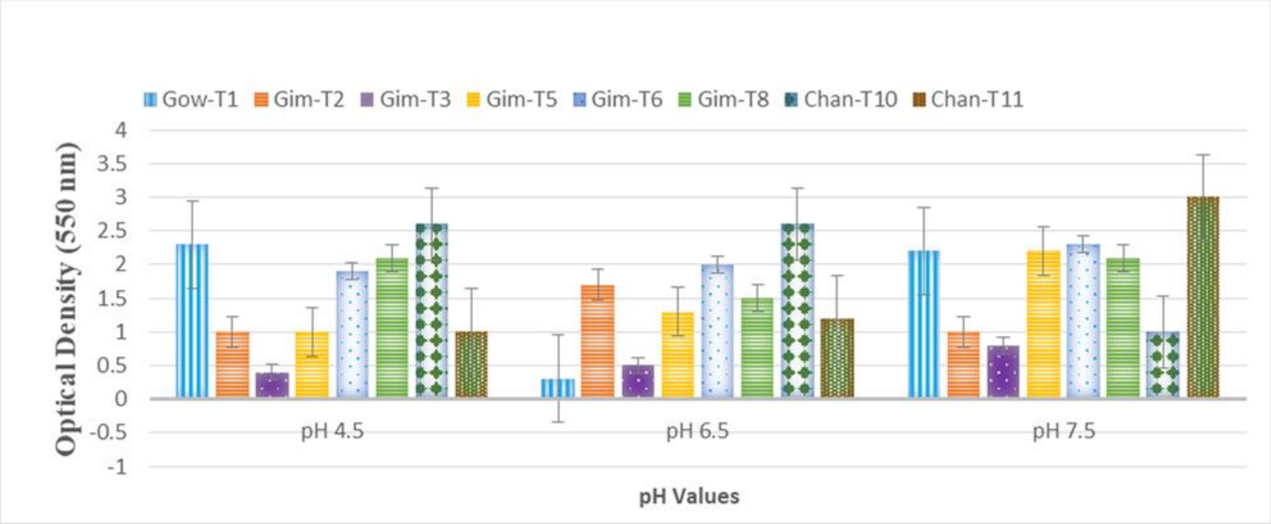


Figure 9. Influence of pH on the spore yield of *Trichoderma* isolates after 10 days in batch culture

The growth and conidiation of *Trichoderma* was influenced by some known environmental factors include the ambient pH of the medium, temperature, physical injury to the mycelium and the presence of fungal-derived volatile organic compounds (Steyaert *et al.*, 2010a). In our study, different pH and temperature conditions affected the mycelial growth and conidiation of *Trichoderma isolates.* The result of this study showed that the highest conidia/ml was obtained at pH 7.5 by isolate Gim-T05 followed by Gim-T06 (Fig. 10). In this study the tested pH range favored the mycelial growth, spore yield and conidiation of *Trichoderma* isolates with different values. It has been demonstrated that *Trichoderma* isolates were active under a wider range of pH (Kredics *et al.*, 2003) and from our analysis it can be reduced that *Trichoderma* isolates grow and sporulated maximally at a medium ambient pH.

A similar response was observed in another study, it is found that *T. harzianum* grow in wide range of pH 2.0 to 6.0, with maximum growth at 4.0 (Kredics, 2004). The initial pH of the medium has also been demonstrated to have an effect on mycelial growth and conidiation, and unlike the C: N ratio, pH levels which favor conidiation have been shown to favor mycelia growth as well (Bastos, 2001; Brian and Hemming, Steyaert *et al.*, 2010b). In contrast to this study, Benitez (2004) reported that growth of *Trichoderma* is more efficient in acidic than alkaline soils and they modify the rhizosphere soil by acidifying the soil. Low ambient pH of the growth medium has been demonstrated to result in intracellular acidification in *Aspergillus* *niger* and *Saccharomyces cerevisiae* (Brodeur, 2012). This explains the reason why the *T. harzianum* strain preferred acidic pH.

In general, commercial preparations of *Trichoderma* isolates for biological control consist of bulk-produced conidia (asexual spores), whereas good biocontrol activity in the environment relies upon the fungus remaining vegetative and thus antagonistically active. The high spore yield obtained was at the expense of greatly reduced mycelia, resulting in a high spore yield.

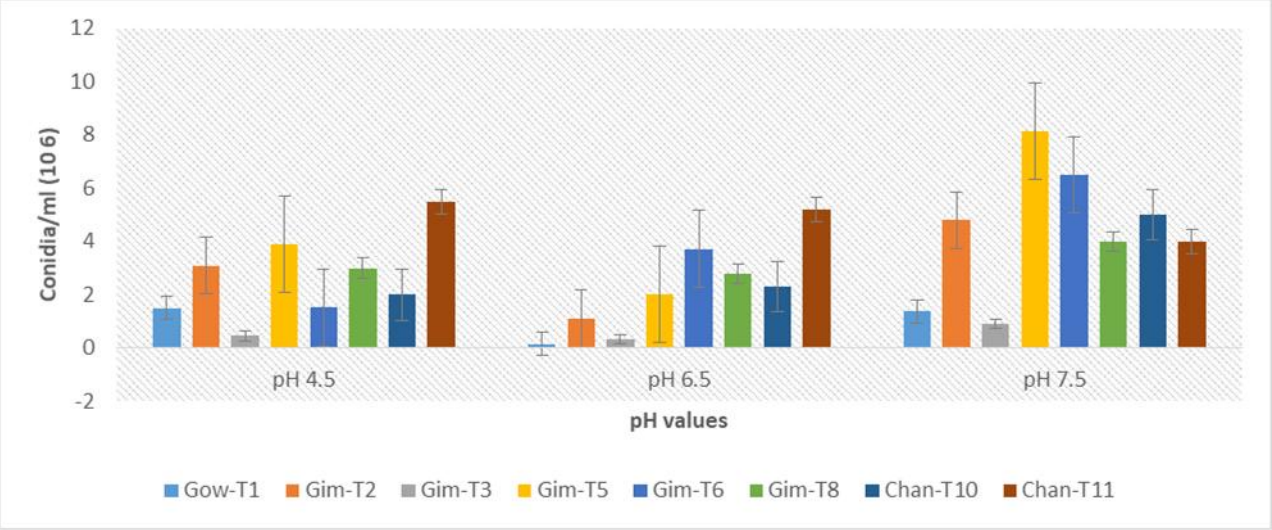


Figure 10. Influence of pH on the conidial production of *Trichoderma* isolates after 10 days in batch culture

Almost all *Trichoderma* isolates grown in the PDA plates at 25^{O}C showed confluent growth within 4 days because of their higher growth rates (Table 5). But there were slight variations among some of the isolates. Among them, isolates Gim-T5, Chan-10 and Chan-T11 were significantly expressed the highest growth. Meanwhile, the slowest growth rates were expressed by isolate Gow-T1. Analysis revealed that 25^{O}C was the optimum temperature for mycelial growth in batch culture. In contrast to the above mentioned temperature, isolate Gim-T03 grown on PDA plates showed confluent growth within 5 days at 37^{O}C whereas the other isolates grow up to 1 cm on the Petri dish (9 cm). On the other hand, all isolates couldn't grow at 4^{O}C. Therefore, the improvement of stress tolerance in *Trichoderma* isolates could result in increasing their efficacy against plant pathogenic fungi even under unfavorable environmental conditions. So, for exploiting the optimal antagonistic potential of *Trichoderma* which is to be applied as biocontrol agent (BCA), the effect of pH on their mycelial growth should be tested. Similarly, in major parts of the country, high soil temperature is an important factor for the survival of *Trichoderma* species. Temperature could affect their metabolic activity especially the production of volatile antibiotics and enzymes (Dennis and Webster, 1971). Though temperature plays an important role in the growth of organisms, at elevated level, it damages the organisms by denaturing enzymes, transport carriers, integrity of cell membrane (Prescott *et al.*, 2002). In general, this work has shown that batch culture system at pH range from 4.5 to 7.5 and temperature range of $25^{O}C$ was the best production strategy for maximum spore production of *Trichoderma* for BCA application than batch culture system.

Table 5: The average colony diameters of each *Trichoderma* isolates grown in PDA plates

S.No	**Isolate code**	**Average colony diameter (cm)**			
		Day 1	**Day 2**	**Day 3**	**Day 4**
1	Gow-T1	2.8 ±0.15	4.5±0.07	6.8±0.12	8.5±0.08
2	Gow-T2	3.3±0.01	6.2±0.09	8.6±0.03	9.00
3	Gim-T3	2.9±0.15	6.3±0.14	8.5±.0.12	9.00
4	Gim-T5	3.2±0.05	7.2±0.05	8.2±0.13	9.00
5	Gim-T6	3.6±0.15	8.1±0.05	9.00	9.00
6	Gim-T8	3.5±0.12	7.4±0.06	8.3±0.21	9.00
7	Chan-T10	4.4±0.06	8.0±0.03	9.00	9.00
8	Chan-11	3.8±0.21	8.4±0.05	9.00	9.00

4.4 *In vitro* evaluation of antagonistic activity of *Trichoderma* isolates on *F. xylarioides*

The experimental results showed that all isolates of *Trichoderma* were able to inhibit the growth of *F. xylarioides* under *in vitro* condition (between 42.2% and 69%) after 10 days of incubation. The highest mean inhibitory effect on the growth of the test pathogen was achieved by Gim-T06 (69%) followed by Gim-T10 (67.6%) and Gim-05 (66.7%) isolates. The mean inhibitory effect against *F. xylarioides* restricted almost completely in plates (Fig.11) as compared to the control, *F. xylarioides*, grown alone. Similar results were obtained using *Trichoderma* isolates with the greatest growth reduction of 77% and 72.9% (Afrasa Mulatu *et al.*, 2013). The action exerted by these isolates was relatively more potent than other *Trichoderma* isolates. The *Trichoderma* isolates formed coiled structures around the hyphae of the test pathogen (*F. xylarioides*) when observed under microscope. This coiling is a characteristic of the interaction between mycoparasitic and phytopathogenic fungi leading to penetration of the cell wall (Harman *et al.*, 2004). The inhibition in radial growth of two interacting organisms in dual culture is attributed to inhibitory substance released by one or both organisms through competition, mechanical obstruction and hyper parasitism (Dennis and Webster, 1971; Tapwal *et al.*, 2015), whereas Gow-01 isolate showed the lowest (42.2%).

Thereafter, the subculture of mycelia of *F. xylarioides* from test Petri dishes showed no growth, indicating the complete lethal effect of both *Trichoderma* isolates over *F. xylarioides.* Similarly, Mokhtar and Aid (2013) have showed that *Trichoderma* species inhibited the growth of the *F. solani* through its ability to grow much faster than the test pathogen thus competing efficiently for space and nutrients and forming coiled structures around the hyphae of the pathogen. Starvation has been regarded as the most common cause of death for microorganisms, so that competition for limiting nutrients and produces metabolites resulted in biological control of fungal phytopathogens (Mokhtar and Aid, 2013).

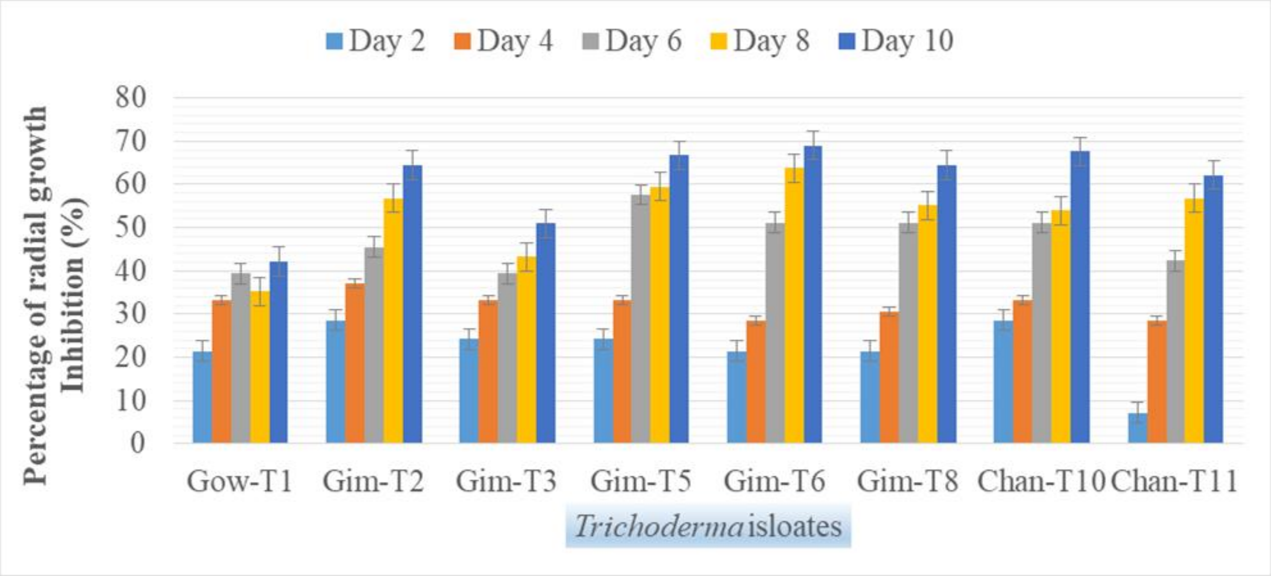


Figure 11. Percentage of radial growth inhibition of *Trichoderma* isolates against *F. xylarioides*

4.5 Effect of volatile and non-volatile compounds of *Trichoderma* isolates on the mycelial growth of *F. xylarioides*

The results indicated that *Trichoderma* isolates apparently produced volatile and nonvolatile substances that suppressed the test pathogen growth. Data presented in Table 6 indicates that Gim-T05 derived both volatile and non-volatile substances caused maximum inhibition of the mycelial growth of *F. xylarioides*.

Results from this work showed that both volatile and non-volatile components of the BCAs could significantly inhibit the mycelial growth of pathogenic *F. xylarioides. Trichoderma* isolate, Gim-T05 caused a maximum inhibition of the mycelial growth of this test pathogen by 71% (non-volatile) and 75.6 % (volatile), Similarly, Afrasa Mulatu *et al.* (2013) have obtained that the highest mean inhibitory effect on the growth of the pathogen was achieved by AUT2 (77.4%) isolate under *in vitro* bioassay. El-Katatny *et al.* (2001) have showed that formation of inhibition zone against *F. xylarioides* in dual cultures could be explained on the basis of production of extracellular hydrolytic enzymes by *Trichoderma*. Similarly, Fajola and Alasoadura (1975) found that culture filtrates of *T. harzianum* inhibit zoospore germination, germ tube elongation and mycelial growth of *P. aphanidermatum* causing the damping-off disease of tobacco. The variations in the inhibitory potential may be due to the differences in the quantity and quality of the inhibitory substances produced by the antagonists as reported by Druzhinina *et al.* (2011) who demonstrated that concentration of the secondary metabolites produced by *Trichoderma* species, determines inhibitory activity of these compounds.

Therefore, it is evident from this result that the antagonistic *Trichoderma* isolates inhibited the growth of *F. xylarioides* through production of volatile and nonvolatile compounds, indicating that antibiosis is one of the mechanisms involved in biocontrol of *F. xylarioides.* Moreover, cell free culture filtrate has been used to demonstrate the possible role of antibiosis in biological control (El_Komy *et al.*, 2015). It is also important to mention that *Trichoderma* species are known to produce a number of antibiotics such as trichodermin, trichodermol, herzianolide, ethylene and formic aldehyde (Fravel *et al.*, 1999; Hermosa *et al.*, 2000; Fravel, 2005). A similar result was recorded by Anita *et al.* (2012) that the antagonistic effect of secondary metabolites secreted by *T. atroviridae* at different concentrations and found rapid concentration dependent decrease in the linear growth of the pathogen.

Table 6. Inhibitory effect of volatile and non-volatile compounds of *Trichoderma* isolates on mycelial growth of *F. xylarioides* in comparison to control after 10 days

S/No.	Isolates	Effect of Non- volatile compound(s) Inhibition (%)	Effect of Volatile compounds (%)
1	Gow-T01	53	42.8
2	Gim-T02	61	46.7
3	Gim-T03	65.5	55.6
4	Gim-T05	71	75.6
5	Gim-T06	64.5	51.1
6	Gim-T08	60	44.5
7	Chan-T10	68.5	67.3
8	Chan-T11	63	60
	Control		-

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Isolation, characterization and morphological description of *Trichoderma* species are important before further dissemination is done leading to the biomass production at different environmental and cultural conditions. *Trichoderma* isolates grow at varying pH and temperature for a better growth of different isolates. As *Trichoderma* is an ecofriendly biological control agent against other soil borne plant pathogens, it is necessary to grow it at suitable conditions before it is used for commercial purposes. Under *in vitro* bioassay, the highest mean inhibitory effect against the growth of the pathogen was achieved by Gim-T06 isolate in dual culture whereas Gim-T05 was the best producer of both volatile and non-volatile compounds.

5.2 Recommendation

Although a substantial amount of work has been carried out, further studies should be carried out to:

1) Confirm the effectiveness of *Trichoderma* isolates, Gim-T05 and Gim-T06.
2) Detection, and elucidation of the structure and analytical characterization of the most active antifungal compounds
3) Finally, evaluate the biocontrol activity in planta under different conditions to control the causative agent of CWD, *F. xylarioides*.

REFERENCES

Ab Rahman, S.F.S., Singh, E., Pieterse, C.M. and Schenk, P.M. (2017). Emerging Microbial Biocontrol Strategies for Plant Pathogens. *Plant Sci.* **12**:57-69.

Afrasa Mulatu, Negussie Megersa and Tesfaye Alemu (2013). Characterization of fungal extracts from *Trichoderma* isolates: their effects against coffee wilt pathogen (*Gibberella xylarioides*). *SINET: Ethiop. J. Sci.* **36**: 81-92.

Ahluwalia, V., Kumar, J., Rana, V.S., Sati, O.P. and Walia, S. (2015). Comparative evaluation of two *Trichoderma harzianum* strains for major secondary metabolite production and antifungal activity. *Nat. Prod. Res.* **29**: 914-920.

Al-Saeedi, S.S. and Al-Ani, B.M. (2014). Study of antagonistic capability of *Trichodermaharzianum* isolates against some pathogenic soil borne fungi. *Agri. Biol. J. North Am.* **5**: 15-23.

Anees, M., Tronsmo, A., Edel-Hermann, V., Hjeljord, L.G., Héraud, C. and Steinberg, C. (2010). Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani. Fungal Biol.* **114**: 691-701.

Aneja, M., Gianfagna, T.J. and Hebbar, P.K. (2005). *Trichoderma harzianum* produces nonanoic acid, an inhibitor of spore germination and mycelial growth of two cacao pathogens. *Physiol. Molecul.Plant Pathol.***67**: 304-307.

Anita, S., Ponmurugan, P. and Babu, R.G. (2012). Significance of secondary metabolites and enzymes secreted by *Trichoderma atroviride* isolates for the biological control of Phomopsis canker disease. *Afric.J. Biotech.***11**: 10350-10357.

Arega Zeru (2006). Diversity of Arabica coffee populations in afromontane rainforests of Ethiopia in relation to *Colletotrichum kahawae* and *Gibberella xylarioides*, M.Sc Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Atanasova, L., Le Crom, S., Gruber, S., Coulpier, F., Seidl-Seiboth, V., Kubicek, C.P. and Druzhinina, I.S. (2013). Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism. *BMC Genom.***14**: 121.

Baek, J.-M., Howell, C.R. and Kenerley, C.M. (1999). The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. *Curr. Gen.* **35**: 41-50.

Baker, P. (2013). The changing climate for sustainable coffee. *Proceedings of the 24th International Conference on Coffee Science*, pp. 603-610.

Barbosa, M.A.G., Rehn, K.G., Menezes, M. and Mariano, R.d.L.R. (2001). Antagonism of *Trichoderma* species on *Cladosporium herbarum* and their enzimatic characterization. *Braz. J. Microbiol.***32**: 98-104.

Beaert, J., De Brabandere, R., Gyselen, A., Steyaert, J. and Verbeke, R. (1957). Functional exploration of the adrenal cortex in pulmonary tuberculosis. *Acta tuberculosea Belgica* **48**: 171.

Biswas, K.K. and Sen, C. (2000). Management of stem rot of groundnut caused by *Sclerotium rolfsii* through *Trichoderma harzianum*. *Ind. Phytopathol.* **53**: 290-295.

Brimner, T.A. and Boland, G.J. (2003). A review of the non-target effects of fungi used to biologically control plant diseases. *Agri. Ecosys. Environ.* **100**: 3-16.

Butt, T.M. and Copping, L.G. (2000). Fungal biological control agents. *Pesticide Outlook* **11**: 186-191.

Cannell, M. (1985). Physiology of the coffee crop. **In**: *Coffee*, pp. 108-134, Springer, CABI.

Chandler, D., Davidson, G., Grant, W.P., Greaves, J. and Tatchell, G.M. (2008). Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability. *Trends in Food Sci. Technol.***19**: 275-283.

Chaverri, P., Branco-Rocha, F., Jaklitsch, W.,Gazis, R., Degenkolb, T. and Samuels, G.J. (2015). Systematics of the Trichoderma harzianum species complex and the re-identification of commercial biocontrol strains. *Mycologia* **107**: 558-590.

Chet, I.,Inbar, J. and Hadar, I. (1997). Fungal antagonists and mycoparasites. *The Mycota IV: Environmental and microbial relationships. Springer-Verlag, Berlin*: 165-184.

De La Cruz, J., Pintor-Toro, J.A., Benitez, T., Llobell, A. and Romero, L.C. (1995). A novel endo-beta-1, 3-glucanase, BGN13. 1, involved in the mycoparasitism of Trichoderma harzianum. *J. Bacteriol.***177**: 6937-6945.

De la Cruz Quiroz, R., Roussos, S., Hernández, D., Rodríguez, R., Castillo, F. and Aguilar, C.N. (2015). Challenges and opportunities of the bio-pesticides production by solid-state fermentation: filamentous fungi as a model. *Crit. Rev. Biotechnol.***35**: 326-333.

Dean, R.,Van Kan, J.A., Pretorius, Z.A., Hammond- Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J.,Dickman, M.,Kahmann, R. and Ellis, J. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molec. Plant Pathol.***13**: 414-430.

Dennis, C. and Webster, J. (1971). Antagonistic properties of species-groups of Trichoderma: II. Production of volatile antibiotics. *Transact.Brit. Mycol. Soc.***57**: 41-44.

Derso, E. (1997). Coffee diseases and their significance in Ethiopia. *Colloque Scientifique International Sur Le Cafe*, pp. 723-726.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V. and Kubicek, C.P. (2011). *Trichoderma*: the genomics of opportunistic success. *Nature reviews. Microbiology* **9**: 749.

Dubey, S.C., Suresh, M. and Singh, B. (2007). Evaluation of *Trichoderma* species against *Fusariumoxysporum f. sp. ciceris* for integrated management of chickpea wilt. *Biol. Cont.* **40**: 118-127.

El-Katatny, M., Gudelj, M., Robra, K.-H., Elnaghy, M. and Gübitz, G. (2001). Characterization of a chitinase and an endo-β-1, 3-glucanase from *Trichoderma harzianum Rifai* T24 involved in control of the *Phytopathogen Sclerotium rolfsii*. *Appl.Microbiol. Biotechnol.* **56**: 137-143.

El-Mougith, A. (1999). Effect of benomyl on the growth and lipid composition of *Trichoderma koningii*. *Folia Microbiol.* **44**: 41-44.

El_Komy, M.H., Saleh, A.A., Eranthodi, A. and Molan, Y.Y. (2015). Characterization of novel *Trichoderma asperellum* isolates to select effective biocontrol agents against tomato *Fusarium* wilt. *Plant Pathol. J.* **31**: 50-61.

Eshetu Derso (2000). Preselection methods for coffee berry disease (CBD) resistance in Ethiopia.

Fajola, A. and Alasoadura, S. (1975). Antagonistic effects of *Trichoderma harzianum* on *Pythium aphanidermatum* causing the damping-off disease of tobacco in Nigeria. *Mycopathologia***57**: 47-52.

Fernández, C.A.M. (2006). Trading Coffee Through the Ethiopia Commodity Exchange. Addis Ababa, Ethiopia

Flood, J. (2010). *Coffee Wilt Disease*. CABI.

Fravel, D. (2005). Commercialization and Implementation of biocontrol. *Annu. Rev. Phytopathol.***43**: 337-359.

Fravel, D.R., Rhodes, D.J. and Larkin, R.P. (1999). Production and commercialization of biocontrol products. **In**: *Integrated pest and disease management in greenhouse crops*, pp. 365-376, Springer, New York.

Getachew Gashaw, Tesfaye Alemu and Kassahun Tesfaye (2014). Evaluation of disease incidence and severity and yield loss of finger millet varieties and mycelial growth inhibition of *Pyricularia grisea* isolates using biological antagonists and fungicides *in vitro* condition. *J. Appl. Biosci.***73**: 5883-5901.

Geiser, D.M., Lewis Ivey, M.L., Hakiza, G., Juba, J.H. and Miller, S.A. (2005). *Gibberella xylarioides* (anamorph: *Fusarium xylarioides*), a causative agent of coffee wilt disease in Africa, is a previously unrecognized member of the *G. fujikuroi* species complex. *Mycologia* **97**: 191-201.

Sihen Getachew, Girma Adugna, Fikru Lemessa and Hindorf, H. (2012). Coffee wilt disease (*Gibberella xylarioides* Heim and Saccas) in forest coffee systems of southwest and southeast Ethiopia. *Plant Pathol. J.* **11**: 10-17.

Girma Adugna. (1997). Status and economic importance of *Fusarium* wilt disease of Arabica coffee in Ethiopia. *Proceedings of the first regional workshop on coffee wilt disease (tracheomycosis)*, pp. 28-30.

Girma Adugna, Chala Jefuka, Demelash Teferei and Arega Zeru. (2008). Multiple resistances to Coffee Berry Disease, Coffee Wilt and Leaf Rust in Coffea arabica populations of Ethiopia. *XXII ASIC, Campinas, SP, Brazil*.

Girma Adugna and Hindorf, H. (2001). Tracheomycosis (*Gibberella xylarioides*) on coffee (*Coffea arabica*). *Conference on International Agricultural Research for Development. Deutscher Tropentag*, pp. 9-11.

Girma Adugna, Million Adana, Hindorf, H., Arega Zeru, Demelash Teferi and Chala Jefuka. (2010). Coffee Wilt Disease in Ethiopia. **In**: *Coffee Wilt Disease*, pp.63-78.

Girma Adugna, H., H. (2001). Recent investigation on coffee tracheomycosis, *Gibberella xylarioides* (*Fusarium xylarioides*) in Ethiopia. *19th International scientific colloquium on coffee (ASIC)*, pp. 165-171.

Girma Adugna, S. (2004). *Diversity in Pathogenicity and Genetics of Gibberella xylarioides (Fusarium xylarioides) Populations and Resistance of Coffea spp. in Ethiopia*. Cuvillier Verlag.

Ha, T.N. (2010). Using *Trichoderma* species for biological control of plant pathogens in Vietnam. *Journal ISSAAS* **16**: 17-21.

Haas, D. and Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas spp*. and relevance for biological control of plant disease. *Ann.Rev.Phytopathol.***41**:117-153.

Hagn, A., Pritsch, K., Schloter, M. and Munch, J.C. (2003). Fungal diversity in agricultural soil under different farming management systems, with special reference to biocontrol strains of *Trichoderma* spp. *Biol. Fert. Soils* **38**: 236-244.

Harman, G., Jin, X., Stasz, T., Peruzzotti, G., Leopold, A. and Taylor, A. (1991). Production of conidial biomass of *Trichoderma harzianum* for biological control. *Biol. Cont.***1**: 23-28.

Harman, G.E. (2006). Overview of mechanisms and uses of *Trichoderma spp*. *Phytopathology* **96**: 190-194.

Harman, G.E., Howell, C.R.,Viterbo, A., Chet, I. and Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.***2**: 43-47.

Hein, L. and Gatzweiler, F. (2006). The economic value of coffee (*Coffea arabica*) genetic resources. *Ecol. Econ.* **60**: 176-185.

Hermosa, M., Grondona, I., Iturriaga, E.t., Diaz-Minguez, J., Castro, C., Monte, E. and Garcia-Acha, I. (2000). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Appl. Environ. Microbiol.***66**: 1890-1898.

Hhmau, H., Wijesundera, R., Chandrasekharan, N., Wijesundera, W. and Kathriarachchi, H. (2015). Isolation and characterization of Trichoderma erinaceum for antagonistic activity against plant pathogenic fungi. *Current Research in Environmental & Applied Mycology***5**: 120-127.

Hindorf, H. and Omondi, C.O. (2011). A review of three major fungal diseases of *Coffea arabica* L. in the rainforests of Ethiopia and progress in breeding for resistance in Kenya. *J. Advan. Res.***2**: 109-120.

Hocking, D. (1965). Fungicides for Arabica Coffee in East Africa. *Pest Articles and News Summaries. Plant Dis. Cont.l***11**: 397-418.

Hoitink, H., Madden, L. and Dorrance, A. (2006). Systemic resistance induced by *Trichodermaspp*.: interactions between the host, the pathogen, the biocontrol agent, and soil organic matter quality. *Phytopathology*, **96**: 186-189.

Howell, C. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.***87**: 4-10.

Junaid, J.M., Dar, N.A., Bhat, T.A., Bhat, A.H. and Bhat, M.A. (2013). Commercial biocontrol agents and their mechanism of action in the management of plant pathogens. *Int. J. Mod. Plant Anim. Sci.***1**: 39-57.

Khalid M. (2009). Novel plant bio-protectants based on Trichoderma spp. strains with superior characteristics. PhD Thesis. Universita' Degli Studi Di Napoli "Federico II".

Khulbe, A. (2009). Variability in Alternaria brassicae (Berk.) Sacc., the causal agent of black spot of rapeseed mustard and evaluation of different components of host resistance, GB Pant University of Agriculture and Technology, Pantnagar-263145 (Uttarakhand),

Kimani, M., Little, T. and Vos, J.G. (2002). Introduction to coffee management through discovery learning. *CAB Bioscience IPM.*

Kranz, J. and Mogk, M. (1973). *Gibberella xylarioides* Heim et Saccas on arabica coffee in Ethiopia. *J. Phytopathol.***78**: 365-365.

Lewis Ivey, M., Miller, S., Hakiza, G., Erbough, M. and Geiser, D. (2002). Diversity of the coffee wilt pathogen, *Fusarium xylarioides* in Uganda. *Proceedings on IPM conference for sub-Saharan Africa*, pp. 3.

Lewis Ivey, M., Miller, S., Hakiza, G. and Geiser, D. (2003). Characterization of the coffee wilt pathogen in Uganda. *Phytopathology,* **93**: S50.

Masoko, P. and Eloff, J. (2005). The diversity of antifungal compounds of six South African *Terminalia* species (*Combretaceae*) determined by bioautography. *Afri.J.Biotech.***4**: 25-37.

Meera, T. and Balabaskar, P. (2012). Isolation and characterization of *Pseudomonas fluorescens* from rice fields. *Int. J. Food Agric. Vet. Sci.* **2**: 113-120.

Mogk, M.H. (1975). Losses caused by coffee berry disease (*Colletotrichum coffeanum*) in different stages of berry development. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutzv. 82 (4) p. 193-200.*

Mokhtar, H. and Aid, D. (2013). Contribution in isolation and identification of some pathogenic fungi from wheat seeds, and evaluation of antagonistic capability of *Trichoderma harzianum* against those isolated fungi *in vitro*. *Agric. Biol. JN Am.***4**: 145-154.

Musoli, C.P., Pinard, F., Charrier, A., Kangire, A., Ten Hoopen, G.M., Kabole, C., Ogwang, J., Bieysse, D. and Cilas, C. (2008). Spatial and temporal analysis of coffee wilt disease caused by *Fusarium xylarioides* in Coffea canephora. *Eur. J.Plant Pathol.***122**: 451-460.

Naglot, A., Goswami, S., Rahman, I., Shrimali, D., Yadav, K.K., Gupta, V.K., Rabha, A.J., Gogoi, H. and Veer, V. (2015). Antagonistic potential of native *Trichoderma viride* strain against potent tea (*Camellia sinensis* (L.) O. Kuntze) fungal pathogens in North East India. *Plant Pathol. J*. **31**: 1-13.

Negash Hailu, (2007). Isolation, Identification of Fusarium xylarioides from Southern Ethiopia And its response to Fungal Biocontrol Agents. Msc Thesis, Addis Ababa University.

Pakdaman, B., Goltapeh, E., Soltani, B., Talebi, A., Nadepoor, M., Joanna, S., Sebastian, P., Sabrina, S. and Giovanni, V. (2013). Toward the quantification of confrontation (Dual Culture) test: a case study on the biological control of *Pythium aphanidermatum* with *Trichoderma asperelloides.J. Biofertil. Biopestici.***4**: 2-10.

Papavizas, G. (1985). *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Ann.Rev. Phytopathol.***23**: 23-54.

Paulos Dubale and Demil Tektay. (2000). The need for forest coffee germplasm conservation in Ethiopia and its signifcance in the control of coffee diseases.

Petit, N. (2007). Ethiopia's coffee sector: A bitter or better future? *J. Agrarian Change***7**: 225-263.

Phiri and Baker. (2009). Coffee wilt disease in Africa; a synthesis of the work for the Regional Coffee Wilt Programme (RCWP), Final Technical Report. CABI, UK.

Phiri, N. and Baker, P. (2009). A synthesis of the work of the Regional Coffee Wilt Programme 2000–2007, CABI, UK.

Phiri, N., Kimani, M., Negussie, E., Simons, S. and Oduor, G. (2010). Management of Coffee Wilt Disease. *Coffee Wilt Disease*, CABI, UK 137.

Pieters, R. and Van der Graaff, N. (1980). Resistance to *Gibberella xylarioides* in *Coffea arabica*: evaluation of screening methods and evidence for the horizontal nature of the resistance. *Neth.J. Plant Pathol.***86**: 37-43.

Rakh, R., Raut, L., Dalvi, S. and Manwar, A. (2011). Biological control of Sclerotium rolfsii, causing stem rot of groundnut by *Pseudomonas cf. monteilii*.*Rec. Res. Sci.Technol.***3**: 44-51.

Ramanujam, B., Prasad, R., Sriram, S. and Rangeswaran, R. (2010). Mass production, formulation, quality control and delivery of *Trichoderma* for plant disease management. *J. Plant Protect. Sci.***2**: 1-8.

Rutherford, M.A. (2006). Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. *Phytopathology* **96**: 663-666.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. and Latha, L.Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *Afri.J. Trad. Compl.Altern. Med.***8**:71-82.

Scala, F., Raio, A., Zoina, A., Lorito, M., Chincholkar, S. and Mukerji, K. (2007). Biological control of fruit and vegetable diseases with fungal and bacterial antagonists: *Trichoderma* and Agrobacterium. **In**: *Biological control of plant diseases*, pp. 151-190, The Haworth Press New York, USA.

Scherm, B., Balmas, V., Spanu, F., Pani, G., Delogu, G., Pasquali, M. and Migheli, Q. (2013). *Fusarium culmorum*: causal agent of foot and root rot and head blight on wheat. *Molec. Plant Pathol.***14**: 323-341.

Schuster, A. and Schmoll, M. (2010). Biology and biotechnology of Trichoderma. *Appl. Microbiol. Biotechnol.***87**: 787-799.

Shahverdi, A.R., Abdolpour, F., Monsef-Esfahani, H.R. and Farsam, H. (2007). A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. *J. Chromat. B.* **850**: 528-530.

Sharma, S.,Srivastava, S. and Saxena, M. (2016). Integrated Management of Fungal Diseases of Soybean [*Glycine max* (L.) Merrill] Occurring in India. Fungi: Applications and Management Strategies. Pp. 432.

Sharon, E., Bar-Eyal, M., Chet, I., Herrera-Estrella, A., Kleifeld, O. and Spiegel, Y. (2001). Biological control of the root-knot nematode Meloidogyne javanica by *Trichoderma* harzianum. *Phytopathology* **91**: 687-693.

Shentu, X., Zhan, X., Ma, Z.,Yu, X. and Zhang, C. (2014). Antifungal activity of metabolites of the endophytic fungus *Trichoderma* brevicompactum from garlic. *Braz.J. Microbiol.* **45**: 248-254.

Siameto, E., Okoth, S., Amugune, N. and Chege, N. (2011). Molecular characterization and identification of biocontrol isolates of *Trichoderma harzianum* from Embu district, Kenya. *Trop. Subtrop. Agroecosys.***13**:23-38.

Silva, M.d.C.,Várzea, V., Guerra-Guimarães, L., Azinheira, H.G., Fernandez, D., Petitot, A.-S., Bertrand, B., Lashermes, P. and Nicole, M. (2006). Coffee resistance to the main diseases: leaf rust and coffee berry disease. *Braz. J. Plant Physiol.* **18**: 119-147.

Srivastava, R., Khalid, A., Singh, U. and Sharma, A. (2010). Evaluation of arbuscular mycorrhizal fungus, *fluorescent Pseudomonas* and *Trichoderma harzianum* formulation against *Fusarium oxysporum f. sp*. l*ycopersici* for the management of tomato wilt. *Biol. Cont.***53**: 24-31.

Stewart, A., Brownbridge, M., Hill, R.A. and Jackson, T.A. (2010). Utilizing soil microbes for biocontrol. **In**: *Soil Microbiology and Sustainable Crop Production*, pp. 315-371, Springer.

Stewart, R.B. (1958). Some plant diseases occurring in Kaffa province, Ethiopia.

Steyaert, J., Hicks, E., Kandula, J., Kandula, D., Alizadeh, H., Braithwaite, M., Yardley, J., Mendoza-Mendoza, A. and Stewart, A. (2016). Methods for the Evaluation of the Bioactivity and Biocontrol Potential of Species of *Trichoderma*. **In**: *Microbial-Based Biopesticides*, pp. 23-35, Springer.

Steyaert, R. (1948). Contribution à l'étude des parasites des végétaux du Congo belge. *Bulletin de la Société Royale de Botanique de Belgique/Bulletin van de Koninklijke Belgische Botanische Vereniging* **80**: 11-58.

Suleiman, M.M., McGaw, L., Naidoo, V. and Eloff, J. (2010). Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. *Afri. J. Trad. Complem. Altern.Med.***7**:31-49.

Summerell, B.A., Leslie, J.F., Liew, E.C., Laurence, M.H., Bullock, S., Petrovic, T., Bentley, A.R., Howard, C.G., Peterson, S.A. and Walsh, J.L. (2011). *Fusarium* species associated with plants in Australia. *Fungal Div.***46**: 1-27.

Sundaramoorthy, S. and Balabaskar, P. (2013). Biocontrol efficacy of *Trichoderma spp*. against wilt of tomato caused by Fusarium oxysporum f. sp. *lycopersici*. *J. Appl. Biol. Biotechnol.***1**: 36-40.

Tapwal, A., Thakur, G., Chandra, S. and Tyagi, A. (2015). *In-vitro* evaluation of *Trichoderma* species against seed borne pathogens. *Int. J. Biol. Chem. Sci.***1**: 14-19.

Tarman, K. (2011). *Biological and chemical investigations of Indonesian marine-derived fungi and their secondary metabolites*. Cuvillier Verlag.

Tarman, K., Lindequist, U., Wende, K., Porzel, A., Arnold, N. and Wessjohann, L.A. (2011). Isolation of a new natural product and cytotoxic and antimicrobial activities of extracts from fungi of Indonesian marine habitats. *Marine Drugs,* **9**: 294-306.

Tefestewold Biratu. (1995). Studies on *Colletotrichum* population of *Coffea arabica* L. Ethiopia and evaluations of the reactions of coffee germplasm. PhD Dissertation, University of Bonn: 231.

Temesgen Belayneh, Kubicek, C.P. and Druzhinina, I.S. (2010). The rhizosphere of *Coffea arabica* in its native highland forests of Ethiopia provides a niche for a distinguished diversity of *Trichoderma*. *Diversity***2**: 527-549.

Tesfaye Alemu (2012). A review of coffee wilt disease, *Gibberella xylarioides (Fusarium xylarioides)* in Africa with special reference to Ethiopia. *Ethiop.J. Biol. Sci.***11**(1): 65-103.

Tesfaye Alemu and Kapoor, I. (2004*). In vitro* evaluation of *Trichoderma* and *Gliocladium spp*. against *Botrytis* corm rot of gladiolus. *Pest Manag. J. Ethiop.* **8:** 97-103.

Tijerino, A., Cardoza, R.E., Moraga, J., Malmierca, M.G., Vicente, F., Aleu, J., Collado, I.G., Gutiérrez, S., Monte, E. and Hermosa, R. (2011). Over expression of the trichodiene synthase gene tri5 increases trichodermin production and antimicrobial activity in *Trichoderma brevicompactum. Fungal Gen. Biol.* **48**: 285-296.

Van der Graaff, N.A. (1981). Selection of arabica coffee types resistant to coffee berry disease in Ethiopia. Mededelingen Landbouwhogeschool. Wageningen. Nederland. Pp.110.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R.,Woo, S.L. and Lorito, M. (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biol. Biochem.* **40**: 1-10.

Viterbo, A., Ramot, O., Chernin, L. and Chet, I. (2002). Significance of lytic enzymes from *Trichoderma spp*. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek* **81**: 549-556.

Vos, C.M., De Cremer, K., Cammue, B. and De Coninck, B. (2015). The toolbox of *Trichodermaspp*. in the biocontrol of *Botrytis cinerea* disease. *Molec.Plant Pathol.* **16**: 400-412.

Waller, J.M., Bigger, M. and Hillocks, R.J. (2007). *Coffee pests, diseases and their management*. CABI.

Workafes Woldetsadik and Kasu Kebede. (2000). Coffee production systems in Ethiopia.

Workafes, W. and Kassu, K. (2000) Coffee production systems in Ethiopia. In: Proceedings of the Workshop on Control of Coffee Berry Disease (CBD) in Ethiopia. 13–15 August 1999. Addis Ababa, Ethiopia, pp. 99–107.

Wrigley, G. (1988). Coffee: Tropical Agriculture Series London. John Wilyand Son. *Inc., New York, USA*. Pp. 639.

Yedidia, I., Srivastva, A.K., Kapulnik, Y. and Chet, I. (2001). Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and soil* **235**: 235-242.