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Evaluation of antifungal activity of *Trichoderma* Isolates and their crude extracts on Coffee Wilt pathogen (*Gibberella xylarioid*es)

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Micro gram
µl	Micro liter
AAU	Addis Ababa University
AUT	Addis Ababa University *Trichoderma isolate*
BCAs	Biological Control Agents
C3H6O	Acetone
CBD	Coffee Berry Disease
$CHCl_3$	Chloroform
CLR	Coffee Leaf Rust
CWD	Coffee Wilt Disease
CWDEs	Cell Wall Degrading Enzymes
C_6H_{14}	n-Hexan
CZI	Clear Zone of Inhibition
EtOAc	Ethyl acetate
EtOH	Ethanol
F254	Fluorescent light at 254 wavelengths
HCN	Hydrogen Cyanide
IAA	Indole Acetic Acid
INT	p-iodonitrotetrazolium violet
IPM	Integrated Pest Management
JARC	Jimma Agricultural Research Center
MEA	Malt Extract Agar
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
Min	Minute
NAGase	4-N-acetyl-glucosaminidase
nm	Nano meter
PAP	6-Penthyl-alpha-pyrone

PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGPR	Plant Growth Promoting Rhizobacteria
PIRG	Percentage Inhibition of Radial Growth
Ppm	parts per million
Rf	Retention factor
rpm	Revolution per Minute
SS	Solvent System
TLC	Thin Layer Chromatography
TTB	Tetrazolium Bromide
TTC	2, 3, 5-tripheny tetrazolium chloride
UV	Ultra Violet Light

ABSTRACT

Coffee wilt disease (CWD) caused by the fungus *Fusarium xylarioides* is an important soil-borne disease reducing coffee (*Coffea arabica* L.) production yields at up to 60%. The present study aimed to evaluate, test and characterize potential biocontrol of rhizosphere *Trichoderma* isolates and their most active antifungal crude extracts against coffee wilt pathogen (*Gibberella xylarioides*). *In vitro* antagonistic bioassays were performed to evaluate and determine the potentiality of *Trichoderma* isolates as biocontrol agents against *F. xylarioides.* For extraction of antifungal crude extracts from fungal mycelia organic solvents such as chloroform, ethanol, methanol, ethyl acetate, n-hexane and acetone were used. A direct bioautographic procedure, involving spraying suspension of *F. xylarioides* on TLC plates developed in solvents of varying polarities to detect a number of antifungal substances present in the extracts. 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 54.8% to 83.2% after 10 days of incubation. The highest mean inhibitory effect on the growth of the test pathogen was achieved by Addis Ababa University *Trichoderma* isolate 71 (AUT-71) (83.2%) followed by AUT-03 (72.8%) and AUT-86 (67.2%), while AUT-89 isolate showed the lowest (54.8%) mean inhibitory effect restricting it almost completely in plates as compared to the control consisting of *F. xylarioides* growing alone. AUT-32 and AUT-66 isolates produced zones of inhibition which is an indicative of the production of secondary metabolite(s) inhibiting the mycelial growth of *F. xylarioides*. Bioautography assay revealed that 94 zones of inhibition spots and the highest inhibition zone was observed by SS1 in isolates AUT-21 (53 mm) at R_f (0.75), and AUT-66 and AUT-89 (35 mm) with chloroform extract at R_f (0.51) and at R_f (0.55), respectively. 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*, Coffee wilt disease, TLC, *Trichoderma* isolates

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 its 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 Regional States 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 *coffee 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 *G. 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. Coffee quality may also be affected through, for example, premature ripening of the berries (Rutherford, 2006)

A number of other *Fusarium* species are pathogenic to coffee: *F. oxysporum, F. solani, F. stilboides, F. lateritium* and *F. decemcellulare* (Summerell *et al*., 2011). 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; Getachew *et al.*, 2012). To control the pathogen with fungicides and cultural practices are 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). Nowdays, 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.

According to Temesgen Belayneh *et al.* (2010), *Trichoderma* spp 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 its 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). A number of *Trichoderma* species has a promising potential for biological control of plant pathogenic fungi (Dennis and Webster, 1971;Dubey *et al.*, 2007; Chandler *et al.*, 2008; Druzhinina *et al.*, 2011). *Trichoderma* has been used against the wilt diseases of tomato (Srivastava *et al.*, 2010), melon and cotton (Sundaramoorthy and Balabaskar, 2013) and *Fusarium culmorum* on wheat (Scherm *et al.*, 2013). *Trichoderma* species, especially, *T. harzianum*, grown on solid substrates have been tested with varying diseases *Trichoderma harzianum* gave 60 to 83% control *Fusarium* diseases in naturally infected field soil (Khulbe, 2009)

Many species of *Trichoderma* namely *Trichoderma harzianum, Trichoderma viride* and *Trichoderma virens* from rhizosphere were found to have good antagonistic potential against many soil born fungi, such as *Fusarium oxysporum (Dubey et al., 2007), Sclerotium rolfsii* (Biswas and Sen, 2000) and *Rizoctonia solani* (Anees *et al*., 2010). As an antagonist *Trichoderma* species produce antibiotics and antifungal toxic metabolites and inhibit pathogens by secreting enzymes like glucanase, cellulases, chitinase and protease which disintegrate the cell wall of pathogen and act as a competitor for mineral nutrients (Baek *et al.*, 1999; De La Cruz *et al.*, 1995; Dennis and Webster, 1971).

1.2 Statement of the Problem

Coffee is vital to the economy of Ethiopia providing a major source of foreign exchange earnings and as a cash crop, supporting the livelihood of millions of people who are involved in cultivation, processing, marketing, and export. Ethiopia is believed to be the country of origin of *C. arabica* that makes over 90% of the world's production (Paulos Dubale and Demil Tektay, 2000). However, the production of coffee is hampered by various biotic and abiotic factors. The abiotic factors include temperature, relative humidity, soil pH and mineral deficiency (Tesfaye Alemu 2012). 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).

Coffee wilt disease caused by *G. xylarioides*, a soil-inhabiting fungus, is endemic in several African countries, affecting commercially important coffee species and causing serious economic losses to coffee producers. One of the limiting factors for coffee production in Ethiopia is *tracheomycosis*/vascular wilt disease caused by *Fusarium xylarioides* Steyaert imperfect stage (*Gibberella xylarioides* Heim and Saccas perfect stage), which caused yield losses in the major coffee growing areas of western, southern and eastern parts of Ethiopia. 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 or severity by direct or indirect manipulation of antagonists (Tesfaye Alemu and Kapoor, 2004).

Therefore, the main purpose of this study is to evaluate the interaction between the antagonists *Trichoderma* spp. and against the test pathogen of coffee wilt disease (*G. xylarioides*) in *in-vitro* by means of dual culture and to evaluate, characterize effective anti-fungal extracts from *Trichoderma* isolates against coffee wilt pathogen (*G. xylarioides*).

1.3 Significance of the Study

The use of *Trichoderma* isolates to control the fungal pathogens such as *G. xylarioides* will go a long way in assisting farmers to realize effective and efficient coffee production. *Trichoderma* species acts both as a biological fungicide without residual toxic effects in the soil. However, the use of these chemicals in coffee production has no effect in controlling CWD due to the nature of the pathogen. Control strategies for CWD pathogen with fungicides and cultural practices are very difficult, because the pathogen infects and is found in the vascular systems of the coffee trees, and in the soil, as soil borne disease. Various studies indicated that there is no resistant variety that stands against *G. xylarioides,* and no curative control methods and current recommendations to control this disease, that are limited to phytosanitary measures against the source and spread of diseased material from infected trees.

Since previous study has shown *Trichoderma* species have proved to be an efficient biological control agent of soil borne pathogens, it is important to establish the interaction between *Trichoderma* species and *G. xylarioides*. It is therefore important to determine the antagonistic effect of *Trichoderma* isolates and their antifungal extracts against the test pathogen. Therefore, the focus of the present study was evaluation and characterization of rhizosphere *Trichoderma* isolates and the investigation of their antifungal crude extracts. The research aimed at finding *Trichoderma* isolates being able to produce novel and biologically active antifungal crude extracts for biocontrol of CWD purposes.

1.4 Research question and hypothesis

1.4.1 Research questions

- ✓ Are indigenous *Trichoderma* isolates effective against coffee wilt disease, *G. xylarioides* under *in vitro* condition?
- ✓ Do the antifungal crude extracts obtained from *Trichoderma* isolates have an antagonistic activity on TLC- directed Bioautography against *G. xylarioides*?

1.4.2 Hypothesis

- ✓ *In vitro* evaluation of Trichoderma isolate will be effective in suppression or inhibition of mycelial growth of *G. xylarioides* under in vitro condition.

1.5 Research objectives

1.5.1 General objective

- To screen, evaluate, characterize, and determine the efficacy of indigenous *Trichoderma* isolates for the biocontrol of CWD (*G. xylarioides)* under *in vitro* conditions

1.5.2 Specific objectives

- To test biocontrol potential of rhizosphere *Trichoderma* isolates against *G. xylarioides* under *in vitro* conditions
- To characterize the most active antifungal crude extracts from *Trichoderma* isolates against *G. xylarioides* by means of TLC- directed bioautography

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 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 (*G. 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

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*

Source: Negash Hailu (2007).

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 *G. 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 (*G. 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 (G. 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 *G. 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 according to (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 *G. 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

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).

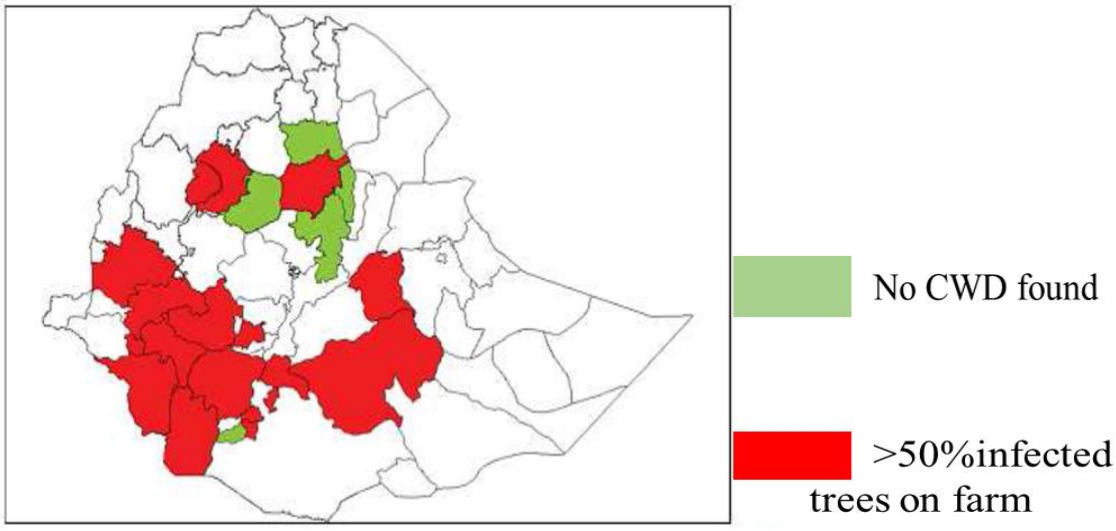


Figure 2. Distribution of coffee wilts disease (CWD) in Ethiopia

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 plantation coffee had been abandoned completely. Girma Adugna (2001) confirmed that the disease was severe in plantation coffee at Bebeka, Teppi, Gera and Jimma.

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

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 of in Coffee Research Centers

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 *G. 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 were reported. The leaves feel limp 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 3. 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 6 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 restricted as much as possible (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. This involves the destruction of all affected coffee in border areas and encouragement of farmers to grow crops other than coffee (Girma Adugna, 2004; Rutherford, 2006; Flood, 2010).

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, *G. 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% WP) 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

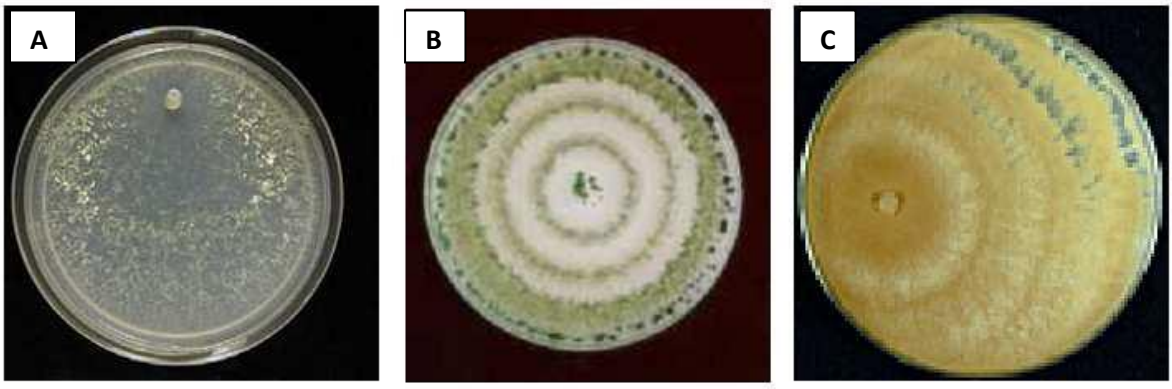
Trichoderma are free living, asexually reproducing and filamentous fungi. They are members of a genus belonging to a group of largely asexually reproducing fungi that includes a wide spectrum range from very effective soil colonizers with high biodegradation potential to facultative plant symbionts that 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 ^{0}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 4. *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, phosphorus, 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).

2.11 Extraction of Antifungal compounds

The analyses of antifungal compounds in fungal cultures usually include extraction of the analytes from culture medium or mycelium, purification from matrix components, analytes enrichment, and eventually chromatographic separation and detection. Successful determination of biologically active compounds from fungal species is largely dependent on the type of solvent used in the extraction procedure (Shentu *et al.*, 2014). Properties of a good solvent in *Trichoderma* isolates extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate (Ahluwalia *et al.*, 2015).

2.12 Thin-Layer Chromatography (TLC) and Bioautography for Antifungal Analysis

A number of bioautographic assays have been developed, which can be divided into three groups. These include direct bioautography; where the microorganisms grow directly on thin layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography (Suleiman *et al.*, 2010).

2.12.1 Contact Bioautography

In contact bioautography antimicrobials diffuse from a TLC plate or paper to an inoculated agar plate. The chromatogram is placed face down onto the inoculated agar layer and left for some minutes or hours to enable diffusion. Then the chromatogram is removed and the agar layer is

incubated. The inhibition zones are observed on the agar surface in the places where the spots of antimicrobials are stuck to the agar (Shahverdi *et al.*, 2007). The method resembles a disk assay. The disadvantages of contact bioautography were difficulties in obtaining complete contact between the agar and the plate and adherence of the adsorbent to the agar surface. These shortcomings were avoided by applying for chromatography silicic acid-glass fibre sheets, ChromAR. Still, the basis of the method was the same and antimicrobials had to be transferred from the sheet to agar causing their loss and dilution (Tarman *et al.*, 2011).

2.12.2 Immersion/agar overlay Bioautography

In immersion bioautography the chromatogram is covered with a molten, seeded agar medium. After solidification, incubation and staining (usually with Tetrazolium dye) the inhibition or growth bands are visualized. Sometimes, before incubation, plates are left for several hours at low temperature to enable diffusion. Agar-overlay is a hybrid of contact and direct bioautography. Antimicrobials are transferred from the TLC plate to the agar layer as in the contact assay but during incubation and visualization the agar layer stays onto the plate as in direct bioautography. The main disadvantage of this method is lower sensitivity caused by dilution of antibacterial in the agar layer compared with direct bioautography. Agar overlay is advised especially when direct bioautography is impossible to perform. Nowadays direct bioautography prevails over them (Shahverdi *et al.*, 2007).

2.12.3 Direct Bioautography

In direct bioautography a developed plate is dipped in the suspension of microorganisms growing in a suitable broth or this suspension is sprayed onto the plate. The plate is incubated and microorganisms grow directly on it. Hence, separation, preconditioning, incubation and visualization are performed directly on the plate. For location and visualization of antibacterial and antifungal activities, Tetrazolium salts are usually used, which are converted by the dehydrogenases of living microorganisms to intensely coloured formazan (Aneja *et al.*, 2005; Anita *et al.*, 2012). The bacteria/fungi are killed by antimicrobials on the TLC plate so color is not produced in the places of antibacterial /antifungal spots and so-called zones of inhibition that are pale on a coloured background are formed (Shahverdi *et al.*, 2007).

In an effort to discover new lead compounds, many research groups screen fungi extracts to detect secondary metabolites with relevant biological activities. In this regard, several bioassays were developed for screening purposes. Once the technique has been mastered, bioautography is a highly efficacious assay for the detection of antifungal compounds because it allows localization of activity even in a complex matrix, and therefore facilitates the target-directed isolation of the active constituents.

Bioautography has enabled rapid progress for quick detection of new antifungal compounds from fungi and other natural products. This technique allows the localization of antifungal activity directly on a chromatographic plate where the organism is applied. The method is fast, cheap, and permits a better bioassay-directed fractionation of bioactive compounds (Afrasa Mulatu *et al.*, 2013). Bioautography is particularly important to avoid the time-consuming isolation of inactive compounds. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture. Suleiman *et al.* (2010) discuss the factors influencing bioautography results and concluded that because of their diversity and variability, it is difficult to standardize the bioautographic methods. Still, the attempts to standardize these parameters or at least to estimate their influence on the final results were made.

The factors influencing direct bioautography are: mobile phases and their additives, type of adsorbent, test microorganism, living conditions for test fungi (e.g., type of broth, density of inoculums), precondition of plates, mode of development, drying, and post chromatographic conditioning, post-chromatographic detection (e.g., humidity of the chamber, time of incubation, fungi concentration, detecting reagent). The quantitative bioautographic analysis is usually done by the *regression analysis* of the inhibition zone sizes. According to some papers, the relationship between the diameter and the area of inhibition zone plotted against the logarithm of the concentration of the antimicrobial applied is linear. For a wider range of concentrations exponential relation fits better (Tarman, 2011).

3. MATERIALS AND METHODS

3.1 *Trichoderma* isolates and test pathogen

Twelve isolates of *Trichoderma* were obtained from Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU). All *Trichoderma* isolates used in this study were previously isolated from soil collected from major coffee growing areas of southern and southwestern Ethiopia which represent different agronomical management practices and levels of soil fertility (Afrasa Mulatu *et al.*, 2013). The coffee wilt pathogen (*Fusarium xylarioides*) was also obtained from the above mentioned laboratory (MCMB) and it is used as a test pathogen. All the isolates were designated as AUT in which AUT stands for Addis Ababa University *Trichoderma* isolate (Table 4). Characterization and *in vitro* antagonistic evaluation of potential antifungal compounds, compound extraction and purification by chromatographic separation and detection were performed in the Mycology Laboratory, Department Microbial, Cellular and Molecular Biology from 2016 to2018.

Table 4. *Trichoderma* isolates studied and their identification

S/No	***Trichoderma* Isolates**	**Isolate Designation**	**Place of Collection**
1	Addis Ababa University Trichoderma 03	AUT-03	Gera
2	Addis Ababa University Trichoderma 11	AUT-11	Gera
3	Addis Ababa University Trichoderma 16	AUT-16	Gera
4	Addis Ababa University Trichoderma 18	AUT-18	Gera plantation site
5	Addis Ababa University Trichoderma 21	AUT-21	Gera
6	Addis Ababa University Trichoderma 29	AUT-29	Gera
7	Addis Ababa University Trichoderma 32	AUT-32	Gera
8	Addis Ababa University Trichoderma 66	AUT-66	Yirga cheffe
9	Addis Ababa University Trichoderma 71	AUT-71	Aleta Wendo
10	Addis Ababa University Trichoderma 72	AUT-72	Aleta Wendo
11	Addis Ababa University Trichoderma 86	AUT-86	Gera plantation site
12	Addis Ababa University Trichoderma 89	AUT-89	Melko

3.2 Sterilization and maintenance of cultures

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. 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.

3.3 Cultural and morphological characterization of *Trichoderma* isolates

The cultural characteristics of *Trichoderma* isolates were studied in PDA/MEA. 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.

3.4 Evaluation of Antagonistic Activity of *Trichoderma* isolates on mycelial growth of *G. xylarioides in vitro*

3.4.1 Dual culture plate testing

The antagonistic effects of 12 Trichoderma isolates were evaluated against *G. xylarioides* in *in vitro* condition using the direct confrontation method. Briefly, two discs (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 ± 2 ^{O}C and the 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 *G. 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 2and 4were 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 *et al.*, 2013):

$$PIRG = \frac{(C-T)}{C} \times 100$$

Where PIRG = Percent Inhibition of pathogen growth by antagonists; C = Radial growth in control (cm); T = Radial growth in the test culture (cm).

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.

Additional biocontrol parameters were determined as described by (Pakdaman *et al.*, 2013) including (i) days required for the two colonies to come into contact (C); (ii) days required for the BCA to fully grow over the pathogen colony (Z); (iii) days required for the BCA to fully grow over the plate (M); (iv) the radial growth distance (in cm) of the test pathogen colony between the point of inoculation and the marginal point of contact with the BCA growth zone (P); (v) the pathogen resistance index (R) to the BCA was defined as a ratio of Z/M based on the periods required for the full growth of a fungal BCA in the presence (Z) and absence (M) of pathogen (Pakdaman *et al.*, 2013). In addition, the Pakdaman's biological control index (PBCI) combining temporal parameters and pathogen growth parameter was calculated following the formula below.

$$\text{PBCI (in cm}^{-1}) = \frac{\text{M (in days)}}{\text{Z (in days) x P (in cm)}}$$

3.4.2 In vitro characterization of different biocontrol mechanisms of Trichoderma isolates

3.4.3 Effect of volatile metabolites of bio agents

Selected *Trichoderma* isolates based on the mycelium inhibition assay against *F. xylarioides* were evaluated for the production of volatile inhibitory substances under *in vitro* conditions following the modified methods of (Dennis and Webster, 1971). Five millimeter disc of *Trichoderma* colony was inoculated centrally in Petri plates containing PDA medium in triplicates. The Petri plates were sealed at the edges and incubated at 25 ± 2 ^{o}C. After 5 days, the test pathogen was inoculated on fresh PDA and the lids of the Petri plates inoculated with antagonist were replaced by the pathogen on PDA. The plates were fixed with cellophane-tape and incubated for another 7 days; whereas, control plates were inoculated with pathogen alone (Dubey *et al.*, 2007). The radius of *F. xylarioides* disc was recorded and 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.

3.4.4 Effect of crude extracts of bio agents 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 ml 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).

3.4.5 Mycoparasitism test using slide culture

For each *Trichoderma* isolate and test pathogen (*F. xylarioides*) interaction, a clean slide was placed in 9 cm diameter plates and then a small amount of autoclaved melted Potato Dextrose Agar medium was spread over the slide to make a thin PDA film on the slide. Five mm disc of both pathogen and antagonist's isolates were paired on slide 3 cm apart on PDA surface. Distilled water was poured in Petri plates to avoid drying (incubated at 28 +2 ^{o}C 3 to 5 days according to interactions). Meeting area was observed microscopically by staining with lacto phenol and cotton blue for presence of mycelia penetration and cell wall disintegration in the area of interaction (Schuster and Schmoll, 2010).

3.5 Hydrogen Cyanide (HCN) Production

For HCN production, *Trichoderma* species was grown on Tryptic Soy Agar (TSA) supplemented with 4.4 g /L of glycine for two days. White filter paper discs was cut in the same size and soaked in picric acid solution (0.5% picric acid in 2% (w/v) sodium carbonate in 1 L of water). The sheets of filter papers were placed on the upper lid of each plate. The plates were sealed with Para film and incubated for seven days at 25°C. After incubation, HCN production were observed by the colour changes of the filter paper from yellow to light brown or reddish brown which indicated the production of HCN (Meera and Balabaskar, 2012). Three Petri plates without inoculation of *Trichoderma* isolates were considered as control. The scores including 0, 1 and 2, represented no ability, medium and very high ability of HCN production, respectively.

3.6 Evaluation of Antifungal crude Extracts from *Trichoderma* Isolates

3.6.1 Extraction of Antifungal Crude Extracts from Solid Culture Media

For extraction of antifungal crude extract from fungal mycelium or culture media different organic solvents: chloroform (99.9%), absolute ethanol (99.9%), methanol (99.9%), ethyl acetate (99.9%), hexane (99.9%) and Acetone (99%) were used. All organic solvents used in this study were of analytical grade reagent (Sigma-Aldrich (Bangalore, Karnataka, India). To extract antifungal crude extract from solid culture media, 3 Petri dishes were prepared for each of *Trichoderma* isolates. During harvesting process, 25 ml/Petri dish of each organic solvent was added to eight day's old cultures of *Trichoderma* isolates in flasks and placed on a shaker at 121 rpm for 30 min and finally filtered using Whatman No. 1 filter paper under aseptic condition.

The culture filtrate was then extracted by using rotary evaporator and further purification of the crude extract was achieved by using Thin Layer chromatography (TLC) (Afrasa Mulatu *et al.*, 2013). Several steps were followed in order to obtain crude antifungal extracts from *Trichoderma* isolates mycelia according to the scheme shown in Fig. 5.

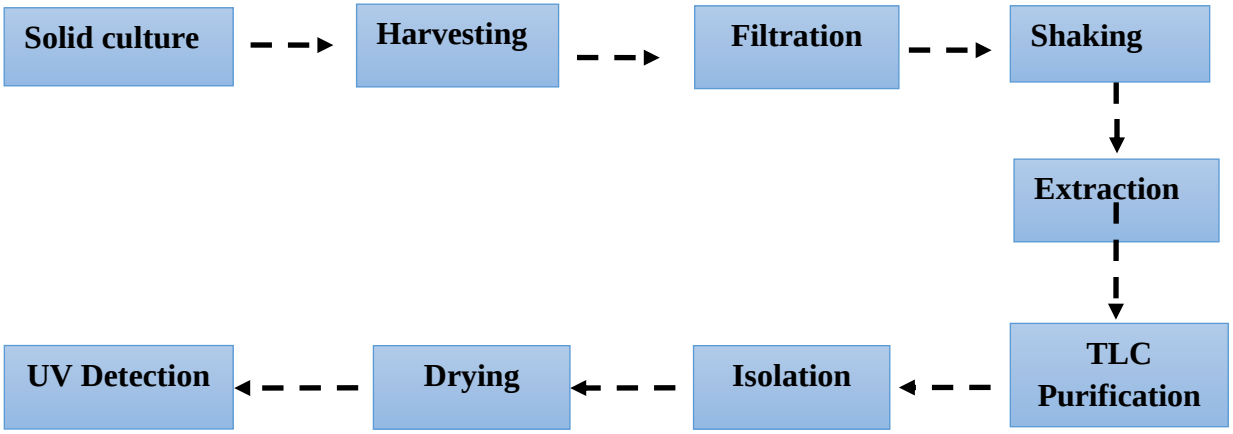


Figure 5. General overview of extraction of antifungal compounds from *Trichoderma* isolates

3.6.2 Detection and Assaying of Active Antifungal Extracts using TLC

Thin Layer Chromatography was used for separation of extracts and to screen the qualitative purity of the crude extracts by using TLC aluminum sheets silica gel 60 with fluorescent indicator (F_{254}) pre-coated 20 x 20 cm as described by (El-Mougith, 1999). Attempts were made to isolate and detect active antifungal crude extracts and for this the dissolved crude residue was subjected to TLC analysis. Standard chromatograms of fungal crude extracts were prepared by applying 20 µl solution using a capillary tube to a silica gel TLC plate (aluminum sheet Silica gel 60 F_{254}, Merck). For successful detection and separation of antifungal metabolites, different solvent systems with different ratio were tested using TLC plate.

Table 5. Solvent mixtures screened for TLC analysis of antifungal extracts from *Trichoderma* isolates

Solvent System (SS)	Composition of Solvent System	Ratio (v/v)
SS1	Hexane : Benzene : Methanol	3:2:5
SS2	Acetone: Acetic Acid: Toluene	1:1:8
SS3	Hexane : Ethanol : Methanol	5:3:2
SS4	Hexane: Benzene: Chloroform	2:5:3
SS5	Hexane: Benzene: Toluene	3:3:4
SS6	Toluene : Chloroform: Benzene	2:2:1

TLC was performed in sealed glass chambers and organic solvents were added separately into the chamber to a depth of about 2 cm. A piece of filter paper was placed at the center of the chamber and was left for 10 min to saturate the atmosphere of the chamber before adding a TLC plate. TLC plates were cut to an appropriate size (10 cm x 10 cm) and 20 µl crude samples were spotted on a line 2 cm from the bottom edge. The TLC plate was then immersed in the closed chamber and left till the solvent almost reached the opposite side of the plate. The chromatographs /crude extract was then detected by their UV absorbance at the wavelength 254 nm and/or by spraying Tetrazolium salts (2, 3, 5- Tri-phenyl-Tetrazolium Chloride (TTC) on the TLC plates with spraying reagents followed by heating at 110 ^{o}C (Afrasa Mulatu *et al.*, 2013). The distances of the spots and the solvent were measured to calculate the retention factor (R_f). Active spots were located and detected from a developed TLC by direct bioautography.

$$\textbf{R}_f \textbf{ value} = \frac{\text{distance moved by crude extract from the origin to the spot center}}{\text{Distance moved by solvent system from the origin to solvent front}}$$

3.7 The use of TLC with Direct bioautography for antifungal analysis

The direct bioautography was applied by transferring the test pathogen directly on TLC plate. Active antifungal extracts were transferred from the stationary phase to the agar layer (which contains the microorganism, *G. xylarioides*) by a diffusion process. After incubation, the plate is sprayed with a Tetrazolium salts (2, 3, 5- Tri-phenyl-Tetrazolium Chloride (TTC), which is converted to a formazan dye by the microorganism (Suleiman *et al.*, 2010).

Screened solvent systems with optimum R_f were added into a chamber to a depth of 2 cm. Two TLC plates (10 x 10 cm) were spray with 100 µl of each of the extracts with a micropipette and subsequently developed using appropriate solvent system. Organic solvents, which cause inactivation of enzymes or death of living organisms, were completely removed before biological detection. About 25-30 ml inoculums solution spray was prepared containing 1 x 10^6 spores/ml of 7 day test fungus, *G. xylarioides* (Afrasa Mulatu *et al.,* 2013).

The plates were sprayed lightly 3 times with concentrated spore suspension and incubated on a sheet of filter paper in a moist Petri dish for 24 h in darkness at 25 ± 2 ^{0}C and then sprayed with 2 mg/ml solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) as a growth detector (Silva *et al.*, 2006). A moist chamber was further incubated for 3-7 days to allow the fungal growth of the pathogen. Finally, the TLC plate was dried and visualized using visible light, UV-light at 254 nm. The diameter of the inhibition zone was measured in mm and the active spots were observed under UV light. The antifungal activity of the separated compounds was recorded as absence of growth over the active principle (white clear zone) (Afrasa Mulatu *et al.,* 2013). The R_f of the inhibition zones on the plate (plate B) was compared with the R_f of the reference chromatogram (plate A) to determine the R_f of the active compound.

3.8 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).

4. RESULTS AND DISCUSSION

4.1 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 whitish.

4.2 *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 54.8% and 83.2%) after 10 days of incubation. The highest mean inhibitory effect on the growth of the test pathogen was achieved by AUT-71 (83.2%) followed by AUT-03 (72.8%) and AUT-86 (67.2%) isolates. The mean inhibitory effect against *F. xylarioides* restricted almost completely in plates (Fig. 7) 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. AUT-71, AUT-03 and AUT-86 isolates of *Trichoderma* were rapidly grown in the form of powdery and spread widely and fully occupied the Petri plates within 3 to 4 days (Fig. 7). 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 AUT-89 isolate showed the lowest (54.8%).

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.* However, AUT-03 and AUT-71 isolates failed to develop inhibition zones, since they grew in the form of wide spread powders and occupied all the spaces on the Petri dish and the test fungi was inhibited

and overgrown by *Trichoderma* isolates within 4 days (Fig. 6). This could be due to competition for space and nutrients, and antibiosis rather than forming inhibition zones. On the other hand, AUT-32, AUT-66 and AUT-72 isolates produced zones of inhibition to *F. xylarioides* an indicative of the formation of secondary metabolites inhibiting the mycelial growth of test pathogen.

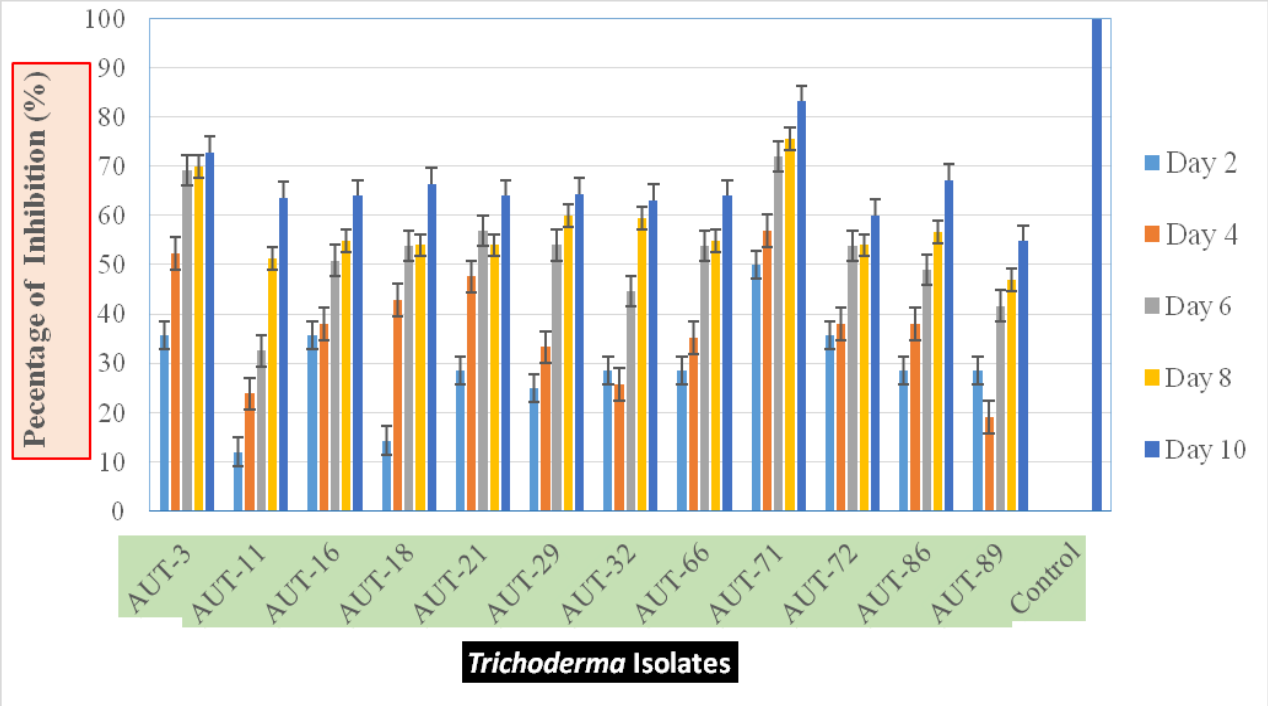


Figure 6. Percentage of radial growth inhibition of Trichoderma isolates against *F. xylarioides* (AUT- Addis Ababa University *Trichoderma* isolates)

Additional biocontrol parameters for *Trichoderma* isolates were assessed as presented in Table 6. The result indicated that the BCA grew fastest and sporulated on the pathogen and consequently restricted the growth of the test pathogen compared to the negative control. The comparison of mean C values indicated different speeds of *Trichoderma* isolates in growth toward *F. xylarioides* colonies. The difference observed in Z values after treatments indicated that AUT-03 and AUT-71 *Trichoderma* isolates fully grew over *F. xylarioides* colonies (as illustrated in Fig.7) after 4 days compared to 5 days for AUT-29 and 6 days for AUT-16 isolates (Table 6). However, other temporal parameter (M) for *Trichoderma* isolates was found to be 2 to 4 days, indicating that the *Trichoderma* isolates fully grew over the plate within different time period. With the parameter R, *F. xylarioides* exhibited highly significant deference in its resistance against different *Trichoderma*

isolates. The pathogen resistance index (R values) as the ratio of Z/M was found in the ranges of 0.9-2.5 as shown (Table 6).

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 resulted in biological control of fungal phytopathogens (Mokhtar and Aid, 2013).

Table 6. Biocontrol Parameters (C, Z, M, P, R and PBCI) obtained from the dual culture of biocontrol agents (BCAs) and *F. xylarioides* after 10 days of incubation

Isolates	Mean C	Mean M	Mean Z	Z score*	Mean P	Mean R	PRGI (%)	PBCI (cm^{-1})
AUT-03	2	4	4	2	5.3	1	72.8	0.2
AUT-11	3	4	5	1	5.1	1.25	63.2	0.17
AUT-16	4	5	6	2	5	1.2	64	0.17
AUT-18	3	4	4	1	5.2	1	66.4	0.19
AUT-21	4	4	10	1	5.4	2.5	64	0.07
AUT-29	2	4	5	2	5.4	1.25	64.4	0.37
AUT-32	2	4	0	0	5.6	2.25	63.2	ND
AUT-66	4	6	0	0	5.2	1.7	64	ND
AUT-71	2	3	4	2	5.2	1.3	83.2	0.14
AUT-72	6	6	0	0	5.5	1.7	60	ND
AUT-86	3	4	10	1	5.6	2.5	67.2	0.07
AUT-89	8	10	9	1	5.5	0.9	54.8	0.2
Control	-	-	-	-	-	-	0.00	-

Note: *C= days required for the two colonies to come into contact; Z= days required for the BCA to fully grow over the pathogen colony; M= days required for the BCA to fully grow over the plate; P=the radial growth distance (in cm) of the test pathogen colony between the point of inoculation and the marginal point of contact with the BCA growth zone; R= the pathogen*

*resistance index to the BCA and PBCI=Pakdaman's biological control index*Z score: 0=not over grow the test pathogen, 1= moderately overgrow the pathogen and 2= fully overgrow the pathogen, PRGI= Percentage of radial growth inhibition and ND= no determined.*

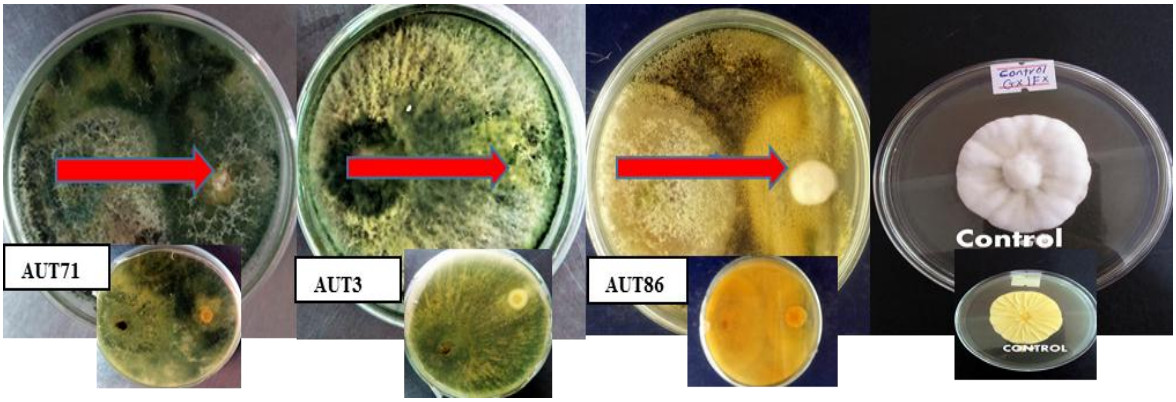


Figure 7. Antagonistic effects of *Trichoderma* isolates on test pathogen diagonally inoculate were to assess the biological control potential of *Trichoderma* isolates.

4.3 Effect of non-volatile compounds on the mycelial growth of *G. xylarioides*

The results indicated that *Trichoderma* isolates apparently produced nonvolatile substances that suppressed the test pathogen growth. Data presented in Table 7 indicates that AUT-71 derived non-volatile substances caused maximum inhibition of the mycelial growth of *F. xylarioides*.

Table 7. Inhibitory effect of non-volatile compounds of *Trichoderma* isolates on mycelial growth of *F. xylarioides* in comparison to control after 10 days and their HCN production

S/No.	Isolates	Non- volatile compound(s) Inhibition (%)	HCN production
1	AUT-03	67	+ + +
2	AUT-11	66.2	+
3	AUT-16	65.5	+ +
4	AUT-18	62.5	++
5	AUT-21	64.5	+ + +
6	AUT-29	68.5	+ + +
7	AUT-32	68.5	+
8	AUT-66	63	+ + +
9	AUT-71	71	+ + +
10	AUT-72	70.5	++
11	AUT-86	61.5	+
12	AUT-89	62.5	+ + +
	Control		-

Note: *+= Weak production, ++ =Moderate production and +++= High/strong production*

Results from this work showed that non-volatile components of the BCAs could significantly inhibit the mycelial growth of pathogenic *F. xylarioides. Trichoderma* isolates, AUT-71 (71%) caused a maximum inhibition of the mycelial growth of this pathogen. 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.* (200) 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.

4.4 Mycoparasitism test on slid culture

Microscopic observations showed that *Trichoderma* isolates were capable of overgrowing and degrading *F. xylarioides* mycelia and sclerotia, coiling around the hyphae of the test pathogen with appressoria and hook-like structures (Fig. 8). Microscopically, these isolates also performed well against *F. xylarioides* with levels of antagonistic activity. On the other hand, microscopic observation of *Trichoderma* isolates and *F. xylarioides* interaction on slide culture indicates that cell wall appeared thick and dark in *F. xylarioides* mycelia. *Trichoderma* isolates showed heavy sporulation where it encountered metabolites of *F. xylarioides*.

In the present study, microscopic observation of the interaction between *Trichoderma* isolates and the test pathogen revealed that *Trichoderma* had strong antagonistic potential. This may be due to different mechanisms such as mycoparasitism. Accordingly, Al-Saeedi and Al-Ani (2014) and Hhmau *et al.* (2015) found similar mechanisms of actions. Mokhtar and Aid (2013) also stated that mycoparasitism has been proposed as the major antagonistic mechanism displayed by *Trichoderma* species. After host recognition, *Trichoderma* spp. attaches to the host hyphae via coiling, and penetrate the cell wall by secreting cell wall-degrading enzymes. Moreover, coiling is characteristic of the interaction between mycoparasitic and phytopathogenic fungi, leading to penetration of the cell wall of pathogen followed by the breakdown of chitin by production of chitinase and glucanase enzymes at the site of endophyte penetration (Naglot *et al.*, 2015).

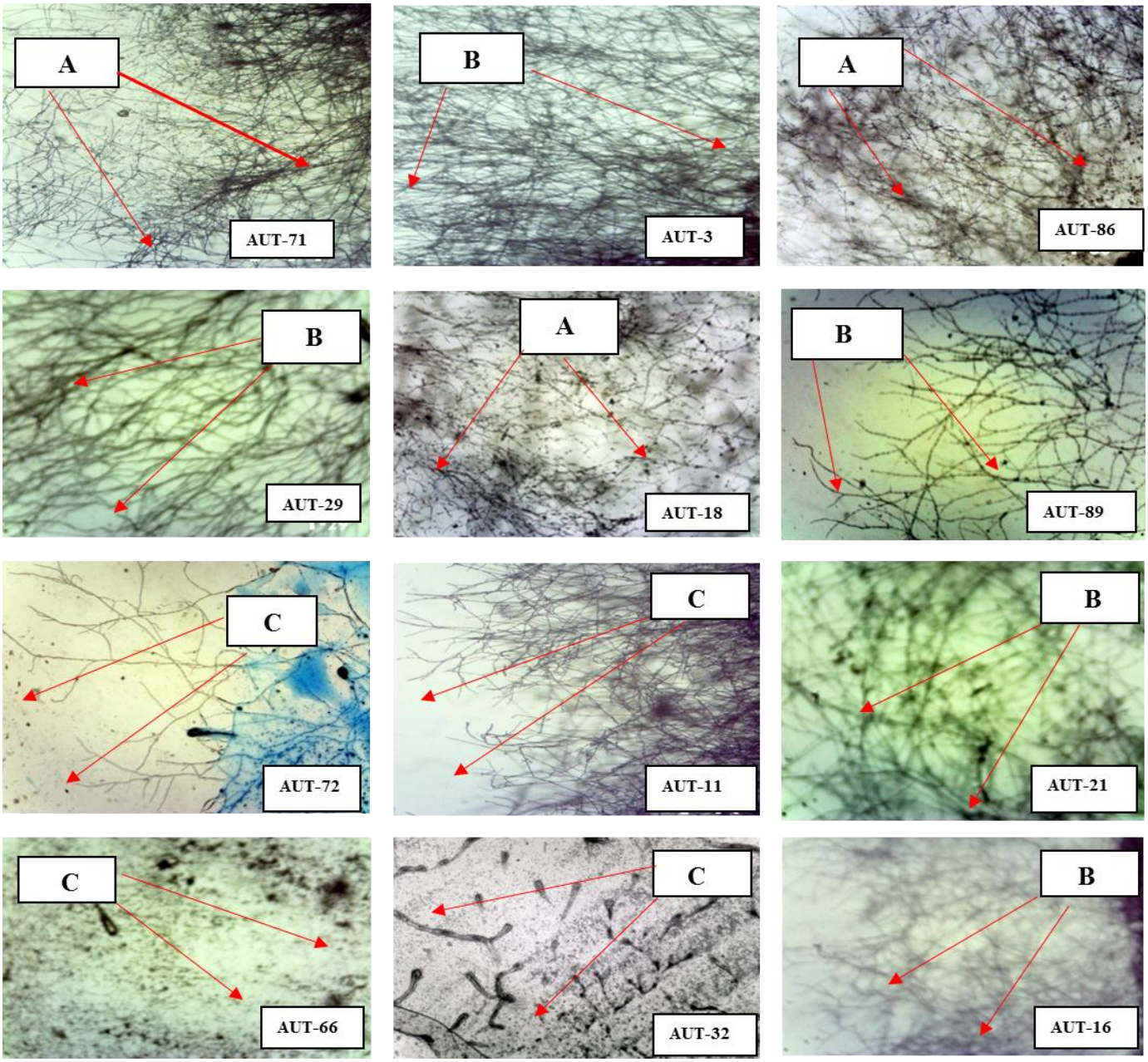


Figure 8. Interaction of *Trichoderma isolates* and *F. xylarioides and their* mycoparasitism effect using slide culture under microscope, the coil structure formation (A), hook-like structure (B) and clear zone (C).

4.5 Hydrogen Cyanide (HCN) Production

In the present study, all *Trichoderma* isolates produced HCN and converted yellow color of the filter paper to brownish or reddish color when compared to the control. Among twelve *Trichoderma* isolates, six isolates were strong producer of HCN while three isolates were moderate and the remaining three isolates were weak in production of HCN (Table 7 and Fig. 9). As described by Rakh *et al.* (2011) HCN producers formed orange brownish or reddish compound with sodium picrate and the intensity of the color increased with the amount of HCN. Hence, the production of HCN contributes to the effective inhibition of radial mycelial growth of *F. xylarioides* under *in vitro* condition. This is due to the fact that HCN could effectively block the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentration. This study is in agreement with the previous research findings who reported that over 30% of *Trichoderma* species were cyanogenic (Rakh *et al.*, 2011).

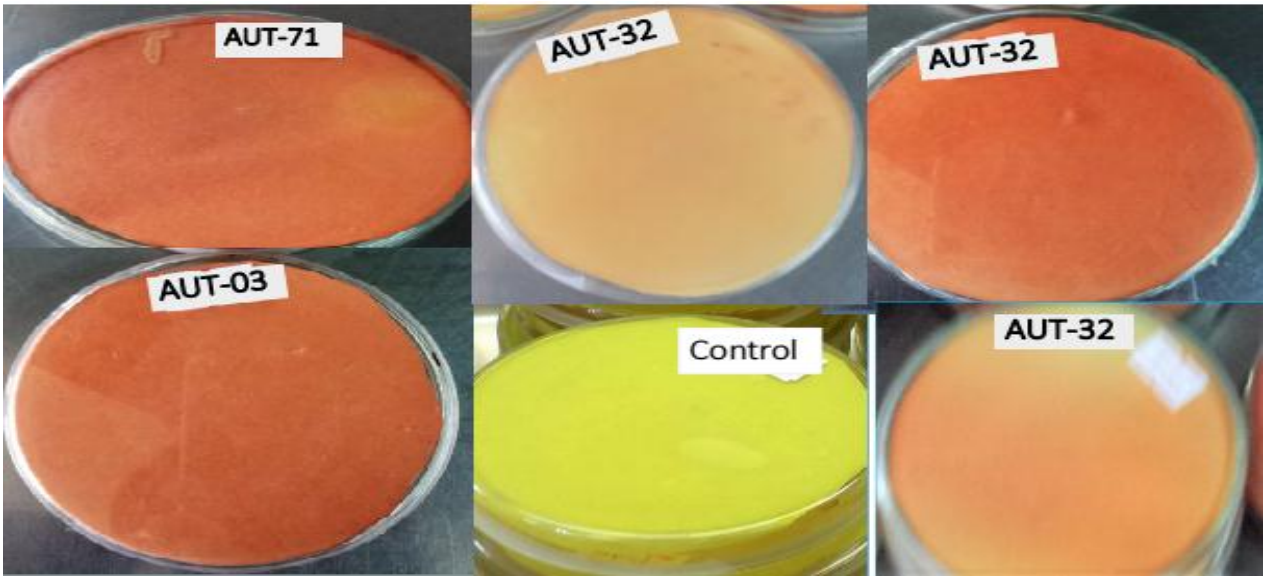


Figure 9. HCN production by different *Trichoderma* Isolates (AUT- Addis Ababa*Trichoderma*)

4.6 Extraction of Antifungal Compounds from *Trichoderma* isolates

All organic solvents were successful in extraction of the crude extracts from the antagonist and there was a high miscibility between the solvent and fungal mycelium during harvesting. In this study, different organic solvents separated antifungal crude extracts and the highest antifungal activity was achieved with chloroform, ethanol and ethyl acetate on TLC coupled with direct bioautography. As *Trichoderma* isolates are a promising antagonistic for major soil borne diseases, studies on their antifungal substances have extensively made in order to find out the best active compound and the mode of action (Kimani *et al.*, 2002). The type and composition of the eluent in TLC is one of the variables influencing the separation processes, since it shows differences in chemical composition of the antifungal extracts. The solubility, affinity and the resolution of the solvent system greatly determined the R_f values of the crude extracts (Tarman *et al.*, 2011).

4.7 Analysis of crude extracts and detection of R_f values of antifungal extracts on TLC

In this study, 12 solvent systems (Appendix 3) were tested of which six were selected for further TLC and bioautographic analyses (Table 8). Only three extraction solvents exhibited the optimum R_f values: $CHCl_3$, EtOH and EtOAc. These organic solvents were further used in Direct Bioautography. This study agreement with (Afrasa Mulatu *et al.*, 2013) Methanol, hexane and acetone crude extracts were not eluted sufficiently and their R_f values approach zero and/or 1. This may due to the high affinity of crude extracts to the stationary phase than the mobile phase in which the crude sample was remain at the origin (R_f value was too low). On the other hand, if the extract had high affinity for the mobile phases than stationary phase, the crude samples would have reached the solvent front; the condition related to the high R_f value. An important factor in quantifying the movement of a compound on a stationary phase with a certain solvent system is the retardation factor (R_f value). Crude extracts of *Trichoderma* isolates were subjected to TLC bioautography to measure their retention frequency, inhibition zones and to count the active band(s). According to the findings of this study, the highest inhibition zone on Bioautographic assay was observed by AUT21 at R_f value of 0.55, AUT-66 R_f value of 0.51 and AUT-89 R_f value of 0.55 with chloroform extract respectively. Since, the R_f value is constant for the same compound under defined conditions; the presence of clear spots with the same R_f value may mean that the same compounds are probably responsible for the antifungal activity. Bioautography assay revealed 60 zones of inhibition spots and the highest inhibition zone was observed in AUT5 (51 mm) and AUT6 (44 mm) with ethanol extract at Rf value of 0.43 (Afrasa Mulatu *et al*., 2013).

Table 8. Solvent system and R_f *values* of the antifungal extracts on TLC analysis

Solvent System (SS)	Composition of SS	Ratio	Types of Extract	R_f value of Antifungal crude Extracts											
				AUT3	**AUT11**	**AUT16**	**AUT18**	**AUT21**	**AUT29**	**AUT32**	**AUT66**	**AUT71**	**AUT72**	**AUT86**	**AUT89**
SS1	n-Hexane: Ethanol: Methanol	1:1:8	$CHCl_3$	0.72	0.57	0.28	0.13	0.63	0.8	0.73	0.56	0.57	0.67	0.66	0.57
			EtOH	0.47	0.48	0.67	0.51	0.48	0.48	0.5	0.5	0.47	0.5	0.57	0.57
			EtOAc	0.65	0.63	0.64	0.65	0.16	0.69	0.65	0.67	0.83	0.84	0.84	0.84
SS2	Acetone: Acetic acid: Toluene	1:1:3	$CHCl_3$	0.68	0.66	0.24	0.15	0.84	0.84	0.86	0.69	0.67	0.67	0.8	0.37
			EtOH	0.58	0.48	0.67	0.47	0.32	0.16	0.23	0.47	0.51	0.57	0.48	0.47
			EtOAc	0.67	0.81	0.67	0.57	0.51	0.48	0.57	0.51	0.67	0.8	0.63	0.57
SS3	n-Hexane Benzene Methanol	3:2:5	$CHCl_3$	0.72	0.57	0.28	0.13	0.63	0.8	0.73	0.56	0.57	0.67	0.66	0.57
			EtOAc	0.32	0.48	0.32	0.81	0.81	0.32	0.2	0.27	0.38	0.21	0.32	0.32
SS4	n-Hexane Benzene Chloroform	2:5:3	$CHCl_3$	0.56	0.64	0.24	0.13	0.83	0.73	0.67	0.51	0.54	0.73	0.58	0.26
			EtOH	0.66	0.6	0.49	0.21	0.67	0.76	0.77	0.61	0.67	0.68	0.51	0.54
			EtOAc	0.69	0.8	0.48	0.51	0.47	0.57	0.67	0.65	0.83	0.84	0.57	0.48
SS5	Hexane Benzene Toluene	3:3:4	$CHCl_3$	0.83	0.4	0.84	0.73	0.67	0.71	0.83	0.84	0.81	0.67	0.57	0.51
			EtOH	0.67	0.65	0.51	0.66	0.64	-	0.67	0.64	0.67	0.51	0.48	0.57
			EtOAc	0.51	0.56	0.67	0.64	0.48	0.61	0.51	0.64	0.68	0.71	0.57	0.48
SS6	Toluene Chloroform Benzene	2:2:1	$CHCl_3$	0.51	0.48	0.17	0.48	0.51	0.67	0.71	0.67	0.83	0.71	0.51	0.68
			EtOAc	0.32	0.32	0.27	0.26	0.32	0.48	0.51	0.51	0.71	0.67	0.51	0.48

4.8 The use of TLC with direct bioautography for antifungal analysis

Many compounds present in the extracts inhibited the growth of *F. xylarioides*. AUT-71 appeared to be the *Trichoderma* isolate having crude extract with the highest number of inhibition spots compared with the other isolates tested against the test fungus whereas, the lowest numbers of inhibition spots were observed in AUT-03. On the other hand, the highest inhibition zone was observed by SS1 in isolates AUT-21 (53 mm) at R_f (0.75) and AUT-66 and AUT-89 (35 mm) with chloroform extract at R_f (0.51) and at R_f (0.55), respectively. The lowest inhibition zone on bioautography was 4 mm (Table 9).

TLC bioautographic methods combine chromatographic separation and *in situ* activity facilitating the localization and target directed isolation of active constituents in a mixture (Sasidharan *et al.*, 2011; Shahverdi *et al.*, 2007). In some cases, *F. xylarioides* did not grow well and it was difficult to detect inhibition zones. The inhibition was only detected early during incubation. Subsequent growth of mycelia on top of an active band made it difficult to see the inhibition. There were a few cases where the test fungus did not grow well on certain parts of the Bioautogram, making it difficult to evaluate the number of antifungal compounds (Sasidharan *et al.*, 2011). The non-activity of the other *Trichoderma* isolate extracts by using bioautography assays could be explained by a weak activity of the extracts against the test pathogenic fungus with the disruption of synergism between active constituents caused by TLC separation, or the low concentration of the active compounds in the crude extract under the tested conditions. In other cases, there were growths but no inhibitions were observed.

The non-activity of these extracts in bioautography may possibly explain by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC separation (Masoko and Eloff, 2005). The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the fungus because of the presence of compound that inhibits their growth. Actively growing microorganisms have the ability to reduce TTC to a purple red colour (Suleiman *et al.*, 2010). In the presence of active compounds on the chromatograms, the growth of the organism was inhibited.

However, in some cases no inhibition of microbial growth ware observed. The absence of activity could be due to the evaporation of the active compounds or alternatively due to very little amount of the active compound (Shahverdi *et al.,* 2007). Most of the antifungal agents detected in this study were present in extracts of relatively non-polar solvents such as chloroform. These findings are in good agreement with the previously published results (Afrasa Mulatu *et al.,* 2013; Masoko and Eloff, 2005) that showed the substances responsible for the antifungal activity were mainly non-polar in nature.

However, the ethanol and ethyl acetate crude extracts of *Trichoderma* isolates, in contrast to the chloroform crude extracts, had good activity against plant pathogenic fungus. AUT-71 had the highest number of inhibition spots (18) against the test pathogen (fungus).While AUT3 had the lowest number of inhibition spots (13) against the test fungus. It is important to note that bioautography is not a quantitative measure of antifungal activity, since it only indicates the number of compounds that were separated with antifungal activity and further analysis and elucidation of the most active spots detected on the Bioautogram should be done.

Table 9. Inhibition diameter on bioautographic TLC plates by 3 extracts of Trichoderma isolates against *F. xylarioides*

		AUT-71				AUT-03				AUT-21				AUT-29				AUT-89				AUT-66			
Solvent Systems	Extracts solvent	R_f values		Inhibition \	Active Spots	R_f values		Inhibition \	Active Spots	R_f values		Inhibition \	Active Spots	R_f values		Inhibition \	Active Spots	R_f values		Inhibition \	Active Spots	R_f values		Inhibition \	Active Spots
		A	B			A	B			A	B			A	B			A	B			A	B		
SS1	$CHCl_3$	0.57	0.64	12	2	0.72	0.55	20	2	0.63	0.75	53	1	0.8	0.11	20	1	0.57	0.51	35	2	0.56	0.55	35	1
	EtOH	0.47	0.57	4	1	0.65	0.11	8	1	0.48	0.40	5	1	0.69	0.77	11	1	0.57	0.52	6	1	0.48	0.57	5	1
	EtOAc	0.83	0.41	20	2	0.68	0.47	33	1	084	063	16	1	0.48	0.17	8	1	0.84	0.17	9	1	0.67	0.37	16	1
SS2	$CHCl_3$	0.67	0.68	9	1	0.67	0.28	6	1	0.51	0.32	8	1	0.84	0.63	11	1	0.37	0.45	22	1	0.69	0.32	23	2
	EtOAc	0.67	0.28	4	1	0.72	0.54	18	1	0.32	0.40	6	1	0.48	0.87	13	2	0.57	0.22	7	1	0.51	0.55	8	1
	EtOH	0.51	0.48	10	1	0.32	0.25	5	1	0.63	0.67	11	1	0.16	0.17	5	1	0.47	0.41	5	1	0.47	0.55	5	1
SS3	$CHCl_3$	0.57	0.42	13	1	0.56	0.41	16	1	0.81	0.51	6	1	0.8	0.77	16	1	0.57	0.44	16	1	0.56	0.36	25	1
	EtOAc	0.38	0.47	5	1	0.69	0.31	6	1	0.83	0.71	13	1	0.32	0.48	11	1	0.35	0.51	4	1	0.27	0.25	12	1
SS4	$CHCl_3$	0.54	0.44	20	1	0.83	0.44	15	1	0.47	0.40	7	1	0.73	0.67	12	1	0.26	0.31	10	1	0.51	0.38	16	1
	EtOAc	0.83	0.47	8	1	0.51	0.32	8	1	0.67	0.26	6	1	0.57	0.51	9	1	0.48	0.38	7	1	0.65	0.35	12	1
	EtOH	0.67	0.47	4	1	0.51	0.51	29	1	0.67	0.51	14	1	0.76	0.73	8	1	0.54	0.57	5	1	0.61	0.51	6	1
SS5	$CHCl_3$	0.81	0.58	10	1	0.32	0.28	10	1	0.47	0.32	5	1	0.71	0.67	12	1	0.51	0.47	21	1	0.84	0.77	23	1
	EtOAc	0.68	0.41	8	1	0.72	0.55	20	2	0.51	0.48	16	1	0.61	0.48	10	1	0.48	0.42	6	1	0.64	0.51	5	1
	EtOH	0.67	0.55	4	1	0.65	0.11	8	1	0.32	0.47	6	1	0.67	0.32	9	1	0.57	0.52	4	1	0.67	0.48	16	1
SS6	$CHCl_3$	0.83	0.65	4	1	0.68	0.47	33	1	0.63	0.75	53	1	0.48	0.51	8	1	0.68	0.51	10	1	0.51	0.63	4	1
	EtOAc	0.71	0.38	9	1	0.67	0.28	6	1	0.48	0.40	5	1	0.8	0.11	20	1	0.48	0.55	6	1	0.56	0.55	35	1
Total					18				13				14				16				17				16

It has been demonstrated, in this study, that there were 94 zones of inhibition detected with different extracts, test fungus and solvent systems used in *Trichoderma* extracts. Different solvent systems were optimized and analyzed to determine which TLC solvent system separated the highest number of active compounds. Solvent system 1 (SS1) was the best solvent (21 active spots) followed by SS2 (19 active spots) to separate the active antifungal compounds from *Trichoderma* isolates (Appendis.6). it si clearly observed thtat chloroform was the best extraction solvent with more antifungal extracts from *Trichoderma* isolates (Afrasa Mulatu *et al.*, 2013). This implies that non- polar systems separated less active compounds compared to the polar system. Moreover, the best extracting solvent of active antifungal compound was chloroform (40 zone of inhibition) followed by ethyl acetate (37 zone of inhibition) and ethanol (17 zone of inhibition). With R_f values of the reference chromatogram (A), active antifungal compound on Bioautogram (B) inhibition zones (mm) and number active spots present in different extracts eluted with different solvents from AUT-29 against *F. xylarioides* were presented.

In general, chloroform was the best extraction solvent with more antifungal extracts from *Trichoderma* isolates. Most compounds were extracted in low polarity solvent (chloroform), fewer in high polarity solvent (ethyl acetate) and least in intermediate polarity solvent (ethanol) solvents.

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Under *in vitro* bioassay, the highest mean inhibitory effect against the growth of the pathogen was achieved by AUT-71 isolate in dual culture. The comparison of biological parameters indicate that *Trichoderma* isolates fully over grown and restricted the growth of the test pathogen within 4 days after incubation. The optimum R_f value was obtained by only three extraction solvents; *viz.*, chloroform, ethyl acetate and ethanol out of the six pre-screened solvent systems. Bioautographic method was found to be very useful in isolating active compounds with antifungal activity since the R_f value of the active compounds can be used in bioassay guided fractionation instead of requiring labour intensive determination of activity of *Trichoderma* isolates crude extracts. Thin-Layer Chromatography (TLC) direct bioautography tests revealed that most of the crude extracts from *Trichoderma* isolates exhibited antifungal activity toward *F. xylarioides*. The best extraction solvent for the active antifungal compounds was chloroform.

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, AUT-71, AUT-29 and AUT-89 under greenhouse and field conditions.
2) Detection, and elucidation of the structure and analytical characterization of the most active antifungal compounds
3) Finally, much more work needed to develop stable, cost effective, easy to produce and easy to apply formulations from these *Trichoderma* isolates.

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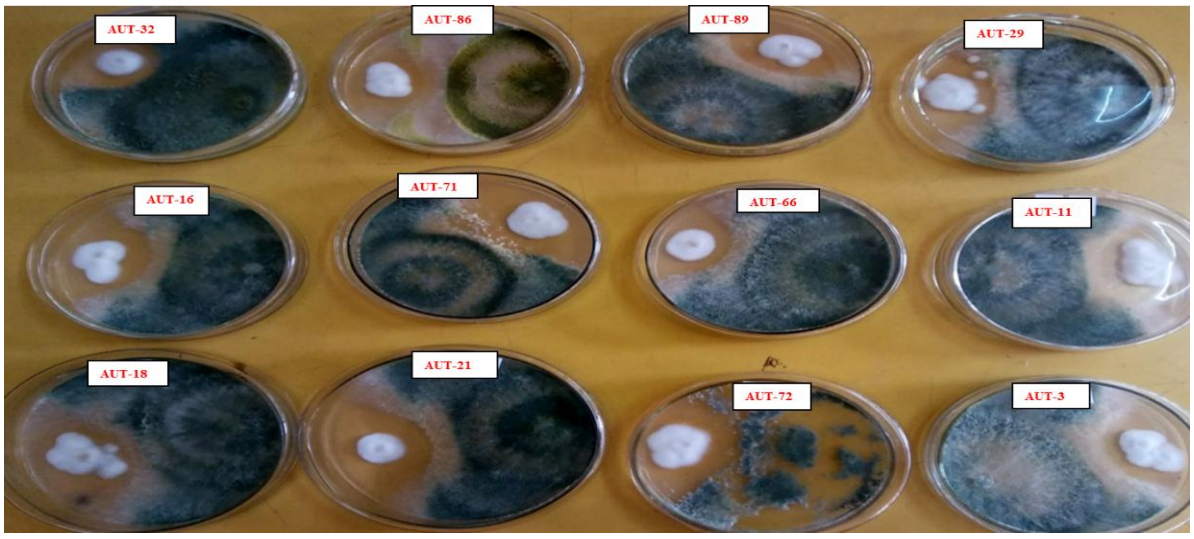
APPENDIXES

Appendix 1. Dual culture of *Trichoderma* Isolates and *F. xylarioides* mycelia radial growth in the test culture (cm) for 10 days

	Dual culture of *Trichoderma* Isolates and *G. xylarioides* mycelia.														
***Trichoderma* Isolate ID**	**Radial growth in the test culture (cm) for 10 days**														
	Day2			**Day4**			**Day6**			**Day 8**			**Day 10**		
	Rep -I	**Rep -II**	**Rep -III**	**Rep -I**	**Rep -II**	**Rep -III**	**Rep -I**	**Rep -II**	**Rep -III**	**Rep -I**	**Rep -II**	**Rep -III**	**Rep -I**	**Rep -II**	**Rep -III**
AUT-3	0.55	0.45	0.65	0.6	0.5	0.73	0.6	0.5	0.65	0.6	0.65	0.55	0.6	0.75	0.7
AUT-11	0.53	0.7	0.55	0.75	0.8	0.7	0.75	1.1	0.85	0.75	0.9	1.05	0.8	0.95	1
AUT-16	0.5	0.45	0.55	0.7	0.65	0.75	0.75	0.8	0.7	0.78	0.83	0.75	0.9	0.85	0.95
AUT-18	0.53	0.6	0.5	0.75	0.6	0.73	0.78	0.75	0.83	0.8	0.85	0.78	0.85	0.8	0.86
AUT-21	0.45	0.5	0.55	0.6	0.55	0.65	0.75	0.7	0.78	0.88	0.85	0.93	0.9	0.85	0.95
AUT-29	0.5	0.45	0.55	0.8	0.7	0.9	0.7	0.65	0.75	0.88	0.85	0.95	0.88	0.86	0.93
AUT-32	0.58	0.5	0.45	0.69	0.78	0.65	0.78	0.9	0.75	0.9	0.75	1.1	0.92	0.75	1.1
AUT-66	0.48	0.5	0.48	0.65	0.68	0.73	0.75	0.75	0.73	0.83	0.83	0.85	0.93	0.85	0.93
AUT-71	0.35	0.35	0.4	0.4	0.45	0.4	0.4	0.45	0.4	0.45	0.45	0.45	0.4	0.4	0.45
AUT-72	0.48	0.45	0.5	0.68	0.65	0.7	0.73	0.75	0.68	0.95	0.85	0.9	1	0.95	1.05
AUT-86	0.45	0.5	0.45	0.65	0.65	0.63	0.75	0.83	0.7	0.75	0.8	0.8	0.8	0.85	0.8
AUT-89	0.55	0.5	65	0.75	0.85	0.7	1.05	0.95	1	1.05	0.98	1.1	1.15	1.1	1.15
Control	0.6	0.7	0.65	1	1.05	1.1	1.4	1.63	1.5	1.6	1.85	1.9	2.2	2.8	2.5

Appendix 2. Twelve different plates showing antagonistic effect of *Trichoderma* isolate with test pathogen



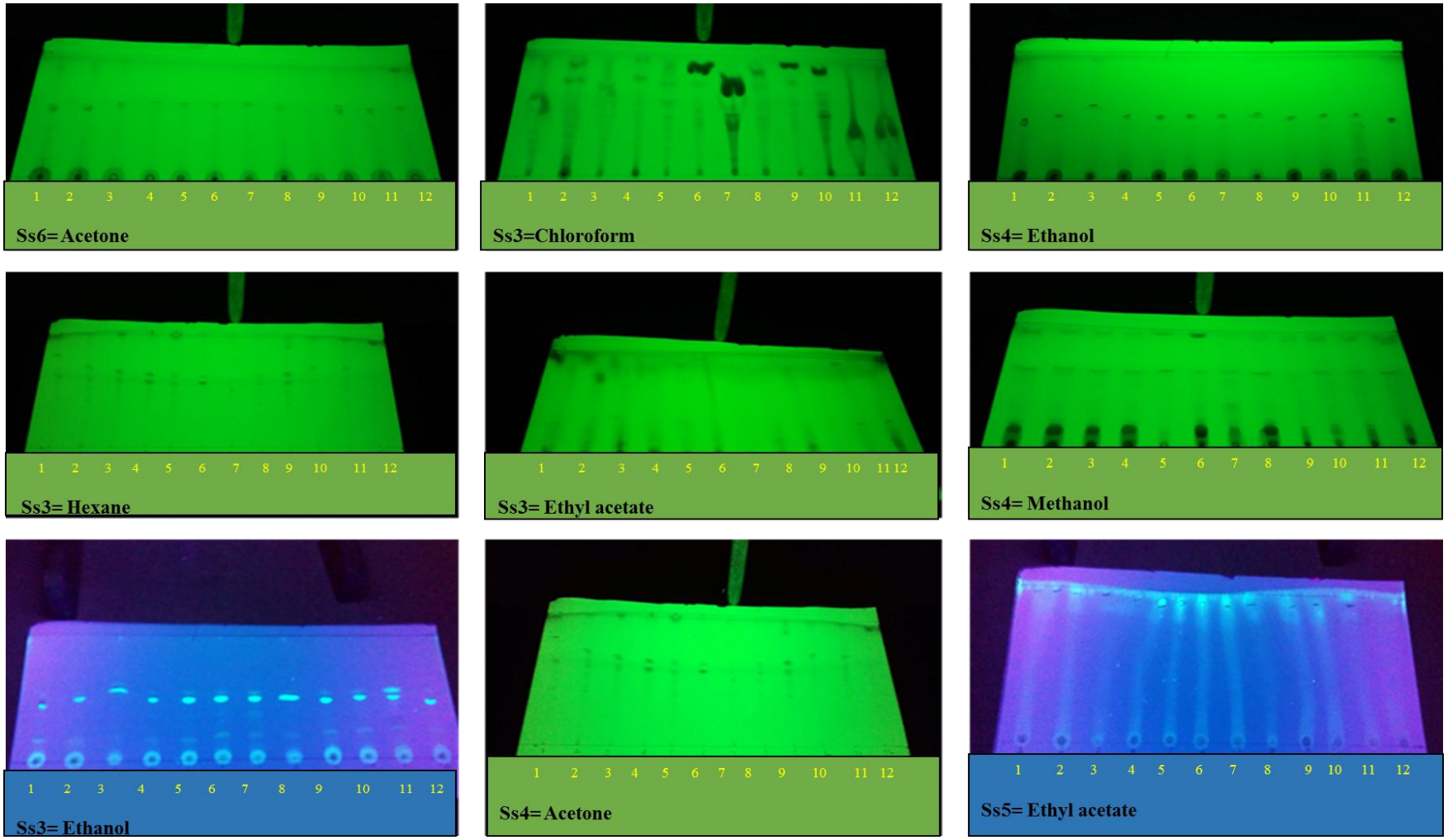
Appendix 3. Culture broth for extraction of antifungal compounds from isolated *Trichoderma* Isolates

Appendix 4. Optimum Solvent system and R_f values of the antifungal extracts on TLC analysis.

Solvent System (SS)	Composition of Solvent	Ratio (SS)	Types of Extract	R_f Value of Antifungal crude Extracts											
				AUT3	AUT11	AUT16	AUT18	AUT21	AUT29	AUT32	AUT66	AUT71	AUT72	AUT86	AUT89
SS1	n-Hexane: Ethanol: Methanol	5:5:40	CHCl3:	0.72	0.57	0.28	0.13	0.63	0.8	0.73	0.56	0.57	0.67	0.66	0.57
			EtOH:	0.47	0.48	0.67	0.51	0.48	0.48	0.5	0.5	0.47	0.5	0.57	0.57
			EtOAc	0.65	0.63	0.64	0.65	0.16	0.69	0.65	0.67	0.83	0.84	0.84	0.84
SS2	Acetone: Acetic acid: Toluene	10:10:30	CHCl3:	0.68	0.66	0.24	0.15	0.84	0.84	0.86	0.69	0.67	0.67	0.8	0.37
			EtOH:	0.58	0.48	0.67	0.47	0.32	0.16	0.23	0.47	0.51	0.57	0.48	0.47
			EtOAc	0.67	0.81	0.67	0.57	0.51	0.48	0.57	0.51	0.67	0.8	0.63	0.57
SS3	n-Hexane Benzene Methanol	15:10:25	CHCl3:	0.72	0.57	0.28	0.13	0.63	0.8	0.73	0.56	0.57	0.67	0.66	0.57
			$EtOA_c$	0.32	0.48	0.32	0.81	0.81	0.32	0.2	0.27	0.38	0.21	0.32	0.32
SS4	n-Hexane Benzene Chloroform	10:25:15	CHCl3:	0.56	0.64	0.24	0.13	0.83	0.73	0.67	0.51	0.54	0.73	0.58	0.26
			EtOH:	0.66	0.6	0.49	0.21	0.67	0.76	0.77	0.61	0.67	0.68	0.51	.54
			EtOAc	0.69	0.8	0.48	0.51	0.47	0.57	0.67	0.65	0.83	0.84	0.57	0.48
SS5	Hexane Benzene Toluene	15:15:20	CHCl3:	0.83	0.4	0.84	0.73	0.67	0.71	0.83	0.84	0.81	0.67	0.57	0.51
			EtOH:	0.67	0.65	0.51	0.66	0.64	-	0.67	0.64	0.67	0.51	0.48	0.57
			EtOAc	0.51	0.56	0.67	0.64	0.48	0.61	0.51	0.64	0.68	0.71	0.57	0.48
SS6	Toluene Chloroform Benzene	20:20:10	CHCl3	0.51	0.48	0.17	0.48	0.51	0.67	0.71	0.67	0.83	0.71	0.51	0.68
			$EtOA_c$	0.32	0.32	0.27	0.26	0.32	0.48	0.51	0.51	0.71	0.67	0.51	0.48

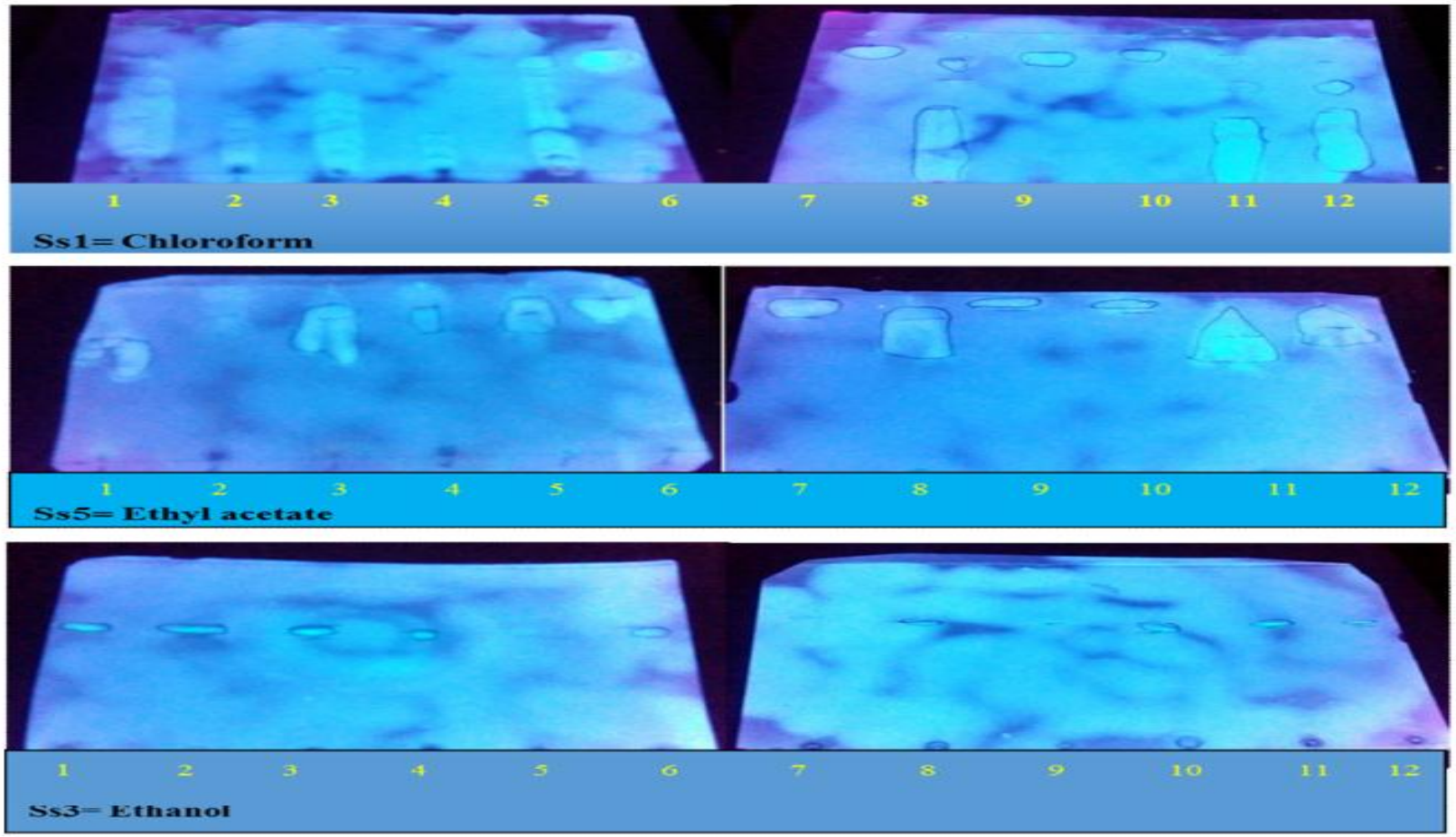
SS7	**n-Hexane Benzene Methanol**	**15:10:25**	EtOAc	0.91	0.92	0.95	0.97	0.92	0.97	0.97	0.97	0.97	0.97	0.97	0.97
SS8	**Acetone Acetic acid Toluene**	**5:5:40**	CH3OH	0.87	0.85	0.66	0.64	0.6	0.63	0.67	0.64	0.32	0.66	0.57	0.93
			C6H14	0.77	0.71	0.67	0.64	0.67	0.61	0.51	0.64	0.68	0.71	0.69	0.83
SS9	**n-Hexane Ethanol Methanol**	**25:15:10**	C3H6O	0.9	0.88	0.93	0.9	0.9	0.87	0.87	0.86	0.9	0.91	0.9	0.93
			EtOH	0.47	0.48	0.67	0.51	0.48	0.48	0.5	0.5	0.47	0.5	0.57	0.57
SS10	**n-Hexane Benzene Chloroform**	**10:25:15**	CHCl3	0.72	0.57	0.28	0.13	0.63	0.8	0.73	0.56	0.57	0.67	0.66	0.83
SS11	**Hexane Benzene Toluene**	**15:15:20**	EtOH	0.78	0.64	0.68	0.57	0.53	0.48	0.59	0.62	0.41	0.65	0.71	0.73
SS12	**Toluene Chloroform Benzene**	**20:20:10**	C6H14	0.37	0.28	0.35	0.31	0.38	0.38	0.51	0.35	0.47	0.34	0.51	0.49

Appendix 5. TLC and Direct Bioautography for the detection of antifungal activity against test fungus *F. xylarioides u*nder (UV)

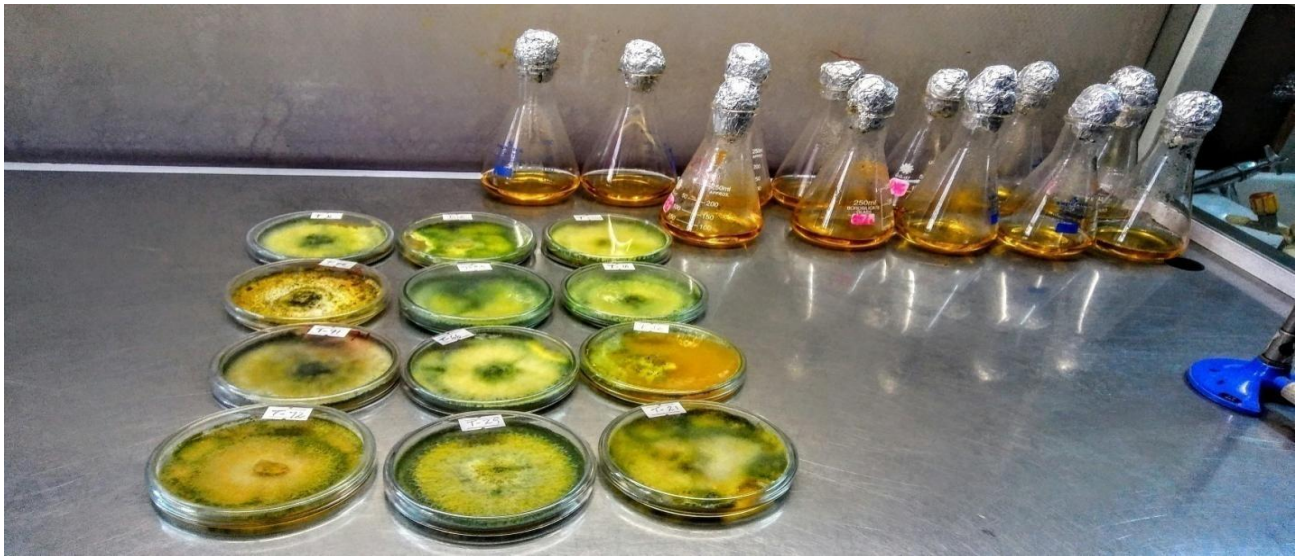


Appendixes 6. Bioautographic for the detection of antifungal activity against test pathogenic fungus *F. xylarioides.*

A



Appendix 7. Solid culture medium preparation for the filtration with PDA



Appendix 8. Crude antifungal Extraction from 12 isolated *Trichoderma* with different solvent mixtures

Appendix 9. Rota vapor for extraction of antifungal compounds with different *Trichoderma* isolates

DECLARATION

I, the undersigned, declare that this thesis work is present in the entitled "Evaluation of antifungal activity of *Trichoderma* species and their crude extracts on coffee wilt pathogen, *Gibberella xylarioides*" in partial fulfillment of the requirements for the award of the degree of Masters of Science in Applied Microbiology, Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, is my own original work under the supervision of Dr. Tesfaye Alemu and has not been presented for any other awards at this or other university and all the sources of material used for this thesis have been dually acknowledged.

Name of Candidate: Gizachew G/Michael

Signature: _____

This MSc thesis has been submitted for examination with our approval as University Advisor.

1. Tesfaye Alemu:- (PhD)

   Signature: _____ Date_____

Department: MCMB, College of Natural and Computational Sciences, Addis Ababa University

Date of Submission: _____ /_____/_____