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Characterization and Testing of Antifungal Extracts from *Trichoderma* Isolates against *Fusarium xylarioides*, the causative agent of coffee wilt disease

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Characterization and Testing of Antifungal Extracts from *Trichoderma* isolates against *Fusarium xylarioides,* the Causative agent of Coffee Wilt Disease

By

A f r a s a M u l a t u

A Thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements of the Degree of Masters of Science in Applied Microbiology

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TABLE OF CONTENTS

CONTENTS	Page
ACKNOWLEDGMENT	0
TABLE OF CONTENTS	III
LIST OF TABLES	VI
LIST OF FIGURES	VII
LIST OF ABBREVIATION AND ACRONYMS USED	VIII
ABSTRACTS	X
1. INTRODUCTION	1
2. OBJECTIVES OF THE STUDY	5
2.1 General Objective	5
2.2 Specific Objectives	5
3. LITERATURE REVIEW	6
3.1 Fungal Disease of Coffee Plants	6
3.1.1 Coffee Berry Diseases (CBD)	6
3.1.2 Coffee Leaf Rust (CLR)	7
3.1.3 Coffee Wilt Disease (CWD)	8
3.2 Diversity of Coffee Wilt Pathogen, *F. xylarioides* in Ethiopia	11
3.3 Survival and Spread of Coffee Wilt Disease	11
3.4 Incidence and Distribution of Coffee Wilt Disease (CWD) in Ethiopia	13
3.5 Strategies to Control Plant Diseases	13
3.5.1Chemical Control	13
3.5.2 Biological Control Agents (BCAs) of Plant Disease	14
3.6 Biocontrol of Coffee Diseases	15
3.7 General Characteristics of *Trichoderma* species	17
3.8 Known Application of *Trichoderma* species	18
3.9 *Trichoderma* as Biological Control Agents (BCAs) of Plant Diseases	19

3.10 Mechanisms of Action of *Trichoderma* species as BCAs 20
3.10.1 Competition for Nutrients and Space 21
3.10.2 Antibiosis 21
3.10.3 Mycoparasitism 22
3.10.4 Bio-fertilization and Stimulation of Plant Disease Mechanism 23
3.10.5 Inactivation of Pathogen's Enzymes 23
3.10.6 Fungicide Tolerance 23
3.11 Organic solvents for Antifungal extraction 24
3.12 Thin-Layer Chromatography (TLC) and Bioautography for Antifungal Analysis 25
3.12.1 Contact Bioautography 25
3.12.2 Immersion/agar overlay Bioautography 25
3.12.3 Direct Bioautography 26
4. MATERIALS AND METHODS 28
4. 1 *Trichoderma* isolates and Test pathogen 28
4.2 Sterilization and Maintaining of Cultures 28
4.3 Antagonism of *Trichoderma* isolates against *Fusarium xylarioides* 29
4.4 Mycoparasitism Test using Slide Culture 29
4.5 Antifungal Activity of Crude Extracts 30
4.6 Preparation of Culture Media for the Growth of *Trichoderma* isolates 30
4.7 Extraction Methods for Antifungal Compounds from *Trichoderma* isolates 30
4.7.2 Extraction of Antifungal Compounds from Liquid Culture Media 31
4.7.3 Separation of culture filtrates by Solvent Fractioning 32
4.8 Purification Methods for Antifungal Compounds 34
4.8.1 Detection and Assaying of Active Antifungal Compound(s) by TLC 34
4.8.2 The use of TLC with Direct Bioautography for Antifungal Analysis 35
4.9 Bioassay of Antifungal Compounds from *Trichoderma* isolates 36
4.9.1 Agar Disc Diffusion Assay 36
4.9.2 Effect of Non-Volatile (Culture Filtrate) Compounds from Antagonists on the Radial Growth of *F. xylarioides* 36
4.10 Methods of Data Analysis 37
5. RESULTS 38

5.1 In-Vitro Evaluation of Antagonistic Activity of *Trichoderma* isolates 38
5.2 Mycoparasitism test using slide culture 39
5.3 Antifungal Activity of Crude Extracts 42
5.4 Extraction of Antifungal Compounds from *Trichoderma* isolates 42
5.5 Analysis of Crude Extracts and Detection of R_f Values of Antifungal Compounds on TLC 43
5.6 The use of TLC with Direct Bioautography for Antifungal Analysis 44
5.7 Bioassay of Antifungal extracts from *Trichoderma* isolates 47
5.7.1 Agar Disc Diffusion Assay 47
5.7.2 Effect of Non-Volatile Antifungal Compounds on Radial Growth of *F. xylarioides* 47
6. DISCUSSION 49
7. CONCLUSIONS AND RECOMMENDATIONS 52
7.1 Conclusions 52
7.2 Recommendations 52
REFERENCES 53
APPENDICES 66
DECLARATION 71

LIST OF TABLES

Table 1. Designation of *Trichoderma* isolates 28

Table 2. Solvent systems used for TLC analysis of crude extracts from *Trichoderma* isolates 34

Table 3. In-vitro antagonistic effect of *Trichoderma* isolates on radial and mycelial growth inhibition of *F. xylarioides* on PDA after 8 days of incubation..... 38

Table 4. Antagonistic activity of crude extracts from *Trichoderma* isolates at different concentration against the radial growth of *F. xylarioides* 42

Table 5.Optimum solvent systems and Rf values of antifungal compounds on TLC analysis..... 44

Table 6. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of AUT5 against *F. xylarioides*..... 46

Table 7. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of AUT6 against *F. xylarioides*..... 46

Table 8. Radial growth inhibition of *F. xylarioides* treated with different concentration of *Trichoderma* isolates culture filtrate (non-volatile compounds) after 8 days of incubation..... 48

LIST OF FIGURES

Figure 1. Symptoms of fungal diseases of Coffee. 10

Figure 2. General overview of extraction of antifungal compounds from *Trichoderma* isolates 31

Figure 3. Initial separation procedures for antifungal compounds from the culture filtrate of *Trichoderma* isolates 33

Figure 4. Percentage inhibition of radial growth of *Trichoderma* isolates against *F. xylarioides* 39

Figure 5. Antifungal activity (using the slide culture method) of *Trichoderma* isolates against *F. xylarioides* 41

Figure 6. Percentage inhibition of *Trichoderma* isolates on radial growth of *F. xylarioides* from culture filtrate (non-volatile compounds)……...48

LIST OF ABBREVIATION AND ACRONYMS USED

µg	micro gram
µl	micro liter
AAU	Addis Ababa University
AUT	Addis Ababa University *Trichoderma*
BCAs	Biological Control Agents
CBD	Coffee Berry Disease
$CHCl_3$	Chloroform
CLR	Coffee Leaf Rust
CWD	Coffee Wilt Disease
CWDEs	Cell Wall Degrading Enzymes
CZI	Clear Zone of Inhibition
EtOAc	Ethyl acetate
EtOH	Ethanol
F_{254}	Fluorescent light at 254 wavelengths
IAA	Indole Acetic Acid
INT	p-iodonitrotetrazolium violet
IPM	Integrated Pest/ Pathogen management
JARC	Jimma Agricultural Research Center
MEA	Malt Extract Agar
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
Min	Minute
MTT	3-{4, 5-dimethylthiazol-2-yl}-2, 5-diphenyltetrazolium
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
R_f	Retention factor
rpm	Revolution per Minute
spp	species
SS	Solvent System
TLC	Thin Layer Chromatography
TTB	Tetrazolium Bromide
TTC	2, 3, 5-tripheny tetrazolium chloride
USA	United State of America
UV	Ultra Violet Light

ABSTRACTS

Coffee Wilt Disease (CWD) is a vascular disease caused by the fungal pathogen; Fusarium xylarioides and one of the most important diseases of coffee that was prevalent in Ethiopia. The use of indigenous antagonistic isolates of Trichoderma would be a nature conserving means to combat this disease. The current research work was designed to isolate, extract, characterize, evaluate and determine the antifungal compounds from Trichoderma isolates against F. xylarioides. An experiment was conducted to extract, characterize, purify, evaluate and determine the antagonistic potential of antifungal compounds from six Trichoderma isolates that were inhibitory towards F. xylarioides. For extraction of antifungal compounds from fungal mycelium or culture media different organic solvents: $CHCl_3$, EtOH, MeOH, EtOAc, n-hexane, and butane were used. The chloroform, ethanol and butane extracts were screened for their antifungal activity. A direct bioautographic procedure, involving spraying suspension of F. xylarioides on TLC plates developed in solvents of varying polarities was used to detect a number of antifungal compounds present in the extracts. Moreover, in-vitro antagonistic bioassays were performed to test, evaluate and determine the potentiality of Trichoderma isolates as biological control agents against F. xylarioides. The study indicated that antifungal compounds were successfully extracted from fungal culture media with all organic solvents used except hexane. For purification and separation of crude extracts on a TLC, the optimum R_f value was obtained by only three extracting solvents: $CHCl_3$, EtOH and Butane using seven pre-screened solvent systems. Bioautography assay revealed 60 zones of inhibition spots and the highest inhibition zone was observed in AUT5 (51 mm) and AUT6 (44 mm) with EtOH extract at R_f value of 0.43. In in-vitro bioassay, the highest mean inhibitory effect on the growth of the pathogen was achieved by AUT2 (77.4%) isolate followed AUT3 (72.9) in dual culture. The minimum inhibitory concentration (MIC) of non-volatile compounds was observed in isolate AUT3 and AUT6 to be 5% of culture filtrate. In general, TLC-directed bioautography assay was useful in isolating active compounds with antifungal activity and all Trichoderma isolates were accounted for reduction in mycelial growth of the test pathogen in-vitro.

Keywords**: **Antagonistic activity, Bioassay methods, Bioautography, Organic solvent polarity, Thin layer chromatography

1. INTRODUCTION

Coffee is a tropical and subtropical crop grown in more than 80 countries with a total production close to 108 million ton per year (Temesgen Belayneh *et al*., 2010). Ethiopia is the primary gene center for Arabica coffee (*Coffea arabica* L.) (Fair Trade Farmers in Ethiopia, 2004). It contribute 60% of foreign exchange earnings to the country and nearly 25% of the Ethiopian population depend directly or indirectly on coffee for a livelihood by involving in the production, processing and marketing of coffee as the major contribution to the development of the rural and the national economy (CTA, 1999; Paulos Dubale and Demil Teketay, 2000).

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants, especially in the tropical and subtropical regions (Brimner and Boland, 2003). The major coffee diseases in Ethiopia are coffee Berry Disease (CBD), Coffee Wilt Disease (CWD) and Coffee Leaf Rust (CLR). The most important diseases both in severity and wide distribution are CBD and CWD.

Coffee Wilt Disease (CWD) is currently has a devastating effect on coffee production in parts of Eastern and Central Africa and continues to spread at an alarming rate. Currently, the disease is highly distributed in parts of Eastern and Central African countries such as Ethiopia, Uganda, Republic of Democratic Congo, and Ivory Coast (Rutherford, 2006). It is called Tracheomycosis caused by *Fusarium xylarioides* Steyaert (Teleomorph: *Gibberella xylarioides* Haem and Saccas) (Stewart, 1957). The disease is responsible for the reduction in the production of coffee beans and severely attacks the vascular system of the plant causing wilting and eventually die-back. This is due to the disruption of water transportation from the roots to the leaves that kills the coffee tree. The major factors that predispose coffee to CWD were the lack of nutrients, weed competition and water logging (Pieters and Vander Graaff, 1980).

Coffee vascular disease (Tracheomycosis) was reported in Ethiopia for the first time in the 1970s (Van der Graff and Pieters, 1978; Merdassa Ejetta, 1985) and in 1993 in Uganda were it causes significant losses in Robusta Coffee (Adipala *et al.*, 2001). In major Arabica coffee growing areas of South Western Ethiopia, including Bebeka, Teppi, Jimma and Gera, the incidence of *Fusarium (Gibberella) xylarioides* is reported to be 60% and the fungus is causing significant yield losses due to severe damage and death of millions of trees (Girma Adugna *et al*., 2001). A report from Uganda

(Serani *et al*., 2007) indicates that *F. xylarioides* causes more deaths of young coffee plants than any of other *Fusarium* emphasizing the severity of this pathogen. Coffee wilt disease (CWD) is known to attack all species of *Coffea*, including the wild indigenous lines in Tropical Africa (Wrigley, 1988; Coste, 1992). The disease was reported to be prevalent on *Coffea excelsa* in Central Africa Republic and Cameroon, on Robusta varieties in Zaire and Ivory Coast (Booth, 1971; Coste, 1992) and found on Arabica coffee in Ethiopia, mainly in plantations near Agaro, Jimma and Bonga in early 1970's (Kranz and Mogk, 1973). The disease incidence is high where coffee is grown under advanced cultural practices and minimal in the less managed forest coffee (Van der Graaff, 1981).

Attempts to control CWD are breeding of resistant plants, plant and environmental management, and synthetic fungicides (Chet, 1987; Emmert and Handelsman, 1999). The high cost of pesticides, the emergence of fungicide resistant pathogen biotopes and other social and health related impacts of convectional agriculture on the environment have increased interest in agricultural sustainability and biodiversity conservation (Cook *et al*., 1996; Vander Vossen, 2005). Thus, there is a need for new solutions to plant disease problems that provide effective control while minimizing cost and negative consequences for human health and the environment.

Nowadays, the diseases are managed with the application of chemical pesticides. Use of chemical pesticides causes environmental problem, as most of them do not undergo biodegradation and left toxic residues in the soil. Apart from identifying resistant varieties of coffee against CWD, there is a need to look for the biological control methods that can be effectively used along with other methods of disease control. Consequently, the scientific literature contains a vast body of research on many soil fungi and bacteria with biocontrol abilities. These microorganisms are known by several generic names, including biological control agents (BCAs), plant growth promoting rhizobacteria (PGPR) and biopesticides. Because of their catabolic versatility, their excellent root colonising abilities, and their capacity to produce a wide range of antifungal metabolites, the soil-borne *Trichoderma spp, Gliocladium spp, Actinomycetes, Bacillus spp* and *Pseudomonas spp* have received particular attention (Walsh *et al.*, 2001).

Species of genus *Trichoderma* have been proven as effective biocontrol agents (BCAs) of soil-borne plant pathogen (Jensen and Wolffhechel, 1993). The genus was described by Persoon almost 200 years ago and consists of anamorphic fungi isolated primarily from soil and decomposing organic matter. Isolates of *Trichoderma* are ubiquitous and are relatively easy to isolate and culture

(Harman, 2004). In addition, isolates grow quickly on many substrates, produce metabolites with demonstrable antibiotic activity, and may be mycoparasitic against a wide range of pathogens. Mycoparasitic activity and antibiotic production were first demonstrated in *Trichoderma* by Weindling in 1932 and 1934, and many modern biotechnological applications of these fungi as biocontrol agents are derived from these early works (Hassan *et al.*, 2011). Most species of the genus grow rapidly in artificial culture and produce large numbers of small green or white conidia from conidiogenous cells situated at the ends of widely branched conidiophores.

Trichoderma spp are among the most frequently isolated soil fungi and present in plant root ecosystems (Harman *et al*., 2004). To date, most of the studies on *Trichoderma spp* have been conducted with respect to their activity as biological control agents. Their efficacy has been demonstrated for the management of nematode and fungal diseases under greenhouse and field conditions (Siddiquee *et al.,* 2009). Understanding the mechanisms involved in the antagonistic effect of *Trichoderma spp* against plant pathogen are important in selection of suitable biocontrol agent for effective and safe utilization since different isolates of *Trichoderma* have antagonism against fungal pathogens and effects on the plant health. The modes of action as biological control agents include mycoparasitism, antibiosis, competition; enzyme activity and induced plant defense (Howell, 2003). They were tested for antagonistic capacity against a large number of foliar and soil borne pathogens namely, *Sclerotium rolfsii, Rhizoctonia solani, Fusarium oxysporum and Pythium spp* in vegetables, field, fruit and industrial crops. *T. harzianum* isolates effectively inhibited these pathogens (47–92%) in dual cultures (Tran, 1998; Howell, 2003; Harman, 2004; Ngo *et al.*, 2006). Mycoparasitic *Trichoderma* strains are able to recognize the host hyphae, to coil around them, develop haustoria and penetrate the cell wall of the host (Siddiquee *et al.*, 2009). *Trichoderma spp* would be especially suitable for combating CWD, because many of its species are rhizosphere competent (Bailey and Lumsden, 1998; Whipps, 2001).

Yet the antagonistic efficacy of BCAs in the field was also depend on the environment where it was applied and therefore ideally screening for appropriate biocontrol agents should be done in the same environment (Ojiambo and Scherm, 2006). Most *Trichoderma spp* are antagonistic to phytopathogenic fungi and have been broadly used as the most important biocontrol agent (Mohammad *et al.,* 2011). This study was focused primarily to select *Trichoderma* isolates with antifungal potential using different crude extracts and chromatographic techniques, to obtain

improved isolates with increased activity against the test pathogen, *F. xylarioides* and to optimize culture conditions for the production of antifungal compounds. Therefore, this study was focused on extraction, evaluation, characterization and determination of antifungal extracts activities from *Trichoderma* isolates against *F. xylarioides*.

2. OBJECTIVES OF THE STUDY

2.1 General Objective

- The overall goal of the research was to screen the active *Trichoderma* isolates and extract, characterize, evaluate and determine their antifungal extracts against *F. xylarioides*.

2.2 Specific Objectives

The specific objectives of the present study are:

- ✓ to screen, extract, purify and characterize antifungal compounds with high antagonistic activity from *Trichoderma* isolates.
- ✓ to evaluate, test and determine the in vitro activity of the antifungal compounds against *F. xylarioides*.
- ✓ to determine the antifungal activity of the crude extract(s) and the isolated compound using antifungal bioassay methods and bioautographic techniques.

3. LITERATURE REVIEW

3.1 Fungal Disease of Coffee Plants

The genus *Coffea* is endemic to Africa and a number of species are described in West, Central and East Africa. Due to disease constraints, poor management practices and other factors such as yield, quality and growth habits, only two species are nowadays commercially grown worldwide, namely *C. canephora* (Robusta) in lowlands and *C. arabica* (Arabica) in highlands (Hindorf and Omondi, 2011; Musoli *et al.,* 2001). However, coffee remains the most important export crop for many African countries both in terms of the earnings and its impact on socio-economic life of the rural folk engaged in its production. Many African producer countries depend almost entirely on foreign exchange earnings from coffee exports while large sections of their population draw their livelihood from coffee cultivation, processing and marketing establishments (Kimani *et al*., 2002).

Many abiotic and biotic factors are the major constraints of coffee production in the country. Coffee diseases caused by fungi are the major constraints to reduce coffee production in major coffee producing countries of Africa (Kimani *et al*., 2002). The crop is prone to a number of diseases that attack fruits, leaves, stems and roots, and reduce the yield and marketability (Eshetu Derso, 1997). The major coffee diseases in Ethiopia are coffee berry disease (CBD), coffee wilt disease (CWD) and coffee leaf rust (CLR). Coffee berry disease (CBD) is the top major disease of coffee in Ethiopia, which attack mainly the green berries of coffee. Next to coffee berry disease (CBD), the most limiting factors both in severity and wide distribution for coffee production in Central and East African countries is Tracheomycosis or vascular wilt disease of coffee caused by *Fusarium xylarioides* Steyaert imperfect stage (*Gibberella xylarioides* Heim and Saccas Perfect stage). The major difference between Tracheomycosis and many other coffee diseases is that it kills all affected trees at all stages of development (Girma Adugna, 2004; Negash Hailu, 2007).

3.1.1 Coffee Berry Diseases (CBD)

Coffee Berry Disease (CBD), caused by the fungus *Colletotrichum kahawae,* rots fruits of Arabica coffee trees, leading to harvest losses averaging 40%, but which can reach 80% (Handrof and Omondi, 2011).The disease was reported for the first time in Kenya in 1922

(Gibbs, 1969) Angola around 1930, Zaire in 1937, Cameroon between 1955-1957, Uganda in 1959, Tanzania in 1964, Ethiopia in 1971 (Mulinge, 1973; Van der Graaff, 1981) and in Malawi in 1985 (Lutzeyer *et al.,* 1993). Since then it gradually spread to all the coffee production zones in Africa in which it has been favored by favorable environmental conditions. It is a serious threat to the major arabica production zones throughout the world. It is also prevalent in all coffee growing countries in Africa and causes an average about 30% national yield losses to Ethiopia (Tefesetewold Biratu, 1995; Eshetu Derso *et al.,* 2000).

The causative agent, *Colletotrichum kahawae*, attacks all stages of development from flowers to ripe fruit and occasionally attacks leaves, but maximum crop losses occur after infection of green berries. Infection takes place either early during flower bud formation causing some losses in flowers or remains latent in the inflorescence until the berries start to expand in growth (Tefesetewold Biratu, 1995; Eshetu Derso *et al.,* 2000).

Colletotrichum kahawae is an ascomycete, a member of the sac fungi. One of the features of these fungi is that they generate spores, called conidia or conidiospores that can be easily dispersed by splashing rain. The conidia are also distributed by insects, coffee pickers or other vectors, but never by wind due to a sticky constellation in the pink masses. In the absence of buds and berries the pathogen survives in the maturing bark of secondary branches. The pathogen never attacks mature coffee beans; it remains in the pulp. The losses occur during early infestation by destroying the beans or by preventing proper wet and dry processing since the pulp cannot be removed completely, causing so called ‘‘stinkers’’ in the crop and reducing the quality. An intensive progress of the disease in the expanding stage of the berry development finally produces mummified berries with no economic value at all (Handrof and Omondi, 2011).

3.1.2 Coffee Leaf Rust (CLR)

Coffee Leaf Rust (CLR) is one of the most important diseases of *C. arabica* in the world. It devastated Arabica coffee plantations in Ceylon at the end of the 19th century and was responsible for its replacement with tea plantations. Despite effective fungicides and resistant varieties developed to control rust, yield reductions of 20% or more in various countries are still caused by the pathogen (Waller, 1982). In Brazil, losses have been estimated to be about 30% and an annual loss of about 4500 tons of coffee was estimated in Kenya in the 1960s. The

pathogen prefers a temperature range of 20–28 ^{o}C, needs a leaf wetness period only during spore germination and penetrates with the germination hyphae into the stomata of the host. The fungus tolerates longer seasons without rainfall and spores are wind-borne, only attacking leaves and needs no other host for completing the life cycle. Coffee grown in lower altitudes is more predisposed to the disease and suffers more attacks. A heavy infestation of leaves not only reduces the assimilation area but also results in a complete defoliation diminishing the next year's crop tremendously (Hindorf and Omondi, 2011).

Coffee leaf rust (CLR) was first reported in Ethiopia in 1934 (Hindorf, 1998; Sylvain, 1958) but the disease had existed for a long time in other countries without causing epidemics or eradications of certain varieties of *C. arabica*. The long-term co-existence of coffee and rust coupled with the high genetic diversity of coffee populations and a high level of horizontal resistance might have kept the rust at low levels (Van der Graff, 1981). Other factors such as the low average productivity associated with shade and the existence of biological agents such as the hyperparasite *Verticillium lecanii*, were also believed to play an important role in maintaining CLR at low levels. Coffee leaf rust (CLR) occurs in Ethiopia at tolerable level under a balanced path system and it inflicts minor attack to the crop except in certain areas and some pocket fields planted with homogeneously susceptible cultivars at lower elevations (Meseret Wondimu *et al.,* 1987; Eshetu Derso *et al*., 2000).

According to Girma Adugna *et al* (2008), the effect of shade on the occurrence of CLR could be shown in nursery experiments at the Jimma Agricultural Research Centre (JARC). All young coffee trees grown under the shade were infected more seriously with rust than in the non-shaded sites. Comparing coffee from the different forest regions, the material from Bonga seemed to be more tolerant to rust than others.

3.1.3 Coffee Wilt Disease (CWD)

Coffee Wilt Disease (Tracheomycosis) is a vascular disease caused by the fungal pathogen, *Gibberella xylarioides* Heim and Saccas *(Fusarium xylarioides* Steyaert*)* and is one of the major diseases of coffee and the second most occurring disease next to CBD (Van der Graaff and Pieters, 1978). The pathogen exists on coffee trees in two developing stages: *Gibberella* as the sexual or perfect stage producing wind-borne ascospores and *Fusarium* as the asexual or

imperfect stage with splash-borne conidia. Infection mostly takes place at the imperfect stage penetrating through wounds into the base of the stem. The fungus blocks the water supply in the vascular system and causes a typical brown discoloration. In the field, black to violet perithecia of the perfect stage are formed on or beneath the bark at the base of the stem (Girma Adugna, 2004).

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 *Fusarium xylarioides* by Steyaert (Flood, 1997; Girma Adugna, 1997; Girma Adugna *et al*., 2001). The *Fusarium* wilt disease on *Coffea arabica* was first observed in Ethiopia (in Keffa province) (Steyaert, 1957). He described the wilting symptom and identified the causal organism to be *Fusarium oxysporum* f. sp. *Coffea*. Stewart and Dagnachew Yirgu (1967) had noted the presence of CWD on arabica coffee. Later on, the fungus inciting Tracheomycosis was authentically confirmed to be *G. xylarioides*, of which *F. xylarioides* is the imperfect state. This was based on comparative studies of the isolates collected from dying arabica coffee trees of different origin (including isolates from *Coffea arabica* L., Ethiopia) and different *Coffea spp* (Kranz and Mogk, 1973).

In recent years, the emergence of *Fusarium* wilt (*F. xylarioides*) across East Africa has affected 90% and 30% of farms in Uganda and Ethiopia, respectively (CABI, 2005). According to CABI (2005) 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 % (Kimani *et al*., 2002; Kingori, 2001; Rutherford, 2006). The losses caused by this disease have been estimated that 1% per annum in coffee production since the pathogen was observed in Uganda (Flood and Brayford, 1997).

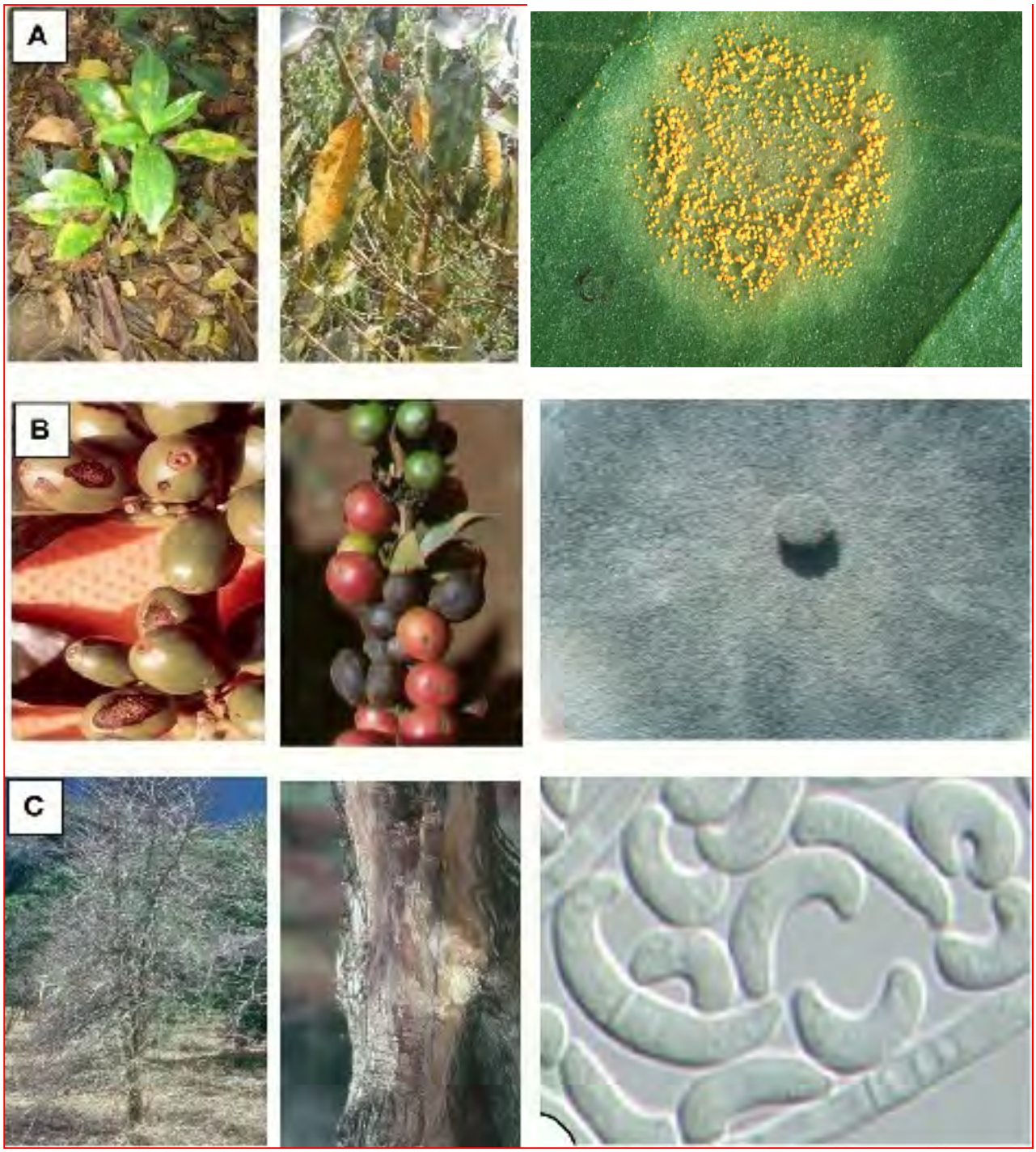

Figure 1. Symptoms of fungal diseases of Coffee. (**A**) Coffee Leaf Rust: on seedlings, older leaves, upper and lower site of the leaf and hyper parasitized by *Verticillium lecanii*. (**B**) Coffee Berry Disease: on green and mummified berries, mycelium color on Malt-Extract Agar. (**C**) Coffee Wilt Disease: dead tree, brownish vascular system on stem, conidia of the imperfect stage *Fusarium xylarioides* (Hindrof and Omondi, 2011).

3.2 Diversity of Coffee Wilt Pathogen, *F. xylarioides* in Ethiopia

Fusarium xylarioides Steyaert (teleomorph: *G. xylarioides* Ham and Saccas) is the etiological agent most often associated with coffee wilt disease or a vascular wilt that has seriously affected coffee production in Africa. Nelson *et al* (1983) listed this species as ‘‘insufficiently documented,’’ and stated that isolates from diseased coffee plants from Ethiopia were similar to a ‘‘female’’ strain described by Booth (1971), but most fusariologists (Booth 1971, Gerlach and Nirenberg, 1982) recognized the species and placed it in *Fusarium* section *Lateritium s*o called ‘‘male’’ and ‘‘female’’ isolates described by Booth (1971) were thought to exhibit dimorphism in conidial and colony characters, with ‘‘male’’ isolates producing thin, elongated, 5-7 septate sporodochial conidia (macroconidia) and ‘‘female’’ isolates producing shorter, highly curved, 0-3 septate conidia (microconidia). In diseased trees, the sexual stage *Gibberella xylarioides* Heim and Saccas forms readily in the cracks of stem bark from the collar region of dying trees. Booth (1971) noted that *G. xylarioides perithecia* form in culture when opposing mating types are brought together, thus inferring that the fungus is heterothallic. The existence of ‘‘male’’ and ‘‘female’’ strains was questioned by Gerlach and Nirenberg (1982), whose description of *F. xylarioides* included only the ‘‘female’’ conidial morphology, as did the original description of this species (Steyaert, 1957).

Other important coffee pathogens reported from Ethiopia include *Fusarium stilboides Wollenw* (teleomorph: *Gibberella stilboides*) and *Fusarium oxysporum Schlechtend* (Silva *et al*., 2006). However, studies reveal that *F. xylarioides* causes more deaths of young coffee plants than any other *Fusarium spp* (Serani *et al*., 2007).

3.3 Survival and Spread of Coffee Wilt Disease

The *F. xylarioides* survives in the soil in the form of microconidia, macroconidia, chlamydospores and perithicium with ascospores. The pathogen appears to be a soil inhabiting fungus which can penetrate through wounds or directly through root hairs and the epidermis of the small roots either above or below ground (Serani *et al*., 2007). Once a wilt pathogen has penetrated a suitable host through wounds, it moves to the vascular tissue. The pathogen then spread throughout the plant by means of mycelia growth or conidia, primarily microconidia, produced in infected xylem vessel elements. As the disease development progresses, the fungus

invades the water conducting system (xylem tissues) such as pith, cambium, phloem, and cortex and blocks the movement of water upwards from the roots to the rest of the plant. The time from first symptoms to death of the tree varies from days in young plants to eight months in trees more than ten years old (Girma Adugna, 2004). Once the fungus infects the coffee tree, all affected trees eventually die. According to Rutherford (2006) some farmers call the disease" Coffee AIDS".

According to Girma Adugna (2004) 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 (Girma Adugna, 2004).

Replanting susceptible cultivars in the infected field increases the fungus inoculums density (CABI, 2005). Pieters and Van der-Graff (1980) reported that among socio-economic factors contributing to the spread of CWD, particularly in Ethiopia, is the frequent replacing with several seedlings (3-8) per uprooted wilted trees. The infection of the young replants undoubtedly suggests that the fungus survives in stumps, root debris or in the soil for 2-3 years (Kimani *et al*., 2002; Stover, 1992).

Perithecia of *Fusarium xylarioides* contain great number of viable ascospores with 95% germinating rate (CABI, 2005) and abundant in the soil, so that these sexual spores are the most important source of inoculums in the CWD epidemics. High infection of susceptible *Coffea arabica* seedlings is observed after inoculating with field-collected ascospores suggesting that the perithecial stage is the primary source of inoculums in the field. The major function of the sexual state of the fungus is largely to serve as a survival mechanism, rather than maintaining diversity in the population structure.

The spores of the fungus can be carried by wind and in water (rain splash and flooding) help to spread the disease from tree to tree. Wind spread may occur over long distances (Flood, 1997; Rutherford, 2006). Human activities, such as pruning, weeding with a hoe and transporting affected trees for use as firewood or fencing can spread the fungus (Rutherford, 2006). When a

tree is deliberately or accidentally wounded, during pruning, weeding around the trees and even harvesting, the fungus may enter and cause disease (Gesier *et al.*, 2005; Rutherford, 2006).

The first signs of CWD are yellowing, folding and curling inward of leaves (Girma Adugna, 1997; Vander-Graff, 1978). 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 (Rutherford, 2006). These signs are known as dieback, often start on the branches on one side of the tree but rapidly spread to the whole tree.

3.4 Incidence and Distribution of Coffee Wilt Disease (CWD) in Ethiopia

Since 1993, the disease is serious in some Eastern and Central African countries (Flood, 1997; Flood and Brayford, 1997; Girma Adugna, 1997; Rutherford, 2006). Pieters and Vander-Graff (1980) have reported that the disease was endemic in all coffee growing areas of Ethiopia and reached epidemic proportions in some areas. The incidence of this disease in Ethiopia is reported to be 60%, with significant yield losses due to very severe damage and ultimate death of millions of coffee bushes (Girma Adugna *et al*., 2001). 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).

According to Girma Adugna (1997) 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 (Girma Adugna, 2004) and CWD became endemic to *Coffea arabica*. As stated by Flood and Brayford (1997) a disease is endemic, when it is always present, but with little damage, a situation characterized by a degree of horizontal resistance in the host and relatively low level of virulence of the pathogen or both.

3.5 Strategies to Control Plant Diseases

3.5.1 Chemical Control

Chemical fungicides and pesticides have been providing effective protection against many diseases but their application results in environmental pollution and emergence of resistant

pathogen strains. In addition, chemical control which adds to the cost of production is often beyond the reach of the small farmers in developing nations. The non-degradable components of these compounds have accumulated over the years and entered the food chain, causing toxicity in animals (Harman *et al*., 2004).

The environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in people's attitudes towards the use of pesticides in agriculture. Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove the most hazardous chemicals from the market (Rutherford, 2006). Additionally, the spread of plant diseases in natural ecosystems may preclude successful application of chemicals, because of the scale to which such applications might have to be applied.

Chemical pesticides suggested as a control measure for CWD such as copper based fungicide has lost their effects due to the vascular nature of the pathogen. The causative agent of coffee, *F. xylarioides* is thought to live in the soil and inside the plant, making it hard to target the fungus even with systemic fungicides (Tesfaye Alemu and Kapoor, 2004). If the fungus carried on coffee seeds, then the treatment of seeds with fungicides may be beneficial (Rutherford, 2006). Considering the cost of pesticides and environmental hazards of the use of these chemicals, the use of microbial antagonists in the control of plant pathogens has received increasing attention throughout the world (Ghaffar, 1988a, b, 1992; Krishna and Gardener, 2006).

3.5.2 Biological Control Agents (BCAs) of Plant Disease

Biological control of plant disease is defined as the involvement and the use of antagonistic microorganisms, such as specialized fungi and bacteria, genetically modified genes or gene products to attack and control plant pathogens and the diseases they cause (Harman, 2006; Papavizas, 1985). Definitions of biocontrol differ depending on the target of suppression, number, type and source of biological agents and the degree and timing of human intervention. Most broadly, biological control is the suppression of damaging activities of one organism by one or more other organisms, often referred to as natural enemies. With regards to plant diseases, suppression can be accomplished in many ways. If growers' activities are considered relevant, cultural practices such as the use of rotations and planting of disease resistant cultivars (whether

naturally selected or genetically engineered) would be included in the definition. Because the plant host responds to numerous biological factors, pathogenic and non-pathogenic, induced host resistance might be considered a form of biological control. More narrowly, "*biological control refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens"*. This may involve the use of microbial inoculants to suppress a single type or class of plant diseases. Or, this may involve managing soils to promote the combined activities of native soil and plant associated organisms that contribute to general suppression. Most narrowly, "*biological control refers to the suppression of a single pathogen (pest), by a single antagonist, in a single cropping system*" (Papavizas, 1985; US Congress, 1995). This strategy of control is ecologically clean and compatible with different models of agriculture: organic, biological and integrated pest/pathogen management (IPM) programmes.

Different biological control agents (BCAs) can be used for the control of plant diseases. These include bacteria, fungi and actinomycetes. The most important BCAs belong to the genus *Trichoderma spp*, *Bacillus spp, Pseudomonas spp* and *streptomycetes.* Biological control of plant pathogens is an attractive alternative to decrease heavy dependence of modern agriculture on costly chemical fungicides, which not only cause environmental pollution but also lead to the development of resistant strains (Harman *et al*., 2004). A variety of biological controls are available for use, but further development and effective adoption will require a greater understanding of the complex interactions among plants, people, and the environment.

3.6 Biocontrol of Coffee Diseases

Phytopathogenic microbes have an immense impact on agricultural productivity, greatly reducing crop yields and sometimes causing total crop loss. Major pathogens induce well known root or vascular diseases with obvious symptoms (Barbosa *et al*., 2001). Pathogenic fungi in general and *Fusarium spp* in particular are highly destructive pathogens of both greenhouse and field-grown major crops under favorable conditions for disease development. The disease caused by this fungus is characterized by yellowing of the older leaves, browning of the vascular system, wilting in a later stage and finally death of the whole plant. Chlamydiospores of the pathogen remain in infested soils for several years and invasion occurs through wounds on the root surface (Antoun and Prevost, 2006).

Currently, control of plant disease is a pressing need for agriculture across the globe, particularly in economically disadvantaged countries. Existing practices for controlling plant disease are fundamentally based on genetic resistance in the host plant, management of the plant and its environment, and synthetic chemicals (Strange, 1993). The high cost of pesticides, the emergence of fungicide resistant pathogen biotypes and other social and health related impacts of conventional agriculture on the environment have increased interest in agricultural sustainability and biodiversity conservation Moreover, many of the synthetic chemicals may lose their usefulness due to revised safety regulations and concern over non-target effects (Vander Vossen, 2005). Thus, there is a need for new solutions to plant disease problems that provide effective control while minimizing cost and negative consequences for human health and the environment. In most systems, the biological elements are the primary factors in disease suppression and the topic of 'biological control of plant pathogens' has gained feasibility in the context of sustainable issues (Weller *et al*., 2002).

The rich diversity of the microbial world provides a seemingly endless resource for this purpose. Biological control is also likely to be more robust than disease control that is based on synthetic chemicals. The complexity of the organism interactions, the involvement of numerous mechanisms of disease suppression by a single microorganism, and the adapteviness of most biocontrol agents to the environment in which they are used all contribute to the belief that biocontrol will be more durable than synthetic chemicals (Serani *et al*., 2007). Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front line defense for roots against attack by pathogens (Weller, *et al*., 2002). The groups of soil microorganisms with antagonistic properties towards plant pathogens are diverse, including plant associated prokaryotes and eukaryotes. Increased plant productivity by biocontrol mechanisms is indirect and results from the suppression of deleterious microorganisms and soil-borne pathogens, by PGPR in particular (Serani *et al*., 2007).

Bacillus/Paenibacillus spp have been tested on a wide variety of plant species for their ability to control diseases. They are appealing candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation (Weller *et al*., 2002). Currently, *Pseudomonas spp* are also receiving much attention as biocontrol agents due to their remarkable potential for

rhizosphere competence. The world wide interest in these groups of bacteria was sparked by studies initiated for sustainable production systems. The *Fluorescent pseudomonads* and *Bacillus spp* (Landa *et al*., 1997) are the main candidates for the biological control of diseases induced by fungal pathogens and they have been applied successfully to suppress *Fusarium* wilts of various plant species. Similarly, among wild Arabica coffee rhizosphere isolates, *Bacillus* and *Pseudomonas spp* in particular showed remarkable inhibition against *F. xylarioides*, *F.stilboides* and *F. oxysporum* under in vitro conditions (Diriba Muleta *et al*., 2007 and Landa *et al*., 1997).

3.7 General Characteristics of *Trichoderma* species

Trichoderma is a fungal genus which include species of free living soil fungi, opportunistic, avirulent plant symbionts (Harman *et al.,* 2004), asymptomatic endophytes (Wilberforce *et al.,* 2003), and parasites of other fungi (Harman, 2006). This fungal genus was described in 1794, including anamorphic fungi isolated primarily from soil and decomposing organic matter (Persoon, 1794). It is often the major component of the mycoflora in soils of various ecosystems, such as agricultural farm soil, grassland, forest, marshes, deserts, and water (Danielson and Davey, 1973; Papavizas, 1985; Zhang *et al.,* 2005). *Trichoderma* species are highly interactive in root, soil and foliar environments. They possess high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, and capability to modify the rhizosphere (Woo *et al.,* 2005).

Trichoderma 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 spores. *Trichoderma* is found in nearly all temperate and tropical soils, where samples contained 10^1-10^3 cultivable propagules per gram of soil. These fungi also colonize woody and herbaceous plant materials, in which the sexual teleomorph (genus *Hypocrea*) has most frequently found (Rutherford, 2006).

In general, the mycelia of *Trichoderma* species on PDA plate cultures is typically fast growing, with the optimal temperatures between 25-30 °C, and growth is usually minimal or absent at temperatures greater than 35 °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. A characteristic sweet or 'coconut' odor is produced by some species such as *T. atroviride*. 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 or are borne along the scant aerial hyphae. Main branches of the conidiophores produce lateral side branches that may be paired or not, the longest branches distant from the tip and often phialides arising directly from the main axis near the tip (Harman *et al*., 2004).

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 (Harman, 2006). For example, numerous *Trichoderma* strains produce hundreds of different metabolites that are also known to have antibiotic activity.

3.8 Known Application of *Trichoderma* species

Trichoderma species have been widely studied and are presently marketed as biopesticides, bio-fertilizers and soil amendments, due to their ability to protect plants, enhance vegetative growth and contain pathogen populations under numerous agricultural conditions (Harman, 2000; Harman *et al*., 2004; Lorito *et al*., 2006). Moreover, this genus plays an important role in the bioremediation of soil that are contaminated with pesticides and herbicides. They have the ability to degrade a wide range of insecticides: organo chlorides, organophosphates and carbonates (Jin *et al.,* 1996).

Due to effective control of plant diseases, several commercial biological products based on *Trichoderma* species are manufactured and marketed in Asia, Europe and United State of America (USA) for use on a wide range of crops. The commercial success of products containing these fungal antagonists can be efficiently used as conidia, mycelium and chlamydospores which are produced in either solid state or liquid fermentation. Several growth media and protocols for *Trichoderma spp* spore production were reported as mass scale production which has great potential for commercial use. At the beginning, *Trichoderma* propagules, in the form of conidia, mycelium and chlamydospores, were mass produced on conventional synthetic media. However the cost of these materials was too high. To overcome cost limitation, alternative substrates such

as rice husks, coffee, sugarcane waste, rice bran and corn meal were used (Harman *et al.*, 2004; Agosin *et al.*, 1997).

The living microorganisms, conserved as spores, can be incorporated into various formulations, liquid, granules or powder and stored for months without losing their efficacy and products can be applied to the soil, used as seed treatment, seedling root dip or added to organic fertilizers or compost (Jin *et al.*, 1996). To date more than 50 different *Trichoderma* based preparations such as Biocontrol Trichoderma 50, Antagon, Binab T, BioFit, BioFungus, Combat, Harzian 20 and Primastop were commercialized and used to protect or increase the productivity of numerous horticultural and ornamental crops (Lorito *et al.*, 2006). The use of *Trichoderma* product has both short effects: immediate control of diseases and growth enhancement of crops as well as long term effects which are demonstrated by the decrease in fungal pathogen inoculums in the field.

3.9 *Trichoderma* as Biological Control Agents (BCAs) of Plant Diseases

Antagonists of phytopathogenic fungi have been used to control plant diseases, and 90% of such applications have been carried out with different strains of the fungus *Trichoderma*. *Trichoderma* species have long been recognized as biological control agents (BCAs) for the control of plant pathogenic fungi and for their ability to increase root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients (Tran, 1998; Samuels, 2006). Strains within this genus include a wide spectrum of evolutionary solutions that range from very effective soil colonizers with high biodegradation potential, to non-strict plant symbionts that colonize the rhizosphere. Species concepts within *Trichoderma* are very wide, which has resulted in the recognition of many infra-specific groups. Some groups of biotypes within this conglomerate are able to antagonize phytopathogenic fungi by using substrate colonization, antibiosis and/or mycoparasitism as the main mechanisms. This antagonistic potential is the base for effective applications of different *Trichoderma* strains as an alternative to the chemical control against a wide set of fungal plant pathogens (Dubey *et al*., 2007).

As a consequence of the variety of activities displayed by the *Trichoderma* strain conglomerate, a large range of applications have been developed: the antagonistic potential is the basis for the

effective control of a wide set of phytopathogenic fungi (Samuels, 2006) and the biodegradative capacity is a source of useful enzymes in different industrial sectors (Harman *et al.,* 2004).

The benefits of using *Trichoderma* in agriculture are multiple, and depending upon the strain the advantages for the associated plant can include: (i) colonization of the rhizosphere by the BCA (rhizosphere competence), allowing rapid establishment within the rhizosphere of a stable microbial community and occupying a physical space and avoiding the multiplication of the pathogens; (ii) control of phytopathogenic and competitive micro flora or fauna by using a variety of mechanisms; (iii) overall improvement of the plant health; (iv) plant growth promotion, by stimulation of above and below ground parts; (v) enhanced nutrient availability and uptake, (vi) producing antibiotics that can kill the pathogens; and (vii) inducing the defensive mechanisms of the plant (Harman *et al.,* 2004; Howell, 2003; Woo and Lorito, 2006).

3.10 Mechanisms of Action of *Trichoderma* species as BCAs

Trichoderma species show different antagonistic mechanisms towards fungal pathogens. These include the production of a variety of antibiotics, mycoparasitism or hyper parasitism, competition for nutrients or space and cell wall lytic enzymes activity. In addition, these fungi may induce systemic resistance in plant which prevents further attacks by pathogens (Vinale *et al*., 2005).

Trichoderma and its products have been studied and used for biocontrol in many countries. The fungi *Trichoderma* has been an exceptionally good model to study biocontrol because it is ubiquitous, easy to isolate and culture, grows rapidly on many substrates, affects a wide range of plant pathogens, acts as a mycoparasite, competes well for food and growth sites, produces antibiotics and has an enzyme system capable of attacking a wide range of plant pathogens (Howell, 2003). Furthermore, *Trichoderma* inhibitors degrade pectinases and other enzymes that are essential for plant pathogenic fungi, to penetrate leaf surfaces. Most of effective *Trichoderma* species produced both cell wall lytic enzymes and secondary metabolites against the disease causing fungi (Harman, 2006).

Knowledge concerning the behavior of these fungi as antagonists is essential for their effective use since they can act against target organisms in several ways. Species of *Trichoderma* can

produce extracellular enzymes and antifungal antibiotics, but they may also be competitors to fungal pathogens, promote plant growth, and induce resistance in plants (Girma Adugna, 1997). The commercial use of *Trichoderma* as BCAs must be preceded by precise identification, adequate formulation, and studies about the synergistic effects of their mechanisms of biocontrol. There are a number of mechanisms whereby these fungi act as biocontrol agents.

3.10.1 Competition for Nutrients and Space

Competition is an indirect effect whereby pathogens are excluded by depletion of food bases necessary for pathogen propagules to germinate near planted seeds or by physical occupation of sites (Maloy, 1993). During this process, the antagonist may suppress the growth of the pathogen population in the rhizosphere and thus reduce disease development. The study of Barbosa *et al* (2001) in the in vitro antagonism of *Trichoderma spp* on *Cladosporium herbarum* revealed that the colonies of *Trichoderma spp* grew always faster than *C. herbarum* in single or mixed culture. *T. viride* compete for the same niches with the pathogens (Flores *et al*., 1997; Okigbo and Ikediugwu, 2000). Thus, the rapid growth of *Trichoderma* gives it an important advantage in the competition for space and nutrients with plant pathogenic fungi (Barbosa *et al*., 2001).

Trichoderma has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain energy from the metabolism of different sugars, such as those derived from polymers, wide spread in fungal environments including cellulose, glucan and chitin among others. *Trichoderma* strongly inhibits *Rhizoctonia solani*, *Pythium ultimum* and *Chalara elegans* when cultured in the same agar medium and the inhibitory action was associated with high rate and extent of CO_2 accumulation in comparison with the plant pathogenic fungi (Ozbay and Newman, 2004).

3.10.2 Antibiosis

Antibiosis occurs during interactions involving low molecular weight diffusible compounds called antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. *Trichoderma* strains are known to produce antibiotics and toxins, which are volatile or non-volatile in nature, and have a direct effect on other organisms. Fungal strains of

this genus are well known producers of secondary metabolites with antibiotic activity. Their production varies in relation to (i) the specific compound; (ii) the strain and the species; (iii) the presence of other microbes; and (iv) balance between elicited biosynthesis and biotransformation rates. Some of these metabolites are Peptaibols (trichorizianines, trichokindins, trichorozins and harzianins, trichothecin), sesquiterpine, harzianic acid, alamethicins, 6-penthyl-alpha-pyrone (6 PAP), massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and Trichodermin that has antimicrobial effect on bacteria and fungi. Peptaibols are thought to act on the membrane of the target fungus to inhibit membrane associated enzymes involved in cell wall synthesis (McIntyre *et al*., 2004; Okigbo and Ikediugwu, 2000).

3.10.3 Mycoparasitism

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack, subsequent penetration and killing of the host pathogen. *Trichoderma spp* may exert direct biocontrol by parasitizing a range of fungi; detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of cell wall degrading enzymes (CWDEs), mostly chitinases, glucanases and proteases and production of secondary metabolites that affect membrane structure (Harman *et al*., 2004). Mycoparasitism involves morphological changes, such as coiling and formation of appressorium like structures, which serve to penetrate the host (Dennis and Webster, 1971; McIntyre *et al*., 2004). *Trichoderma* attaches to the pathogen via its cell wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria and subsequent secretion of CWDEs and Peptaibols (Howell, 2003).

The CWDEs of *Trichoderma* such as different chitinolytic enzymes and glucanases have been suggested as the key enzyme in mycoparasitism (McIntyre *et al*., 2004). Endochitinase (42-kDa), chitobiosidase (40-kDa) and N-acetyl-b-D-glucosaminidase (73-kDa) from *T. atroviride* strain P1 and *T. virens* strain 41 were reported to have a substantial inhibitory effect on the germination of spores and hyphal elongation of several fungal pathogens, viz., *Botrytis cinerea, Fusarium spp*, *Alternaria spp* and virtually on all fungi containing chitin in their cell wall (Harman *et al*., 2004; Lorito *et al*., 2006; McIntyre *et al*., 2004).

3.10.4 Bio-fertilization and Stimulation of Plant Disease Mechanism

With regard to the added secondary benefits of *Trichoderma spp* on plant growth, two main mechanisms of action have been revealed in the past decade: (i) increased plant nutrition through solubilization and/or enhanced uptake of macro and micronutrients and (ii) production of plant growth factors (John *et al*., 2010). These mechanisms are responsible for the so called 'bio-fertilizer effect' of *Trichoderma*.

Trichoderma strains are known to associate with plant roots and root ecosystems. They are also plant symbionts and opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi producing compounds that stimulate growth and plant defense mechanisms (Harman *et al*., 2004). This mechanism includes plant root colonization and rhizosphere modification.

3.10.5 Inactivation of Pathogen's 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 (John *et al., 2010*). Besides, Harman *et al*., (2004) reported that *T. viride* and *T. harzianum* secrete extra cellular cellulase. These lytic enzymes are probably responsible for hyphal lysis through the digestion of major cell wall components which are necessary for pathogens to penetrate plant surfaces (Arst and Penalva, 2003). It is believed that these enzymes act synergistically with the antibiotics to inhibit the growth of fungal pathogens. 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.

3.10.6 Fungicide Tolerance

Some soil borne root infecting fungi are difficult to eradicate because they produce resting structure like sclerotia, chlamydospores or oospores for their survival for a longer period of time under adverse environmental conditions (Omer and Shahzed, 2007). Use of fungicides for the control of soil borne diseases is costly and also produces environment and health hazards to men and also adversely affects the beneficial microorganisms in soil (Dluzniewska, 2003). This has diverted the attention of plant pathologist towards alternate methods for the control of plant diseases.

The combined use of biocontrol agents and chemical pesticides has attracted much attention in order to obtain synergestic or additive effects in the control of soil borne diseases. Reduced amount of fungicide can stress and weaken the pathogen and render its propagules more susceptible to subsequent attack by the antagonist (Hjeljord and Tronsmo, 1998). Srinivas and Ramakrishnan (2002) have reported that integration of biocontrol agents and commonly used fungicides showed positive association by reducing the seed infection compared to fungicide and the fungal antagonists individually.

Trichoderma spp are known to suppress infection of root by soil borne pathogens like *Macrophomina phaseolina, Rhizoctonia solani, Fusarium spp* and *Pythium spp* on various crops (Adekunle *et al*., 2001; Benitez *et al*., 2004). Species of *Trichoderma* also have growth promoting capabilities that may or may not be integral to biological control *T. harzianum* has shown effective control of root infecting fungi and root knot nematodes. *T. harzianum* isolated from rhizome rot suppressive soils reduced the disease and increased plant growth and yield (Benitez *et al*., 2004; Dubey *et al*., 2007). It has been reported that many *Trichoderma* species has an innate and/or induced resistance to many fungicides but the level of resistance varies with the fungicide (Omer and Shahzad, 2007).

3.11 Organic solvents for Antifungal extraction

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. 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 (Howell, 2003).

3.12 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).

3.12.1 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. 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, 2011).

3.12.2 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; Suleiman *et al*., 2010).

3.12.3 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 [3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium (MTT), p-iodonitrotetrazolium violet (INT), 2,3,5-tripheny tetrazolium chloride (TTC), 2,3,5-triphenyltetrazolium bromide (TTB)] are usually used, which are converted by the dehydrogenases of living microorganisms to intensely coloured formazan. 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 (Hamburger and Cordell, 1987). 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 (Shahverdi *et al*., 2007).

Suleiman *et al* (2004) 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).

4. MATERIALS AND METHODS

4. 1 *Trichoderma* isolates and Test pathogen

Biocontrol agents and Coffee wilt disease pathogen were obtained from Mycology Laboratory; Department of Microbial, Cellular and Molecular Biology, College of Natural Sciences, Addis Ababa University. All *Trichoderma* isolates used in this study were previously isolated from soil collected from Mana, Gomma, Kerssa, Gera and Kossa woredas of Jimma zones. All the isolates were designated as AUT1-6 which stands for Addis Ababa University *Trichoderma*. Isolates were different in colony morphology, spore production and color. Screening, testing, characterization and in-vitro antagonistic evaluation of potential antifungal compounds were conducted in Mycology and Applied Microbiology Research Laboratories while extraction and purification processes by chromatographic separation and detection were done in Analytical laboratory, Department of Chemistry, Addis Ababa University.

Table 1. Designation of *Trichoderma* isolates

***Trichoderma* Isolates**	**Designation**
Addis Ababa University *Trichoderma* 1	AUT1
Addis Ababa University *Trichoderma* 2	AUT2
Addis Ababa University *Trichoderma* 3	AUT3
Addis Ababa University *Trichoderma* 4	AUT4
Addis Ababa University *Trichoderma* 5	AUT5
Addis Ababa University *Trichoderma* 6	AUT6

4.2 Sterilization and Maintaining 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 dry in hot air oven at 80 ^{O}C for 45-60 minutes. The maintenance of cultures of *Fusarium xylarioides* isolates and biological control agents (*Trichoderma spp*) were maintained on Potato Dextrose Agar (PDA) slants in the plugged test tubes. The slants were stored in the refrigerator at 4 ^{O}C until further study.

4.3 Antagonism of *Trichoderma* isolates against *Fusarium xylarioides*

Six different isolates of *Trichoderma* were individually tested for their antagonistic property against *F. xylarioides* using the dual culture technique. Agar pieces of 6 mm diameter of *F. xylarioides* and *Trichoderma* isolates were taken from pure cultures of 8 days old, and placed on plates of PDA in a distance 6 cm between *F. xylarioides* and the *Trichoderma* strain. Plates were incubated at room temperature for 8 days. Five plates were prepared for each isolate. Plates inoculated with *F. xylarioides* alone served as control. Clear zone of inhibition (CZI) was also determined by measuring the clearance between the colony margins of the *F. xylarioides* and *Trichoderma* isolates. Radial growth of *F. xylarioides* was measured every 2 days after inoculation. Percentage inhibition of radial growth (PIRG) was calculated following the formula suggested by Rita and Tricita (2004):

$$\% \text{ Mean inhibition effect of isolates} = \frac{\mathbf{R1 - R2}}{\mathbf{R1}} \times 100$$

Where R1 = the colony diameter of the pathogen in the control

R2 = the diameter of the pathogen during antagonistic interaction.

Antagonism was assessed in semi-quantitative means (Rita and Tricita, 2004): > 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.

4.4 Mycoparasitism Test using Slide Culture

For slide cultures, a clean slide was placed on an L-shaped glass rod in a 9 cm diameter Petri dish and autoclaved. Then a small amount of molten water agar was poured and evenly spread over the slide to make a thin agar film on the slide. One end of the slide was kept free of the medium to facilitate handling. The 5 mm discs of one week old growing colonies cut from the margin of *F. xylarioides* and *Trichoderma* isolates were placed on the opposite sides of the slide 3 cm apart on the PDA surface and incubated at 25 ± 2 ^{o}C for 5-7 days. A few ml of sterile water was added to the Petri dish to prevent drying. At the end of incubation period, regions where the

hyphae of *Trichoderma* met the hyphae of the pathogen was observed under Olympus compound microscope for the presence of coil formation and penetration structures, or wall disintegration.

4.5 Antifungal Activity of Crude Extracts

For antifungal activity test, crude extracts obtained from both solid and liquid culture media were mixed and tested against the test pathogen after 250, 500, 750 and 1000 µl of crude sample was amended with 25 ml of PDA media and the test pathogen was seeded on it. The antifungal activity of the crude extract against the test pathogens was recorded as the radial growth inhibition. For positive control, culture media without crude extracts were also seeded with the test pathogen and incubated for 7 days at 25 ^{O}C.

4.6 Preparation of Culture Media for the Growth of *Trichoderma* isolates

To test the production of inhibitory substances in solid media, *Trichoderma* isolates and *Fusarium xylarioides* were grown on Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA). *Trichoderma* isolates were grown on sterilized petri dishes containing 25 ml PDA/ MEA media and incubated at room temperature for 8 days. For liquid culture media, flasks containing 100 ml of PDB in each flask (250 ml) was used to culture the isolates of *Trichoderma* separately. Each flask was inoculated with 5 disks of (5mm diameter each). The inoculate flasks were incubated, at 25 ^{O}C on shaker at 120 revolution per minute (rpm) for 21 days. After 21 days of incubation the broth was filtered through Whatman No. 1 filter paper and subsequently the filtrate of each isolate was centrifuged, at 5000 rpm for twenty minutes (Aneja, 2005) to make it cell free.

4.7 Extraction Methods for Antifungal Compounds from *Trichoderma* isolates

4.7.1 Extraction of Antifungal Compounds from Solid Culture Media

For extraction of antifungal compounds from fungal mycelium or culture media different organic solvents: chloroform ($CHCl_3$), ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc), hexane (n-C_6H_{14}) and butanol (BuOH) were used. Extraction of antifungal compounds was done by using rotary evaporator by taking different boiling point of the solvent systems.

To harvest and extract antifungal compounds 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 or 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 Thin Layer chromatography (TLC). Moreover, the crude extracts were tested against the test pathogen by using bioassay methods. In order to yield pure antifungal compounds, several steps needs to be followed: extraction, isolation, purification, separation and detection of active antifungal compounds from *Trichoderma* isolates mycelia according to the scheme shown in Figure 2.

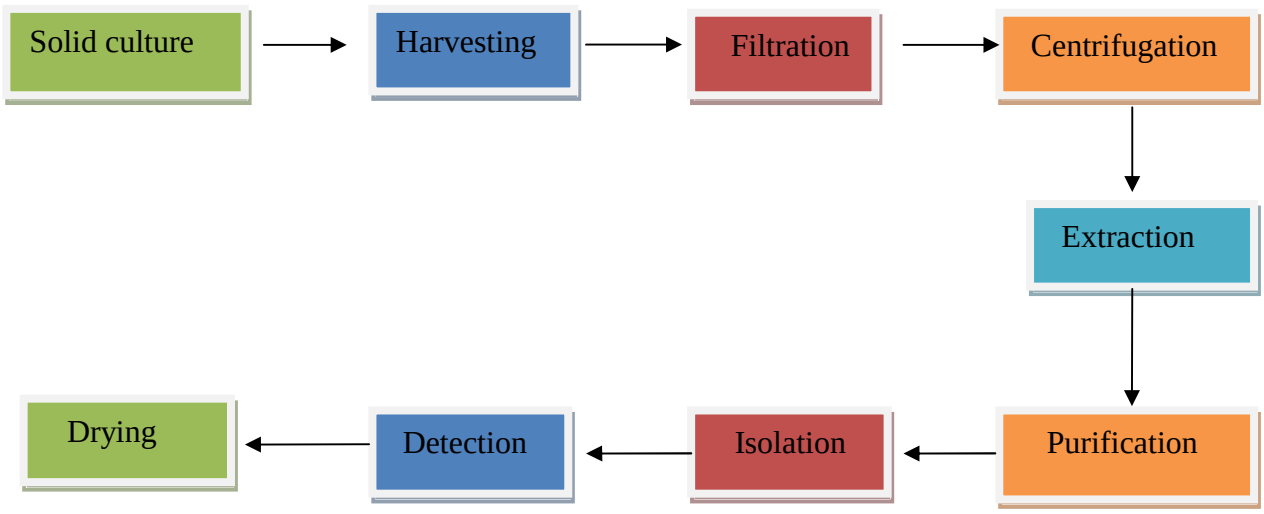

Figure 2. General overview of extraction of antifungal compounds from *Trichoderma* isolates (Dennis and Webester, 1981).

4.7.2 Extraction of Antifungal Compounds from Liquid Culture Media

The *Trichoderma* isolates were cultured in liquid potato dextrose medium at 25 ^{O}C for 21 days. After incubation of 21 days, each broth culture was filtrated twice by using Whatman No.1 filter paper under aseptic condition and centrifuged at 5,000 rpm for 20 min to make it cell free. To 100 ml of culture filtrate, 25 ml of each solvent was added to each broth culture filtrate, shaken for 5 min and the solvent and aqueous layer were separated using solvent fractioning (Howell, 2003). The culture filtrate (solvent layer) was used for extraction of

antifungal metabolites. The culture filtrate crude extract was then examined against the test pathogen by using bioassay methods.

4.7.3 Separation of culture filtrates by Solvent Fractioning

The initial separation of active substances from the culture filtrate follows the solvent fractioning procedure shown in Figure 2 (Dennis and Webster, 1981), using n-hexane, butanol, $CHCl_3$, EtOAc, MeOH and EtOH organic solvents. Briefly, the same amount of each solvent was equaled and mixed by shaking for 5 minutes in a separatory funnel with 150 ml of the culture filtrate and separation was made between the solvent and aqueous layers (non-volatile compounds) (Zigong *et al*., 2000).

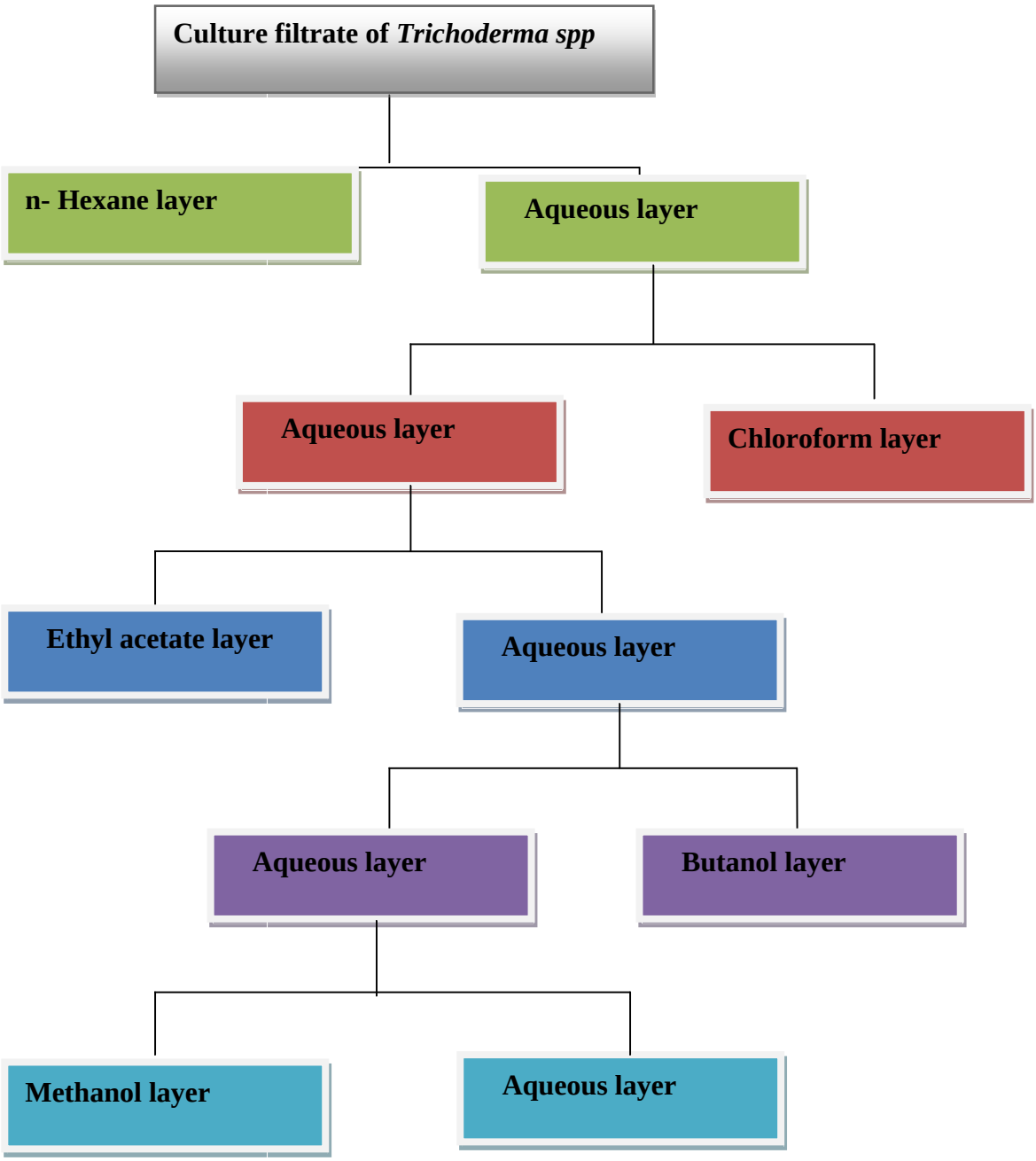

Figure 3. Initial separation procedures for antifungal compounds from the culture filtrate of *Trichoderma* isolates (Bailey and Lumsden, 1998 with some modification)

4.8 Purification Methods for Antifungal Compounds

4.8.1 Detection and Assaying of Active Antifungal Compound(s) by TLC

TLC was used for separation of extracts and fractions, and to screen the qualitative purity of the compound using TLC aluminum sheets silica gel 60 F_{254} pre-coated 20 x 20 cm as described by El-Mougith *et al* (1986). Attempts were made to isolate the active principle of the antifungal compounds and for this the dissolved crude residue was subjected to TLC analysis. Standard chromatograms of fungal extracts was prepared by applying 20 µL solution to a silica gel TLC plate. For successful detection and separation of antifungal metabolites, different solvent systems were tested using TLC (aluminum sheet Silica gel 60 F_{254}, Merck).

Table 2. Solvent systems used for TLC analysis of crude extracts from *Trichoderma* isolates

Solvent System (SS)	**Composition Solvent System (SS)**	**Ratio (v/v)**
1	Hexane: acetone: EtOAc	1:8:1
2	$CHCl_3$: MeOH : EtOAc	2:6:2
3	$CHCl_3$: Hexane: Butane	2:6:2
4	$CHCl_3$: Acetonitrile: DCM	3:4: 3
5	Butane: Acetic acid: H_2O	4:1:5
6	Hexane: MeOH: H_2O	3:3:4
7	Acetone: MeOH: Acetic acid	2:2:6
8	Hexane : Benzene :$CHCl_3$	4: 2 : 4
9	Acetone: Acetic Acid: Toluene	1: 1: 8
10	Hexane : Benzene: $CHCl_3$: Toluene	5: 4: 5: 6
11	Hexane: Benzene: MeOH	3: 2: 5
12	Hexane: EtOH : MeOH	5: 3: 2
13	Hexane: Benzene: $CHCl_3$	2: 5: 3
14	Hexane: Toluene: $CHCl_3$	3: 4: 3

TLC was performed in sealed glass chambers. Solvents were added separately into each 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 30 min to saturate the atmosphere of the chamber before adding a TLC plate. TLC (aluminum sheet Silica gel 60 F_{254}, Merck) 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 /compounds was then detected by their UV absorbance at the wavelength 254 nm and/or by spraying the TLC plates with spraying reagents followed by heating at 110 ^{o}C (Geetha *et al.*, 2003). The distances of the spots and the solvent were measured to calculate the relative mobility of retardation factor (R_f). Active spots were located and detected from a developed TLC by direct bioautography.

$$\mathbf{R_f\ value} = \frac{\text{Distance moved by the crude sample from the origin to spot centre}}{\text{Distance moved by solvent systems from the origin to solvent front}}$$

4.8.2 The use of TLC with Direct Bioautography for Antifungal Analysis

The direct bioautography was applied by transferring the microorganism directly on TLC plate. Active antifungal extracts were transferred from the stationary phase to the agar layer (which contains the microorganism) by a diffusion process. After incubation, the plate is sprayed with a tetrazolium salts such as 2, 3, 5- Triphenyltetrazolium bromide (TTB), 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. 100 µl of each of the extracts were applied on the spots of two TLC plates 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 25-50 ml inoculums solution spray was prepared containing 1 x 10^6 spores/ml of 7 day test fungus, *F. xylarioides*. 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 ^{o}C and then sprayed with 2 mg/ml solution of 2, 3, 5-triphenyltetrazolium bromide (TTB) 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). 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.

4.9 Bioassay of Antifungal Compounds from *Trichoderma* isolates

4.9.1 Agar Disc Diffusion Assay

Crude extracts, fractions and pure antifungal compounds were tested for antifungal activity against *F. xylarioides*. The agar diffusion assay was performed according to Kirby-Bauer method (Howell, 2003). For screening, aliquots of the test solutions was applied to sterile filter-paper discs (6 mm, diameter) to give a final disc loading concentration of 300, 200, 100, 50 and 25 µl/disc) for crude extracts. The antifungal activity was recorded as the zone of inhibition surrounding the disc at which the diameter was measured in mm.

4.9.2 Effect of Non-Volatile (Culture Filtrate) Compounds from Antagonists on the Radial Growth of *F. xylarioides*

The non-volatile **(**culture filtrate) compounds of *Trichoderma* isolates were investigated to determine their influence on the mycelial growth of the test fungus (*F. xylarioides*) on PDA. The sterilized filtrates were amended in PDA to make 5, 10, 20 and 40% (v/v) concentration in Petri plates. The solidified agar plates in triplicates were inoculated at the centre with 5 mm diameter mycelial disc of pathogen and incubated at 25 ± 2 ºC for 8 days. The Colony diameter was measured and percent inhibition of radial growth was calculated. The measurements were recorded after 8 days of inoculation. The percent inhibition of mycelial growth was determined by the formula ((Rita and Tricita, 2004; Tarman, 2011):

$$\% \text{ Mean inhibition effect of isolates} = \frac{\mathbf{R1 - R2}}{\mathbf{R1}} \times 100$$

Where R1 = the colony diameter of the pathogen in the control

R2 = the diameter of the pathogen during antagonistic interaction.

4.10 Methods of Data Analysis

Comparative analysis of experimental results was analyzed by using spreadsheet software (Microsoft Excel 2007).

5. RESULTS

5.1 In-Vitro Evaluation of Antagonistic Activity of *Trichoderma* isolates

Table 3 shows that all isolates of *Trichoderma* tested were able to inhibit the growth of *F. xylarioides* in vitro (between 59.5% and 77%) after 8 days of incubation. The highest mean inhibitory effect the on growth of test pathogen was achieved by AUT2 (77.0%) followed by AUT3 (72.9%) and AUT1 (67.6%) while AUT6 (59.6%) isolate showed the lowest mean inhibitory effect on *F. xylarioides* after 8 days of incubation (Figure 4).

Table 3. In-vitro antagonistic effect of *Trichoderma* isolates on radial and mycelial growth inhibition of *F. xylarioides* on PDA after 8 days of incubation

***Trichoderma* Isolates**	**Percent of Inhibition *(*Days*)***							
	2		4		6		8	
	Radial growth (mm)	PIRG (%)	Radial growth (mm)	PIRG (%)	Radial growth (mm)	PIRG (%)	Radial growth (mm)	PIRG (%)
AUT1	5.0	28.6	5.4	50.9	6.0	57.1	6.00	67.6
AUT2	5.23	25.3	5.75	52.0	4.75	66.0	4.25	77.0
AUT3	4.92	29.7	5.25	52.3	4.70	66.4	5.00	72.9
AUT4	5.75	15.4	6.75	60.7	6.50	53.6	7.00	62.1
AUT5	5.60	20.0	6.25	67.9	6.10	56.4	7.25	60.8
AUT6	5.00	28.6	6.75	60.7	6.2	55.7	7.50	59.5
Control	7.00	-	11.00	-	14.00	-	18.5	-

AUT1, AUT2 and AUT3 isolates were rapidly grow in the form of powdery wide spread throughout the Petri plates. These isolates failed to develop inhibition zone, since they grew in the form of widespread powders and occupied over all the spaces. The interaction was due to the competition for spaces and nutrients rather than forming inhibition zone. On the other hand, AUT4 and AUT5 isolates were produced zone of inhibition indicative of the formation of secondary metabolite(s) inhibiting *F. xylarioides* and there is no complete overgrowth on the test pathogen while AUT6 isolate secretes some oily substances towards the test pathogen and it forms intermediate zone of inhibition as well as overgrew on the hyphae of *F. xylarioides*. The pattern of zone of inhibition between the two organisms clearly indicated that *F. xylarioides* initially posed inhibition to the progress of *Trichoderma* towards itself by secreting deleterious

metabolites but it also suffered from the stress which resulted from metabolites released by *Trichoderma*.

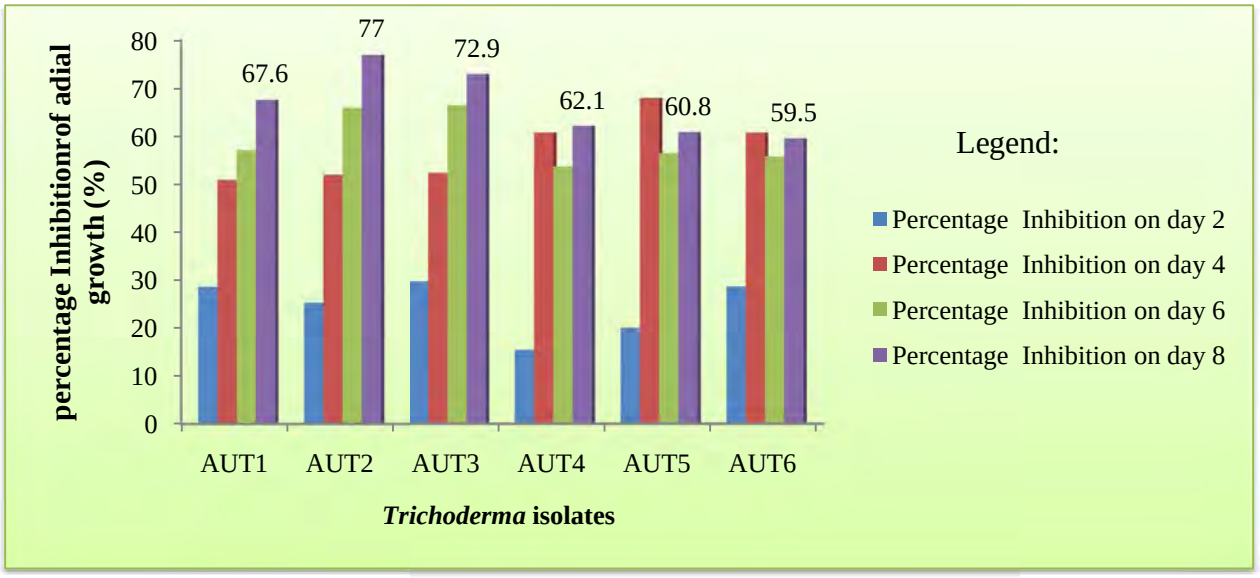

Figure 4. Percentage inhibition of radial growth of *Trichoderma* isolates against *F. xylarioides*

Considering difference in growth of *F. xylarioides* under direct intense influence of *Trichoderma* isolates, due to rapid spread of *Trichoderma* on dual culture plate with the advancement of interaction, the test pathogen was almost encircled by *Trichoderma* isolates such that even the distal side of *F. xylarioides* colony came under influence of *Trichoderma* metabolites. Later, in some cases, *Trichoderma* trapped *F. xylarioides* from all sides. Therefore, no side of *F. xylarioides* colony was free from influence of *Trichoderma* isolates.

5.2 Mycoparasitism test using slide culture

In the slide-culture assay, once the fungal hyphae came into contact, the *Trichoderma* isolates attached to the pathogenic fungus, and were able to coil around it, form hook-like structures and form clear zones that aided in penetrating the host cell wall (Figure 5). Macroscopic observations showed that the borderlines where *Trichoderma* isolates and *F. xylarioides* encountered each other were offensive as well as defensive mechanisms were activated. 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*. *Fusarium* mycelia away from *Trichoderma* showed smooth, hyaline and normal morphology.

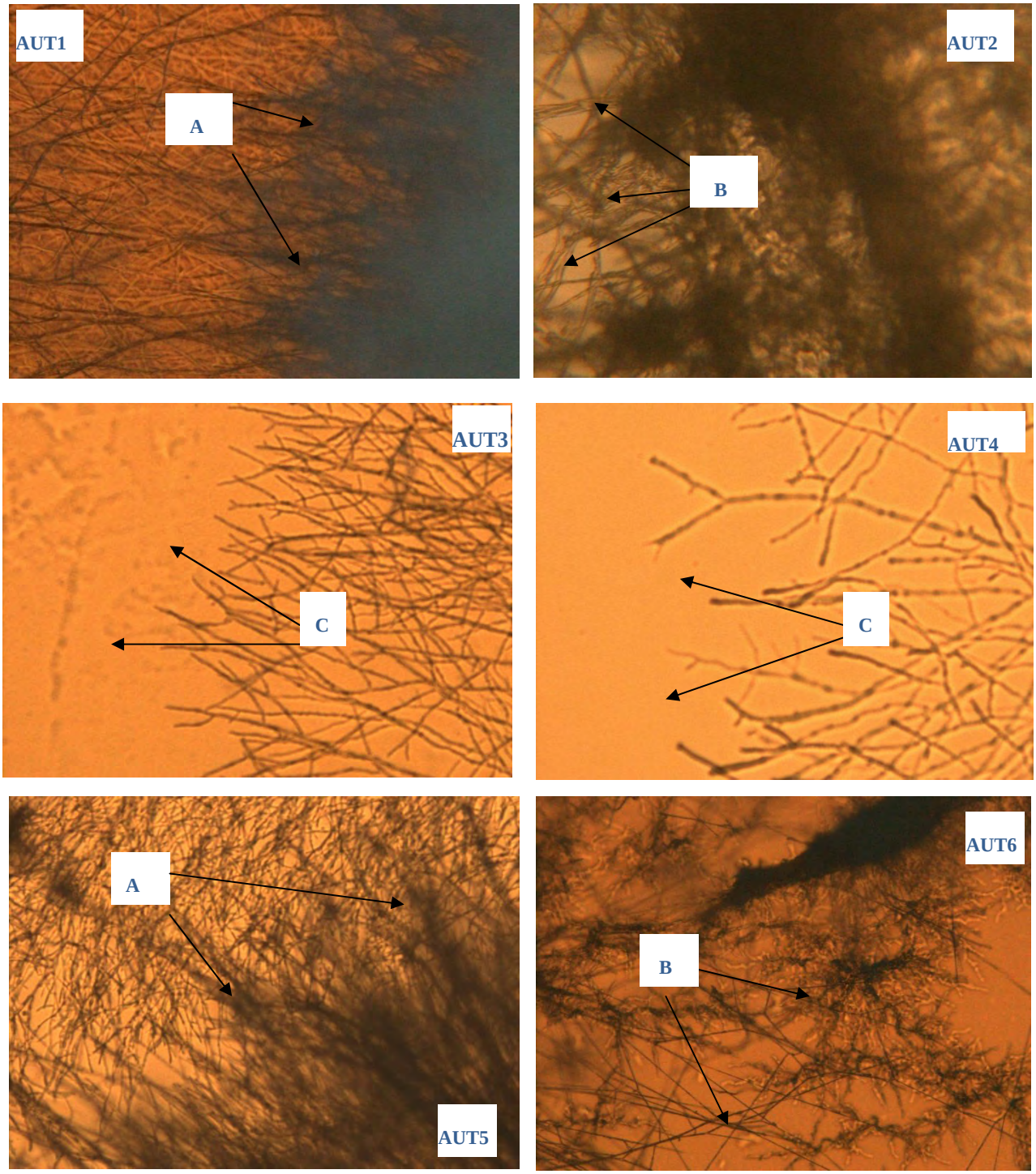

Figure 5. Antifungal activity (using the slide culture method) of *Trichoderma* isolates against *F. xylarioides.* The slide culture of the fungi was incubated on a thin layer of water agar for 7 days at 25 °C. The coil structure formation (A), hook-like structure (B) and clear zone (C) were observed.

5.3 Antifungal Activity of Crude Extracts

The inhibition diameters for the crude extracts were computed against test organism as given in Table 4. Accordingly, isolate AUT3 with hexane extract and AUT4 with butane extract showed complete inhibition while isolate AUT5 with ethanol extract and AUT6 methanol extract exhibited a moderate to high antifungal activity over the tested organism. The crude chloroform extract showed least potent inhibitory effect on the growth of *F. xylarioides* (2.7%) at a concentration of 750 µl (Table 4).

Table 4. Antagonistic activity of crude extracts from *Trichoderma* isolates at different concentration against the radial growth of *F. xylarioides* (CI= indicate Complete Inhibition)

***Trichoderma* Isolates**	***Concentration of Crude Extract* (µl)**								
	Extract	**250**	**PIRG (%)**	**500**	**PIRG (%)**	**750**	**PIRG (%)**	**1000**	**PIRG (%)**
AUT1	$CHCl_3$	15	18.9	14	24.3	18	2.7	16	13.5
AUT2	EtOAc	8	56.8	11	40.5	10	46.0	5	73.0
AUT3	Hexane	CI		CI		CI		CI	
AUT4	Butane	CI		CI		CI		CI	
AUT5	MeOH	10	46.0	6	67.6	5	73.0	5	73.0
AUT6	EtOH	6	67.6	7	62.2	5	73.0	5	73.0
Control	18.5								

5.4 Extraction of Antifungal Compounds from *Trichoderma* isolates

For extraction purpose of antifungal compounds from *Trichoderma* isolates, the preferred solid culture media for the production of dense mycelial cell was MEA than PDA while for bioassay purposes PDA was the best media. All organic solvents were successful in extraction of the crude compounds from the antagonist except hexane which is not successful in extraction process since there is no miscibility between the solvent and fungal mycelium. Among the organic solvents used for separation processes, only $CHCl_3$ and hexane were the best solvent that forms layers readily where others were mixed together and no layer was formed.

5.5 Analysis of Crude Extracts and Detection of R_f Values of Antifungal Compounds on TLC

Separation of crude extracts of *Trichoderma* isolates into its individual components was conducted on TLC using different solvent systems as a mobile phase. The optimum separation of compounds by TLC was usually achieved when R_f values are in between 0.2-0.8. During TLC development choosing appropriate mobile phase was the critical condition to achieve optimum R_f values for crude extracts and the crude sample must have a relatively equal affinity for the solvent and the packing material. In TLC, the type and composition of the eluent is one of the variables influencing the separation processes, since it shows differences in chemical composition of the crude extracts. The solubility, affinity and the resolution of the solvent system greatly determines the R_f values of the crude extracts.

Thin layer chromatography (TLC) is a rapid and effective means of obtaining a characteristic analytical finger print of a crude extract. In this study, 14 solvent systems were primary screened of which seven were selected for further TLC and bioautographic analysis (Table 5). The optimum R_f value was obtained by only three extracting solvents: $CHCl_3$, EtOH and Butane. Methanol and ethyl acetate crude extracts were not eluted sufficiently and their R_f values were approach zero and/or 1. This may be to the high affinity of crude extracts to the stationary phase than to 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 mobile phases than stationary phase, the crude samples reach the solvent front (R_f value was too high).

Table 5. Optimum solvent systems and R_f values of antifungal compounds on TLC analysis

Solvent System (SS)	Composition of solvent system	Ratio of SS (V/V)	Type of Extract	R_f Values of Antifungal Compounds					
				AUT 1	***AUT 2***	***AUT 3***	***AUT 4***	***AUT 5***	***AUT 6***
SS_8	*n-Hexane: Toluene: Chloroform*	3: 4: 3	*$CHCl_3$*	0.89	0.8	0.88	0.84	0.85	0.75
			EtOH	0.8	0.8	0.8	0.8	0.8	0.84
			Butanol	0.8	0.62	0.57	0.77	0.65	0.58
SS_9	*n-Hexane : Benzene : Chloroform*	4: 2 : 4	$CHCl_3$	0.4	0.88	0.46	0.87	0.44	0.48
			EtOH	0.16	0.14	0.14	0.16	0.16	0.16
			Butanol	0.50	0.50	0.54	0.54	0.57	0.57
SS_{10}	*Acetone: Acetic Acid: Toluene*	1: 1: 8	$CHCl_3$	0.82	0.84	0.86	-	0.84	0.84
			EtOH	0.66	0.62	-	-	-	0.50
			Butanol	0.76	0.78	0.76	0.78	0.78	0.78
SS_{11}	*n- Hexane : Benzene: $CHCL_3$: Toluene*	5: 4: 5: 6	$CHCl_3$	0.67	0.64	0.63	0.57	0.64	0.69
			Butanol	0.63	0.65	0.73	0.75	0.79	0.76
SS_{12}	*n-Hexane: benzene: MeOH*	3: 2: 5	*Butanol*	0.72	0.76	0.72	0.68	0.62	0.58
SS_{13}	*n-Hexane: EtOH : MeOH*	5: 3: 2	*EtOH*	0.58	0.58	0.6	0.87	0.88	0.8
SS_{14}	*n-Hexane: Benzene: Chloroform*	2: 5: 3	*$CHCl_3$*	0.35	0.36	0.36	0.4	0.35	0.36
			EtOH	0.18	0.22	0.25	0.83	0.83	0.20

****AUT=** Addis Ababa University *Trichoderma*

5.6 The use of TLC with Direct Bioautography for Antifungal Analysis

Bioautography was used to screen for antifungal compounds to obtain more information on the diversity and active antifungal compounds present in different extracts. Crude extracts of *Trichoderma* isolates were subjected to TLC bioautography to measure their retention frequency (R_f), inhibition zones and to determine the active band (s).

The chloroform, ethanol and butanol extracts of *Trichoderma* isolates were screened for their antifungal activity. A simple bioautographic procedure, involving spraying suspensions of the *F. xylarioides* on TLC plates developed in solvents of varying polarities to detect the number of active antifungal compounds present in the extracts. Bioautography tests revealed that most of the crude extracts from *Trichoderma* isolates exhibited antifungal activity against *F. xylarioides*. This activity was denoted by white spots against a red-purple background on the TLC plates after spraying with TTB. White areas indicate where reduction of TTB to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested fungi, *F. xylarioides*.

Fifteen (15) TLC plates for seven (7) solvent systems were tested in the bioautographic procedure. The growth of the fungus, *F. xylarioides* was inhibited by many compounds present in the extracts. AUT5 (Table 6) appeared to be the *Trichoderma* isolate having crude extract with highest number of inhibition spots when compared with other isolates tested against the test fungi where as the lowest number of inhibition spots where observed in AUT6 (Table 7). On the other hand, the highest inhibition zone was observed in AUT6 (51 mm) (Table 7) with ethanol extract (SS8) at R_f (0.43) followed by AUT5 (44 mm) with ethanol extract (SS8) at R_f (0.43) and AUT1 (41 mm) with butane extract (SS12) at R_f (0.52). The lowest inhibition zone on bioautography was 4 mm (Table 6).

It has been demonstrated in this study there were 60 zones of inhibition detected with different extracts, test fungus and solvent systems used in *Trichoderma* extracts. Different solvent systems were analyzed to determine which TLC solvent system separated the highest number of active compounds. Solvent system 8 (SS8) was the best solvent (15 active spots) followed by SS9 (14 active spots) to separate active antifungal compounds from *Trichoderma* isolates. This implies that polar systems separated less active compounds compared to non-polar system. Moreover, the best extracting solvent of active antifungal compound was $CHCl_3$ (25) followed by EtOH (20) and butane (15). The R_f values of 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 AUT5 and AUT6 against *F. xylarioides* were presented in Table 6 and 7 respectively.

Table 6. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of AUT5 against *F. xylarioides* (A= Represent R_f values of Reference chromatogram, B = Represent R_f values of active compound on Bioautogram)

Solvent system	Extracts	R_f values		Inhibition Diameter (mm)	Active spots	Total Active spots
		A	B			
SS8	EtOH	**0.8**	**0.43**	**44**	**1**	**12**
	Butane	**0.65**	**0.89**	**12**	**1**	
SS9	$CHCl_3$	**0.44**	**0.19**	**14**	**1**	
	EtOH	**0.16**	**0.77**	**10**	**1**	
SS10	$CHCl_3$	**0.84**	**0.93**	**10**	**1**	
	Butane	**0.78**	**0.62**	**17**	**1**	
SS11	$CHCl_3$	**0.64**	**0.66**	**4**	**1**	
	Butane	**0.79**	**0.77**	**4**	**1**	
SS13	EtOH	**0.88**	**0.88**	**5**	**1**	
SS14	$CHCl_3$	**0.35**	**0.4**	**20**	**1**	
	EtOH	**0.83**	**0.24, 0.83**	**14, 5**	**2**	

Table 7. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of AUT6 against *F. xylarioides*

Solvent system	Extracts	R_f values		Inhibition Diameter (mm)	Active spots	Total Active spots
		A	B			
SS8	EtOH	**0.84**	**0.43**	**51**	**1**	**8**
SS9	$CHCl_3$	**0.48**	**0.54, 0.77**	**10, 13**	**2**	
	EtOH	**0.16**	**0.54**	**12**	**1**	
SS10	$CHCl_3$	**0.84**	**0.47**	**17**	**1**	
SS12	Butane	**0.58**	**0.37**	**32**	**1**	
SS13	EtOH	**0.8**	**0.86**	**5**	**1**	
SS14	$CHCl_3$	**0.36**	**0.4**	**13**	**1**	

In some cases *F. xylarioides* does 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. The non-activity of the other *Trichoderma* isolates 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 growth but no inhibition was observed. The non-activity of these extracts in bioautography may possibly be explained by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC separation.

In general, chloroform was the best extractant of more antifungal compounds from *Trichoderma* isolates. Most compounds were extracted in low polarity (chloroform), fewer in high polarity (ethanol) and least in intermediate polarity (butane) solvents. A number of isolates used in this study showed promising biological activity.

5.7 Bioassay of Antifungal extracts from *Trichoderma* isolates

Screening programs for biologically active natural compounds require the right bioassays. A number of bioassay methods were used to test, evaluate and characterize the antifungal activities from *Trichoderma* isolates against the coffee wilt disease causing pathogen, *F. xylarioides* in Ethiopia**.**

5.7.1 Agar Disc Diffusion Assay

The movement of molecules through the matrix that was formed by gelling of the agar under controlled condition was used to test, determine and evaluate the susceptibility or resistance of the test pathogen to the antagonist. In this study, the antagonistic activity of crude extracts of MeOH, EtOH, EtOAc and butane were less effective even if there were diffusible. Moreover, $CHCl_3$ and hexane crude extracts antagonistic activity was null since they do not easily diffuse into the agar. In general, the test pathogen was not susceptible to the antagonists.

5.7.2 Effect of Non-Volatile Antifungal Compounds on Radial Growth of *F. xylarioides*

The culture filtrate of antifungal compounds isolated from *Trichoderma* isolates were used to evaluate the antifungal activity against test organism. Different concentrations comprising of 5, 10, 20 and 40% of the culture filtrate were used to test antifungal activity and the minimum inhibitory concentration was determined. On potato dextrose medium, different *Trichoderma*

isolates show different percentage of inhibition against the test pathogen. The culture filtrate (non-volatile compound) from all the *Trichoderma* isolates exhibited growth inhibition. It was observed that culture filtrate from AUT3 (78.4%) shows high antagonistic activity at a concentration of 40% where as AUT2 exhibited 72.9%. The minimum inhibitory concentration was observed in isolate AUT5 and AUT6 56.8% for both isolates at 5% concentration of culture filtrate. In general, maximum and minimum inhibitions were recorded in isolate AUT3 containing 40% culture filtrate and in isolate AUT1 containing 20% of culture filtrates, respectively (Table 8).

Table 8. Radial growth inhibition of *F. xylarioides* treated with different concentration of *Trichoderma* isolates culture filtrate (non-volatile compounds) after 8 days of incubation

***Trichoderma* Isolates**	**Radial growth of *F. xylarioides*, mm**							
	5%	**PIRG (%)**	**10%**	**PIRG (%)**	**20%**	**PIRG (%)**	**40%**	**PIRG (%)**
AUT1	11	40.5	14	24.3	15	18.9	10	46.0
AUT2	10	46.0	10.5	43.2	9	51.4	5	72.9
AUT3	8	56.8	7	62.2	6	67.6	4	78.4
AUT4	8	56.8	7.5	64.9	9	51.4	10	46.0
AUT5	**6.5**	**64.9**	6.75	63.5	8	56.8	10	46.0
AUT6	**9.5**	**48.6**	11	40.5	12.5	32.4	10.5	43.2
Control	**18.5**							

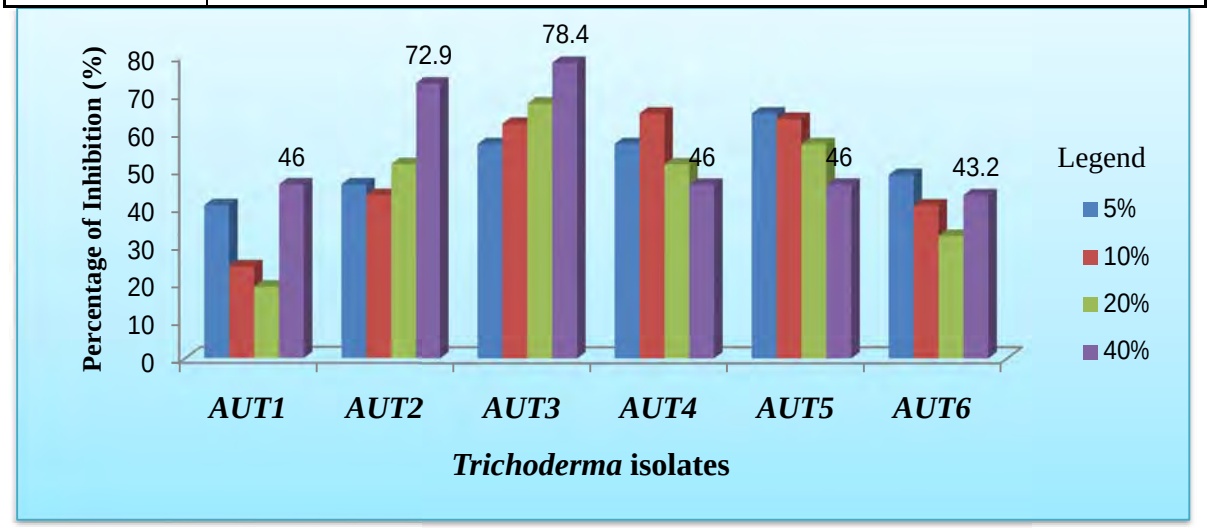

Figure 6. Percentage inhibition of *Trichoderma* isolates on radial growth of *F. xylarioides* from culture filtrate (non-volatile compounds)

6. DISCUSSION

Trichoderma spp biocontrol potential was as a result of a number of qualities which include antagonism, antibiotics and degrading enzymes which digest the cell wall, and similar observations were reported by Kamal (2009); Hanson and Howell (2004). According to Rita and Tricita (2004) (64) semi-qualitative means, all *Trichoderma* isolates tested in this study showed high biocontrol activity against the test pathogen except AUT6 which showed moderate antagonistic activity (Figure 4). Variations in the inhibitory potential may be due to the differences in the quantity and quality of the inhibitory substances produced by the antagonists which agrees with Hanson and Howell (2004).

Similar results showed colonies of *Trichoderma* isolates always grew faster than the test pathogen under in-vitro culture. Rapid growth of *Trichoderma* was observed to be an important condition in competition with plant pathogenic fungi for space and nutrients. Competitiveness is based on rapid growth and the production of various asexual generated conidia and chlamydospores (Chet *et al.,* 1998). The time taken by each *Trichoderma* isolate to parasitize *F. xylarioides* has been given/ indicated in Table 3. *Trichoderma* isolate AUT1 and AUT2 were the quickest of all isolates in crossing the zone of inhibition and parasitizing *F. xylarioides*. On agricultural field, dominance of biocontrol agent through its high growth rate and offensive mechanisms against pathogen was decisive in manifestation of disease control (Zemang *et al*., 1996). Parasitism by *Trichoderma* was its powerful weapon in destruction of pathogen. But, *Trichoderma* must overwhelm pathogen before the pathogen proliferates and infects germinating of the plant seeds. Therefore, a measure of speed with which biological control on pathogen is achieved is important when searching an aggressive strain of *Trichoderma*.

In dual culture, AUT2 and AUT3 isolates inhibited the radial growth of *F. xylarioides* with the greatest growth reduction of 77% and 72.9% respectively. These *Trichoderma* isolates form coiled structures around the hyphae of *F. xylarioides*. This coiling is a characteristic of the interaction between mycoparasitic and phytopathogenic fungi leading to penetration of the cell wall (Harman *et al*., 2004). In general, *Trichoderma* isolates which inhibited the growth of CWD could be used in the biological control of the diseases caused by *F. xylarioides*.

As *Trichoderma* isolates are a promising antagonistic for major soil borne diseases, studies on its antifungal substances have extensively been made in order to find out the best antifungal compound and the mode of its action. In this study, antifungal compounds were extracted by different organic solvents and the highest antifungal activity was achieved by chloroform, ethanol and butane on TLC coupled with bioautography where as hexane and butane crude extracts were highly significant in reducing the mycelial growth of the test pathogen using bioassay techniques in-vitro.

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). According to this study the highest inhibition zone on bioautographic assay was observed in AUT5 and AUT6 with ethanol extract at R_f value of 0.43 (Table 5 and 6). Because, the R_f value is constant for the same compound under defined conditions and the presence of clear spots with the same R_f value may mean that the same compounds are probably responsible for the antifungal activity. TLC bioautographic methods combine chromatographic separation and in-situ activity facilitating the localization and target directed isolation of active constituents in a mixture (Shahverdi *et al*., 2007).

The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the fungus because of presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce TTB to a purple-red colour (formazan) (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 was observed. The absence of activity could be due to evaporation of the active compounds or alternatively due to very little amount of the active compound.

Most of the antifungal agents detected in this study were present in extracts of relatively non-polar solvents such as chloroform and hexane. These findings agreed with previously published results (Masoko and Eloff, 2005) that the substances responsible for the antimicrobial activity were mainly non-polar in nature. However, the ethanol and butane crude extracts of *Trichoderma* isolates, in contrast to the chloroform crude extracts had good activity against plant pathogenic fungus (Shahverdi *et al*., 2007). AUT5 had the highest number of inhibition spots (12 inhibition spots) against test fungi, while AUT6 had the lowest number of inhibition spots (8 inhibition

spots) against test fungi. AUT1, AUT2, AUT3 and AUT4 had 10, 9, 11and 10 inhibition spots respectively against test fungi (Appendix 5-8). It is important to realize that bioautography is not a quantitative measure of antifungal activity. It only indicates the number of compounds that were separated with antifungal activity.

With regard to the antagonistic activity of culture filtrates, the inhibition varied depending on the *Trichoderma* isolates producing the metabolites from 18.9% to 78.4% (Figure 5) in non-volatile compounds on PDA culture media after 8 days of incubation at room temperature. *F. xylarioides* growth was significantly reduced in the presence of metabolites produced by isolate AUT2, 3 and 4 than the other isolates of *Trichoderma*. It has been observed that the crude extracts and culture filtrates from all isolates of *Trichoderma* have antagonistic effect toward the test pathogen. Similar results have been investigated by various authors including Hassan *et al* (2011) who reported that the crude extract of antifungal compounds was active against *F. oxysporum*.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The optimum R_f value was obtained by only three extracting solvents: $CHCl_3$, EtOH and Butane out of the seven pre-screened solvent systems. TLC- direct bioautography (TLC-DB) tests revealed most of the crude extracts from *Trichoderma* isolates exhibited antifungal activity toward *F. xylarioides* in which the highest inhibition zone was observed in AUT5 (51 mm) and AUT6 (44 mm) with EtOH extract at R_f value of 0.43. The best extracting solvent of the active antifungal compound was $CHCl_3$ (25) followed by EtOH (20) and butane (15).

Bioautographic method was found very useful in isolating active compounds with antifungal activity since the R_f value of the active compound can be used in bioassay guided fractionation instead of requiring labour intensive determination of activity of the crude extracts. This could be considered as an important procedure to select *Trichoderma* isolates for further works. One should, however, keep the possibility that volatile compounds should be evaporated from the chromatogram in mind when using this approach.

In in-vitro bioassay, the highest mean inhibitory effect on the growth of the pathogen was achieved by AUT2 (77.4%) isolate followed AUT3 (72.9) in dual culture. The minimum inhibitory concentration (MIC) of non-volatile compounds was observed in isolate AUT3 (56.8) and AUT6 (48.6) at 5% culture filtrate.

7.2 Recommendation

The major emphasis of this study has focused on the antifungal activity produced by *Trichoderma* isolates towards *F. xylarioides*. It may also be of interest to carry out further studies concerning confirmation of the effectiveness of *Trichoderma* isolates, detection, elucidation of the structure and analytical characterization of promising antifungal metabolites excreted especially by the isolates AUT2, AUT3 and AUT5. Further studies would also be of interest and to know the chemical and the physical properties of these compounds.

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APPENDICES

Appendix 1. Culture broth for extraction of non-volatile antifungal compounds

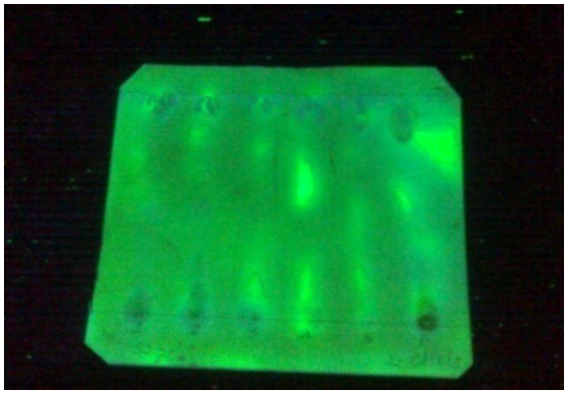
APPENDIXES

Appendix 2. Rota vapor for extraction of antifungal compounds set at 140 rpm

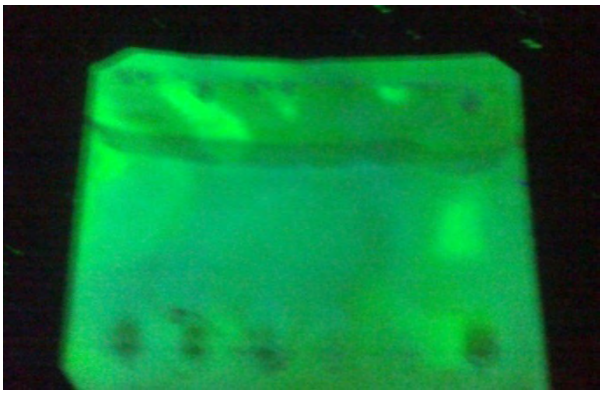
Appendix 3. R_f values of crude extracts obtained from solid culture media, PDA on TLC analysis

Solvent System	Composition of solvent system	Ratio of SS (V/V)	Type of Extract	R_f Values of Antifungal Compounds					
				AUT1	*AUT2*	*AUT3*	*AUT4*	*AUT5*	*AUT6*
SS1	Hexane: acetone: EtOAc	*1:8:1*	*$CHCl_3$*	*0.95*	*0.95*	*0.95*	*0.95*	*0.95*	*0.95*
			EtOH	*0.12*	*0.11*	*0.13*	*0.98*	*0.11*	*0.96*
			Butanol	*0.13*	*0.1*	*0.92*	*0.12*	*0.0*	*0.89*
SS2	$CHCl_3$: MeOH : EtOAc	*2:6:2*	*$CHCl_3$*	*0.11*	*0.13*	*0.23*	*0.2*	*0.3*	*0.24*
			EtOH	*0.88*	*0.1*	*0.0*	*0.0*	*0.88*	*0.88*
			Butanol	*0.12*	*0.1*	*0.12*	*0.13*	*0.0*	*0.0*
SS3	$CHCl_3$: Hexane: Butane	*2:6:2*	*$CHCl_3$*	*0.96*	*0.95*	*0.94*	*0.94*	*0.95*	*0.94*
			EtOH	*0.88*	*0.88*	*0.0*	*0.0*	*0.89*	*0.84*
			Butanol	*0.23*	*0.12*	*0.2*	*0.3*	*0.25*	*0.13*
SS4	$CHCl_3$: Acetonitrile: DCM	*3:4:3*	*$CHCl_3$*	*0.91*	*0.93*	*0.9*	*0.9*	*0.9*	*0.9*
			EtOH	*0.92*	*0.98*	*0.98*	*0.97*	*0.94*	*0.96*
			Butanol	*0.93*	*0.95*	*0.94*	*0.94*	*0.94*	*0.95*
SS5	Butane: Acetic acid: H_2O	*4:1:5*	*$CHCl_3$*	*0.89*	*0.9*	*0.9*	*0.94*	*0.87*	*0.88*
			EtOH	*0.23*	*0.1*	*0.14*	*0.19*	*0.15*	*0.1*
			Butanol	*0.86*	*0.23*	*0.96*	*0.98*	*0.93*	*0.88*
SS6	Hexane: MeOH: H_2O	*3:3:4*	*$CHCl_3$*	*0.12*	*0.1*	*0.4*	*0.26*	*0.1*	*0.1*
			EtOH	*0.23*	*0.12*	*0.1*	*0.4*	*0.4*	*0.4*
			Butanol	*0.14*	*0.21*	*0.43*	*0.33*	*0.68*	*0.2*
SS7	Acetone: MeOH: Acetic acid	*2:2:6*	*$CHCl_3$*	*0.21*	*0.15*	*0.15*	*0.15*	*0.14*	*0.15*
			EtOH	*0.31*	*0.34*	*0.13*	*0.26*	*0.12*	*0.12*
			Butanol	*0.4*	*0.25*	*0.13*	*0.24*	*0.20*	*0.18*
SS_8	n-Hexane: Toluene: Chloroform	3: 4: 3	*$CHCl_3$*	*0.38*	*0.35*	*0.38*	*0.0*	*0.30*	*0.35*
			EtOH	*0.0*	*0.3*	*0.0*	*0.0*	*0.28*	*0.3*
			Butanol	*0.6*	*0.62*	*0.57*	*0.63*	*0.63*	*0.58*
SS_9	n-Hexane : Benzene : Chloroform	4: 2 : 4	$CHCl_3$	*0.4*	*0.88*	*0.46*	*0.87*	*0.44*	*0.48*
			EtOH	*0.16*	*0.14*	*0.14*	*0.16*	*0.16*	*0.16*
			Butanol	*0.50*	*0.50*	*0.54*	*0.54*	*0.57*	*0.57*
SS_{10}	Acetone: Acetic Acid: Toluene	1: 1: 8	$CHCl_3$	*0.82*	*0.84*	*0.86*	*0.0-*	*0.84*	*0.84*
			EtOH	*0.66*	*0.62*	*0.0*	*0.0*	*0.0*	*0.50*
			Butanol	*0.76*	*0.78*	*0.76*	*0.78*	*0.78*	*0.78*
SS_{11}	Hexane : Benzene: Chloroform: Toluene	5: 4: 5: 6	$CHCl_3$	*0.67*	*0.64*	*0.63*	*0.57*	*0.64*	*0.69*
			Butanol	*0.63*	*0.65*	*0.73*	*0.75*	*0.79*	*0.76*
SS_{12}	n-Hexane: benzene: MeOH	3: 2: 5	*Butanol*	*0.72*	*0.76*	*0.72*	*0.68*	*0.62*	*0.58*
SS_{13}	n-Hexane: EtOH : MeOH	5: 3: 2	*EtOH*	*0.58*	*0.58*	*0.6*	*0.87*	*0.88*	*0.8*
SS_{14}	n-Hexane: Benzene: Chloroform	2: 5: 3	*$CHCl_3$*	*0.35*	*0.36*	*0.36*	*0.4*	*0.35*	*0.36*
			EtOH	*0.18*	*0.22*	*0.25*	*0.83*	*0.83*	*0.20*

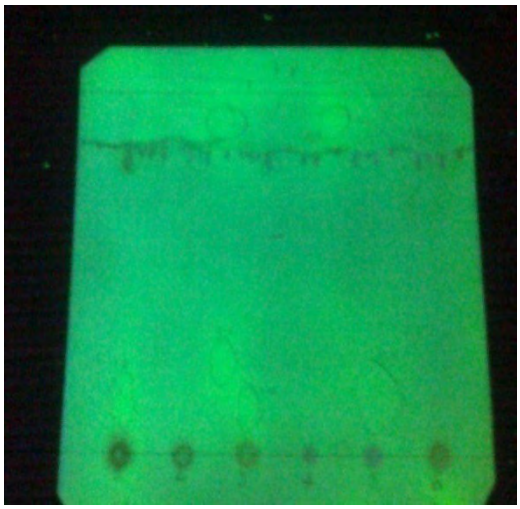
Appendix 4. Direct TLC Bioautography for the detection of antifungal activity against test pathogenic fungus *F. xylarioides*



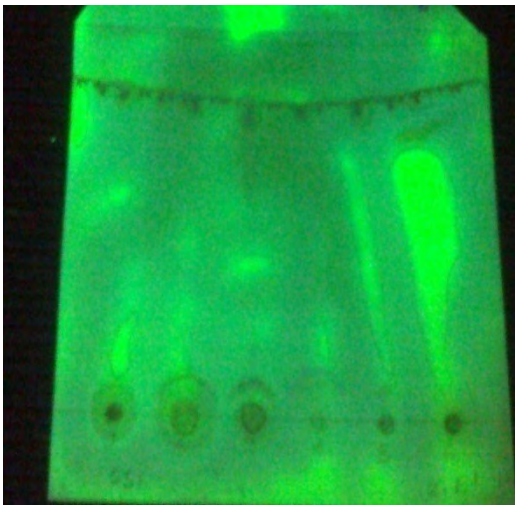
SS2 ($CHCl_3$)



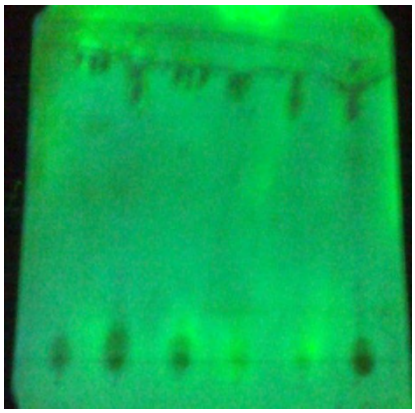
SS3 ($CHCl_3$)



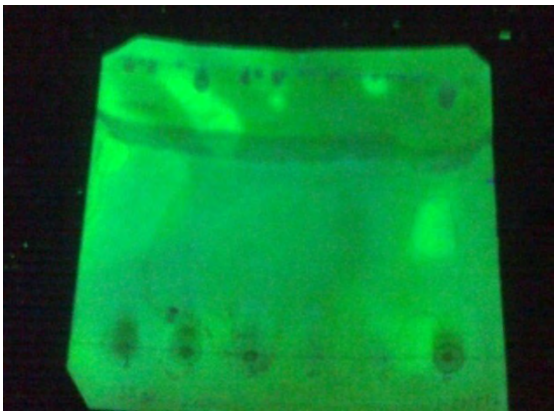
SS1 (Butane)



SS1 (EtOH)



SS1 ($CHCl_3$)



SS3 (EtOH)

Appendix 5. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of **AUT1** against *F. xylarioides* (A= Represent R_f values of Reference chromatogram, B = Represent R_f values of active compound on Bioautogram)

Solvent system	Extracts	R_f values		Inhibition diameter (mm)	Active spots	Total Active spots
		A	B			
SS8	$CHCl_3$	**0.89**	**0.89**	**10**	**1**	**10**
	EtOH	**0.8**	**0.12, 0.53**	**12, 5**	**2**	
	Butane	**0.8**	**0.22, 0.8**	**5, 6**	**2**	
SS9	$CHCl_3$	**0.4**	**-**	**-**	**0**	
	EtOH	**0.16**	**-**	**-**	**0**	
	Butane	**0.5**	**0.87**	**11**	**1**	
SS10	$CHCl_3$	**0.82**	**0.66**	**10**	**1**	
	EtOH	**0.66**	**-**	**-**	**0**	
	Butane	**0.76**	**-**	**-**	**0**	
SS11	$CHCl_3$	**0.67**	**-**	**-**	**0**	
	Butane	**0.63**	**-**	**-**	**0**	
SS12	Butane	**0.72**	**0.52**	**41**	**1**	
SS13	EtOH	**0.58**	**0.87**	**8**	**1**	
SS14	$CHCl_3$	**0.35**	**0.27**	**5**	**1**	
	EtOH	**0.18**	**-**	**-**	**0**	

Appendix 6. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of **AUT2** against *F. xylarioides*

Solvent system	Extract	R_f values		Inhibition zone (mm)	Active spots	Total Active spots
		A	B			
SS8	$CHCl_3$	**0.8**	**0.78**	**6**	**1**	**9**
	EtOH	**0.8**	**-**	**-**	**0**	
	Butane	**0.62**	**-**	**-**	**0**	
SS9	$CHCl_3$	**0.88**	**0.81**	**7**	**1**	
	EtOH	**0.14**	**0.4, 0.85**	**17,12**	**2**	
	Butane	**0.5**	**-**	**-**	**0**	
SS10	$CHCl_3$	**0.84**	**0.8, 0.93**	**6, 8**	**2**	
	EtOH	**0.66**	**-**	**-**	**0**	
	Butane	**0.78**	**0.6**	**10**	**1**	
SS11	$CHCl_3$	**0.64**	**-**	**-**	**0**	
	Butane	**0.65**	**-**	**-**	**0**	
SS12	Butane	**0.76**	**0.47**	**25**	**1**	
SS13	EtOH	**0.58**	**0.88**	**6**	**1**	
SS14	$CHCl_3$	**0.36**	**-**	**-**	**0**	
	EtOH	**0.22**	**-**	**-**	**0**	

Appendix 7. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of **AUT3** against *F. xylarioides*

Solvent system	Extracts	R_f values		Inhibition zone (mm)	Active spots	Total Active spots
		A	B			
SS8	$CHCl_3$	**0.88**	**0.84**	**8**	**1**	**11**
	EtOH	**0.8**	**0.4**	**4**	**1**	
	Butane	**0.57**	**0.19, 0.3**	**9, 10**	**2**	
SS9	$CHCl_3$	**0.46**	**0.21**	**6**	**1**	
	EtOH	**0.14**	**0.78**	**10**	**1**	
	Butane	**0.54**	**-**	**-**	**0**	
SS10	$CHCl_3$	**0.86**	**0.93**	**6**	**1**	
	EtOH	**0.0**	**-**	**-**	**0**	
	Butane	**0.76**	**-**	**-**	**0**	
SS11	$CHCl_3$	**0.63**	**0.19**	**10**	**1**	
	Butane	**0.73**	**-**	**-**	**0**	
SS12	Butane	**0.72**	**-**	**-**	**0**	
SS13	EtOH	**0.6**	**0.33, 0.85**	**16, 7**	**2**	
SS14	$CHCl_3$	**0.36**	**0.48**	**35**	**1**	
	EtOH	**0.25**	**-**	**-**	**0**	

Appendix 8. Inhibition of growth (diameter) on Bioautographic TLC plates by 3 extracts of **AUT4** against *F. xylarioides*

Solvent system	Extracts	Rf values		Inhibition zone (mm)	Active spots	Total Active spots
		A	B			
SS8	$CHCl_3$	**0.86**	**0.53**	**7**	**1**	**10**
	EtOH	**0.8**	**-**	**-**	**0**	
	Butane	**0.77**	**0.8**	**4**	**1**	
SS9	$CHCl_3$	**0.87**	**0.21, 0.62**	**12,13**	**2**	
	EtOH	**0.14**	**-**	**-**	**0**	
	Butane	**0.54**	**0.31**	**13**	**1**	
SS10	$CHCl_3$	**0.0**	**-**	**-**	**0**	
	EtOH	**0.0**	**-**	**-**	**0**	
	Butane	**0.78**	**-**	**-**	**0**	
SS11	$CHCl_3$	**0.57**	**0.9**	**10**	**1**	
	Butane	**0.75**	**-**	**-**	**0**	
SS12	Butane	**0.68**	**0.31**	**4**	**1**	
SS13	EtOH	**0.87**	**0.87**	**7**	**1**	
SS14	$CHCl_3$	**0.4**	**0.4**	**19**	**1**	
	EtOH	**0.83**	**0.83**	**16**	**1**	

DECLARATION

I and the undersigned declare that the work which is being presented in the thesis entitled, "Characterization and Testing of Antifungal Extracts from Trichoderma isolates against F. xylarioides, the causative agent of coffee wilt disease" in partial fulfillment of the requirements for the award of the degree of Masters of Science in Applied Microbiology, Department Microbial, Cellular and Molecular Biology, College of Natural Sciences, Addis Ababa University; is an authentic record of my own work during a period of ten months (September 2011 to June 2012) under the supervision of Dr. Tesfaye Alemu and Dr. Negussie Megersa. All materials used in this thesis have been dually acknowledged.

Candidate Name: Afrasa Mulatu

Signature: _____

Date of Submission_____

The work has been done under the supervision of:

Dr. Tesfaye Alemu *Signature: _____ Date_____*

Dr. Negussie Megersa *Signature: _____ Date_____*