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Isolation, Identification of *Fusarium Xylarioides (Gibberella Xylarioides)* From Southern Ethiopia And Its Response To Fungal Biocontrol Agents

By
Negash Hailu

A Thesis submitted to the School of graduate studies of Addis Ababa University in partial fulfillment of the requirements of the Degree of Master of Science in Biology (Applied Microbiology)

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Abbreviations and Acronyms

µm=micrometer
AAU=Addis Ababa university
ANOVA= Analysis of variance
CABI=Center for Agriculture and Bioscience
CBD= Coffee berry disease
CLR= Coffee leaf rust
CTA= Coffee and Tea Authority
CWD=Coffee wilt disease
CDA = Czapeck Dox Agar
DRC=Democratic Republic of Congo
FSA=Fusarium Selective Agar
Fx =*Fusarium xylarioides*
GLM=General Linear Model
IAR=Institute of Agricultural Research
lb =Pound
ISR= Induced systemic resistance
masl = meters above sea level
MEA=Malt Extract Agar
ml= milliliter
mm= millimeter
NAO=New Agriculturalist Online
Nm=nanometer
OD=Optical density
PDA=Potato Dextrose Agar
Rpm= rovolutio per minute
SDW= Sterile Distilled Water
SNA=Synthtic-low Nutrient Agar
SNNP = South Nation, Nationalities and Peoples
SPSS= Statistical program for social sciences
Th= *Trichoderma harzianum*
Tv =*Trichoderma viride*
UV=Ultraviolet light

Abstract

Five isolates of Fusarium xylarioides form five coffee growing Weredas in southwestern Ethiopia were isolated from Arabica coffee trees showing sever symptoms of vascular wilt. These strains were characterized based on vegetative and spore morphology and vegetative growth on different growth media, temperature and pH ranges. These strains produced both microconidia and macroconidia on Potato Dextrose Agar (PDA) and Synthetic low-nutrient Agar (SNA). Microconidia were unicellular, allantoids and had 0-1 septate. Macroconidia were strongly curved with marked foot cells and had 1-4 septate. Pathogenicity test was performed for the subsets of isolates by inoculating 20 ml, 40 ml, and 60 ml of spore suspensions. Symptoms developed on the 30^{th} day of inoculation concentration wisely. All seedlings died on the 90 day of inoculation. The controls were drenched with equivalent amount of distilled water and no symptoms developed until the completion of the study. In vitro evaluation of antagonistic test of dual culture of the test isolates with two Trichoderma species showed that the radial growth inhibition of the pathogens. The cell free culture filtrates of different concentration also showed the radial growth inhibition of the test pathogens.

Key words: *Fusarium xylarioides, Trichodrma harzianum, Trichoderma viride,* Biological control.

1. INTRODUCTION

Coffee belongs to the family Rubiaceae, which is widely distributed throughout the tropical region. Although there are many species of coffee, the only two commercially important ones are (*Coffea arabica*) and (*Coffea robusta)* (Pieters and Vander-Graff, 1980). Both species can grow best on deep, free- draining, loamy soils, with a good water holding capacity and a slightly acid soil (PH 5-6)(CTA, 1999; Kimani *et al*., 2002; Lewis Ivey et *al*., 2003) and soil fertility is important for good production.

According to Kimani *et al*. (2002) about 60% of the world coffee production is Arabica coffee, which is supposed to be of higher quality and can get much more in the market compared to Robusta. However, *Coffea arabica* is susceptible to most diseases attacking coffee compared to other Coffea species (Vander Graff and Pieters, 1978; Pieters and Vander-Graff, 1980; Bertrand et al., 2001).

Ethiopia is considered not only the primary origin but also the diversity of Arabica coffee (Girma Adugna 1997; Tewoldeberhan Gebere Egziabher, 1990; Girma Adugna and Hindorf, 2001; Girma Adugna, 2004). Coffee is produced in South West, East and some areas of Northern parts of Ethiopia. The major coffee producing areas are Oromia, SNNP and Gambella. It has been reported by CTA (1999), Girma Adugna (2004) the total area covered by coffee is about 400,000 hectares, with a total production of 200,000 tones of coffee per year.

Coffee is the most important commercial crop in the national economy of Ethiopia, contributing 60% of its foreign exchange earnings and nearly 25% of Ethiopian population depends, 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).

Coffee production is hampered by various biotic factors and abiotic factors in the Country. Diseases are the most important factors that contribute to the reduction of coffee production. Tracheomycosis/ coffee wilt disease caused by *Fusarium xylarioides* attacks coffee trees in the major coffee growing areas of Ethiopia. Therefore the aim of this study concentrated on the morphological and physiological characterization of *F. xylarioides* in the laboratory and to inquire the controlling mechanisms.

2. OBJECTIVE OF THE STUDY

2.1. General objective

- To isolate, identify and characterize the pathogens by using different physiological parameters.
- To evaluate antagonistic activities of *Trichoderma* species as biological control agents against the test pathogen.

2.2. Specific Objectives

- To isolate, identify and characterize the casual agents of the coffee wilt cultivars of *Fusarium species* from coffee growing areas of the country based on temperatures, PH, media and other growth parameters.
- To study the pathological variability and characterize the pathogen of coffee wilt disease isolates.
- To evaluate *In Vitro* antagonistic effects of *Trichoderma species* against the test pathogen.

3. REVIEW LITERATURE

3.1. Fungal Diseases of coffee

Coffee diseases caused by fungi are the major constraints to reduce coffee production in major coffee producing countries of Africa (Kimani *et al*., 2002). Coffee diseases especially caused by fungi are the major constraints to reduce the coffee production in major coffee producing countries of Africa. Next from coffee berry Disease (CBD) the most limiting factors 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. Currently, in Ethiopia it has been reported that more than 45 fungal pathogens of coffee. The following table summarizes some of the fungal diseases of coffee that were recorded in Ethiopia.
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). Coffee berry disease is the most serious disease to *Coffea arabica* causes on average about 30% national yield losses to Ethiopia (Tefesetewold Biratu, 1995; Eshetu Derso *et al.*, 2000).

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, and Hindorf, 2001; Oduor *et al*, 2003).

Table 1.Some important fungal diseases of coffee in Ethiopia.

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

The disease had first been recorded in Ethioipia (Kaffa Province) in 1957, and the causal organism was identified as *Fusarium oxysporum f sp. Coffeae* (Stewart, 1957). Kranz and Mogk (1973) isolated F. xylarioides from infected coffee. The pathogen also attacks *Coffea arabica* and is endemic in all coffee growing areas of Ethiopia (Flood. 1997; Girma Adugna and Hindorf, 2001; Girma Adugna, 2004; Lepoint *et al.*, 2005). During the 1950s and 1960s, it was considered to be the most serious disease of coffee in Africa and destroyed millions of coffee trees (Oduor *et al.*, 2003; Girma Adugna, 2004). Systematic elimination of affected plants over vast areas combined with the development of breeding programmes effectively reduced its impact to a minor disease (Kimani *et al*., 2002; Lewis Ivey *et al.*, 2003). However, the incidence has begun to increase dramatically and spread throughout Central and East Africa (Lewis Ivey *et al.*, 2003; Rutherford, 2006). Since 1993, farmers began reporting a wilt disease of coffee in western Uganda near the border with the Democratic Republic of Congo and later in

1995, in Central Africa Republic (Lewis Ivey *et al*., 2003; Geiser *et al*., 2005; Rutherford, 2006).

This disease is clearly becoming a serious threat to coffee production in Africa and the cause of its reemergence is due to the arising of a new, aggressive strain or biotypes of the pathogen. Isolates from other species of coffee (*C. arabica, C. excelsa*) and parts of Africa (Ivory Coast, Ethiopia) gave different band patterns. These results are surprising for a heterothallic fungus which produces its sexual stage in nature and support the hypothesis that a new, more aggressive strain of the pathogen may have arisen within the wider gene pool of the pathogen population in Africa (Lewis Ivey *et al*., 2003; Geiser *et al*., 2005; Lepoint et *al.,* 2005)

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 (2003) 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 traceomycosis with an incidence of up to 40 % (King`ori, 2001; Kimani *et al*., 2002; 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).

From about 20 species of *Fusarium* have been recorded from coffee worldwide, only four species are known to be pathogenic to coffee (Barnett and Hunter, 1972; Flood and Brayford, 1997; Flood, 2003; Gesier *et al.*, 2005). These are *F. solani* causing lethal root disease; *F. stillboides* inciting bark disease; *F. oxysporum* and *F. xylarioides* causing wilt diseases (Waller and Brayford, 1990; Stover, 1992; Waller and Holderness, 1997; Gesier *et al.,* 2005). Formae speciales of *F solani* and *F. Oxysporum* can be recovered from coffee root, husks and soil samples obtained from infected trees with wilt disease (Flood, 1997) and inducing different types of wilting on coffee in different geographical regions.

Fusarial bark diseases of coffee caused by the same *Fusarium stillboides* is an important factor limiting Arabica coffee production in the low and medium altitude distinct of Kenya (Flood and Brayford, 1997; King`ori, 2001).

3.2. Biology and Taxonomy of *Fusarium xylarioides (Gibberella xylarioides*

The sporodochial macro conidia are 1-3 septated, frequently falcate slightly curved with distinct visible foot and basal cells (Janzac *et al.,* 2005; Lepoint *et al.,* 2005). Microconida of the aerial mycelia are usually 0-1 septated, and often variable in shape from slightly curved to allantoidal, and comma or U- shaped (Lewis Ivey *et al.,* 2003; Geiser *et al.,* 2005; Janzac *et al.,* 2005; Lepoint *et al.,* 2005).

According to Rutherford (2006) although little is known about the fungus that causes tracheomycosis, it lives in the soil, on infected debris, in an alternative hosts or as resistant propagules of species and enters the coffee tree through wounds in the base of the tree or in the roots (Flood, 2003; Janzac *et al.,* 2005; Lepoint *et al.,* 2005). Three asexual spores (macroconidia, microconidia and chlamydospores) and the fourth sexual spore (ascospores) allow the pathogen for the production of highly variable population, in addition to the parasexual cycle (Flood, 2003; Girma Adugna, 2004; Rutherford, 2006). The fungus produces special survival spores that are thick walled resting spores (chlamydospores) and survive for many years (Booth, 1971; Fisher *et al*., 1982; Girma Adugna, 2004; Rutherford, 2006).The sporulating stage of each fungus develops within one or two days on the split stem of diseased coffee, provided that stems are kept moist. *Fusarium* produce sickle-shaped conidia on sporodochia. *Fusarium xylarioides* survives for two to eleven years or five to ten years in the soil as “saprophyte” because it produces resting spores or chlamydospores. Moreover, the sexual spores (ascospores) produced in the perithecia may be able to act as survival spores (Flood, 2003).

The taxonomy of *Fusarium* is based on the morphological characters including the presence or absence, the shape and the dimensions of micro conidia, macro conidia basal cells and chlamydospores, and the growth and color development on different media are used as markers in practice (Gerlach, 1978; Flood and Brayford, 1997; Lewis Ivey *et al*., 2003; Gesier *et al*., 2005). All *Gibberella species* are sexual states or teleomorphs of *Fusarium species*, which are destructive plant pathogens (Samuels *et al.*, 2001: Desjardins, 2003). The anamorphic stage (G. *xylarioides*) *F. xylarioides* was first described by Steyaert from stem samples of diseased coffee trees obtained from *coffea excelsa (*Lewis Ivey *et al*., 2003). The teleomorph form was observed by Saccas in 1949 on dead trees of *C. noearnolandiana* and described and renamed as the *Gibberella xylarioides* (Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983). The fungus was indicated as one of heterothallic ascomycetes having male and female strains, which can be identified based on the colony appearance and conidial morphology (Barnett and Hunter, 1972; Gerlach, 1978; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983; Girma Adugna and Mengsitu Huluka, 2000).

3.3. Survival and Spread of the Pathogen

The pathogen 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 either above or below ground. Inside the coffee the fungus invades the water conducting system (xylem) and blocks the movement of water upwards from the roots to the rest of the plant. The timing from first symptoms to death of the tree varies from days in young plants to eight months in trees more than ten years old (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 Wrigley (1988) the lateral and feeder roots of coffee spread on the surface plate parallel to soil surface for a distance of 1.2 to 1.8 meters from the trunk, and F. *xylarioides* is abundantly recovered from root parts of symptomatic and asymptomatic trees (Flood,1997; Flood and Brayford,1997; Girma Adugna *et al*., 2001; Girma Adugna, 2004). The pathogen spreads 2 meters up to four plants on either sides of the inoculated focus plant through the infection of the roots in greenhouse experiment (Lewis Ivey *et al.*, 2002; Rutherford, 2006). Closely spaced trees are more liable to wounding and cross inoculation while slashing or hoeing coffee fields. 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).

When seedlings with healthy roots are transplanted into either naturally or artificially infested soils, no wilting symptoms appeared. Infection exhibits when the tap roots are injured and transplanted into naturally or artificially infested soils, and also only on those seedlings inoculated by stem wounding through ditching with *F. xylarioides* infested scalps or by injecting the conidial suspensions with needles (Lewis Ivey *et al.*, 2002). The stem nicking or root drenching inoculation methods also elaborate the roles of contaminated farm implements in cross inoculating coffee trees as well as disseminating the coffee wilt pathogen in the field (CABI, 2003; CABI, 2005).

Replanting susceptible cultivars in the infected field increases the fungus inoculum density (CABI, 2003). Pieters and Van der-Graff (1980) reported that among socioeconomic 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 (Stover, 1992; Miville-de-chene, 1999; Kimani *et al.*, 2002).

Perithecia of *Fusarium xylarioides* containing great number of viable ascospores with 95% germinating rate (CABI, 2003) and abundant in the soil, so that these sexual spores are the most important source of inoculum 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 inoculum 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; Flood, 2003; 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 (Flood, 2003; 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).

3.4. Occurrence and Distribution of Coffee Wilt Disease 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. 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 CABI (2003) most farmers observed the disease 40 years ago and since then the disease increased at lower rate. Its spread and control methods are not well known by farmers, extension workers and agricultural officers, although the observation by farmers coincided more or less with the first record of disease by Stewart (1957) who first discovered symptoms of wilting in Ethiopia. Kranz and Mogk (1973) observed the

disease on a few single trees scattered in some plantations around Agaro, Jimma and Bonga.

During initial surveys made in 1973, the presence of *Gibberella xylarioides* from Jimma, Gera, Manna, Gomma, Mettu, Sidamo (IAR, 1974); Dembidollo, and Wondoguenet (IAR, 1980). 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 Vander Plank (1975), 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.

It has been reported by Girma Adugna (2004) that CWD is found to be more prevalent in the plantations, either in small scale farmer's fields, research plots or large-scale commercial farms followed by garden and semi forest production systems. The disease is more severe in Yirgacheffe than in Kochore and Wenago areas of Southern Region. In addition to less heterogeneity in the local cultivars or landraces and relatively intensive agronomic activities, the fungus is more aggressive to cause high CWD incidence in the garden production systems (Girma Adugna, 2004).
trees.

3.5. Disease Symptoms

The first signs of CWD are yellowing, folding and curling inward of leaves (Vander-Graff, 1978; Girma Adugna 1997). The leaves feel limp to touch, then dry up and feel papery and then turn brown. Eventually, the leaves drop off leaving the infected trees completely bare. Affected branches may turn black brown or blackish and dry up (Lewis Ivey *et al*., 2002; Flood, 2003; Lepoint *et al.*, 2005; 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.

The bark on the trunk, especially near the base of the tree, may become swollen and have many vertical and spiral cranks. Underneath the bark the wood appears blue-black in color (Flood, 2003; Lewis Ivey *et al*., 2003; Lepoint *et al.*, 2005; Rutherford, 2006). Towards the end of the rainy season black structures resembling soil occur on the bark, usually at the base of the plant (Lewis Ivey *et al.*, 2002; Girma Adugna, 2004; NAO, 2003). These structures are dark-violet perithecia; contain spores (ascospores) of the fungus that enable it to spread to other coffee trees and to survive in the soil or on plant material (Miville-de-chene, 1999; Girma Adugna and Hindorf, 2001; Kimani *et al*., 2002). In the roots, a moist black rot is observed (Kimani *et al.*, 2002; NAO, 2003).

Another important early sign of CWD is that berries on infected trees turn red prematurely and appear to ripen early. Most affected trees die 2-3 months after the first symptoms are observed (Rutherford, 2006). Although other symptoms are caused by other problems, only CWD causes the blue-black discoloration of the wood (Lewis Ivey *et al.*, 2003; Rutherford, 2006).

3.6. Method of study of Coffee Wilt Disease

The process of isolation begins with the collection of symptomatic plant materials. Plant tissues are first washed in sterile distilled water (SDW) and undergo surface sterilization in 2% NaOCl (Sodium hypochloride) for one minute, 96% ethanol for ten seconds or 0.5% cupric chloride, 70% alcohol each for one minute (Gesier *et al.*, 2005; Summerell *et al.*, 2006). This is followed by rinsing the plant material in sterile distilled water and allowed to dry on sterile tissue paper (Dhingra and Sinclair, 1993; Aneja, 2005; Gesier, *et al*., 2005).

There are two types of isolation methods proposed, which are direct, indirect. In direct ways of isolation, the fruiting bodies or small-excited plant parts from the margins of healthy and infected part are transferred into general or selective media (Vannini and Vettraino, 2000).

The indirect means of isolation can be done at least in two ways. The first one is done by attacking symptomatic organs disc to the cover of Petri plates with the pseudothecia

facing downwards in order to release its spores directly to the isolation media (Lewis Ivey *et al.*, 2003; Jansen, 2005). Secondly, segments from infected plant body are transfered into moist chamber to promote growth of mycelium, fruiting bodies or to sporulate out of infected tissue (Roux *et al.*, 2004; Gesier, *et al*., 2005). After the isolates are inoculated to the Petri plates that contain suitable media they will be incubated and subsequent routine subculture will be done until pure colony is obtained.

3.7 Factors Affecting the Growth of the Pathogen

The major factors affecting growth are medium, temperature, light, aeration, pH and water activity among which some are mentioned below.

Media

The growth requirements for fungi may vary from strain to strain, although cultures of the same species and genera tend to grow best on similar media (Dhingra and Sinclair, 1993; Aneja, 2005). The source of isolates can give an indication of suitable growth conditions. Cultures grow more satisfactorily on media freshly prepared in the laboratory, especially natural media such as vegetable decoctions. These are usually easy and relatively cheap to prepare and preparation can be carried out with limited facilities (Dhingra and Sinclair, 1993; Aneja, 2005). However, synthetical media are often useful and can be very important in replicating work of others. Media affects colony morphology and color, whether particular structures are formed (Dhingra and Sinclair, 1993; Burgess *et al*., 1994; Burgess and Wingfield, 2002; Aneja, 2005). The best media for mycelia growth of *Fusarium xylarioides* are PDA, MEA and FSA and for sporulation SNA.

Temperature

The majority of filamentous fungi are mesophilic, growing at temperatures within the range of 10-35°C, of which most grow with temperatures between 15 and 30°C (Kapoor and Kar, 1989; Dhingra and Sinclair, 1993; Smith and Onions, 1994); Burgess and

Wingfield, 2002). According to Nelson *et al*. (1983) the suitable temperatures for the growth of *Fusarium xylarioides* is 25±1^{O}C.

pH

Filamentous fungi vary in pH requirements. Most common fungi grow well over the range pH 3 to 7, although some can grow at pH 2 (Smith and Onions, 1994; Aneja, 2005; Burgess *et al.*, 1994). It has been reported by Kapoor and Kar (1989) the suitable pH for the growth of mycelium and sporulation of *Fusarium oxysporum f. sp. lycopersici* on tomato is 3-6. However; the best pH for this pathogen was found to be 5.5 during their investigation.

Near ultraviolet light (black light)

Fungi which require near ultraviolet (near UV and often referred to as black light) light (wavelength 300-380 nm) for sporulation must be grown in plastic Petri dishes or plastic universal bottles for 3-4 days before irradiation (Smith and Onions,1994; Aneja, 2005; Burgess *et al.*,1994). Glass is not suitable, as it is often opaque to ultraviolet light. Rich growth media should be avoided, as they may give rise to excessive growth of mycelium; nutritionally weak media such as Potato Carrot Agar (PCA) are more suitable for inducing sporulation (Nelson *et al*., 1983).

3.8. Sporulation

This is one of the important steps in the process of identification, characterization and preparation of spores for pathogenicity tests. Spores often are produced under conditions that are not suitable for vegetative growth (Dhingra and Sinclair, 1993; Nelson *et al.*, 1983; CABI, 2005). Factors affecting the sporulation such as ultraviolet light, fluctuating temperature conditions (Nelson *et al*., 1983), high humidity or nutrient poor medium (Nelson *et al*., 1983; Vettraino *et al.*, 2002).

The use of near ultra violet (black light) would be best if it is alternatively used on the basis of 12 hrs light and 12 hrs dark inside an incubator (Nelson, *et al*., 1983). Regarding the sporulation media, many species sporulate better on natural substrata such as macerated leaves and lupine stems than Agar (Lewis Ivey *et al.*, 2003; Gesier *et al*., 2004). In addition to this, nutritionally weak media such as Potato Carrot Agar, Oat Agar, and Tap Water Agar are recommended for many fungi to sporulate successfully (CABI, 2005).

3.9. Pathogenicity tests

In order to substantiate an organism, group of organisms or combination of organisms and environmental factors are the causal agents for a disease, it must fulfill Koch's postulates (Patridge, 1999; Agrios, 2004; Pethybridge *et al.,* 2004; Aneja, 2005). The pathogenicity test begins with preparation of the sporulated spore or raw mycelium from the test organism. Different authors used different amount of spore loads during this test: 2-4x10^5 spores/ml of *Phytophtora ramorum* (Denman *et al.,* 2005), 5x10^5 spores/ml of *Paulonia spp* (Ray *et al.,* 2005), 10^4-10^5 conidia/ ml of *Alternaria alternaria* (Belisario *et al.,* 1999), 5x10^4 (Bohar and Schwazinger, 1999), 10^4-10^5 conidia/ml (Holdenrieder, and Kowalski, 1989) and 5x10^4 propagules/ ml (Hutton and Mayers, 1988)

The given spore or mycelium could be loaded with or without wounding the plant (Denman *et al.,* 2005). The non-wound trial helps to determine whether infection could develop without previous damage (Luque *et al.,* 2000 Gesier *et al*., 2005). During inoculation of stems, the adjusted spore suspension should be placed on the surface of the soil by little wounding of feeder roots of coffee.

3.10. Disease management

3.10.1. Quarantine measures

For races currently free from tracheomycosis strict quarantine measures, which help to prevent its entry and spread. Movement of coffee materials (seedlings, husks, and other organs) between affected and unaffected areas should be restricted as much as possible (Hakiza, and Mwebesa, 1997). These measures need to be backed up with dissemination of information about the disease to farmers, extension workers, scientists and the general public. Dissemination of information on the symptoms of the disease is essential to allow monitoring and early detection of the disease. For countries bordering affected countries cordon sanitors can be constructed. This involves the destruction of all affected coffee in border areas and encouragement of farmers to grow crops other than coffee (Flood, 1997; Girma Adugna, 2004; Rutherford, 2006).

3.10.2. Resistant varieties

Production of resistant cultivars is the best option for controlling CWB in the longer term. This method was very successful in controlling outbreaks of the disease in 1950s and 1960s in West and Central Africa, where affected coffee is uprooted and destroyed and the fields replanted with resistant cultivars of *C. canephora* such as cultivar `robust`, but recently resistance is broken down due to emergence of a new form of the fungus (Meseret Wondimu *et al.*, 1987; Flood and Brayford, 1997).

In Ethiopia, breeding programmes were initiated for *C. arabica* (Vander-Graff and Pieters, 1978; Pieters and Vander-Graff, 1980) but the disease remains a problem in some areas of the country. Some farmers are conducting their own selection since, even in very badly affected areas, a few trees may survive. Farmers are replanting with seeds from these plants might be resistant.

3.10.3. Cultural practice

Systematic elimination of affected plants over vast areas combined with the development of breeding programmes effectively reduced its impact (Flood, 1997; Hakiza, and

Mwebesa, 1997; Hindorf, 1998). Affected trees and trees adjacent to affected trees should also be uprooted and burnt although appear healthy because while symptoms of the disease may not be visible, the fungus may be inside the plant (Rutherford, 2006).

When symptoms are recognized quickly and uprooting and burning done efficiently, the farmers may save some of the crops (Flood and Brayford, 1997; Girma Adugna, 1997; Lepoint *et at.,* 2005; Leslie *et al.,* 2005). If the farmers delay, the infected trees act as source of inoculum to other trees and leads to whole crop losses. Trees cut down as control measure should not be used as fuel as affected trees drugged through healthy trees in the farm will aggravate the spread of the disease. Diseased trees must be burnt where they are uprooted. To prevent spread from one field to another in large plantation, it is recommended that a 300 m strip of land should be cleared of coffee (by uprooting & burning) ahead of the disease front (Girma Adugna, 1997, Hakiza, and Mwebesa, 1997; Flood, 2003; Rutherford, 2006).

Any kind of wounding to the tree will allow the fungus to enter. Wounding may occur through weeding and pruning with machete or hoe, or even by livestock feeding on and around the tree (Flood, 2003; Rutherford, 2006). Great care should be taken to minimize damage to the tree and all tools should be sterilized with fire or with disinfectant before moving to another tree.

Mulches and soil amendments including cow dung and urine have been claimed to control the disease, but bring only temporal improvement to infected trees by increasing plant vigor and stimulating new growth of roots, shoots, and leaves (Flood, 1997; Hakiza, and Mwebesa, 1997; Rutherford, 2006). Improvement will also be partially due to the encouragement of organisms such as *Trichoderma* and *Aspergillus* in the soil that compete with the wilt fungus (Thangavelu *et al*., 2003). Mulches and soil amendments are therefore unlikely to control the disease in already infected trees, but may be useful in preparing the land for replanting after affected trees have been uprooted and burnt (Cooney and Lauren, 1998; Rutherford, 2006).

Following destruction of the diseased trees and preparation of the land, replanting should not be carried out for at least two years to allow the inoculum of the fungus in the soil to decrease (Girma Adugna and Mengistu Huluka, 2000; Girma Adugna, 2004; Rutherford, 2006). Replanting should be done with plants raised from the disease free cuttings and seeds collected from areas that are free from the disease (Girma Adugna and Mengistu Huluka, 2000).

3.10.4. Chemical Control

The pathogen 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 (Lewis Ivey *et al*., 2003; Rutherford, 2006).

3.10.5. Biological control

"Biological control is the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms accomplishing naturally or through manipulation of the environment, host or antagonists, or by mass introduction of one or more antagonists"(Baker and Cook, 1974).

Biological control is the strategy for reducing disease incidence or severity by direct or indirect manipulation of microorganisms (Papavizas, 1985; Zhang *et al*., 1994; Paullitz and Bekanger, 2000; Tesfaye Alemu and Kapoor, 2004). Antagonists that produce antibiotics kill pathogens and eradicate or control them from substrate. Some microorganisms occupy the niches and exclude pathogens from becoming established, thereby protecting plants from infection. Biological control has attracted great interest because of increasing regulation and restriction of fungicides or unnecessary control attempts by other means. It is especially attractive for soil borne diseases because it needs critical evaluation of economics of the country and the pathogens are difficult to reach with specific fungicides (Montealegre *et al.*, 2003).

A number of *Trichoderma* spp. has a promising potential for biological control of plant pathogenic fungi (Papavizas, 1985; Perez-Vicente *et al*., 2003). *Trichoderma harzianum* and *T. viride* are the most studied of all the *Trichoderma* species for biological control

and the most effective in reducing diseases caused by soil borne plant pathogens (Baker, 1987; Cortes *et al.*, 1998; Rocco and Perez, 2001; Tesfaye Alemu and Kapoor, 2004).

Trichoderma has rapid growth and development, and also produces a large number of enzymes, induced by the presence of phytopathogenic fungi. Its high tolerance to extreme environmental conditions and habitat, where fungi are the cause of various diseases, makes it an efficient agent of control; equally, it can survive in media with high levels of pesticides and other chemicals. So the application of *Trichoderma* directly on the soil offers greater protection to the crops (Cooney and Lauren, 1998).

Trichoderma spp stimulates plant growth by producing substances that stimulate plant growth and development. These substances act as catalysts or accelerators in the primary meristem tissues in the young parts of plants, accelerating cell reproduction, so that the plants achieve faster growth than those which have not been treated with this microorganism (Dennis and Webster, 1971; Baker, 1987; Mazzola, 1998).

3.11. Mechanism of action of *Trichoderma species* as bioagents

The success of *Trichoderma spp.* as a biocontrol agent is believed to involve various modes of action, including antibiotic production, secretion of lytic-enzymes, mycoparasitism, competition for space and nutrients, and induction of systemic resistance (Cortes *et al*., 1998; Rocco and Perez, 2001). A given *Trichoderma*-host interaction may involve any of these mechanisms individually or encompass more than one of them acting simultaneously (Cortes *et al*., 1998) and in fact it seems advantageous for a biocontrol agent to suppress a plant pathogen using multiple mechanisms (Estrella and Chet, 1998).

3.11.1. Antibiosis

Both volatile and non-volatile antibiotics are known to be produced from *Trichoderma species* (Okigbo and Ikediugwu, 2000). Peptaibols (trichorizianines, trichokindins, trichorzins, trichorozins and harzianins), a class of antibiotics, are produced by most

species and strains of *Trichoderma*. They generally exhibit antimicrobial activity against fungi and gram positive bacteria. Peptaibols are thought to act on the membrane of the target fungus to inhibit membrane-associated enzymes involved in cell wall synthesis (Okigbo and Ikediugwu, 2000). The antibiotics trichodermin, trichodermol, harzianum A and harzianolide are also known to be produced from *T. viride* and other species of *Trichoderma* (Barbosa *et al*., 2001). Moreover, Lin *et al*. (1994) reported a ribosome-inactivating antifungal agent, Tricholin from *T. viride*.

3.11.2. Lytic enzymes

Studies have shown that mycoparasitic strains of *Trichoderma* produce a complex set of extra cellular enzymes including β-(1,3)-glucanase, chitinases, lipases and proteases when grown on isolated cell walls of pathogenic fungi (Cortes *et al*., 1998; Estrella and Chet, 1998). Besides, Barbosa *et al.* (2001) 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 (Cortes *et al*., 1998; Thangavelu *et al*., 2003). It is believed that these enzymes act synergistically with the antibiotics to inhibit the growth of fungal pathogens (Mora and Earle, 2001). 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.

3.11.3. Mycoparasitism

Mycoparasitism occurs when one fungus exists in intimate association with another from which it derives some or all of its nutrients while conferring no benefit in return (Estrella and Chet, 1998). Perhaps the best-known mycoparasite is the fungus *Trichoderma species* (Campbell, 1989). This is because *Trichoderma* spp attacks a great variety of phytopathogenic fungi that are responsible for most important diseases of major economic importance worldwide (Estrella and Chet, 1998).

It appears that mycoparasitism is a complex process involving several steps (Chet, 1987). The mycoparasitic relationship between *Trichoderma spp* and its potential host might involve biochemical and physiological interactions that lead the microscopically visible

phenomena of hyphal coiling, appressorium formation, penetration and cytoplasmic degradation (Cortes *et al*., 1998).

3.11.4. Competition

Competition is an indirect effect whereby pathogens are excluded by depletion of food bases or by physical occupation of sites (Maloy, 1993). The study of Barbosa *et al*. (2001) in the *in vitro* antagonism of *Trichoderma* species on *Cladosporium herbarum* revealed that the colonies of *Trichoderma* species grew always faster than *C. herbarum* in single or mixed culture. *T. viride* compete for the same niches with the pathogens (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).

In the rhizosphere competition for space as well as nutrients is one of major importance of microbial interaction. Thus, an important attribute of a successful rhizosphere biocontrol agent would be the ability to remain at high population density on the root surface, providing protection of the whole root for the duration of its life (Estrella and Chet, 1998).

In addition to their biocontrol effects, the ability of *Trichoderma species* to increase the rate of plant growth and development has been known for many years. It was found that a number of *Trichoderma* strains were simultaneously plant growth promoters in vegetables and various seedlings and biocontrol agents (Chet, 1987; Naseby *et al*., 2000). *Trichoderma spp* may affect minor pathogens in the soil but it may also directly affect the plant by excreting a regulating hormone which may, in turn, increase the growth rate or the efficiency of nutrient uptake (Chet, 1987).

4. MATERIALS AND METHODS

4.1. Experimental site

All experiments were carried out in the mycological research laboratory and experimental plots of the Department of Biology, at Addis Ababa University. It is found at the altitude of 2444 meters above sea level with the mean annual rainfall of 1196 mm and located $9^{o}01^{!}$ N and $038^{o}45^{!}$ E. The minimum and maximum temperatures were 9.9^{O}C and 24.6^{O}C, respectively

4.2. Study areas and Sample collection

Fusarium xylarioides and *Gibberella xylarioides* are used interchangeably. Five isolates of *Fusarium xylarioides (Gibberella xylarioides)* were selected from center collection of the mycology laboratory, Department of Biology, AAU. They had been collected from different coffee growing weredas from SNNP ,Oromia and Gambella Regional states.

4.3. Sterilization and Maintaining of cultures

The sterilization of media and glasswares (wrapped in brown papers/Kaki papers) was done by autoclaving at 121^{O}C temperature and 15 lb pressure for 15 minutes. Autoclaved glasswares were dried 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 species*) were maintained on Potato Dextrose Agar (PDA) slants in the plugged test tubes. The slants were stored in the refrigerator at 4^{O}C for further study.

4.4. Isolation preparation of inoculum and pathogenicity test

4.4.1. Isolation of *Fusarium xylarioides*

Diseased leaves, stems, and soil were collected from different coffee growing weredas in order to isolate the causative agents of coffee wilt disease. Of these plant materials small leaves and stems were taken from the margin of infected and healthy regions, washed in tap water for two minutes in separate plates in order to minimize surface contaminants**.** Subsequently, dipped in 70% ethanol for one minute to sterilize the surface and rinsed for three times in sterile distilled water to remove the remaining contaminants from the diseased materials to be seeded on the Potato Dextrose Agar (Dhingra and Sinclair, 1993; Aneja, 2005).

After completely sterilized, samples were directly transferred in to growth media containing 2% Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) amended with 60 mg/l streptomycin to suppress the growth of bacteria.

Isolation of *Gibberella xylarioides* isolates from the soil was done by serial dilution agar plating method according to Aneja, (2005). Soil samples were serially diluted by adding 1 gram of each soil sample into 9 ml of sterile distilled water (10^{-1}). The dilution process was continued until the dilution factors of 10^{-5}. All dilutions were streaked on to Czapek Dox Agar plates and incubated for 7 days at 25^{O}C. Dilution of 10^{-3} and 10^{-4} were found to form better colonies and were purified by further streaking into agar plates until pure isolates were confirmed morphologically from grown colonies. The single colony was transferred to PDA in order to purify the desired fungus for further identification.

The stems further kept in separate moist chamber in order to observe growth of mycelium and induce sporulation of the pathogen. Each growth chamber was supplied with sufficient amount of sterile water on sterilized filter paper inside the jar.

All of these groups were incubated in dark incubator at $25^{o}C\pm1$ for 7 days and allowed to grow until the emergence of mycelia and sporulation of the isolates. The emerging

mycelia or spores were directly transferred from moist chamber to fresh MEA. The pure colony from each isolate was obtained and compared and also identified as *Gibberella xylarioides* isolates. Among the various isolates, five isolates were selected and designated as Fx.3, Fx.16, Fx.20, FX.22 and Fx.25, which employed through out the present studies for detailed morphological, cultural, physiological, biological control management and coffee seedlings pathogenicity test studies.

4.4.2. Characterization

The characterization of isolates was made based on morphological characterization that emphasizes on colony characteristics and conidial features.

4.4.2.1. Cultural characteristics

In the cultural characteristics surface texture, pigmentation, growth rate at different temperatures, growth media and pH were used as criteria for authentication of the *Fusarium xylarioides (Gibberella xylarioides)*. Conidial characteristics including spore septation, shape, size and color were examined. All the isolates were classified as *Fusarium xylarioides* based on the size, color, and shape of mycelia, microconidia, macroconidia and sporodochia. Based on these features the five isolates were identified to the species level using keys developed by Nelson *et al*. (1983), Rutherford (2003, 2006)

4.4.2.1.1. The Effect of Temperature on the growth of Gibberella xylarioides.

The five isolates of *Fusarium* xylarioides were grown on MEA for determining the comparative growth rates. In each of the Petri plates, 20 ml of media was poured. Mycelial disks of each isolate, 5 mm in diameter, were taken from the edge of 7-day-old colony grown on PDA and invertly placed at the center of 90 mm diameter Petri plates containing freshly prepared MEA. The plates were incubated in darkness at four different temperature (20^{O}C, 25^{O}C, 30^{O}C, and 35^{O}C). Colony diameters of each isolate in plates was measured in millimeter at right angle with a clear plastic ruler at two days intervals and the actual measurements were recorded at the 8^{th} day of incubation.

4.4.2.1.2. The Effect of PH on the growth of Gibberella xylarioides *isolates.*

The Potato Dextrose broth (PDB) (200g potato, 20g dextrose per liter of sterilized water) was prepared and adjusted to different pH levels (3.5, 4.5, 5.5, 6.5, and 7.5) in order to obtain the optimum and suitable pH value for the growth of the test pathogens. These pH ranges were adjusted using 1N HCl and 1N NaOH. The media were sterilized and five discs, of 5 mm diameter inoculum, taken from the margin of 7 days old culture grown on PDA were inoculated into 250 ml flasks containing 100 ml PDB and these were replicated three times for each. Each replicates of isolate combination were inoculated.

All these treatments were put on to rotary shaker operating 120 rpm. After ten days, the mycelia were separated from the filtrate using Whatman number 42 filter paper. The mycelial mats were harvested on these filter papers washed three times with distilled water so as to wash out the adhering salts. In order to measure the dry weight of the mycelium, each isolate with replicates was kept in an oven having the temperature of 65^{O}C for 48 hours. The dry weight of each isolate was measured with electrical sensitive balance of Wagtech Was 220/ C/2. Similarly the pH of each culture filtrate was measured immediately after being filtered to see the changes/drifts in pH from its initial as a result of the activity of each isolate.

4.4.3. Conidial Morphology

Each isolate was grown on PDA and incubated at, 25^{O}C for 7 days. Blocks of cultures were transferred into sterilized water agar containing 20g/l for sporulation. Sporulation was induced by subjecting the isolates to UV rays of 366 nm in an alternative cycle of 12 hours UV and 12 hours continuous darkness (Sutton, 1980; CABI International, 2005). The radiation was made using UV-LW 366 nm (Konrad Benda 6908 Wiesloch). The spores were observed on slides after staining with lacto phenol cotton blue under (Florescent microscope). The measurements of the spores were made by using digital solutions for imaging and microscopy, soft image system.

4.4.4. Pathogenicity test

Two years old coffee seedlings of *coffea arabica* were used to determine /evaluate the pathogenicity test of the coffee wilt disease during the present studies. The coffee seedlings were supplied by Professor Legesse Negash from greenhouse of the. Department of Biology, Science Faculty, at Addis Ababa University. The pathogenicity test was conducted both in the laboratory and outside under shade condition.

Pathogenicity test was conducted to confirm that *Fusarium xylarioides* isolates were the causal agents of coffee wilt disease. For the pathogenicity test, 2 years old coffee seedlings obtained in plastic bags (16 mm) were inoculated with a spore suspension created by drenching /flooding of *Fusarium xylarioides* isolates cultures with sterile distilled water and adjusting the concentration of spore suspension to be (10^5) spores /ml for all isolates. The spore suspension contained a mixture of micro and macro conidia. Each coffee seedling was inoculated with 20, 40 and 60 ml of the spore suspension in order to cause the infection through the roots of coffee seedlings.

Control coffee seedlings were inoculated with the equivalent amount of sterile distilled water. Three coffee seedlings per treatment and also three coffee seedlings were inoculated as control. After inoculation, the coffee seedlings were grown under shade, until symptoms started to develop. As soon as symptoms were developed on the coffee seedlings, the diseased parts were taken to the laboratory. Subsequently, small cut of diseased parts were made in order to isolate the test pathogen. The diseased parts were surface sterilized using 70% ethanol, 1% sodium hypochloride (1% NaOCl) for one minute to disinfect the surface and rinsed three times and put on sterile filter papers to dry off the moisture. The steriled pieces were then mounted on PDA and incubated, at $25^{O}C \pm 1$ until the growth of the test pathogen isolates appeared on the PDA. After the appearance of the mycelial growth, the single spore isolation technique was employed to purify the fungal spores and to obtain the pure culture of the test pathogen. Simultaneously, the spores were transferred to PDA and continuous observation was made on the sporulation, pigmentation, cultural characteristics, conidial structures and other parameters and compared with the original cultures of *G. xylarioides* isolates.

Besides these, the soil samples were taken from coffee seedlings, in order to confirm the isolates of the test pathogen. To isolate the pathogen from the soil, the serial dilution methods were utilized according to methods employed by Aneja (2005). Identification of isolates was done according to the methods employed by Booth (1971) and Rutherford (2003).

4.4.4.1. Preparation of inoculum of the isolates of F. xylarioides.

The spore suspension of each isolate of *F. xylarioides* was prepared by growing the isolates on PDA for 7 days, at $25^{O}C \pm 1$ in incubator. Then they were transferred to 20g/l water agar medium and subjected to sporulation under UV rays. Spore suspension of each isolate was collected by washing the media with sterilize distilled water. The concentration of spores of different isolates were adjusted to be (10^5) spores /ml for the isolates (Fx.3, Fx.16, and Fx.25). The spore suspension counting was done by using Haemocytometer according to the method employed by Summerell *et al. (*2006) for inoculation of F. *oxysporum* spores to sweet basil (*Oncinum basilicum*).

4.4.4.2. Inoculation and Pathogenicity study of *Fusarium xylarioides* isolates

4.4.4.2.1. Inoculation on cut stems in growth chamber

Non-symptomatic and healthy stems were collected from coffee trees planted in nursery at AAU, in the Campus of Science Faculty. The stems were chopped at equal lengths of 10 cm. The pieces of stems were thoroughly washed in running tap water for 10 minutes to remove dust particles. They were surface sterilized by immersing stem pieces in 70% ethanol for 2 minutes and rinsed for 3 times with sterile distilled water and blotted on sterilized filter paper to remove moisture. The surface sterilized and dried stems were immersed into the prepared spore suspensions for 2 hours (Lewis Ivey *et al.,* 2003;

Summerell *et al.,* 2006). The control groups were immersed in sterile distilled water. All experimental stems were aseptically transferred into sterilized jars. The jars were regularly supplied with sufficient amount of sterilized water on the surface of filter paper to keep for the humidity high in side the jars. The stems with in the jars were incubated, at 25^{o}C for three months. Each of these treatments was performed in three replicates (Jackson *et al.,* 2004). The purpose of this experiment was to check whether the isolates were the causative agents of coffee wilt disease (treacheomycosis) or not.

4.4.4.2.2. Inoculation on coffee seedlings in polyethylene bags.

In the nursery of Science Faculty, AAU inoculation experiment was made on 2 years old *Coffea arabica* seedlings on January 16, 2007. Three non-symptomatic seedlings per each treatment /isolate were prepared for inoculation. Additional three seedlings were included as controls. The feeder roots of coffee seedlings were wounded and inoculated with spore suspensions by drenching method (Lewis Ivey *et al*., 2003). Different inoculum load/ level (20 ml, 40 ml and 60 ml) was used for each isolate as demonstrated by Summerell *et* a*l.* (2006) on sweet basil (*Oncinum basilicum*).

Before inoculation, the soil in which the seedlings were grown was tested for the presence of the pathogens and other microorganisms. The spore suspension was drenched on the soil surface of seedlings after wounding the roots of seedlings. The controls were inoculated with sterile distilled water. Each treated seedlings was placed under shades of trees to avoid over heating and washing of inoculums by rain splash and to provide favorable environment for penetration pegs (Pethybrigde *et al.,* 2004). Each treated subject was marked and lesion/ symptoms developments were evaluated after 60, and 90 days of inoculation. Symptomatic leaves, stems, branches and the soil in which seedlings were particularly selected and undertake re-isolation. The symptomatic leaves, branches and stems were washed and undergone similar procedures as stated in the above isolation technique as before. The re-isolated fungi were compared with the original isolates used for pathogenicity tests according to the method employed by Summerell *et* a*l. (*2006).

4.5. *In Vitro* evaluation of Antagonistic Activity of *Trichoderma species*

Two species of *Trichoderma (T. harzianum*, Th 2895 and *T. viride,* Tv 1433*)* from culture collection of the mycology laboratory were used to evaluate the antagonistic potentials of *Trichoderma spp* against the test pathogen (*F. xylarioides or G. xylarioides*).

4.5.1. *In Vitro* evaluation of *Trichoderma spp* against *G. xylarioides* isolates.

Dual culture method (Sivakumar *et al*., 2000) was employed to evaluate the antagonistic potential of *T. harzianum* and *T. viride.* A 5 mm diameter mycelial disc from the periphery of 7-day-old culture of bioagent was placed on the opposite side of the pathogen isolates on PDA. The *F. xylarioides* isolates were inoculated 12 hours prior to the placement of the *Trichoderma spp* to establish the growth of the test fungus F. *xylarioides*. The experiment was arranged in three replicates, additional plates having only the test isolates were used as control. All plates were incubated, at $25^{o}C \pm 1$. Visual observations of growth inhibition were recorded every two days and the final measurements were recorded at the 8^{th} days of inoculation. Radial growth reduction (percentage of inhibition) was calculated according to Montealegre *et al*. (2003) in relation to growth of the control. The experiments were replicated three times with appropriate control. The percent inhibition (radial mycelial growth reduction) was calculated by the following formula.

$$\textbf{Per cent of inhibition} = \frac{(C-T)}{C} \times 100$$

Where C is radial growth measurement of the pathogen in the control plates and T is radial growth of the pathogen in the experimental plates.

4.5.2. Assay of culture filtrates of *Trichoderma species* on the mycelial growth of test isolates

To test the production of inhibitory substance by the two selected species of *Trichoderma*, Potato Dextrose Broth (PDB) was used to grow them for testing their potential to produce such substances. Two hundred fifty ml flasks containing 100 ml of PDB in each flask was used to culture the two *species* of *Trichoderma* separately. Each flask was inoculated with 5 disks of (5mm diameter each). The inoculated flasks were incubated, at 20^{O}C on shaker at 120 rpm for 20 days. After 20 days of incubation the broth was filtered through Whatman Number 42 filter paper and subsequently the filtrate of each isolate was centrifuged, at 10000 rpm for ten minutes (Aneja, 2005) to make it cell free.

The cell free culture filtrates of the two *species* of *Trichoderma* were examined to determine their influence on the mycelial growth of the test fungus (G. *xylarioides*) isolates namely Fx.3, Fx.16, Fx.20, Fx.22 and Fx.25 on PDA. For this purpose, 3ml, 4ml and 5 ml of culture filtrates of the two *species* of *Trichoderma* were mixed with sterilized and cooled PDA (45^{o}C) and added in to the flasks which contained 20 ml melted and cooled PDA. Twenty-three, 24 and 25 ml mixed media were aseptically poured into Petri plates (three replicates for each isolate).

A 5 mm disk taken from the periphery of a 7 days old culture of isolates of *G. xylarioides* were placed in the center of each plate. The treatments were done in three replications and controls were inoculated in PDA without culture filtrates. All the Petri plates were incubated, at $25^{O}C \pm 1$ for 8 days. The measurements were recorded every two days. The percent of mycelial growth was calculated by the formula developed by Montealegre *et al*. (2003).

4.6. Statistical analysis

The statistical analysis of growth characteristics of isolates at different media, temperature, and pH and mean comparisons of isolates based on different parameters were conducted using the GLM (General Linear Model, multivariate) and (compare means one way ANOVA) procedures of SPSS statistical analysis software (SPSS institute Inc., Cary, NC) version 13. Differences between treatments were determined by using least squared means comparisons with ($P<0.05$).

5. RESULTS

5.1. Symptoms of coffee wilt disease (G. *xylarioides*).

The test coffee plants showed symptoms typical of a vascular wilt, chlorosis of the leaves that become flaccid followed by desiccation and abscission, dieback of the branches at the top of the tree and spread downwards. The terminal buds were completely dried and bluish black lesions were observed on the leaves of the seedlings. Internal blackening of stems and black lesions were formed on the stems. The leaves turned yellow and finally to black brown as infection progresses. The infection was spreaded upwards through the xylem tissues and blocking transport of nutrient materials to the leaves (Fig 1)

Fig 1. Pathogenicity test A represents the control group of seedlings where as B represents experimental groups treated with Fx.3, Fx.16 and Fx.25 respectively

5.2 Characterization

5.2.1 Cultural characterstics

5.2.1.1. Colony morphology

Isolates Fx.3 and Fx.16 of *G xylarioides* showed relatively slow growth less than 7 cm on PDA after 10 days of incubation with light purple color and did not show aerial mycelium. The color of the colony from below was white to light purple diffusing into agar (Fx.20 and Fx.22). Fx.25 isolate of *Fusarium xylarioides* showed relatively slow growth, less than 7 cm in diameter after 10 to 14 days of incubation. The color of aerial mycelium of Fx.25 was white, tan to carmine red according to age, and when observed from below, light purple color may diffuse into agar. Sporodochoia were observed in all of the five isolates.

5.2.1.2. Effect of Temperature on the mycelial growth of G. xylarioides isolates on MEA

Fusarium xylarioides isolates showed differences in their growth patterns at different temperature ranges from 20^{O}C to 35^{O}C. The isolates managed to grow best between temperatures 20^{o}C and 30^{O}C except Fx.22 that showed slow growth on these temperature ranges. Isolates Fx.3, Fx.16, Fx.20 Fx.22 and Fx.25 showed maximum growth at 25^{O}C. All the isolates did not show growth at 35^{o}C.

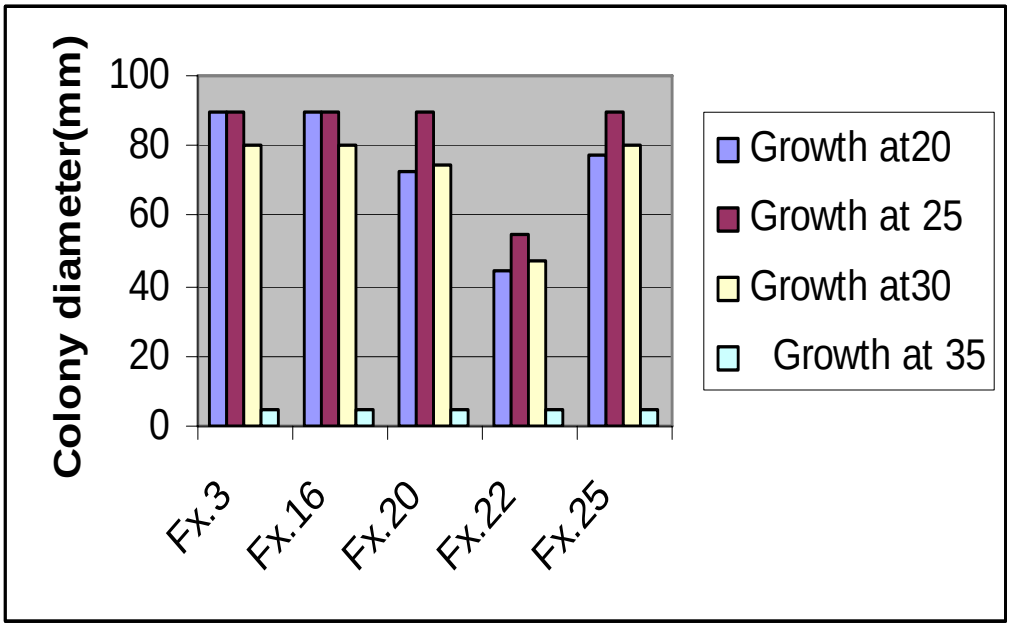

Fig 2. Growth of *F. xylarioides* isolates at different temperatures at the 8th day of incubation on MEA.

5.2.1.3. The effect of pH on mycelial growth of the isolates of G. *xylarioides.*

Five isolates of *G. xylarioides* were grown on Potato Dextrose Broth medium adjusted to different pH values ranging from3.5 to 7.5. The results of Table 3 have indicated that the different isolates of *F. xylarioides* could grow at a very wide range of pH from 3.5-7.5. The best growth of isolates Fx.3 and Fx.16 was obtained at pH 4.5, where as isolates Fx.20 and Fx.22 exhibited best growth at pH 7.5 and Fx.25 exhibited best growth at pH value of 6.5. Among the pH values maximum growth of Fx.3 and Fx.16 was obtained at pH 4.5, which yielded biomass of 323 mg and 359 mg, respectively (Table 2).The minimum growth ofFx.3, Fx.16, Fx.20, Fx.22 and Fx.25 were at pH 7.5, 3.5, 5.5, 5.5, and 7.5, respectively. The isolates changed the original pH values towards the middle value or specific for their growth

Table 2. The effect of pH on the mycelial growth of five isolates of *F. xylarioides* and pH drift on the Potato dextrose Broth medium.

pH	Isolates									
	Fx.3		Fx.16		Fx.20		Fx.22		Fx.25	
	Av. Dry wt (mg)	p_H of filtrate	Av. Dry wt (mg)	p_H of filtrate	Av. Dry wt (mg)	p_H of filtrate	Av. Dry wt (mg)	p_H of filtrate	Av. Dry wt (mg)	p_H of filtrate
3.5	222	3.92	109	3.49	295	3.9	111	3.69	169	3.76
4.5	323	4.48	359	4.46	298	3.93	170	3.95	216	4.39
5.5	220	5.58	313	4.96	232	5.32	103	4.4	131	4.43
6.5	303	5.18	237	5.07	230	5.08	163	4.11	273	4.49
7.5	216	5.32	196	4.93	299	5.19	207	4.45	78.5	4.64

5.2.2. Conidial characteristics

Description of morphological characteristics of microconidia and macroconidia indicated that the above five isolates were grouped into *G. xylarioides*, which coincided with the earlier cultural categorization. The spores appeared in the form of small slimy clusters of microconidia at the apex of short, cylindrical condiogeneous cells on the vegetative mycelium. Microconidia were abundant and strongly curved or in the form of colorless slimy droplets at the apex of elongated condiogeneous cells.

Table 3. Average length and width of conidia (µm) of five isolates of *F. xylarioides,* after 12 days of growth on PDA.

Isolate	Macroconidia		Microconidia	
	Length	Width	Length	Width
Fx.3	15.0-30.5	3.0-3.25	5.0-10.0	2.0-2.5
Fx.16	17.5-27.5	3.0-3.5	5.0-7.5	2.0-2.5
Fx.20	12.5-25.0	2.75-3.75	5.0-10.5	2.25-2.5
Ex.22	20.0-25.0	2.70-3.50	7.5-12.5	2.0-2.5
Fx.25	15.0-17.5	2.70-3.25	7.50-10.0	2.5-2.7
Mean	16.1-25.1	2.83-3.45	6.0-10.1	2.15-2.54
Range	12.5-30.5	2.7-3.75	5.0-12.5	2.0-2.7

The results of Table 4 have shown that the mean conidial dimensions of the isolates ranges from 16.1-25.1µm X 2.83- 3.45µm in macroconidia and from 6.0-10.1µm X 2.15-2.54µm in microconidia (Table 3).

5.3. Sporulation pattern

The sporulation of the five isolates was observed on Potato Dextrose Agar (PDA), Water Agar (WA) and Synthetic low Nutrient Agar (SNA). Some isolates produced their conidiospores on PDA within 10-14 days. Isolates Fx.3, Fx.20 and Fx.25 sporulated with in 10 days while isolates Fx.16, and Fx.22 needed longer time for sporulation on PDA medium. The size of conidia varied within the isolates. When the environment was suitable for mycelial growth, the conidial sizes were very small and the viceversal` was true.

5.4. Pathogenicity test

Pathogenicity of each isolate of *F. xylarioides* was tested on coffee seedlings that were planted in polyethylene bags in soil inoculated with the test isolates. Virulence was determined on the basis of coffee seedling mortality as well as foliage symptoms and disease development of the three isolates (Fx.3, Fx.16 and Fx.25). The experimental data of pathogenicity test showed that the disease symptoms and development of *F. xylarioides* isolates increased up to 90 days of drenching of the inocula of isolates of Fx.3, Fx.16 and Fx.25.The lesions were observed on all of the coffee seedlings treatments and the mycelial growth was observed on stem pieces kept in growth chamber experiment (Fig.4). Since the feeder roots were wounded positive re-isolations coincided from all parts of the coffee seedlings and from the soil, where the seedlings were grown.

5.4.1. Inoculation of spore suspensions on chopped stems in growth chamber

White mycelial growths were observed on chopped stems of coffee trees inoculated with five isolates of F. *xylarioides* on the surface of barks and on the cut surfaces (xylem tissues), after 7 days of incubation. All isolates showed white raised cottony mycelia, which were similar throughout the treatments. No mycelial growth was observed on the controls immersed in sterilize distilled water. During the first 10 days, all isolates didn't produce conidia and developed after 15 days of incubation, at 25^{o}C until the end of the treatment for about 3 months (Fig 4).

Fig 3. The mycelial growth of five isolates of *F .xylarioides* in the growth chamber at 30th days of inoculation

5.4.2. Inoculation of coffee seedlings with spore suspensions in Polyethylene bags.

The results of inoculation of coffee seedlings showed lesions/symptoms development. The typical symptoms dark brown wilting was developed on the 30th days of inoculation. The re-isolated *Fusarium species* from both growth chamber and inoculated seedlings exactly matched with the characterstics of original isolates. This indicted that all isolates were the causative agents for vascular wilt disease of coffee. The percentage of disease incidence and severity of leaves increased with inoculum level or amount and long inoculation time (Table 4). The experimental coffee seedlings completely died at the 90th days of inoculation.

Table 4. The pathogenicity of infected leaves by *F. xylarioides* isolates (percent of coffee seedlings infected) on 60th day of inoculation

Isolate	Load of inoculation (ml)	Infected leaves (Av.)	Normal leaves(Av.)	Infected leaves (%)
Fx.3	20	4.3	24.7	15
Fx.3	40	10.3	52.0	17
Fx.3	60	41.0	42.0	49
Fx.16	20	7.7	47.7	14
Fx.16	40	19.3	57.7	25
Fx.16	60	32.3	46.3	43
Fx.25	20	15.3	45.0	25
2Fx.25	40	17.3	40.7	30
Fx.25	60	32.3	53.0	38
Control	60 water	0.0	32.0	0.0

The maximum percentage of leaf infection was obtained from high concentration 60 ml by Fx.3 (49%) followed by Fx.16 (43 %) and Fx.25 (38%). The minimum percentage of

leaves infection was displayed by lower concentration 20 ml of spore suspensions by Fx.16 (14%) followed by Fx.3 and Fx.25 (having 15% and 25%), respectively.

On average the maximum leaf infection was obtained by isolate Fx.25, which has shown 31%, at 60th day. The highest incidence was recorded by isolate Fx.25 from 60th day to 90th day so that isolate Fx.25 was the most aggressive in causing infection of coffee seedlings followed by Fx.16 and Fx.3 (Table 4). At the end of the 90th day all of the experimental seedlings died out while the control treatments were healthy.

5.5. *In vitro* evaluation of antagonistic activity of *Trichoderma species*

5.5.1. Evaluation of dual cultures

In Vitro antagonistic activity of the two biological agents (*T. harzianum* and *T. viride*) was evaluated against the five isolates of *G. xylarioides*. The results of this study showed differences to inhibit the mycelial growth rate of the isolates. *T. harzianum* has rapid growth as compared to all the test isolates of *F. xylarioides* and it did not show any clear inhibition zone. Therefore, the result indicated that the interaction between *T. harzianum* and the isolates was competition for space, nutrients and intermingling of mycelium than forming inhibition zones.

T. Viride has rapid growth, in the form of powdery widespread throughout the Petri plates. As that of *T. harzianum, it* failed to develop inhibition zone, since it grew in the form of widespread powders, occupied all the spaces. The interaction was due to the competition for spaces and nutrients rather than forming inhibition zone. The growth inhibition of the test isolates by *T. viride* and *T. harzianum* was calculated by the formula mentioned in section 4.6.1.on page 33. T. *Viride* showed maximum growth inhibition (71 % and 68 %) on *F. xylarioides* isolates (Fx.22 and Fx.3), respectively. The minimum percent of growth inhibition (50) of *T. viride* was observed on Fx.25. *T. harzianum* showed maximum inhibition (66%) on Fx.20 and minimum percent of growth inhibition (55) on Fx.16 and Fx.25 as the same range (Table 5).

Table 5. The antagonistic effect of *T. harzianum* and *T. viride* on the mycelial growth of *F. xylarioides isolates* on dual culture on PDA

Isolate	Control	*T. harzianum*		*T. viride*	
		Growth	*% inhibition*	*Growth*	*% inhibition*
Fx.3	55	19.7	64	17.7	68
Fx.16	46	20.67	55	21.3	54
Fx.20	66	22.3	66	26.7	60
Fx.22	55	22	60	16	71
Fx.25	46	20.7	55	23	50

5.5.2. Inhibition of culture filtrates

The two species of *Trichoderma* (*T. harzianum* and *T. viride*) produced inhibitory substances, which is evident from the growth inhibition of the test isolates (Table 6). In general maximum and minimum inhibitions were recorded in plates containing 5 ml and 3ml of culture filtrate the two *Trichoderma* s*pecies, respectively*. *T. Viride* showed maximum percent of (64%) inhibition on the test isolate Fx.22; where as *T. harzianum* produced minimum percent of inhibition (5%) on isolate Fx.16.

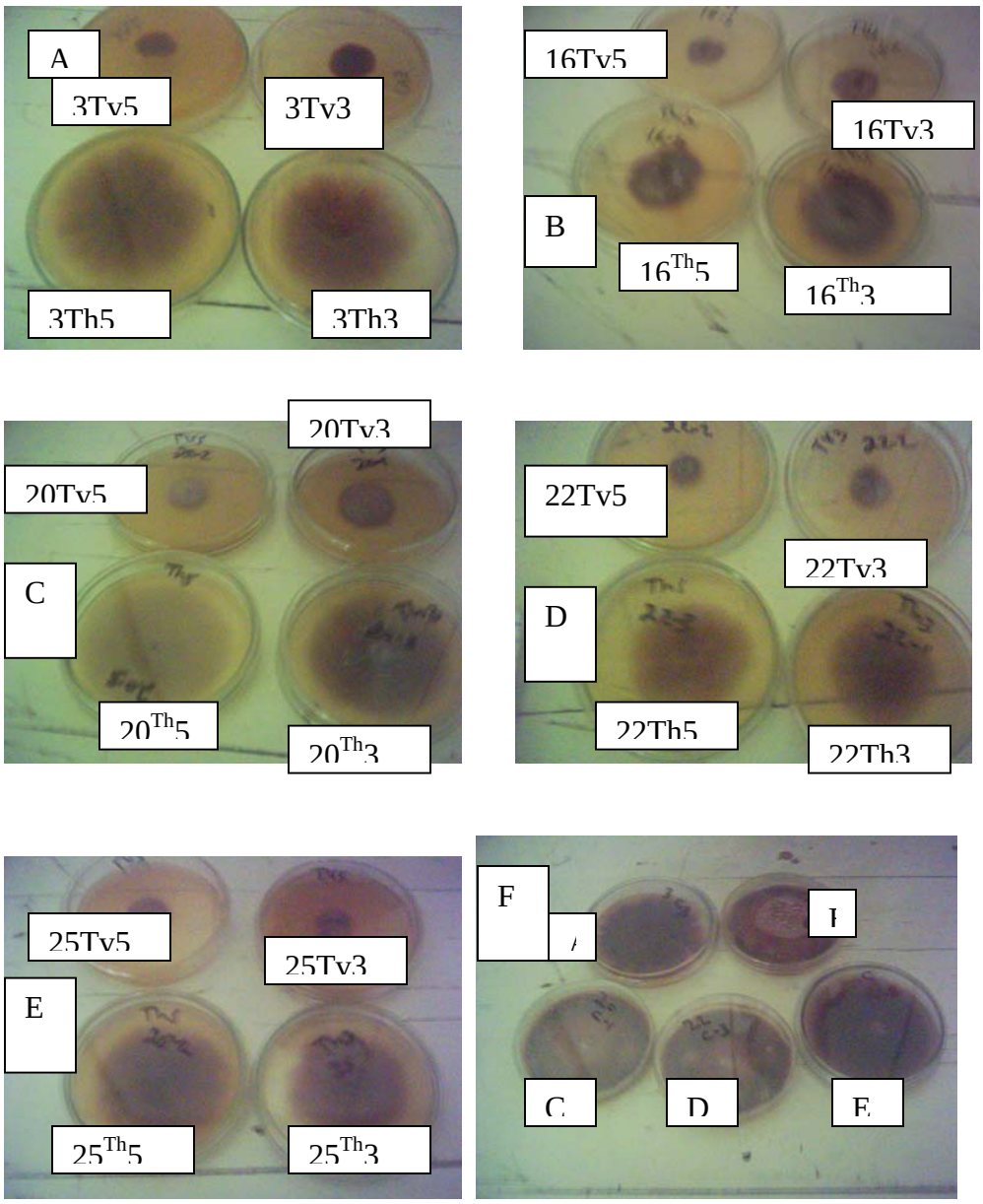

Fig 4. The effect of culture filtrates on mycelial growth A represents the effect on Fx.3, B represents the effect on Fx.16, C represents the effect on Fx.20, D represents the effect on Fx.22, and E represents the effect on Fx.25 concentration wise and F represents the controls of each isolates respectively

Table 6. The effect of culture filtrates of *Trichoderma harzianum* and *T. viride* on the growth of *F. xylarioides* isolates on PDA at 8th day of incubation(mm).

Isolate	Control (mm)	Concentrations of culture filtrates of *Trichoderma species*						Av. % of inhibition over control	
		T. harzianum			*T. Viride*			*T.harz*	*T.*
		5ml	4ml	3ml	5ml	4ml	3ml	*-ianum*	*Viride*
Fx.3	55	37	37.7	38.5	21.7	24.2	26.7	31	56
Fx.16	46	42.7	43.5	44.3	21.7	25.7	29.7	5	44
Fx.20	66	32.3	37.2	42	19.7	25.4	31	44	62
Fx.22	55	44	46	48	19	20	21	16	64
Fx.25	60	29	30	31	21.7	26.2	30.7	50	56

On Average *T. viride* has better inhibition than *T. harzianum* for all test isolates. The percent of inhibition for the two *Trichoderma species* increased when the concentrations/ amounts of culture filtrates increased. Isolate Fx.16 showed higher resistance for culture filtrate of *T. harzianum* (5%) where as Isolate Fx.20 showed higher susceptibility to culture filtrate of *T. harzianum* (44%). All isolates are highly susceptible to culture filtrates of *T. viride*. *Trichoderma viride* produces higher amount of antifungal compounds than *T. harzianum*.

6. DISCUSSION

Coffee is the most important commercial crop in the national economy, contributing 60% of its foreign exchange earnings. Among the major constraints in the successful production of coffee, tracheomycosis caused by (*Fusasrium xylarioides* Steyaert), is highly devastating in the major coffee growing regions of Ethiopia.

Five isolates from different coffee growing regions of Ethiopia were collected from CWD infected coffee and characterized on the basis of cultural, physiological and conidial characters. Colonies of isolates were pale to colorless becoming light purple to orange on the onset of microconidia production on PDA.

Microconidia were unicellular, allantoid, had 0-1 septate, and ranged 5.0-12.5 μm in length and 2.0-2.7 μm in width. Macroconidia were strongly curved with marked foot cells, had 1-4 septate, and ranged 12.5-30.5 μm in length and 2.7-3.75 μm in width. In this experiment, chlamydospores and perithecia were not observed because the shortage of materials limited for storing of cultures in the refrigerator for over seasoning of the isolates for chlamydospores and perithecia are not found in the laboratory conditions (found only in nature). The mean growth of isolates (Fx.3, Fx.16, Fx.20, Fx.22, and Fx.25) on PDA ranged 3.9-6.54 mm/day (3.9, 4.68, 6.54, 4.52, and 5.1), respectively. These results are similar with that of Lewis Ivey *et al*. (2002), which ranged 3.8-4.9 mm/day on PDA.

The suitable temperature for the cultural growth of all isolates was $25^{o}C\pm1$, while most grew best at the temperature range of $20^{o}C$-$30^{o}C$. No growth was observed at $35^{o}C$ in all isolates. From this point of view, the isolates of survive at room temperature and may be found in soils for several years in coffee growing regions of Ethiopia. This is in line with the conclusions of Nelson *et al* (1983) that 25^{o}_{C} is suitable for growth of *Fusarium species.*

With regard to the pH, growth occurred at the range of pH 3.5-7.5. The optimum pH for isolates Fx.3 and Fx.16 was 4.5 where as the optimum pH for isolate Fx.25 was 6.5 and the optimum pH for isolates Fx.20 and Fx.22 was 7.5. This showed that each isolate has specific pH to provide the maximum yield of mycelial growth. The minimum growth of isolates Fx.3, Fx.16, Fx.20, Fx.22, and Fx.25 exhibited at pH values of (7.5, 3.5, 6.5, 5.5, 7.5), respectively. This was correlated with the findings of Kapoor and Kar (1989) who reported that 3-6 (optimum 5.5) for *F. oxysporum f. sp lycopersici* on potato.

The white mycelial growth of each isolate was observed on the chopped stems of coffee inoculated with spore suspensions showed that the isolates were the causative agents of coffee wilt disease. The inoculated isolates have shown white aerial mycelial growth because the coffee bark and stem used as the natural media for the growth of *F. xylarioides* isolates. Spore production was observed after 15 days of incubation because the nutrient components were used up by mycelia of the test isolates during the growing time. When the nutrients used up the mycelial growth of all isolates was reduced and over seasoned as chlamydospores.

The inoculated coffee seedlings with the spore suspensions of three *G. xylarioids* isolates aggravated the disease severity with increasing concentrations of spore suspensions and time after inoculation. Symptom developments were observed after 30^{th} day of inoculation and complete death of the experimental seedlings were exhibited at the end of 90 days, which was coincided with the conclusions of Rutherford (2003), Rutherford (2006) “infected coffees died from 2-3 months depending on the age of coffee plants. On average, higher percentage of leaf infection was observed on seedlings drenched with 60 ml of Fx.3, Fx.16 and Fx.25 in the increasing orders (27%, 27% and 31%), at the end of 60^{th} days of drenching.

T. harzianum and *T. viride* overgrew throughout the Petri plates and occupied all the spaces and competed for nutrients hence hindered the growth of the test isolates of F. *xylarioids.* Since the growth of *Trichoderma species* was fast, the clear inhibition zones were not formed in all incubated Petri plates. The interaction between the F. *xylarioids*

and *Trichoderma species* was competition for space, nutrients and intermingling of mycelia rather than forming clear inhibition zones. The isolates Fx.3, and Fx.22 showed higher mycelial growth or lower percent of inhibition by *T. harzianum* where as the isolates Fx.16, Fx.20 and Fx.25 have shown lower mycelial growth or higher percent of inhibition by *T. viride.*

With regard to the culture filtrates of *T. harzianum* and *T. viride*, the culture filtrates contained inhibitory substances (antibiotics) that hindered the mycelial growth of *F. xylarioides* isolates. The percentage of inhibition was increased with increasing the concentration of culture filtrates in *T. viride* while in *T. harzianum* no significance difference was observed (Table 6). Relatively T. *viride* exhibited higher percentage of inhibition than *T. harzianum* against the test isolates. From these results, it is possible to conclude that *T. viride* produces larger amount of inhibitory subsatnaces coincided with the results of Lin *et al*. (1994) and Barbosa *et al*. (2001). Lin *et al*. (1994) reported that *T. viride* produced a ribosome-inactivating antifungal agent (Tricholin).

7. CONCLUSIONS

Coffee wilt disease (*F. xylarioides*) is one of the highly destructive diseases, which affects plantation and small holder coffee and reduced its production and quality. At present there is no cure for coffee wilt diseases but good agricultural practices (weeding, manuring, and pruning) have been showed to reduce the number of newly infected coffee trees and the spread of the pathogen. The maximum percent of leaves infection of *F. xylarioides* isolates were 49, 43 and 38 for Seka-Chekora, Bako Gazer and Goderie, respectively at 60^{th} day of inoculation.

In this study, smaller proportions of culture filtrates were used. Thus, perhaps a much better could be obtained by using appropriate concentrations. The results of the antagonistic activities of *Trichoderma species* revealed that *T. viride* and *T. harzianum* have good potential in inhibiting the mycelial growth of *F. xylarioides* isolates.

The present study was conducted under greenhouse and laboratory condition has shown good results. Therefore corresponding field trials should be carried out for further confirmation of the effectiveness of *Trichoderma species*

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APPENDICES

Appendix 1. Composition and preparation of different growth media.

1Czapek_Dox's Agar (PH. 6.8)

Sodium nitrate----------2.0g
Dipotassium hydrogen phosphate----------1.0g
Magnesium sulphate----------0.5g
Potassium chloride----------0.5g
Ferrous sulphate----------0.01g
Sucrose----------30g
Agar----------15g
Distilled water----------1000.0ml
Dissolve all ingredients except phosphate in half of the water and add sucrose. Dissolve phosphate separately and add to the rest. Make the volume to 1 liter. Sterilize by autoclaving at 121^{o}C and 15 Ib for 15 minutes.

2. Fusarium Selective Agar (FSA)
Dextrose (alpha-D-Glucose) ----------20g
Potassium biphosphate----------0.5g
Sodium nitrate----------2.0g
Hydrated magnisium sulphate----------0.5g
Yeast extract----------1.0g
1% ferrous sulphate solution----------1.0ml
Agar----------20.0g
Distilled water----------1000.0ml
After autoclaving, cool to about 45oC and add 5 ml of a 1 % suspension of Alisan (contains 50% w/w/ 2.6-dichloro-4nitroaniline) plus 0.1 streptomycin sulphate and 0.01g Aureomycin sulphate.

3**. Malt Extract Agar (MEA**)
Malt extract----------30g
Dipotassium hydrogen phosphate----------2.0g
Ammonium chloride----------1.0g
Citric acid 1 N----------15.0ml
Agar----------12g
Distilled water----------1000.0ml

3 Potato Dextrose Agar (PDA) PH.5.6
Potato peeled----------200.0g
Dextrose----------20.0g
Agar----------15.0g
Distilled water----------1000.0ml
Peel the skin of potato, cut into small pieces and boil (200g) in 500 ml of water, till they are easily penetrated by a glass rod. Filter through cheese cloth. Add dextrose to the filtrate .Dissolve agar in water and bring up to the required volume by the addition of water. Autoclave at 15 Ib pressure and 121^{o}C temperature for 15 minutes.

4. Synthetic-low nutrient Agar (SNA)
Potassium biphosphate---------1.0g
Potassium nitrate----------1.0g
Hydrated Magnesiun sulphate-0.5g
Potassium chloride----------0.5g
Glucose----------0.2g
Sucrose----------0.2g
Agar----------20.0g
Distilled water-------1000.0ml
Autoclave at 121^{o}C for 15 minute. .Place pieces (1cm2) of sterile filter paper on to agar surface in order to inhance sporulation. It aids for identification and induces sporulation and regular spore shape. It is transparent and isolates grow very sparsly.

Appendix.2. ANOVA of the effect of Media on growth of F. xylarioids

		Sum of Squares	df	Mean Square	F	Sig.
2nd	Between Groups	34.214	3	11.405	8.678	
	Within Groups	21.027	16	1.314		
	Total	55.241	19			
4th	Between Groups	457.018	3	152.339	6.185	.005
	Within Groups	394.057	16	24.629		
	Total	851.075	19			
6th	Between Groups	1084.550	3	361.517	5.710	.007
	Within Groups	1012.922	16	63.308		
	Total	2097.472	19			
8th	Between Groups	2266.217	3	755.406	5.013	.012
	Within Groups	2410.917	16	150.682		
	Total	4677.135	19			

Appendix 3. ANOVA of PH Test

		Sum of Squares	df	Mean Square	F	
PH drift	Between Groups	5.291	4	1.323	9.013	.000
	Within Groups	2.935	20	.147		
	Total	8.226	24			
Net mass in mg	Between Groups	25685.654	4	6421.413	.842	.515
	Within Groups	152451.744	20	7622.587		
	Total	178137.398	24			

Appendix 4. ANOVA of temperature effect data

		Sum of Squares	df	Mean Square	F	Sig.
2nd day	Between Groups	3290.983	3	1096.994	133.354	.000
	Within Groups	460.667	56	8.226		
	Total	3751.650	59			
4th day	Between Groups	17069.667	3	5689.889	112.788	.000
	Within Groups	2825.067	56	50.448		
	Total	19894.733	59			
6th day	Between Groups	36776.600	3	12258.867	106.280	.000
	Within Groups	6459.333	56	115.345		
	Total	43235.933	59			
8th day	Between Groups	58756.467	3	19585.489	108.698	.000
	Within Groups	10090.267	56	180.183		
	Total	68846.733	59			

Appendix 5. ***ANOVA of culture filtrates of Trichoderma species***

		Sum of Squares	df	Mean Square	F	Sig.
Th5	Between Groups	87.505	2	43.753	.144	
	Within Groups	304.304	1	304.304		
	Total	391.809	3			
Th4	Between Groups	158.167	2	79.083	.	.
	Within Groups	.000	0	.		
	Total	158.167	2			
Th3	Between Groups	80.683	2	40.342	.108	.907
	Within Groups	373.464	1	373.464		
	Total	454.148	3			
Tv5	Between Groups	1.490	2	.745	.274	.803
	Within Groups	2.714	1	2.714		
	Total	4.204	3			
Tv4	Between Groups	12.750	2	6.375	.797	.621
	Within Groups	8.000	1	8.000		
	Total	20.750	3			
Tv3	Between Groups	43.134	2	21.567	1.342	.521
	Within Groups	16.074	1	16.074		
	Total	59.209	3			

Appendix 6. ANOVA of Patogenicity of F. xylarioides isolates on coffee

Seedlings

		Sum of Squares	df	Mean Square	F	Sig.
Dead leaves	Between Groups	14.741	2	7.370	.035	
	Within Groups	1250.815	6	208.469		
	Total	1265.556	8			
Normal leaves	Between Groups	184.222	2	92.111	1.028	.413
	Within Groups	537.556	6	89.593		
	Total	721.778	8			
% of leaf infection	Between Groups	12.882	2	6.441	.006	.994
	Within Groups	6532.687	6	1088.781		
	Total	6545.568	8			