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Characterization of antagonistic *Trichoderma* species against Coffee Wilt Disease (*Fusarium xylarioides)*

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Abbreviations

CTA--- Coffee and Tea Authority

CWD---Coffee Wilt Disease

CBD---Coffee Berry Disease

CABI--- Center for Agriculture and Bioscience

IAR--- Institute of Agricultural Research

DRC---Democratic Republic of Congo

UCDA--- Uganda Coffee Development Authority

BCA---biological control agent

ISR--- Induced systemic resistaance

CDA---Czapek-dox agar

PDA---Potato Dextrose Agar

MEA---Malt Extract Agar

NA---Nutrient Agar

PSA---Potato Sucrose Agar

Th---*Trichoderma harzianum*

TV---*Trichoderma viride*

Abstract

In vitro antagonistic activity of *Trichoderma* isolates against coffee wilt disease (CWD), *Fusarium xylarioides* (*Gibberella xylarioides*) was studied. Out of 32 soil samples and 7 coffee tree parts collected from 6 different coffee growing woredas of Jimma Zone, 7 potential/effective *Trichoderma* isolates were isolated and characterized out of 74 fungal cultures. *Trichoderma* isolates were characterized based on spore morphology and vegetative growth characterizations on different growth media, temperature and pH ranges. The most suitable and maximum growth of *Trichoderma* isolates was obsereved at pH 4.5 and 5.5 in all of the isolates. *In vitro* evaluation of dual culture test was done for each of the seven *Trichoderma* isolates against *F. xylarioides*. It was observed that all of the seven *Trichoderma* isolates have shown 63.33 to 70.95 percent of mycelial growth inhibition of *F. xylarioides* after 6 days of incubation at 25^0C on potato dextrose agar medium. *Trichoderma* isolate AUT4 was the most effective biocontrol agent which exhibited the maximum inhibition of 70.95%. Whereas, the least percent of mycelial growth inhibition 63.33% was obtained by *Trichoderma* isolate AUT3. In addition to the above, the seven isolates of *Trichoderma* were then tested for their production of volatile antifungal compounds against *F. xylarioides*. They produced volatile compounds which inhibited the mycelial gorwth of the test pathogen from 66 to 80.60%. *Trichoderma* isolate AUT1 produced the most effective volatile compounds which inhibited growth by 80.60%. Therefore, the application of *in vitro* evaluations of seven *Trichoderma* isolates has proved to be highly potential/effective in the control of coffee wilt diseases (*Fusarium xylarioides*).

Key words: Biological Control agents, cofffee wilt disease, *Fusarium xylarioides*, *Trichoderma* isolates.

1. Introduction

Coffee belongs to the family Rubiaceae, which is widely distributed throughout the tropical region. Although there are many species of coffee, the two commercially important ones are *Coffea arabica* and *Coffea robusta* (Pieters and Van der 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) and soil fertility is important for good production of coffee (CTA, 1999; Kimani *et al*., 2002; Lewis Ivey *et al*., 2003).

Rutherford (2006) has reported that, coffee is a commodity that represents 1% of the total value of global imports and exports. Rutherford (2006) also reported that more than 80 countries are involved in coffee production, marketing and processing; and more than 60 million people depend on production and export of coffee for income throughout the world. Coffee also provides employment through cultivation, processing, marketing, transportation, and exportation of the crop (CTA, 1999; Paulos Dubale and Demil Teketay, 2000). Developing countries earnings from coffee alone amount to more than US$10 billion per year. Production of the crop is currently based on two species, *Coffea arabica* L. (Arabica coffee) and *C. canephora* Pierre ex. Froehner (Robusta coffee), which represent approximately 67% and 33% of global production, respectively (Tshilenge *et al*., 2009).

Kimani *et al*. (2002) have reported that, 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 various diseases attacking coffee compared to other *Coffea* species (Van der Graff and Pieters, 1978; Pieters and Van der Graff, 1980; Bertrand *et al*., 2001). Ethiopia is considered not only the primary origin but also the diversity of Arabica coffee (Girma Adugna 1997; 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) and 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.

Ethiopian coffee is immense national and global significance. Ethiopia holds the great majority of the Arabica gene pool, the first country to develop a coffee culture, and the country where the

largest number of smallholders depend upon it for their livelihood. Coffee is a source of livelihood for more than 25 million people engaged in production, processing, trading and marketing of the crop (Girma Adugna 2004).

Phiri and Baker (2009) reported that Ethiopia is the largest coffee producer in Africa, and for 2007/2008, the world's fifth largest. They also reported that coffee accounted for some 60% of Ethiopia's foreign exchange revenue in 2007/2008, when it earned more than 25 million US Dollars from exports of 170,888 tonnes of mostly high-quality Arabica beans. More than 90% of the production is from the garden, semi-forest and forest coffee systems of small-scale farmers while the remaining 10% comes from large-scale coffee plantation. Semi-forest coffee is estimated to contribute 35% to Ethiopia's total coffee (Paulos Dubale and Demel Teketay, 2000), even though yields are low.

However, coffee production is hampered by various biotic and abiotic factors. Diseases are the most important factors that contribute to the reduction of coffee production. Various coffee diseases are reported on coffee arabica, such as fungal, bacterial, nematodes and viral. Among the fungal diseases, the most divastating is tracheomycosis/coffee wilt disease, caused by a pathogen; *Fusarium xylarioides.* Coffee wilt disease (tracheomicosis) attacks all parts of coffee trees at all stages of development in the major coffee growing areas of the country.

The coffee wilt disease (CWD) was found to occur in all of the production systems in Ethiopia; forest, Semi-forest, garden and plantation coffee (Merdassa Ejeta, 1986; Girma Adugna and Hindorf, 2001; Girma Adugna, 2004). It has been reported by Girma Adugna (2004) that CWD is found to be more prevalent in the plantations, either in small scale farmers' 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).
Since 1993, the disease is serious in some Eastern and Central African countries (Flood, 1997; Flood and Brayford, 1997; Girma Adugna, 1997; Rutherford, 2006). Cofee producers all over the

world suffer heavy loss due to coffee wilt disease. Saccas (1951) reported that, plantation of 280 hectares of Central African Republic was completely destroyed by the disease in one year. The Uganda Coffee Development Authority (UCDA) (1993) (in annual report) reported that, the coffee wilt mainly affects the native, low land robusta variety. Since 1993, it has destroyed over 12 million coffee plants. Uganda's economy was highly affected by coffee wilt disease and production has declined from 4.4 million bags in 1996-97 to 3.6 million bags in 1997-98 (Flood, 1997). A decresae in coffee yield of over 50% has been observed in Haut Congo over the period from 1988 to 1995 (Flood and Brayford, 1997). The value of coffee exports of Rwanda has declined from US $104 million in 1985 to US $27 million in 1996. Coffee wilt disease causes yearly losses of around US$9.6 million in Uganda and US$3.8 million in Ethiopia (Kimani, 2005).

It is very difficult to control the pathogen by cultural and chemical control mechanisms once the pathogen gets into the coffee tree system. However applcation of biological antagonists against coffee wilt disease reduces incidence and severity of the pathogen and also increases the yield and quality of coffee beans. A number of *Trichoderma* spp. have a promising potential for biological control of plant pathogenic fungi (Papavizas, 1985; Perez-Vicente *et al*., 2003).The present study was carried out to isolate, characterize, screen, evaluate and test *Trichoderma* isolates to control *F. xylarioides*. We hypothesized that In vitro evaluation and testing of *Trichoderma* isolates inhibit the mycelial growth and reduce the incidence and severity of *F. xylarioides* on culture media.

2. Objetives of the study

2.1. General objective:

To isolate, characterize, evaluate and test *Trichoderma* isolates as biological control agents against *F. xylarioides* in *In Vitro* condition.

2.2. Specific objectives:

1. To study the cultural and morphological characteristics of *Trichoderma* isolates.
2. *In Vitro* screening, evaluation and testing of *Trichoderma* isolates against *F. xylarioides*.

3. Literature Review

3.1. Fungal diseases of coffee

Fungal diseases of coffee are the major constraints to reduce coffee production and quality in major coffee producing countries of Africa (Kimani *et al*., 2002). Next to 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 (Kimani *et al*., 2002).

Currently, in Ethiopia it has been reported that there are more than 45 fungal pathogens of coffee. The following table summarizes some of the fungal diseases of coffee that were recorded in Ethiopia.

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)*
Coffee wilt disease (Tracheomycosis)	*Gibberella xylarioides*
Coffee leaf rust	*Hemileia vastatirx*
Stem blight dieback (Ascochyta blight)	*Ascochyta tarda*
Brown eye spot (Berry bloch or berry spot)	*Cercospora coffeicola*
Anthracnose (Twig dieback or stalk rot of berries)	*Colletotrichum gloeosporioides*
Damping off	*Rhizoctonia salani, Pythium& 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*

Source: Negash Hailu (2007).

3.1.1. Coffee Wilt Disease (Tracheomycosis)

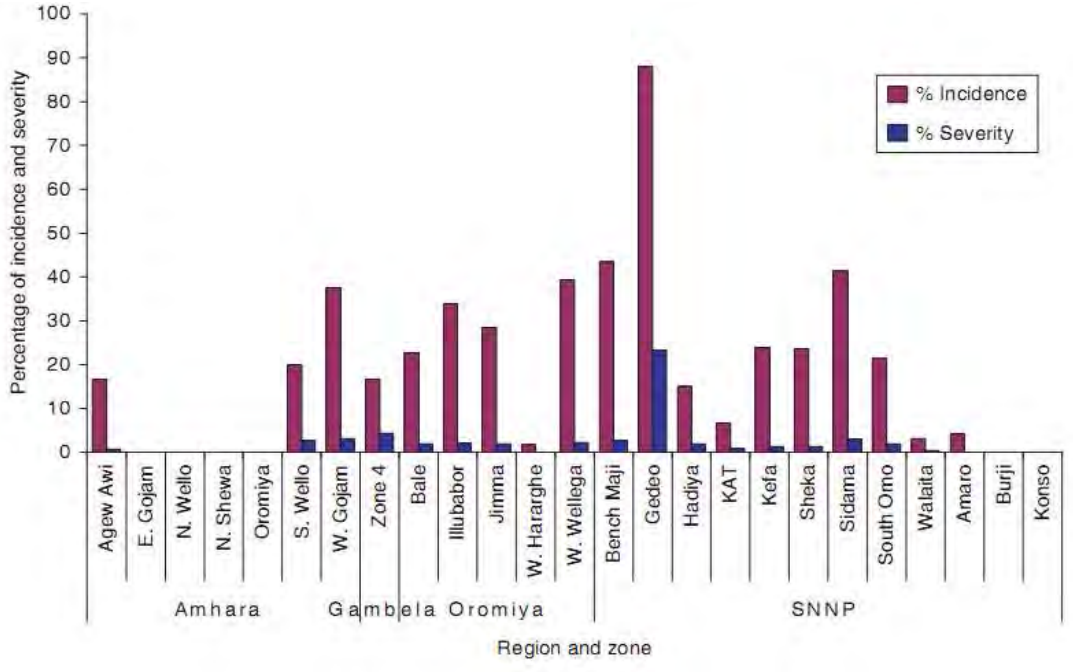
Tracheomycosis or vascular wilt of coffee historically was first observed in 1927 on *Coffea excelsa* in Central Africa Republic and first reported in 1946 and the causal agent was identified as (*Fusarium xylarioides*) by Steyaert (1948) (Flood, 1997; Girma Adugna 1997; Girma Adugna and Hindorf, 2001; Oduor *et al.*, 2003). CWD was first observed in Ethiopia in the Kaffa Province by Stewart (1957), who described a wilting of *C. arabica* and mistakenly classified it as *Fusarium oxysporum* f. sp. *coffeae*. Lejeune (1958) also noted the presence of this disease on Arabica coffee. Later, the causative agent of the disease was confirmed to be *Fusarium xylarioides* (Kranz and Mogk, 1973). 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).

Tracheomycosis is a typical vascular disease syndrome of coffee incited by a fungal pathogen, *Fusarium xylarioides*. The fungus was earlier reported to be a well-known pathogen of other *Coffea* species in West and Central Africa in the 1950s (Booth 1971). The disease was observed again in Zaire (Congo) in the early 1980s and noticed for the first time in Uganda in 1993, it is now causing economic losses to Robusta coffee in both countries (Flood 1996, 1997; Lukwago and Birikunzira, 1997). In Ethiopia, the occurrence of *Fusarium xylarioides* on *C. arabica* was established in the early 1970s by Kranz and Mogk (1973). Since then survey works have demonstrated that the disease is becoming the main factor of coffee tree death in Ethiopia. (Van der Graaff and Pieters 1978, Girma *et al*., 2001).

The incidence and distribution of coffee wilt disease (CWD) have been remarkably increasing throughout the major coffee-producing districts in the South and South-West of Ethiopia (Girma Adugna *et al.*, 2001). The range of incidence and severity of infection in Ethiopian coffee regions is shown below (Fig. 1).

Systematic elimination of affected plants over vast areas combined with the development of breeding programme 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 coffee wilt disease 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).



Source: (Phiri and Baker, 2009).

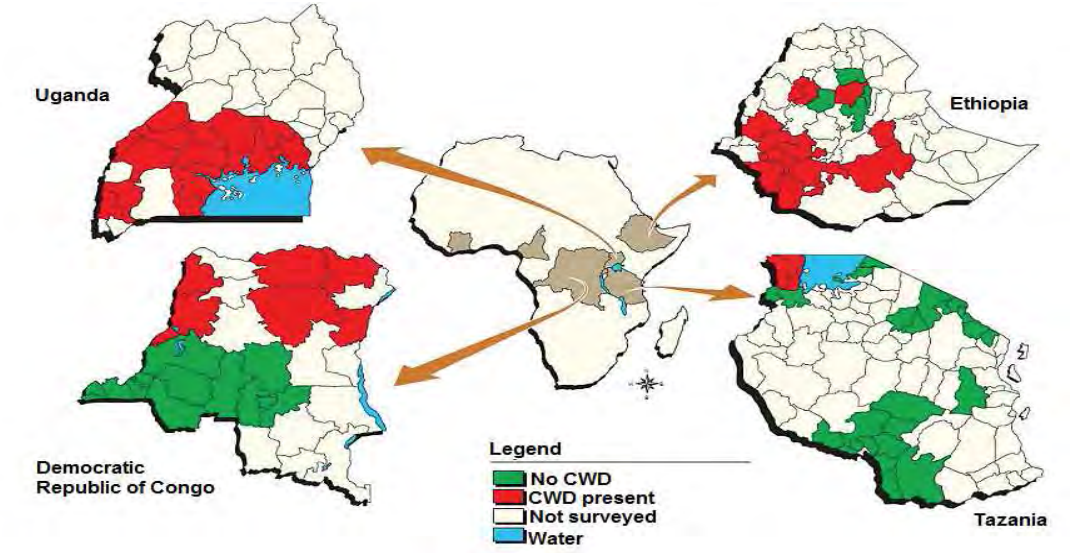
Fig. 1. Range of incidence and severity of infection in Ethiopian coffee regions.

Coffee wilt disease is present in four African countries: Democratic Republic of Congo, Uganda, Tazania and Ethiopia, and absent from the other countries surveyed Rwanda, Cote d'Ivoire and Cameroon (Phiri and Baker, 2009) (Fig. 2).

Until the mid-1900s, coffee production in Africa was based largely on *C. excelsa* A. Chev. and *C. arabica*. However, *C. excelsa* has since been replaced by *C. canephora*, primarily due to the impact of coffee wilt disease (CWD), a vascular wilt disease also referred to as *Fusarium* wilt disease (tracheomycosis) of coffee. CWD is currently having a devastating effect on coffee production in parts of eastern and central Africa and continues to spread at an alarming rate. Unlike many other diseases of coffee, CWD will rapidly kill an infected mature tree, often within as little as 6 months following appearance of the first external symptoms, and thus ultimately

result in total yield loss. Coffee quality may also be affected through, for example, premature ripening of the berries.

The 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 strain or biotypes of the pathogen. Isolates from other species of coffee (*C. arabica* and *C. excelsa*) and parts of Africa (Ivory Coast, Ethiopia) gave different band patterns. Since CWD was first discovered in 1927, it has become apparent that it exists in different forms or strains, as coffee species and even varieties of the same species can be resistant to one strain but susceptible to another. This immediately presents a problem for developing a resistant variety that would have to resist infection for many years, during which time it might come into contact with different races or even species of the disease.



Source: (Phiri and Baker 2009).

Fig. 2. Current coffee wilt disease (CWD) distribution in Africa

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). It has been estimated that affected coffee households are facing a reduction by a third of their income due to coffee wilt disease (CABI, 2003). The level of infection by this pathogen has confirmed the presence of traceomycosis with an incidence of up to 40 % (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 the 20 species of *Fusarium* 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; *Fusarium stillboides* inciting bark disease; *F. oxysporum* and *F. xylarioides* causing wilt diseases (Waller and Brayford, 1990; 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 and inducing different types of wilting on coffee in different geographical regions (Flood, 1997). Fusarial bark diseases of coffee caused by the same *F. stillboides* is an important factor limiting Arabica coffee production in the low and medium altitude distinct of Kenya (Flood and Brayford, 1997).

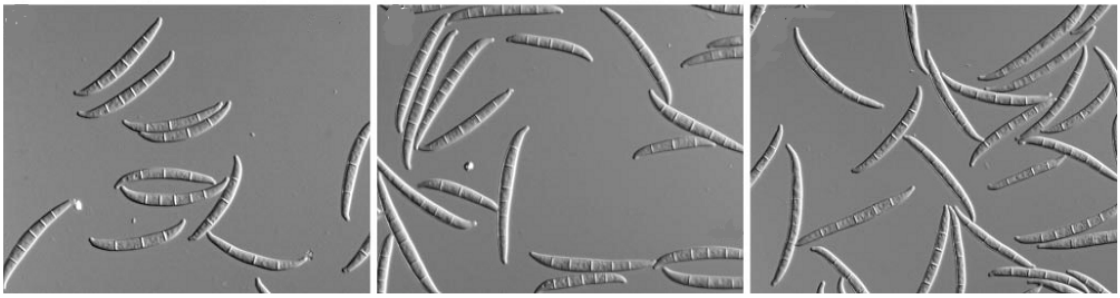
3.1.2. Biology and Taxonomy of *Fusarium xylarioides*

Gibberella xylarioides (the sexual or perfect stage) and *Fusrium xylarioides* (the imperfect/asexual stage) belongs to Kingdom:Fungi, Phylum: Ascomycota, Order: Hypocreales Family: Hypocreaceae and Genus: *Hypocrea*. The sporodochial macroconidia are 1-3 septated, frequently falcate slightly curved with distinct visible foot and basal cells (Fig. 3) (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; Lepoint *et al*., 2005).

Rutherford (2006) has obsereved that a 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; 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* species produce sickle-shaped

conidia on sporodochia. *F. 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). Sickle-shaped condidia of *Fusarium xylarioides* (Fig 3).



Source: David *et al*. (2005).

Fig. 3. Sporodochial conidia from *Fuarium xylarioides*

The taxonomy of *Fusarium* is based on the morphological characters including the presence or absence, the shape and the dimensions of microconidia, macroconidia basal cells and chlamydospores, and the growth and color development on different media are used as markers in practice (Gerlach, 1978; Lewis Ivey *et al*., 2003; Gesier *et al*., 2005). *G. xylarioides* was considered by Booth (1971) as heterothallic, with sex-linked morphological characteristics. “Female” strains produced highly curved, 0-3-septate conidia, and masses of small bluish-black stromata, some of which represented perithecial initials. “Male” strains had a slimy appearance due to the presence of pionnote sporodochia containing long, thin, 5-7-septate conidia. All *Gibberella* species are sexual states or teleomorphs of *Fusarium* species, which are destructive plant pathogens (Samuels, 2006). The anamorphic stage *F. xylarioides* was first described by Steyaert (1948) from stem samples of diseased coffee trees obtained from *C. excelsa* (Lewis Ivey *et al*., 2003). The teleomorph form was described and renamed as the *G. xylarioides* (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; Nelson *et al*., 1983; Girma Adugna and Mengsitu Huluka, 2000).

3.1.3. 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. Once the fungus infects the coffee tree, all affected trees eventually die (Girma Adugna, 2004).

Wrigley (1988) has observed that the lateral and feeder roots of coffee spread on the surface plate parallel to soil surface for a distance of 1.2 to 1.8 meters from the trunk, and *F. xylarioides* is abundantly recovered from root parts of symptomatic and asymptomatic trees (Flood,1997; Flood and Brayford,1997; Girma Adugna *et al*., 2001; Girma Adugna, 2004). The pathogen spreads two meters up to four plants on either sides of the inoculated focus plant through the infection of the roots in greenhouse experiment (Lewis Ivey *et al*., 2002; Rutherford, 2006). Closely spaced trees are more liable to wounding and cross inoculation while slashing or hoeing coffee fields. Girma Adugna (2004) reported that almost all coffee trees have wounds at the crown level or few centimeters above, and on average healthy trees have 1-3 wounds per coffee stem. Weeds are slashed frequently, some times more than ten times a year, depending on the dominating weed flora in plantation coffee. Most of coffee trees are found with wound at least once at all locations, where slashing is employed to control coffee weeds.

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

Perithecia of *F. 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 *C. 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).

In Ethiopia, most of the farmers uprooted the wilted coffee trees; however, instead of burning the wilted trees on the spot, they were dragged to the farmers' kitchens where they were used or sold for firewood. Dragging of these trees may serve to spread the disease in the farm. Plots where wilted trees were uprooted were frequently replanted with several coffee seedlings. About 80% of the farmers used the wood for fencing, 26% used it for constructing houses and animal sheds, 10% gave surplus wilted coffee trees to their neighbors for firewood and 2% sold them (Fig. 4).

Source: Phiri and Baker (2009)

Fig. 4. Infected coffee stems as support for other crops and infected coffee trunks for sale

3.2. Coffee Wilt Distribution

Weeding by slashing is done once a year around the picking season. Semi-forest coffee is estimated to contribute 35% to Ethiopia's total coffee (Paulos Dubale and Demel Teketay, 2000), even though yields are low. Coffee, intercropped with a variety of other crops inhances spread of disease where more intensively managed by slashing and pruning, together with some mulching and other organic materials (Workafes and Kassu, 2000). The coffee wilt disease (CWD) was found to occur in all of the production systems; forest, Semi-forest, garden and plantation coffee (Merdassa Ejeta, 1986; Girma Adugna and Hindorf, 2001; Girma Adugna, 2004).

Phiri and Baker (2009) have reported that, CWD incidence in Ethiopia is greatly affected by the farming system, with relatively low rates of infection in forest and semi-forest coffee and much higher rates in garden and plantation coffee. This may be due to the greater level of intervention in the latter, which gives increased opportunity for the fungus to spread, and may also be related to the greater genetic homogeneity of the coffee planted.

Pieters and Vander-Graff (1980) have reported that the disease was endemic in all coffee growing areas of Ethiopia and reached epidemic proportions in some areas (Tables 2 and 3). Although CWD is not the major constraint to coffee production until recently, it existed in

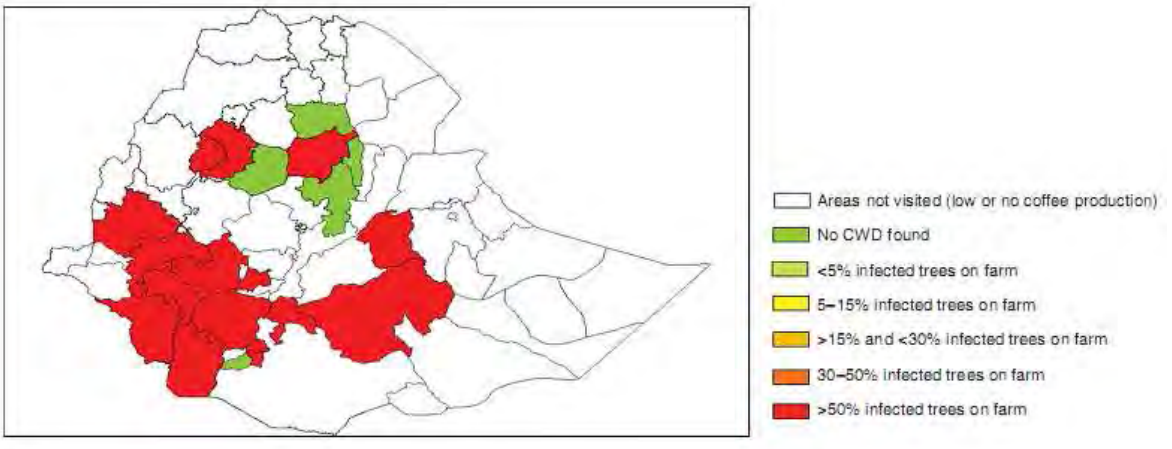
Ethiopia for many years, and yet at present the disease is less noticed by farmers in semi- forest than garden and plantation coffee (Girma Adugna, 2004).

3.2.1. CWD in the forest and semi-forest coffee

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

3.2.2. CWD in garden coffee

CWD is prevalent in the three major quality coffee-producing districts of the southern region, i.e. Wonago, Kochore and Yirgacheffe of Sidama and Gedeo zones, with the highest incidence in Yirgacheffe. The severity of wilting seen in Yirgacheffe varied between 27 and 44% (Girma Adugna, 2004). Disease incidence varied widely across coffee growing areas of the Southern Nations and Nationalities and Peoples state (SNNP) region. It was especially high in Sidama and Gedeo zones, with an incidence rate above 90% and severity of 25%. The incidence of CWD was above 35% in garden coffee of West Gojam Zone of Amhara regional state but it was very low in Wolaita (SNNP) and West Harerghe (Oromiya) (CAB International, 2003) (Fig.5).



Source: Phiri and Baker (2009).

Fig. 5. Distribution of coffee wilt disease (CWD) in Ethiopia.

3.2.3. CWD in plantation coffee

Incidence is severe in both plantation coffee and research centre plots. CWD is commonly encountered in the research plots at Gera and Jimma amounting to 43 and 48%, respectively. On plantations in Gera, Chira and Gechi districts, mean incidence ranged respectively from 22 to 26%, 33 to 77% and 35 to 60%. The overall tree loss in farmers' plantations was more than 30% and a small amount of plantation coffee had been abandoned completely. Girma *et al*. (2001) confirmed that the disease was severe in plantation coffee at Bebeka, Teppi, Gera and Jimma.

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

Location	Field	Estimated area (ha)	Incidence (%)	
			Range	Mean
Gera	Gicho 1	1.0	11.5-35.0	24.5
	Gicho 2	1.5	8.7-38.0	21.7
	Sedi-Loya	1.0	23.9-27.1	25.5
Chira	Gure-Genji	5.2	38.0-75.0	51.5
	Chira 1	4.5	55.0-89.0	77.0
	Chira 2	1.5	14.0-42.0	32.3
Tobba	Yachi	0.3	12.1-20.8	16.5
	Kilole	0.4	14.6-23.9	19.3
	Ageyu	0.2	8.3-27.0	16.1
Gomma	Shashamene	0.5	12.7-19.4	10.8
	Echemo	0.3	12.5-15.5	13.6
	Sombo	0.2	25.8-34.2	29.2
Gechi	Camp	0.5	25.0-70.0	48.9
	Mine-kobba	5.0	15.0-55.0	35.0
	Asendabo	5.0	37.7-78.6	59.7
Yayo	Jitto	1.0	11.0-34.0	22.5
Mettu	Sor	0.5	8.0-33.3	20.4
Mean of ranges and means	(Total 17)	(Total 28.6 ha)	8.3-89.0	30.9 ± 18.2

Source: (Phiri and Baker, 2009).

Table 3. Prevalence and incidence of CWD in experimental plots of Ethiopian Coffee Research Centres

Research centres/ stations	Number of fields (*n*)	Incidence (%)		Altitude (m)
		Range	Mean and SD[a]	
Jimma	10	19.8-82.0	48.2 ± 23.1	1750
Agaro	3	5.2-12.1	8.7 ± 3.4	1650
Gera	15	21.0-61.1	42.5 ± 18.7	2000
Mettu	3	23.3-30.9	27.1 ± 5.4	1550
Teppi	3	6.5-13.4	10.0 ± 4.9	1200
Wenago	3	5.7-14.6	9.8 ± 4.5	1850
Mean of range and means		5.2-82.0	24.4 ± 17.7	

Source: (Phiri and Baker, 2009).

CABI (2003) reported that 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, *Fusarium xylarioides* was observed in Jimma, Gera, Manna, Gomma, Mettu, Sidamo (IAR, 1974); Dembidollo, and Wondoguenet (IAR, 1980). Girma Adugna (1997) has reported the disease outbreaks are observed on some trees at Bebeka and in the Baya at Tepi in 1992. Later the disease distributed to Chira, Gechi, Choora, Yayo districts and other coffee growing regions of Ethiopia (Girma Adugna, 2004) and CWD became endemic to *Coffea arabica*. Van der Plank (1975) has indicated that, 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.

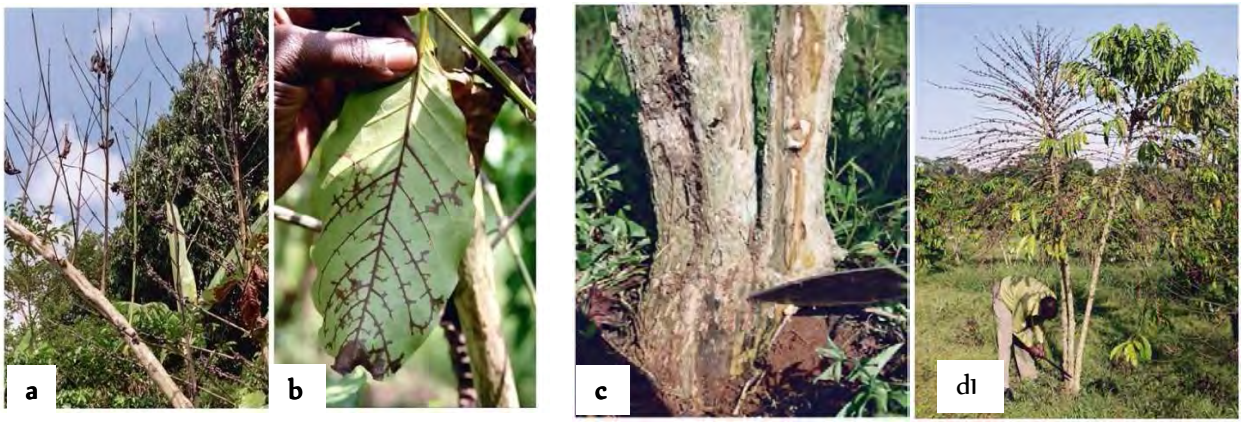
3.2.4. Status of CWD in Ethiopia:

The Arabica strain of the disease is present only in Ethiopia, and although it has been there since 1957, the incidence and severity of the disease is mostly less acute than Democratic Republic of Congo (DRC) or Uganda. Van der Graff and Pieters (1978) reported that coffee lines of *C. arabica* in Ethiopia differed widely in their resistance to *F. xylarioides* and considered that these

differences provided an excellent opportunity to control the disease using resistant varieties. It is extremely worrying that CWD is found in forest coffee, which must be considered a threat to the genetic base of Arabica, which is already under threat because of land-use change and climate change (Phiri and Baker 2009).

3.3. **Symptoms of Coffee Wilt Disease**

The first signs of CWD are yellowing, folding and curling inward of leaves (Van der 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 (Fig. 6).



Source: (Rutherford, 2006)

Fig. 6. Symptoms of wilt disease on coffee in Uganda. From left to right: **a.** defoliation and dieback; **b**. necrosis of leaf veins; **c.** blue black staining of wood (vessels) beneath the bark; and **d**. lateral defoliation of coffee tree (Rutherford, 2006).

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). 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 (Girma Adugna and Hindorf, 2001; Kimani *et al*., 2002). In the roots, a moist black rot is observed (Kimani *et al*., 2002).

Another important early sign of CWD is that berries on infected trees turn red prematurely and appear to ripen early. Most affected trees die within 6 months after the first external symptoms are observed (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).

3.4. Losses of Coffee Wilt Disease

In DRC, two of the six coffee producing provinces, to the Northeast of the country and forming the major production area, were affected with more than 90% of farms experiencing difficulties. A similar situatuation was found in Uganda, where all 27 *C. canephora* growing districts were affected and yield losses averaged 70%. In Ethiopia, 28% of *C. arabica* producing farms were affected, including those in the major coffee areas (Kimani, 2005).

Reports show that, in Tanzania approximately 160, 000kg of clean coffee lost due to the death of 54,133 trees from CWD. It is estimated that the disease has caused a financial loss of approximately US$316,137 over the last 10 years (Phiri and Baker, 2009).

The differences between countries in terms of progression of the disease are therefore striking, and reasons include: different environmental conditions, different agronomic actions, different tree stock, different social effects, different rate of movement of people and plant materials. It is clear that further studies are needed to update the spread of the disease and, for instance, its prevalence in wild coffee areas.

3.5. Management of Coffee Wilt Disease

3.5.1. Quarantine measures

For races currently free from tracheomycosis strict quarantine measures, which help to prevent its entry and spread must be followed. Movement of coffee materials (seedlings, husks, and other organs), soil and farm implements between affected and unaffected areas should be restricted as much as possible (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).

3.5.2. Resistant varieties

These are undoubtedly the most feasible option for controlling CWD in all affected countries. Use of resistant cultivars was found to be highly effective when combined with other control measures during the previous outbreak of the disease. The combined use of selected cultivars and biocontrol agents can provide better disease control than the use of any of them alone (Dik *et al*., 1998). It has been reported that varietal differences in resistance to the pathogen and suggested the use of resistant varieties as a means of control (Bouriquet 1959).

However, developing resistant varieties is long-term and requires considerable resources (human, facilities and financial). Megan *et al.,* (2006) reported that Uganda has advanced further with its CWD breeding programme, using single-tree selection, and some of the more promising selections are currently being evaluated on-farm. The DRC is also trying to select varieties for CWD-resistance.

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). Hybridization can be made between the CWD-resistant clones possessing complementary traits. The hybrid progenies generated in such crosses are also evaluated as individual trees for resistance against CWD and for field performance in the other traits. Good performing individuals can be selected, cloned and planted in multi-location trials for adaptation and adoption tests. (Phiri and Baker 2009).

In Uganda, an intensive Robusta coffee breeding programme since 1996 in search of varietal resistance to coffee wilt disease has led to the development of thousands of lines that are currently screening and evaluating against the pathogen.

Grafting is another valuable technique for the production of resistant plants, on a shorter time scale than growing from seed. Arabica coffee stems, which produce coffee of high quality, can be grafted onto root stock of Robusta coffee which is resistant to the white stemborer (Kimani *et al.*, 2002).

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

Frequent inspection of the crop, along with uprooting and burning infected material at the spot where they were uprooted, minimises disease spread. In addition, replanting should not be done for 2-3 years after uprooting infected bushes to allow the viability of the soil inoculum to decline (Wrigley 1988).

When symptoms are recognized quickly and uprooting and burning done efficiently, the farmers may save some of the crops (Flood and Brayford, 1997; Girma Adugna, 1997; Lepoint *et al*., 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). Rose *et al*. (2003) have indicated that improvement could be partially due to the encouragement of organisms such as *Trichoderma* and *Aspergillus* in the soil that compete with the wilt fungus. 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).

3.5.4. Chemical Control

Fungicides are powerful chemicals which may have a significant impact on the environment, in either a positive or negative way. Some of the advantages of Fungicides are that they are fast-acting, they can control large infestations, they are easy to obtain and apply, they may increase crop production by reducing crop losses. Some of the disadvantages of pesticides are that they may damage and/or accumulate within the environment, they may kill non-target species, they

may be dangerous to users/pets/native species, and they can drift from their original point of application (Grays and Pacific) (http://cru66.cahe.wsu.edu/Label Tolerance.htm).

There are several reports where fungicides have been used for the control of diseases caused by soil-borne pathogens (Seoud *et al*., 1982; Iliesea *et al*., 1985). Captan and Benomyl have been used successfully against several seed-borne fungi under laboratory and field condition (Goulart, 1992). The fungicide Banodenil and Pentachloronitobenzene (PCNB) were found effective in delaying on set of southern blight of apple seedling and root rot and in slowing disease progress (Conway *et al*., 1996).

Commonly used fungicides which are effective against *Fusarium* species are: Carbendazim, Dithane M-45, Thiovit and Thiophanate-methyl significantly reduced the growth of *F. oxysporum (*Abdul *et al.,* 2006). Aminoglycosides: amikacin, gentamicin, kanamycin A, kanamycin B, neomycin, and ribostamycin showed the best fungicidal activities *against F. graminearum* and suppressed fungal infection (Yukie, 2008).

The pathogen, *Fusarium xylerioides* 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). Fungicide stem spray – copper oxychloride (50% WP) sprayed on to the stem only, to be diluted at the rate of 40 g per 7.5 l of water and applied once a month during the rainy season, and once every 3 months during the dry season (Phiri and Baker, 2009). If the fungus carried on coffee seeds, then the treatment of seeds with fungicides may be beneficial (Lewis Ivey *et al*., 2003; Rutherford, 2006).

3.5.5. Biological control

Biological control is the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control diseases, insect pests and weed populations. The organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA) Kamal and Brian (2006). Biological Control is the reduction of inoculum density or disease producing activities of a pathogen or a parasite in its active or dormant state, by one or more organisms,

accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists (Baker and Cook, 1974).

More broadly, the term biological control also has been applied to the use of the natural products extracted or fermented from various sources. These formulations may be very simple mixtures of natural ingredients with specific activities or complex mixtures with multiple effects on the host as well as the target pest or pathogen **(**Kamal and Brian, 2006).

With regards to plant diseases, suppression can be accomplished in many ways. Biological control refers to the purposeful utilization of inroduced 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 (Kamal and Brian, 2006).

3.5.6. Characteristics of Biological Control Agents

The BCAs exhibit different modes of action and hence, a good testing program should elucidate all the mechanisms involved in the biocontrol activity of the BCA. Apart from biocontrol ability, the BCAs possess other traits such as rhizosphere competence, tolerance of fungicides, saprophytic competitive ability, ability to tolerate high and low temperatures, adaptability to different edaphic conditions, good searching ability, host specificity, high reproduction rate, short life cycle, adaptability, well adapted to different stages of life cycle of target host, able to maintain itself after reducing host population (Harman *et al*., 2004; Kok and Victoria, 1999). These traits are useful for good BCA as they help in the establishment of the BCA in a given agro-ecological region.

3.5.7. Antibiotic-mediated suppression

Antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. Most microbes produce and secrete one or more compounds with antibiotic activity. Antibiotics produced by microorganisms have been shown to be particularly effective at

suppressing plant pathogens. Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens. *Bacillus cereus* strain UW85 is known to produce both zwttermicin and kanosamine. The ability to produce multple antibiotics probably helps to suppress diverse microbial competitors, some of which are likely to be plant pathogens (Pal and Mcspadden Gardener, 2006).

Trichoderma have long been recognized as agents for the biocontrol of plant diseases. The potential of *Trichoderma* species as biocontrol agents of plant pathogens was first recognized in the early 1930s (Weindling, 1932) *Trichoderma* spp. can directly affect mycelia or survival propagules of other fungi through production of toxic secondary metabolites, formation of specialized structures, and secretion of cell wall-degrading enzymes (Sarrocco *et al*., 2006).

Mycoparasitic activity of *Trichoderma* spp. against phytopathogenic fungi and oomycetes due to lytic activity of cell wall-degrading enzymes has been widely studied. In addition to mycoparasitism, other mechanisms have been proposed to account for biocontrol of plant disease by *Trichoderma* spp., including the induction of resistance in the host plant and competition for nutrients and potential infection sites (Harman *et al*., 2004).

Trichoderma are widely used in agriculture, and some of the most useful strains demonstrate a property known as rhizosphere competence, the ability to colonize and grow in association with plant roots (Harman, 2000). *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; Tesfaye Alemu and Kapoor, 2004).

Trichoderma would be especially suitable for combating coffee wilt disease because many of its species are rhizosphere competent (Whipps, 2001), and the coffee roots are the first target for the attack by pathogens Bowen and Rovira (1974); Weller (1988). In support of this hypothesis, *Trichoderma* spp. have already been applied successfully to suppress *Fusarium* spp. causing *Asparagus* root rot (Rubio-perez *et al*., 2008), bean root rot (Gilardi *et al*., 2008), and carnation wilt (Shanmugam *et al*., 2002).

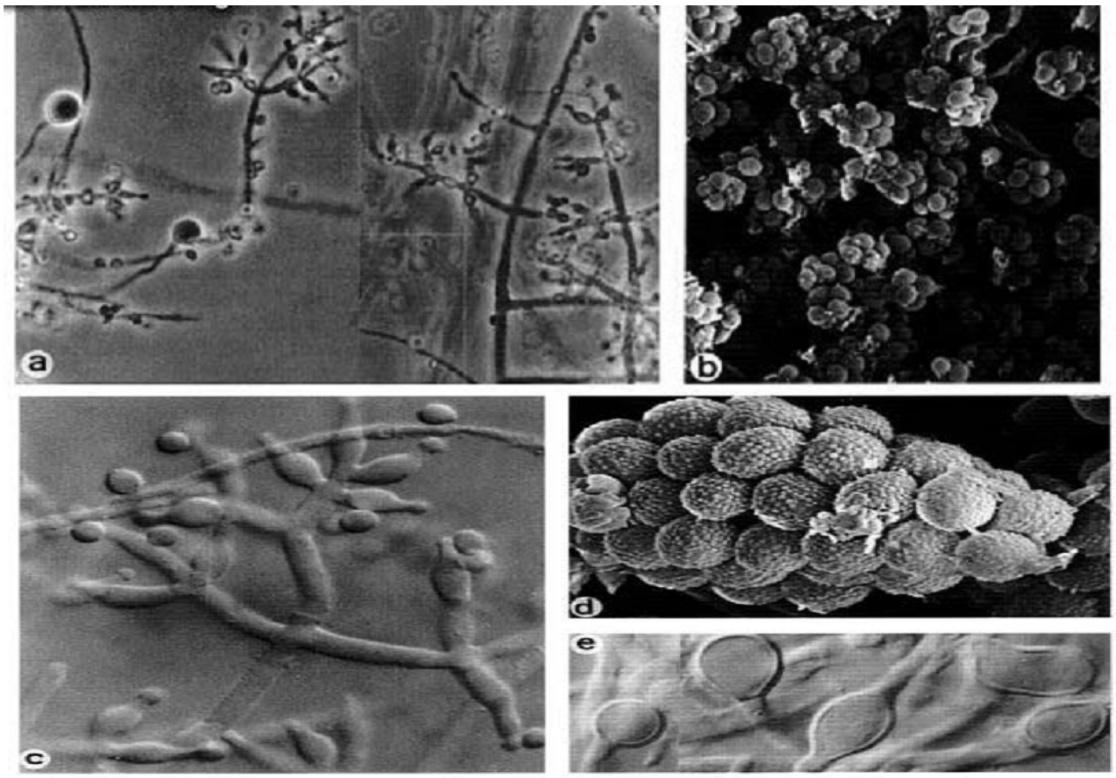
Various strains of the filamentous fungus *Trichoderma* spp. are considered to be among the most useful fungi in industrial enzyme production, agriculture and bioremediation. More recently, these fungi have been utilized extensively as model microorganisms in studies in order to analyze and improve the understanding of the role that these antagonistic fungi have an important biological interactions with crop plants and phytopathogens (Marra *et al*., 2006; Woo *et al*., 2006).

Trichoderma spp. have been widely studied, and are presently marketed as biopesticides, biofertilizers and soil amendments, due to their ability to protect plants, enhance vegetative growth and contain pathogen population under numerous agricultural conditions (Harman, 2000; Harman *et al*., 2004; Lorito *et al*., 2006; Vinale *et al*., 2008). 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 and Kubicek, 1998).

3.6. Morphology and distribution of *Trichoderma* Species

Trichoderma are filamentous fungi commonly found in the soil community that are facultative saprophytes. They are members of a genus belonging to a group of largely asexually reproducing fungi that includes a wide specturm of micromycetes that range from very effective soil colonizers with high biodegradation potentioal to facultative plant symbionts that colonize the rhizosphere.

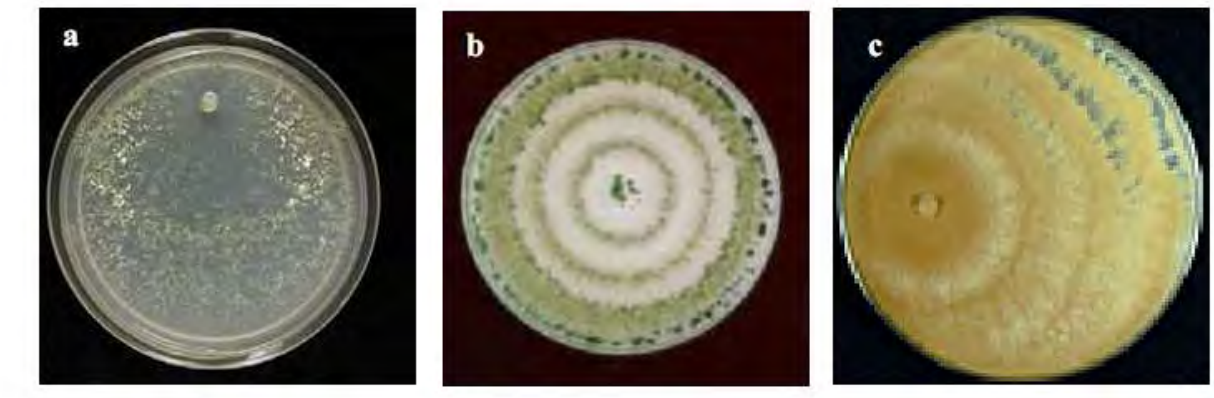
Trichoderma is usually recognized by the presence of fast-growing colonies producing white, green, or yellow cushions of sporulating filaments, the fertile filaments or conidiophores produce side branches bearing whorls of short phialides that support the spherical to ovoid green colored (Fig. 7). *Trichoderma* 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.



Source: Khalid (2009)

Fig. 7. *Trichoderma viride* IFO 30498. a-d Conidiophores and conidia, e chlamydospores.

The mycelia of *Trichoderma* spp. on potato dextrose agar (PDA) plate cultures is typically fast growing, with the optimal temperatures between 25-30^0 C. The hphae are initially transparent or whitish, and depending upon the species, the mycelium become greenish, yellowish or less frequently white within one week (Fig. 8). 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.



Source: Khalid (2009)
Fig. 8. *Trichoderma* spp grown in culture media. a. *T. atroviride*; b. *T. viride*; c. *T. harzianum*.

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* spp directly on the soil offers greater protection to the crops (Cooney and Lauren, 1998).

The mechanisms that *Trichoderma* spp uses to antagonize phytopathogenic fungi include competition, colonization, antibiosis and direct mycoparasitism (Howell, 2003). This antagonistic potential serves as the basis for effective biological control applications of different *Trichoderma* strains as an alternative method to chemicals for the control of a wide spectrum of plant pathogens (Chet, 1987).

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).

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

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; (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, and (vi) induced systemic resistance (ISR) similar to that stimulated by beneficial rhizobacteria (Harman *et al*., 2004; Howell, 2003)

Trichoderma biocontrol strains utilize numerous mechanisms for both attacking other soil organisms and enhancing plant and root growth (Harman, 2000; Harman *et al*., 2004; Vinale *et al*., 2008). The colonization of the root system by rhizosphere competent strains of *Trichoderma* results in increased development of root and/or aerial systems and crop yields (Harman and Kubicek, 1998; Yedidia *et al*., 2001). *Trichoderma* has also been described as being involved in other biological activities such as the induction of plant systemic resistance and antagonistic effects on plant pathogenic nematodes (Sharon *et al*., 2001).

Some strains of *Trichoderma* spp have also been noted to be aggressive biodegraders in their saprophytic phases (Wardle *et al*., 1993), in addition to acting as competitors to fungal pathogens, particularly when nutrients are a limiting factor in the environment (Simon and Sivasithamparam, 1989). These facts strongly suggest that in the plant root environment *Trichoderma* actively interacts with the components in the soil community, the plant, bacteria, fungi, other organisms, such as nematodes or insects, that share the same ecological niche.

Trichoderma spp. is important participants in the nutrient cycle. They aid in the decomposition of organic matter and make available to the plant many elements normally inaccessible. Yedidia *et al*. (2001) noted that the presence of the fungus increased the uptake and concentration of a variety of nutrients (copper, phosphorus, iron, manganese and sodium) in the roots of plants

grown in a liquid medium. These increased concentrations indicated an improvement in plant active-uptake mechanisms.

Corn that developed from seeds treated with *T. harzianum* strain produced higher yields, even when a fertilizer containing 40% less nitrogen was applied, than the plants developed from seed that was not treated (Harman 2000; Harman and Donzelli, 2001). This ability to enhance production with less nitrate fertilizers provides the opportunity to potentially reduce nitrate pollution of ground and surface water, a serious adverse consequence of large-scale maize culture. In addition to effects on the increase of nutrient uptake and the efficiency of nitrogen use, the beneficial fungi can also solubilize various nutrients in the soil that would be otherwise unavailable for uptake by the plant (Altomare *et al*., 1999).

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). 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).

3.6.1.1. 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, harzianins 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.*

3.6.1.2. 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). 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.

Trichoderma spp also produce both volatile and non-volatile compounds that inhibit the growth of the mycoparasites. Gary *et al.,* (2001) identified five classes of volatile compounds, such as alcohols, esters, ketones, acids and lipids, produced by some fungi and bacteria.

3.6.1.3. 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). 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).

3.6.1.4. 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 (Okigbo and Ikediugwu, 2000). Thus, the rapid growth of *Trichoderma* spp 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).

3.6.2. Induction of host resistance

Plants actively respond to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability. Plants also respond to a variety of chemical stimuli produced by soil and plant associated microbes. Molecules produced by *Trichoderma* and/or its metabolic activity also have potential for promoting plant growth (Yedidia and Chet, 1999). Applications of *T. harzianum* to seed or the plant resulted in improved germination, increased plant size, augmented leaf area and weight, greater yields (Vinale *et al*., 2008). Metabolic changes occur in the root during colonization by *Trichoderma* spp., such as the activation of pathogenesis-related proteins (PR-proteins), which induce in the plant an increased resistance to subsequent attack by numerous microbial pathogens (Table 4). Such stimuli can either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens. Induction of host defenses can be local and/or systemic in nature, depending on the type, source, and amount of stimuli. Induced

systemic resistaance (ISR) has been used to describe the systemic resistance induced against pathogens by nonpathogenic or plant growth-promoting rhizobacteria Hoffland *et al.* (1996).

Table 4. Evidence for, and effectiveness of, induced resistance in plants by *Trichoderma* species

Species and strain	Plant	Pathogens	Evidence or effects	Time after application	Efficacy
T. virens G-6, G-6-5 and G-11	Cotton	*Rhizoctonia solani*	Protection of plants; induction of fungitoxic terpenoid phytoalexins	4 days	78% reduction in disease; ability to induce phytoalexins required for maximum biocontrol activity
T. harzianum T-39	Bean	*Colletotrichum lindemuthianum*; *Botrytis cinerea*	Protection of leaves when T-39 was present only on roots	10 days	42% reduction in lesion area; number of spreading lesions reduced
T. harzianum T-39	Tomato, pepper, tobacco, lettuce, bean	*B. cinerea*	Protection of leaves when T-39 was present only on roots	7 days	25–100% reduction in grey-mould symptoms
T. asperellum T-203	Cucumber	*Pseudomonas syringae* pv. lachrymans	Protection of leaves when T-203 was present only on roots; production of antifungal compounds in leaves	5 days	Up to 80% reduction in disease on leaves; 100-fold reduction in level of pathogenic bacterial cells in leaves
T. harzianum T-22; *T. atroviride* P1	Bean	*B. cinerea* and *Xanthomonas campestris* pv. phaseoli	Protection of leaves when T-22 or P1 was present only on roots; production of antifungal compounds in leaves	7–10 days	69% reduction in grey-mould (*B. cinerea*) symptoms with T22; lower level of control with P1. 54% reduction in bacterial disease symptoms.
T. harzianum T-1 & T22; *T. virens* T3	Cucumber	Green-mottle mosaic virus	Protection of leaves when *Trichoderma* strains were present only on roots	7 days	Disease-induced reduction in growth eliminated
T. harzianum T-22	Tomato	*Alternaria solani*	Protection of leaves when T-22 was present only on roots	3 months	Up to 80% reduction in early blight symptoms from natural field infection
T. harzianum T-22	Maize	*Colletotrichum graminicola*	Protection of leaves when *Trichoderma* strains were present only on roots	14 days	44% reduction of lesion size on wounded leaves; no disease on non-wounded leaves
Trichoderma GT3-2	Cucumber	*C. orbiculare*, *P. syringae* pv. lachrymans	Protection of leaves when *Trichoderma* strains were present only on roots; induction of lignification and superoxide generation	1 day	59% and 52% protection from disease caused by *C. orbiculare* or *P. syringae*, respectively
T. harzianum	Pepper	*Phytophthora capsici*	Protection of stems when *Trichoderma* strains were present only on roots; enhanced production of the phytoalexin capsidiol	9 days	~40% reduction in lesion length
T. harzianum NF-9	Rice	*Magnaporthe grisea*; *Xanthomonas oryzae* pv. oryzae	Protection of leaves when NF-9 was present only on roots	14 days	34–50% reduction in disease

Source: (Harman et al., 2004).

3.7. Isolation of fungal biological control agents from soil samples

The method used to isolate microorganisms is a soil dilution technique. Dried, crushed and seived soil samples (10 g) were are shaken in 90 ml of sterile water for 10 min then left standing for a further 20 min. A dilution series was made up to 10^{-6}. Aliquots (0.5 ml) are spread onto three Czapek-dox agar (CZA) plates and incubated at 25°C for 2 weeks (Jones and Stewart 1997). Resulting colonies are purified on PDA plates and identified using standard mycological keys.

3.8. Testing of Biological Agents

Testing must begin with the identification of the BCA and ciontinue up to the commercial product. In Vitro tests are tests which have been designed for identification or selection of potential BCAs and elucidate biocontrol mechanisms of known BCAs. Dual Culture methode is also known as biculture, cross culture, or paired culture Baker and Cook (1974), has been extensively used for preliminary screening of large populations of fungal, bacterial, and actinomycetous BCAs in a petri dish under optimum conditions for both the pathogen and the BCA. The inhibition is rcorded either in the form of the inhibition zone produced or the overgrowth of the pathogen by the BCA.

3.9. Environmental impacts of Biological Control Methods

To achieve successful biological control, good knowledge of the host-pathogen-environment interaction is required in specific agro-ecosystems in which the biological control agent has to act. The interactions between microbial biological control agents, the target species to be controlled, the host and the environment can be complex and require a good research foundation prior to attempting formulation **(**Mausam *et al*., 2007).

Biocontrol agents that have successfully made the difficult transition from lab to field conditions, the record for transfer from experimental field conditions to on-farm use has been abysmal. Many agents that perform better under controlled conditions demonstrate little or no efficacy under agricultural production conditions. A significant barrier appears to be survival of the biocontrol agents when they are fermented, formulated, and applied in scale. Under experimental conditions, it is feasible to prepare the inoculum within 24-48 hours of planting, whereas under

production conditions the inoculum often must survive transportation on seed or in packages. Gains have been made in this area by providing stabilizing and nutritional agents for fungi and bacteria in the formulations such as spores (Handelsman 1996).

Community dynamics- the first step in understanding community function is to describe the membership of the community of the rhizpsphere or phyllosphere before application of the biocontrol agents Handelsman (1996).

Safety-many biocontrol agents are closely related to opportunistic human pathogens. Examples of biocontrol organisms of questionable safety abound. *Pseudomonas aeruginosa*, a biocontrol agent of gray leaf spot on turf is a virulent opportunistic pathogen infecting surgical wounds and severe burns. *Burkholderia cepacia*, a highly successful biocontrol agent of pea root rot and other diseases, is associated with opprtunistic lung infections of patients with cystic fibrosis. *Trichoderma viride* is an opportunistic human pathogen and is on the biological warfare list in some countries Handelsman (1996). Therefore, it is important to carry on safety test before application of biological control agents.

4. Materials and Methods used

4.1. Sources of Biocontrol agents and test fungus

The various cultures of isolates of *Fusarium xylarioides (Gibberella xylarioides*) were obtained from Jimma Agricultural Research Center, which were isolated from different major coffee growing regions of the country. Isolation, identification, characterization, screening, testing, in vitro evaluation and morphological study of *Trichoderma* isolates were conducted in Mycology and Applied Microbiology Laboratories, in the Department of Biology, Science Faculty, Addis Ababa University.

4.2. Sterilization and Maintenance of cultures

The sterilization of media and glass wares were done by autocalaving at 121^0C temperature and 15lb pressure for 15 minutes. The maintenance of cultures of *F. xylarioides* isolates and biological control agents (fungal) were maintained on potato dextrose agar (PDA) slants. The slants were stored in the refrigerator at 4^0C for further investigation.

4.3. Methods of isolation of antagonists from soil

Field sampling: The samples were colleced from Gera, Gomma, Mana, Kossa, Seka Chekorssa woredas of Jimma zone. A total of 8 samples of coffee tree parts and 32 soil samples were taken from a depth of 15 cm in the upper sub soil profile from the root zone of diseased and healthy coffee trees. The samples were put in plastic bags and were brought to Mycology Laboratory, Department of Biology, Addis Ababa University, for isolation and characterization of *Trichoderma* isolates.

The soil samples were air-dried under room temperature in the Mycology Laboratory and were ground in to fine particles before isolation. Isolation of biological control agents from the soil was done by serial dilution agar plating method according to Aneja, (2005). In serial dilution agar plate methods, 10g of soil sample was suspended or agitated in 90 ml sterile water to make microbial suspension. Serial dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were made by pipetting measured volume of 1 ml into 9 ml sterile water.

Dilutions 10^{-3} was used to enumerate fungi to their proportion in soil. Finally, 1ml of various dilutions were added to sterile petri dishes (triplicate for each dilution) to which 20-25ml of the sterile, cool, molten (45^0C) media (Czapek-Dox agar) were added. Dilutions were streaked on to specific agar plates and incubated in an inverted position for 2-7 days at 25^0C. The number of colonies of fungal antagonists on dilution plates were picked and purified on PDA media and were maintained in slants. The single colony was trasferred to potato dextrose agar (PDA) media for further morphological identification of antagonists.

4.4. Screening of Biological Agents

For preliminary dual testing of biological antagonists, 5mm fungal agar blocks from the leading margin of cultures of isolates and *F. xylarioides* cultures were placed 4-5 cm apart from each other at the center of pre-solidified PDA medium. Plates were incubated at 25°C for 6 days and potential isolates were selected depending on their degree of inhibition. Isolates that showed positive mycelial growth inhibition on dual culture medium were further tested, according to Dhingra and Sinclair, (1993); Chowdhry, (1996); Aneja, (2005).

4.5. Cultural and morphological characterizations of fungal isolates

Cultural characteriztion of Biocontrol agents was done on the basis of colony growth characterisics and conidial/spore features that were grown on specefic media for each antagonist fungal islates. In the cultural characterisics surface texture, pigmentation, growth rate at different temperatures, growth media and pH were important growth parameters of the biocontrol agents. Five millimeter disc of each of the pure cultures of the seven fungal isolates were inoculated into sterilized pre-solidified potato dextrose agar (PDA) medium and they were incubated at 25^0C. The mycelial growth of the fungal islates was observed every day. All the isolates were microscopically characterized based on the size, color and shape of mycelia, and conidia.

4.6. The effect of temperature on the growth of fungal isolates

Each of the effective biocontrol agents of *Trichoderma* isolates was grown on PDA/MEA and incubated at 4^0, 25^0C, 30^0C and 35^0C for 7 days. Blocks of cultures were trasferred into sterilized PDA/MEA media. The plates were incubated in darkness at four different teperatures (4^0 , 25^0C, 30^0C, and 35^0C). Colony diameter of each of the isolates in plates was measured.

4.7. Evaluation of culture media for the mycelial growth of *Trichoderma* isolates

Seven *Trichoderma* isolates were inoculated on four different media: PDA, MEA, PSA and CDA to evaluate their media preferece. Each of the media was sterilized and was poured into sterilized petridishes. A 5mm diameter agar disc excised from the edge of pure culture of each of the *Trichoderma* isolates was placed at the centre of each agar plate. The plates were incubated at 25^0C incubator. The *Trechoderma* isolates mycelial growth diameter was measured after 6 days incubation of cultures.

4.8. The effect of pH on the growth of *Trichoderma* isolates

Potato dextrose broth (PDB) 20g/l potato, 20g/l dextrose of sterilized water was prepared and adjusted to differnt pH levels: 3.5, 4.5, 5.5, 6.5, and 7.5 in order to obatain the optimum and suitable pH value for the growth of *Trichoderma* isolates. These pH ranges were adjusted using 1N HCl and 1N NaOH. The media were sterilized and five discs of 5mm diameter inoculum were taken from the margin of 7 days old culture grown on PDA were inculated in to 250ml flasks containing 100ml potato dextrose broth in three replicates for each isolates. All treatments were put on to rotary shaker operating 120 rpm, at room temperature. After ten days, the mycelia were separated from the filtrate using Whatman No 42 filter paper. The mycelial mats were harvested on these filter papers and were washed three times with distilled water so as to wash out the adhering salts. In order to measure the dry weight of the mycelia, each biocontrol agent with replicates of three were kept in an oven of tepperature 65^0C for 48 hours. The dry weight of each biocontrol agents was measured with electrical sensitive balance. Similarly, the pH of each culture filtrate was measured immediately after being filtered to see the changes in pH from its initial as a result of the activity of each biocontrol agent.

4.9. *In Vitro* evaluation and testing of antagonistic activity of *Trichoderma* isolates against *G. xylarioides*.

4.9.1. Antagonistic activities of Biocontrol in Dual Culture Test

Dual culture method was employed applied as detailed in Evans *et al*. (2003) to evaluate the antagonistic potential of *Trichoderma* isolates. A 5 mm diameter mycelial disc from the periphery of 7 days old culture of bio-agents were placed on the opposite side of the test pathogen isolates on PDA. The *F. xylarioides* isolates were inoculated 12 hours prior to the placement of the fungal isolates to establish the growth of the test fungus. The experiment was arranged in three replicates, additional plates having only the test isolates were used as control. All plates were incubated at 25^0C. The mycelial growth inhibition was measured after 7 days of inoculation. Percentage of inhibition was calculated according to Montealegere *et al*. (2003) in relaton to growth of the control. The experiment was replicated three times with appropriate control. The percent inhibition was calculated by the following formula:

% inhibition = $\frac{(C-T) \times 100}{C}$ where 'C' is radial groth measurement of the pathogen in the control plates and 'T' is radial growth of the pathogen in the experimental plates.

4.9.2. Testing Production of volatile compounds

Twenty ml malt extract agar (MEA) was poured onto each Petri dish. A 5 mm diameter agar disc excised from the leading edge of pure culture of each of *Trichoderma* isolates cultures was placed at the centre of each agar plate. Next, a disc of the same size was taken from *F. xylarioides* culture, likewise placed on another agar plate. The lids were removed, and *F. xylarioides* culture plate was immediately placed over each of the *Trichoderma* isolates. Plates were held together with adhesive tapes. The head space prevented any physical contact between *F. xylarioides* and *Trichoderma* spp, so that the volatile compounds were formed and confined to the interior atmosphere of the two plates (Siddiquee *et al*., 2009). For the control plate, only *F. xylarioides* was cultured on each Petri dish. The plates were incubated at 25±2ºC for 7 days. The diameters of *F. xylarioides* colony cultures were measured on the 7^{th} day. Three replicate plates were done for each treatment, and the experiment was repeated twice.

4.9.3. Evaluation and Testing of Sancozeb on mycelial growth of *F. xylarioides*

Two grams of a fungicide chemical, Sancozeb was added into a 250ml flask containing 98ml of distilled sterilized water and was placed on a shaker for 15 minutes. Stock solutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} were made by pipetting 10ml in 90ml distilled sterile water to maintain concentration of fungicide, sancozeb. From each of the dilutions 15ml was transferred into three 250ml flask containing 60ml of sterilized PDA medium before it solidifies at 45^0C. After the content was well shaken, 25ml of the medium was poured into sterile petri dishes (triplicate for each dilution). Seventy five millilitre medium with out fungicide was prepared for control. A 5 mm diameter agar disc excised from the edge of pure culture of *F. xylarioides* was placed at the centre of each agar plate. Plates with out fungicide were controls.The plates were placed at 25^0C incubator for 8 days.

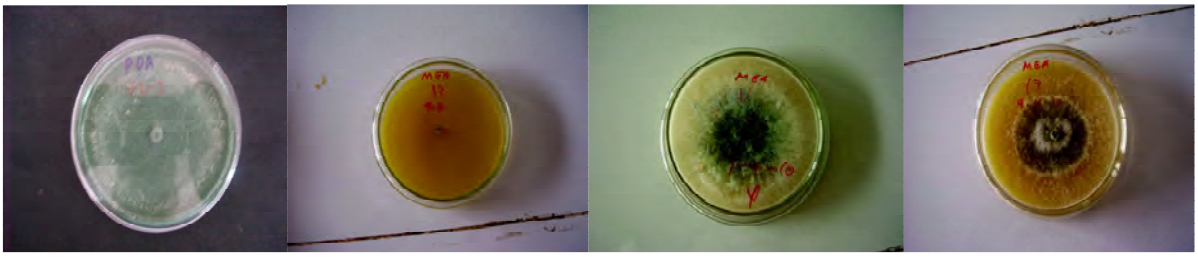
5. Results

5.1. Cultural characterization of *Trichoderma* isolates

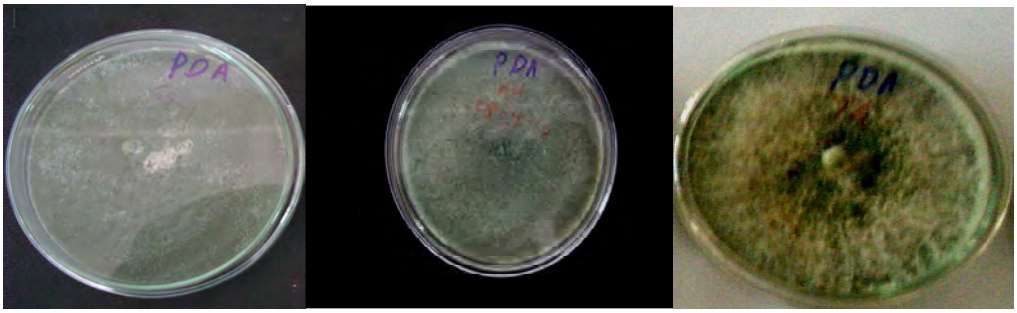
Out of 74 fungal isolates, seven *Trichoderma* isolates showed inhibition durning the preliminary screening of the fungal biocontrol antagonists against *F. xylarioides*. Initially, growth started as light, white mycelia after the second day of inocubation. These then enlaged within 3-4 days and the white color turned to green and yellowish green color which helped in their identification. On the fifth day, all the 7 isolates of *Trichoderma* were 100% grown on 9cm diameter plates. Isolates AUT7 turned to a deep green colour, the mycilia were not raised, they were effused over the media. *Trichoderma* isolate AUT1, AUT5 and AUT2 turned bright green and raised mycilia. Whereas *Trichoderma* isolates AUT4 and AUT6 yellowish green forming ascending rings of spores and *Trichoderma* isolate AUT3 was more yellowish and the mycelia were very light and were not raised. The spores were spreaded all over the plates (Table 5) (Fig. 9).

Table 5 Cultural characteristics of *Trichoderma* isolates

Isolates	Cultural caracteristics colony		
	Colony color	**Conidia shape**	**Mycelial appearance**
AUT1	bright green	Oval	Raised, no rings
AUT2	bright green	Oval	Raised with rings
AUT3	yellow	Oval	Effused & light
AUT4	yellow	Oval	Raised with rings
AUT5	green	Oval	Raised, no rings
AUT6	yellowish green	Oval	Raised, no rings
AUT7	deep green	Oval small and numerous	Effused, no rings



Isolate AUT6 Isolate AUT3 Isolate AUT2 Isolate AUT4



Isolate AUT7 Isolate AUT5 Isolate AUT1

Fig. 9. Mycelial coloration of *Trichoderma* isolate on PDA media

Microscopic examinations showed that the conidiophores typically formed paired branches along the length of the main axis. Phialides were typically enlarged in the middle and cylindrical. The terminal chlamydospores were observed as oval-shaped and colorless (Fig. 10).

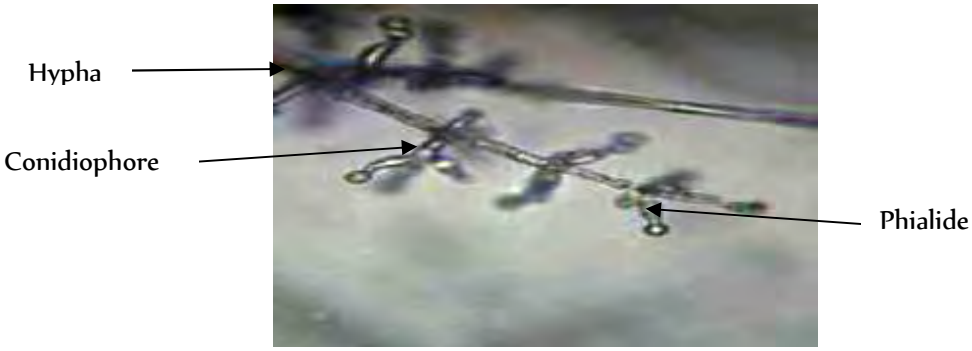


Fig. 10. Microscopic examination of *Trichoderma* hypha, conidiophore and phialide.

5.2. The effect of teperature on the growth of *Trichoderma* isolates

The seven *Trichoderma* isolates grew best at both temperature rages of 25^0C and 30^0C. Trichoderma isolates AUT3, AUT4, AAUT7 and AUT6 have 100% growth at 35^0C as well. *Trichoderma* isolates AUT5, AUT1 and AUT2 shown limited mycelial growth of 1.5, 3.5 and 5.1 cm respectively at 35^0C (Fig. 11). At lower temperature of 4^0C none of the isolates has grown.

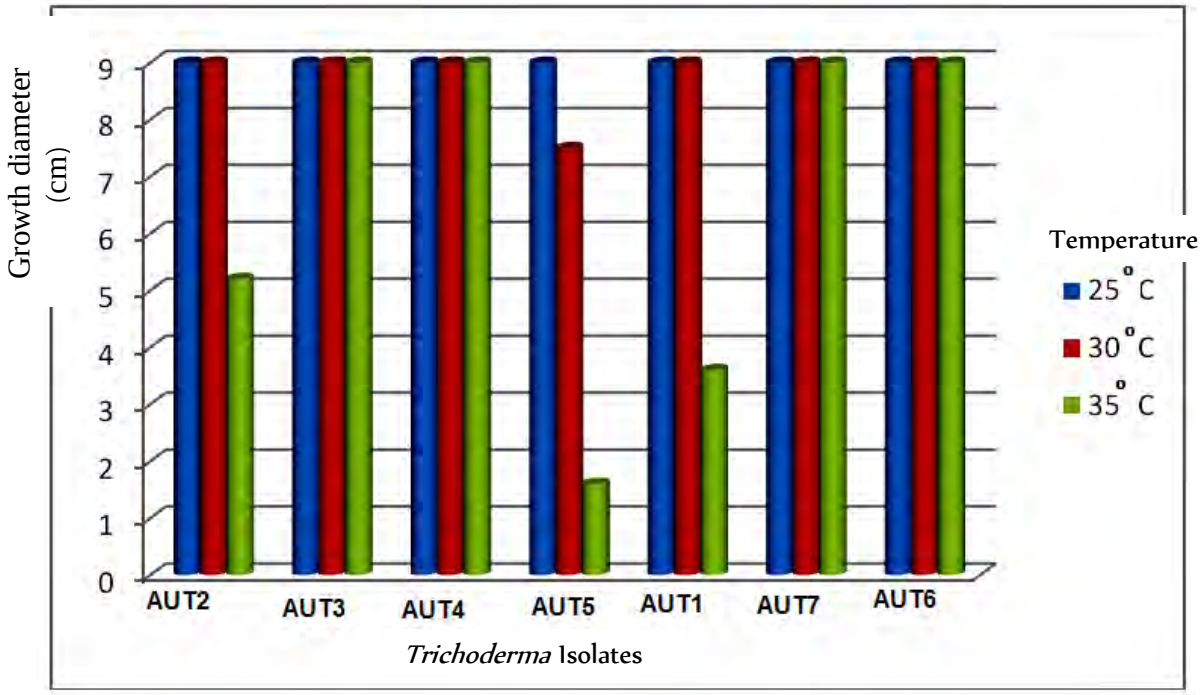
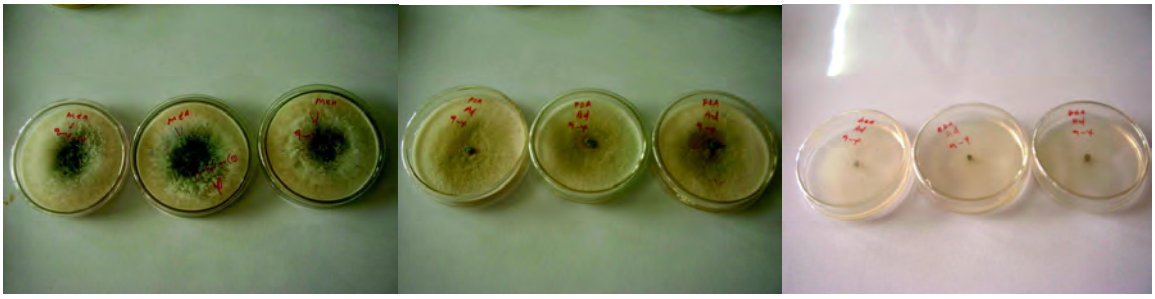


Fig. 11 The effect of temperature on the growth of *Trichoderma* isolates

5.3. Evaluation of media preference of *Trichoderma* isolates

Trichoderma isolates were grown on four different media and all the isolates grown 100% on a 9cm diameter plate of potato dextrose agar (PDA) and malt extract agar (MEA). Their growth on potato sucrose agar (PSA) shown limited growth ranging from 5cm to 7.8cm diameter on a plate of 9cm diameter. The growth of all the isolates on Czapek-Dox Agar (CDA) medium was very much limited growth ranging from 0-3cm(Fig. 12). Mycelial growth measurements of *Trichoderma* isolates on 4 different culture media is shown in (Fig. 13).



MEA PDA CDA

PSA PSA MEA

Fig. 12 Mycelial pattern of Growth of *Trichoderma* isolates on different culture media

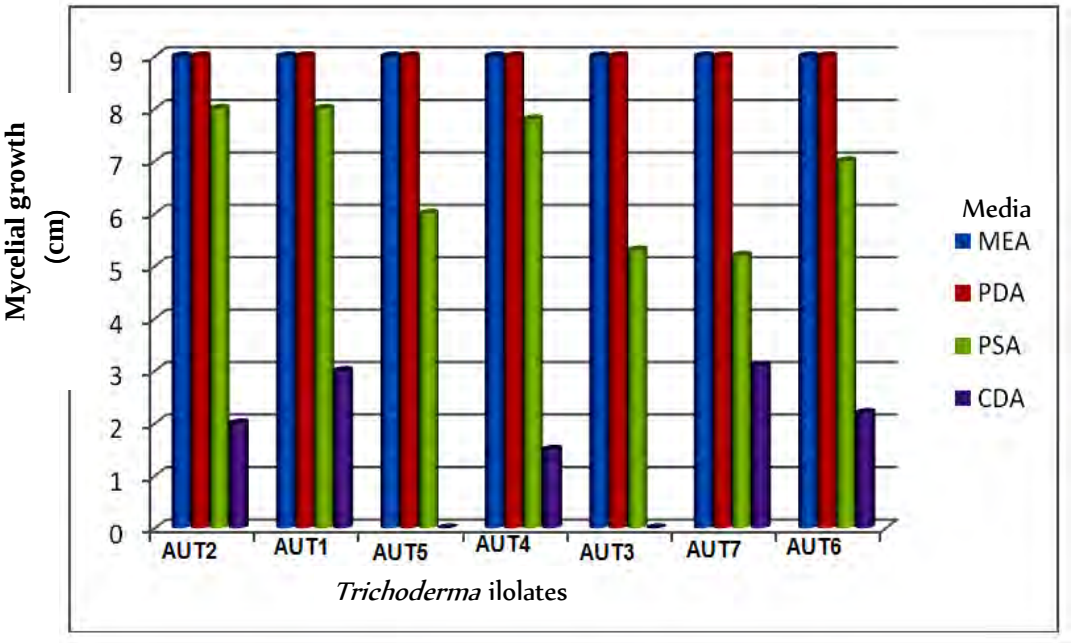


Fig. 13 Mycelial growth measurements of *Trichoderma* isolates on 4 different culture media

5.4. The effect of pH on the growth of *Trichoderma* spp

The Seven isolates of *Trichoderma* were grown in Ptato Dextrose Broth adjusted to pH values ranging from 3.5-7.5 and the dry weight of the macylial mat was measured in grams after 15 days of growth at 25^0C. The results showed that all of the *Trichoderma* isolates could grow in this range of pH values. Maximum growth was obsereved at pH 4.5 and 5.5 in all of the isolates. None of the isolates indicated limited growth in either of the pH values as the mean values of the dry mycelial mat indicates (Table 6). These property of *Trichoderma* isolates enable them to survive in wide range of soil pH.

Table 6. Mean dry weight of the mycelial mat at different pH (g)

Isolates/pH	3.5	4.5	5.5	6.5	7.5
AUT4	0.48	0.61	0.86	0.74	0.73
AUT3	0.58	0.56	0.77	0.56	0.53
AUT2	0.91	1.07	0.95	0.96	0.82
AUT5	0.73	0.72	0.76	0.61	0.58
AUT1	1.11	0.78	0.75	0.83	0.65
AUT7	0.57	0.70	0.63	0.57	0.51
AUT6	0.49	0.64	0.68	0.65	0.54

pH of the filtrate was measured immediately after filtration and changes in pH was recorded. The pH change is not that significant exept in *Trichoderma* isolate‘AUT1’ and ‘‘AUT2’’ of which change in pH shows more variation than other isolates (Table 7).

Table 7 Change in PH of the filtrate of Trichoderma isolates after ten days growth

Isolates	Initial PH	PH after 15 days of growth	Change in PH
AUT3	3.5	3.43	-0.07
	4.5	4.80	+0.30
	5.5	5.85	+0.35
	6.5	6.28	-0.22
	7.5	6.67	-0.83
AUT4	3.5	3.40	+0.10
	4.5	4.81	+0.31
	5.5	6.07	+0.57
	6.5	7.06	+0.56
	7.5	7.06	-0.44
AUT2	3.5	3.03	-0.47
	4.5	7.32	+2.82
	5.5	7.38	+1.88
	6.5	7.33	+0.83
	7.5	7.84	+0.34
AUT5	3.5	4.56	+1.06
	4.5	6.16	+1.66
	5.5	5.57	+0.07
	6.5	5.50	-1.00
	7.5	6.52	-0.98
AUT1	3.5	4.07	+0.57
	4.5	6.81	+2.31
	5.5	6.62	+1.12
	6.5	7.24	+0.74
	7.5	6.70	-0.80
AUT7	3.5	3.07	-0.43
	4.5	3.44	-1.06
	5.5	3.78	-1.72
	6.5	4.58	-1.92
	7.5	4.19	-3.31
AUT6	3.5	3.70	+0.2
	4.5	4.70	+0.2
	5.5	5.37	-0.13
	6.5	5.71	-0.79
	7.5	5.98	-1.52

5.5. Dual Culture Test

All of the *Trichoderma* isolates were able to overgrow and parasitize the mycelia of *F. xylarioides*. The antagonistic potential of *Trichoderma* isolates was determined by the nutritional condition, growth rate, and production of diffusable compounds that inhibit growth by the antagonists and the susceptibility of the pathogen (Fig. 14). All the isolates have shown better inhibition on the dual culture test between 60%-75% (Table 8). The fast growing *Trichoderma* isolates overgrown surrounding the fungus in test and showed no inhibition zone.

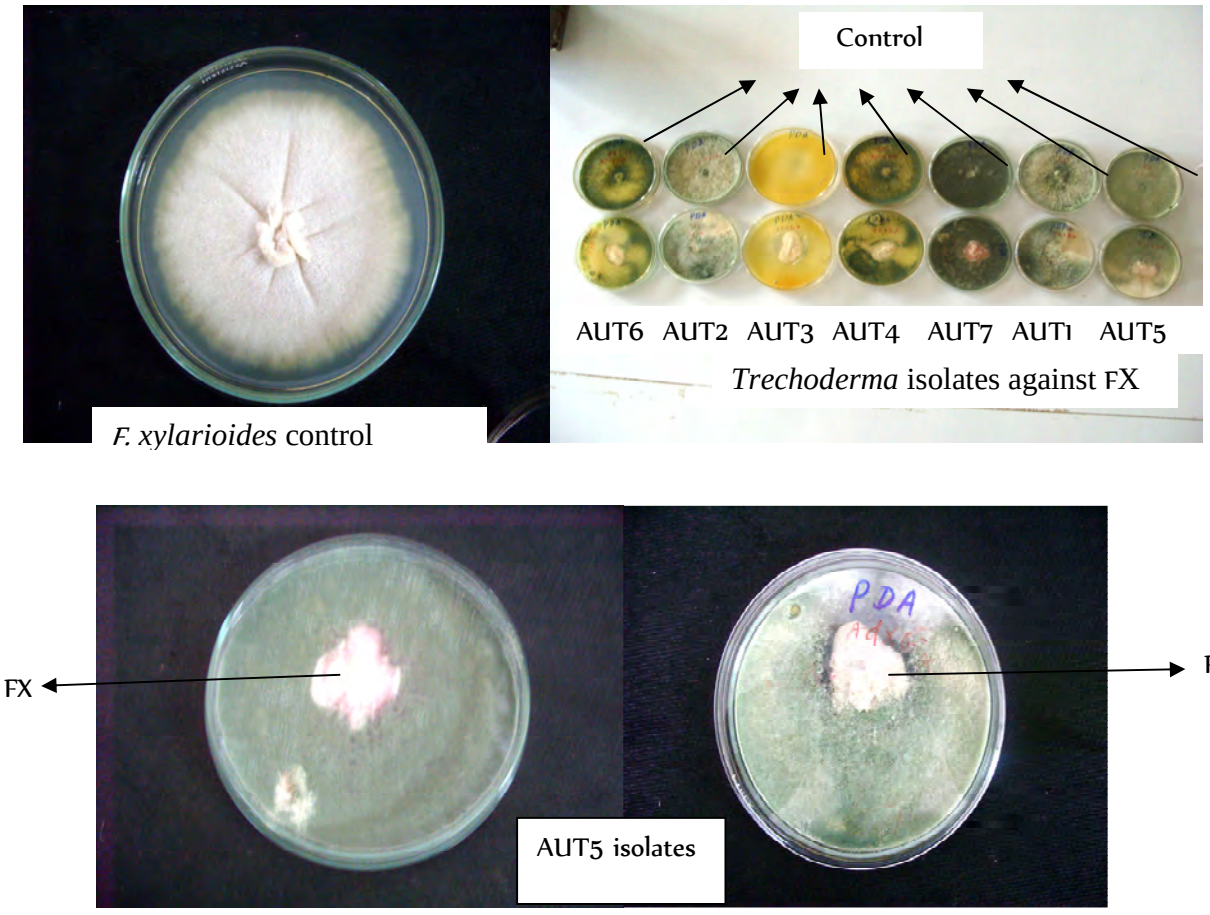


Fig. 14. Mycelial Growth of *F. xylarioides* inhibited by *Trichoderma* isolates

The maximum mycelial inhibition of 79.95% was obtained by *Trichoderma* Isolate AAUT4. The least Percent of mycelial growth was obtained by isolate AAUT3 that is 63.33%. *Trichoderma* isolates AAUT7, AAUT6, AAUT2, AAUT1, and AAUT5 indicated mycelial inhibition not significantly different (Table 8).

Table 8 Percentage of mycelial growth inhibition of dual culture test

Percent inhibition of *Trichoderma* Isolates	
Trichoderma Isolates	% inhibition
AUT7	66.19
AUT6	64.70
AUT2	65.71
AUT1	67.62
AUT3	63.33
AUT4	79.95
AUT5	65.71

5.6. Evaluation of Sancozeb on mycelial growth of F. xylarioides

The fungicide, Sancozeb used during the present studies showed inhibition on growth of *F. xylarioides*. Inhibition decreased as the concentration of the fungicide decreased. The first two; fungicide dilutions 10^{-2} and 10^{-3} showed complete (100%) inhibtion of the pathogen's growth. However, inhibition by the fungicide dilutions 10^{-4}, 10^{-5} and 10^{-6} was limited. The percentage of inhibition is shown in table 6.

Table 9. The effect of Fungicide, Sancozeb on the mycelial growth of *F. xylarioide*

Percent inhibition of sancozeb	
Concentration g/100ml	Percent of inhibition
0.02	100
0.002	100
0.0002	48.33
0.00002	33.33
0.000002	24.17

5.7. Determination of effect of volatile compounds

In the test for determination of volatile compouns in *Trichoderma* spp., Isolates: AUT2, AUT5, and AUT1 produced volatile compounds that inhibited the growth of *F. xylarioides* by 75%-81%. From these obseration, the three isolates produced more effective volatile compounds than isolates AUT3, AUT4 and AUT6, which inhibited the growth of the pathogen in test by 65%-70% (Fig. 15). All the isolates produced volatile compounds except isolate AUT7.

Table 10. Percent of inhibition of mycelial growth of *F. xylarioides* by volatile compounds

Isolates	Percent of inhibition
AUT6	66
AUT7	8
AUT4	64
AUT3	66
AUT5	76
AUT1	81
AUT2	77

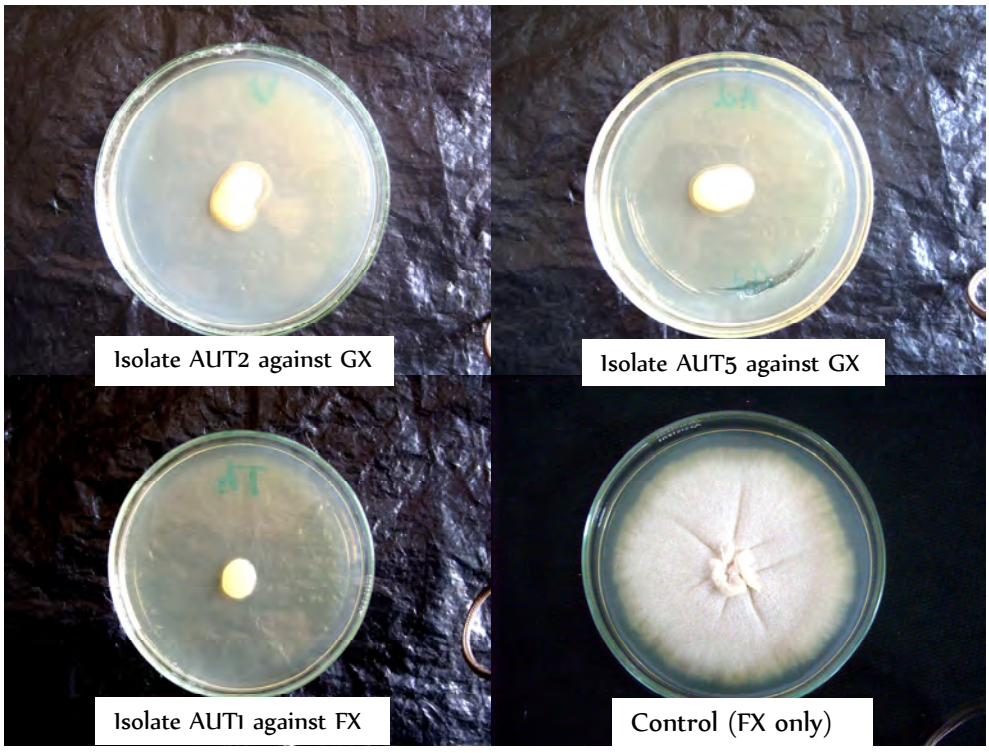


Fig. 15. The effect of volatile compounds on the growth of *F. xylarioides*.

6. Disscussion

Coffee is the most important cash crop in the Ethiopian economy which contributes about 60% of foreign exchange earnings. However, coffee is highly infected by various diseases. Among the major diseases that affect coffee is tracheomycosis, which is caused by *F. xylarioides.* Hence, to minimize the incidences and severity of such serious diseases, different disease control measures are being employed. Among the different control measures of coffee diseases, biological control method is being investigated by many researchers. Because biological Control for soil borne plant pathogens is ideal, cheap, long lasting and eco-friendly as compared to chemicals. In the present study seven *Trichoderma* isolates of *Trichoderma* were tested against *F. xylarioides* for their antagonistic activities and effects on the test pathogen.

Aggregate species of *Trichoderma* can be differentiated on the basis of macroscopic (colour of conidia, sporulation patterns and density) and microscopic (structure and arrangement of phialides, conidial size and shape) features. In the present study it was observed under microscope that all the seven *Trichoderma* isolates had oval-shaped spores which were almost equal in size except isolate AUT7 which had numerous and small-sized spores.

The optimum temperature for growth differs among the *Trichoderma* isolates. In the present study, all isolates of the *Trichoderma* grew best at temperatures 20^0C, 30^0C and 35^0C and the optimum temperature for all *Trichoderma* isolates was 25^0C and 30^0C. Kredics *et al.* (2003) also reported that the optimum temperature for best growth of *Trichoderma* species was 25^0C and 30^0C. Isolates AUT3, AUT4, AUT7 and AUT6 had higher growth rates at 35^0C (Fig.11). However, isolates AUT1, AUT5 and AUT2 had slower growth rates at 35^0C(Fig.11). Negash Hailu (2007) reported that *F. xylarioides* had maximum growth at 25^0C and 30^0C. It has been observed that *F. xylarioides* did not have any growth at 35^0C (Negash Hailu, 2007). Most *Trichoderma* isolates were mesophilic which can grow at 35^0C to 45^0C (Kredics *et al.*, 2003). Kredics *et al.* (2003) also reported that cold tolerant *T. aureoviride*, *T. harzianum* and *T. viride* – grew well at 5^0C. However, at the present study it was observed that none of the *Trichoderma* islates grew at lower temperature of 4^0C.

Another important environmental factor that has effect on the growth of the *Trichoderma* isolates is pH characteristics. pH has effect on mycelial growth and mycoparasitism of *Trichoderma* spp. In the present study the effect of pH on the mycelium growth of the isolates varied slightly among the isolates. Their mycelial dry weight revealed that *Trichoderma* grew well in the pH range of 3.5, 4.5, 5.5, 6.5, 7.5 with slight variation of mean dry weight (Table 5). Isolates AUT2 is the most tolerant which has shown the maximum mycelial dry weight at all pH values evaluated. Isolates AUT4 and AUT6 had the lowest biomass at pH 3.5. They are less resistant to strong acidic conditions. The optimum pH for maximum biomass production is at 5.5-7.5. Kredics *et al.* (2003) reported that the optimum pH range for maximum growth of Trichoderma species was pH 5.5-7.5. The pH of the culture filtrates were measured to observe changes in pH (Table 6).

Trichoderma isolates grew best on PDA and MEA media. As the statistical analysis shows that the mean difference is significant at the ($P< 0.005$) between PDA and PSA, MEA and PSA, PDA and CDA and MEA and CDA media according to the multiple comparison analysis. The mean difference is not significant at ($P< 0.005$) between MEA and PDA media. For *Trichoderma* isolates the most preference media are both PDA and MEA. All isolates have shown a wide range of media preference on PDA and MEA. The mean difference is not significant beween PSA and CDA. Both PSA and CDA did not effectively support growth of all the seven *Trichoderma* isolates. Growth of *Trichoderma* isolates was very limited. This result agrees with the study by Sigdem and Kivan (2003) who reported that all isolates of *Trichoderma* produced colonies larger than 8 cm in diameter on MEA, but they did not grow on Czapek dox agar.

Antagonistic effect based on the dual culture experiments showed that *Trichoderma* isolates significantly inhibited the mycelial growth of *F. xylarioides* ranging from 63%-70.9% against *F. xylarioides* after 7 to 9 days of incubation at 25^0C. No inhibition zones were visibly observed. However, all the Trichoderma isolates have shown over growth on the test fungus in *in vitro* evaluation. Negash Hailu (2007), also reported that *Trichoderma* species against *F. xylarioides* did not show any clear zone but they overgrew the pathogen and occupied all over the media and totally surrounded the fungus in test. Isolate AUT4 gave the highest inhibition percentage value of 70.97% whereas isolate AUT3 inhibited 63% which is the least inhibition after 9 days of

incubation period at 25^0C. Kucuk and Kivanc(2003) reported that the highest mean inhibition values, 88% were obtained against *S. rolfsii* and *R. solani* with T3 and against *R. solani* with T10. In a similar study Temesgen Belayneh *et al.* (2010), have indicated that *in vitro* evaluation of all 10 isolates of *Trichoderma* tested were able to inhibit the mycelial growth of *G. xylarioides* between 55% and 76%. Negash Hailu (2007), also reported percent inhibiton of 66% and 71% for *Trichoderma harzianum* and *Trichoderma viride* against *F. xylarioides.* In similar studies Susanto *et al.* (2005) also documented that *T. harzianum* against *Drechslera triticirepentis* gave the highest inhibition capacity of 97.8% in dual culture analysis. And also Perello *et al.* (2003) reported that *Trichoderma* spp. significantly inhibited *Drechslera triticirepentis* colony growth between 50 and 74% utilizing dual culture techniques on PDA.

Trichoderma spp. produce both volatile and non-volatile compounds that supress the growth of the fungal pathogens. In the present study, production of the volatile compounds was observed. Growth inhibition started after 24 hours of inocubation at 25^0C and increased until fifth day.The growth of *F. xylarioides* was inhibited when exposed to the trapped headspace of volatile compounds produced by *Trichoderma* isolates. Inhibitory effect was due to the production of volatile substances by the Trichoderma isolates. Doi and Mori (1994) observed that volatile compounds from *Trichoderma* spp. impeded the hyphal growth of different fungal pathogens on agar plates. Out of the seven *Trichoderma* isolates, six of them supressed the growth of *F. xylarioides* by produced volatile compounds. Unlike the results of the dual culture test, *Trichoderma* isolate AUT7 inhibited the growth of the pathogen fungus only by 7.5% which is the least inhibition of all the *Trichoderma* isolates. This indicates that, this isolate produced only small amount of the volatile compound. Whereas, isolates AUT1 and AUT2 produced large amount of the volatile compound which inhibited the growth of the *F. xylarioides* by 80.6% and 77.6% respecdtively (table 7). These results are in consistent with the study conducted by Siddiquee *et al*. (2009) that *Trichoderma* spp inhibited the mycelial growth of *G. boninense* up to 70%. Doi and Mori (1994) also reported that *T. viride* produced large amount of volatile compounds to affect the hyphal tips of *Lentinus lepidus* and *Coriolus versicolor*. Kucuk and Kivanc (2003) observed that the volatile metabolites of *T. harzianum* isolates also have inhibitory effects on the growth of the plant pathogens tested.

The effect of a fungicide, sancozeb on the mycelial growth of the pathogen was observed. The results shown that the fungicide was highly effective at higher concentration which inhibited the growth of *F. xylarioides* by 100% at 0.02 g/100ml and 0.002 g/100ml concentration. As the concetration dropped to 0.0002 g/100ml, 0.00002 g/100ml and 0.000002 g/100ml g/100ml, inhibition percentage dropped to 48.33%, 33.33% and 24.17% respectively. Abdul *et al*. (2006) reported that, increase in concentration of fungicides in medium showed significant gradual reduction in the growth of fungus. Fouzia and Saleem (2006) reported that, Sancozeb was found to be the most effective and gave 60% reduction in growth of *Sclerotium rolfsii* when used at 100ppm. Though biological control is not 100% efficient, it is safe, does not pollute the environment and the bioagents can survive and reproduce in the soil for longer period of time and carry on their antagonistic activities. Control levels and specificity of control, can be overcome by the combnation of several other methods like cultural control, using resistance varieties and quarantine. The integration of different disease control methods such as application of Sancozeb and *Trichoderma* species prevent or reduce the possibility of spread and distribution of diseases.

7. Conclusions and Recommendations

- *Trichoderma* isolates are influenced by the substrate or culture media on which they were grown. Temperature and pH also have effect on the mycelial growth of *Trichoderma* isolates. *Trichoderma* isolates grew best on PDA and MEA media in invitro evaluation. pH value of 5.5 is the best for mycelial growth of *Trichoderma* isolates.

- They could colonize soils of differnt pH charactristics and can be used as bioagents in soils of different pH. The optimum temperature for best growth of *Trichoderma* is 20^0C-30^0C. Mycelial growth of *Trichoderma* isolates was limited as temperture increased to 35^0C.

- Seven *Trichoderma* isolates evaluated, performed well as biocontrol agents when tested individually with *F. xylarioides* on culture media. The *in vitro* evaluation of dual culture technique exhibited that the mycelial growth of the pathogenic fungus is suppressed by the production of volatile and non-volatile compounds of *Trichoderma* isolates.

- The future of microbial biological control agents is bright but depends on technological advancements. User acceptance of biological control agents as pest management tools is dependent on the development of low-cost, stable products which provide consistent efficacy Patricia *et. al*. (2003).

Recomendations

- Invivo testing and evaluation of the seven *Trichoderma* isolates to be well studied in the future.
- Isolation, characterization and testing of more fungal, bacterial and actinomycetes antagonists to be followed by invivo evaluation of the antagonists against *F. xylarioides* need to be carried on in the near future studies of this project.
- Evaluation of some locally available and cheap organic substrates for mass production of *Trichoderma* isolates to be carried out in future in the country.
- The results of biocontrol pot experiment and field experiment need to be evaluated at Coffee Research Centers locally and internationally.

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9. Appendices

Appendix 1. ANOVA of the effect of pH on the net weight of mycelial mats

Isolates		Sum of Squares	df	Mean Square	F	Sig.
AUT2	Between Groups	0.079	4	0.020	1.634	0.241
	Within Groups	0.121	10	0.012		
	Total	0.201	14			
AUT3	Between Groups	0.185	4	0.046	1.290	0.337
	Within Groups	0.358	10	0.036		
	Total	0.543	14			
AUT4	Between Groups	0.252	4	0.063	2.589	0.101
	Within Groups	0.243	10	0.024		
	Total	0.495	14			
AUT5	Between Groups	0.109	4	0.027	1.779	0.210
	Within Groups	0.153	10	0.015		
	Total	0.262	14			
AUT1	Between Groups	0.356	4	0.089	2.310	0.129
	Within Groups	0.385	10	0.038		
	Total	0.740	14			
AUT7	Between Groups	0.089	4	0.022	1.535	0.265
	Within Groups	0.145	10	0.015		
	Total	0.235	14			
AUT6	Between Groups	0.109	4	0.027	6.248	0.009
	Within Groups	0.044	10	0.004		
	Total	0.153	14			

Appendix 2. ANOVA of the effect of teperature on growth of *Trichoderma* isolates

ANOVA

The diameter of fungal growth

Isolates		Sum of Squares	df	Mean Square	F	Sig.
AUT2	Between Groups	29.389	2	14.694	75.571	0.000
	Within Groups	1.167	6	0.194		
	Total	30.556	8			
AUT3	Between Groups	0.000	2	0.000	.	.
	Within Groups	0.000	6	0.000		
	Total	0.000	8			
AUT4	Between Groups	0.000	2	0.000	.	.
	Within Groups	0.000	6	0.000		
	Total	0.000	8			
AUT5	Between Groups	91.820	2	45.910	550.920	0.000
	Within Groups	0.500	6	0.083		
	Total	92.320	8			
AUT1	Between Groups	58.320	2	29.160	32.044	0.001
	Within Groups	5.460	6	0.910		
	Total	63.780	8			
AUT7	Between Groups	0.000	2	0.000	.	.
	Within Groups	0.000	6	0.000		
	Total	0.000	8			
AUT6	Between Groups	0.000	2	0.000	.	.
	Within Groups	0.000	6	0.000		
	Total	0.000	8			

Appendix 3. Multiple comparison of media preference by Trichoderma isolates

LSD

Dependent Variable	(I) Type of media	(J) Type of media	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Isolates	MEA	PDA	.000	.632	1.000	-1.26	1.26
		PSA	.000	.632	1.000	-1.26	1.26
		CDA	.000	.632	1.000	-1.26	1.26
	PDA	MEA	.000	.632	1.000	-1.26	1.26
		PSA	.000	.632	1.000	-1.26	1.26
		CDA	.000	.632	1.000	-1.26	1.26
	PSA	MEA	.000	.632	1.000	-1.26	1.26
		PDA	.000	.632	1.000	-1.26	1.26
		CDA	.000	.632	1.000	-1.26	1.26
	CDA	MEA	.000	.632	1.000	-1.26	1.26
		PDA	.000	.632	1.000	-1.26	1.26
		PSA	.000	.632	1.000	-1.26	1.26
The diameter of fungal growth	MEA	PDA	.00000	.21655	1.000	-.4317	.4317
		PSA	2.22857*	.21655	.000	1.7969	2.6602
		CDA	6.73077*	.24763	.000	6.2371	7.2244
	PDA	MEA	.00000	.21655	1.000	-.4317	.4317
		PSA	2.22857*	.21655	.000	1.7969	2.6602
		CDA	6.73077*	.24763	.000	6.2371	7.2244
	PSA	MEA	-2.22857*	.21655	.000	-2.6602	-1.7969
		PDA	-2.22857*	.21655	.000	-2.6602	-1.7969
		CDA	4.50220*	.24763	.000	4.0086	4.9958
	CDA	MEA	-6.73077*	.24763	.000	-7.2244	-6.2371
		PDA	-6.73077*	.24763	.000	-7.2244	-6.2371
		PSA	-4.50220*	.24763	.000	-4.9958	-4.0086

*. The mean difference is significant at the .05 level.

Declaration

I, the undersigned, declare that this thesis is my original work. It never been submitted in any institution and that all sources of materials have been acknowledged.

Name: Yonas Urbanos

Signature: _____

Date: _____