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A Master Thesis Submitted to the Graduate Studies of Addis Ababa University in partial fulfillment of the requirement for a Master of Science Degree in Biology

Biological Control of Anthracnose (*Colletotrichum species*) of Sorghum (*Sorghum Bicolor* L.) using *Trichoderma* isolates under *in vitro* conditions

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October, 2019
Addis Ababa, Ethiopia

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Dr. Tesfaye Alemu, who tirelessly gave me guidance, valuable comments and suggestions right from proposal development up to the end of this study. I also thank him, for his encouragement, technical and material support. I will never forget his endless encouragement and support.

I am grateful to thank and appreciate Mr. Afrasa Mulatu (PhD candidate) for helping me in materials, comments, and encouragement during my study.

It is worthwhile to mention the name of my friends Hadush Goshu, TsegayAsmekash and Tsegay G/Mariam, who encouraged me all the time during my study.

I would like to thank PhD candidates at Mycology laboratory, namely; Mr. Mesele Admassie and Mr. Ayelign Mellese for their collaboration by providing their genuine guidance and support. I am also very grateful to thank Mycology Laboratory technicians W/ro NegatMekonen, W/ro Zenebech Ayetenew and Mr. Kedir Bino for their cooperation by preparing materials in the lab.

Many thanks are due to my family, for their encouragement.

Finally, above all thanks to Almighty God for making possible the once impossible dream I had in mind, when all the possible gates seem closed. Nothing was done without the good will of God!

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LIST OF ACRONYMS AND ABBREVIATIONS

AFLPs: Amplified fragment length polymorphisms

AUC: Addis Ababa University *Colletotrichum* isolate

AUT: Addis Ababa University *Trichoderma* isolates

BCA: Biological Control Agent

CSA: Central Statistical Agency

DAPG: Diacetyl phloroglucinol

DNA: Deoxyribonucleic acid

MSW: Municipal Solid Waste

PDA: Potato dextrose agar

PDB: Potato Dextrose Broth

PIRG: Percent Inhibition of Radial Growth

RAPDs: Random amplified polymorphic DNAs

RFLPs: Restriction fragment length polymorphisms

SDW: Sterilized distilled water

USA: United States of America

ABSTRACT

Biological control with microbial antagonists is considered an alternative approach for controlling plant diseases. *Trichoderma* species are one of the potential fungal biocontrol agents in suppression of soil-borne and foliar pathogens. The present study aimed to evaluate, test and characterize potential biocontrol agents of *Trichoderma* isolates against two pathogenic isolates of *Colletotrichum* species under *in vitro* condition. Infected sorghum leaf, sheath, stalk and soil samples were collected from different ecological habitat of Welkait district for the isolation of *Colletotrichum* isolates. The study examined the effect of pH and temperature on the mycelia growth and spore yield of *Trichoderma* isolates in broth culture. In this study, the *in vitro* potential of 7 *Trichoderma* isolates were evaluated against two pathogenic *Colletotrichum* isolates in dual culture techniques and through production of volatile and non-volatile inhibitors. Pathogenicity test was confirmed *Colletotrichum* isolates as one of the fungal organisms responsible for sorghum anthracnose disease. It was observed that the optimum pH and temperature for maximum mycelial growth and spore yield produced by *Trichoderma* isolates in broth cultures was pH 7.5 and 25^{O}C, respectively. *In vitro* screening results showed that the proportion of isolates with antagonistic activities was the highest for the AUC-1 isolate followed by AUC-2 isolate. *In vitro* confrontation analysis revealed that all *Trichoderma* isolates were highly antagonistic against AUC-1 whereas AUT-97, AUT-131, AUT-11 and AUT-12 isolates displayed over 75% inhibition of mycelial growth of AUC-2 isolate ($P<0.05$). The isolates AUT-11 and AUT-12 showed consistent results in volatile and non-volatile activity under *in vitro* condition against any of the two pathogen isolates tested. Under *in-vitro* dual culture test, all isolates of *Trichoderma* were able to inhibit the growth of both *Colletotrichum* isolates at rates ranging from 58.92% to 90.29% after 8 days of incubation. The highest mean inhibitory effect on the growth of the test pathogens were achieved by AUT-11 isolate (90.29%) against AUC-1 and AUT-97 isolate (81.1%) against AUC-2 while AUT-32 isolate showed the lowest mean inhibitory effect restricting it almost completely in plates as compared to the control consisting of any of the two test pathogens growing alone. In dual culture, volatile and nonvolatile tests, all the *Trichoderma* isolates significantly inhibited the radial growth of the two *Colletotrichum* isolates at ($P<0.05$) compared with control. Thus, the use of novel isolates of *Trichoderma* with efficient antagonistic capacity against *Colletotrichum* isolates is a promising alternative strategy to pesticides for sorghum anthracnose disease management.

Keywords/phrase: Antagonism, *Colletotrichum*, Dual culture, inhibition, *Trichoderma*

1. INTRODUCTION

1.1 Background of the study

Sorghum (*Sorghum bicolor* L.) is one of the most important grain crops grown worldwide for food security. It ranks 5th after wheat, maize, rice, and barley globally and 2nd after maize in Sub-Saharan Africa (DeVries and Toenniessen, 2001; Hadebe *et al.,* 2017). The most important sorghum producers are the United States of America (USA), Nigeria, India, Sudan, Ethiopia, Burkina Faso, China, Tanzania and Niger (Rao *et al.*, 2014). It grows in the tropical and subtropical areas of the world with the four wild and cultivated races differentiated by head type, grain size, yield potential, and adaptation among other traits. The cultivated races include bicolor, guinea, kafir, caudatum, and durra (Supriya *et al.*, 2017).

Sorghum has a wide geographical distribution, being cultivated in the USAs and Asia, as well as its native Africa. Sorghum has become an important industrial crop in many developed countries and a valuable commodity from dry land environments prohibitive to the production of other crops. It is also a very good choice in Africa because it is adapted to low input agriculture, especially in the areas of water scarcity. Over the continent as a whole, sorghum is the second most important cereal after maize occupying 22% of the total area planted to cereals (Rao *et al.*, 2014) In Africa, the area under sorghum production is about 23.14 million hectares (ha) and total production and average yield being 23.35 million metric tons (t) and 1.01 t/ha, respectively. The crop is utilized in different forms, where the grain is used for human food and homemade beverages and to feed. The juice from sorghum can be converted to alcohol using currently available conventional fermentation technology (Reddy *et al.*, 2009).

Ethiopia is the 3rd largest sorghum producer in Africa next to Nigeria and Sudan (Adugna, 2014). It is among the top four cereal crops after teff, wheat and maize in Ethiopia. It ranks 3rd in the area cultivated with cereals and 4^{rth} in total production. It is cultivated annually on 1.8 million ha of land contributing 4.3 million tons to annual grain production of the country. Sorghum production in Ethiopia is affected by different biotic and abiotic constraints. Of the biotic stresses, diseases caused by different fungal pathogens play a significant role in curtailing its production. Anthracnose, smuts, grain mold, downy mildew, charcoal rot and a few others are important

diseases, which are now considered as one of the most destructive diseases of sorghum in major growing regions of the country (Taylor, 2016).

Sorghum anthracnose caused by *Colletotrichum sublineolum* poses a serious threat to sorghum production and profitability (Erpelding *et al.*, 2005). The extent of damage is related to the degree of host susceptibility, the environment, the aggressiveness of the strains and the physiological status of the crop (Agrios, 2005). Anthracnose infection can be observed on all above ground tissues of the sorghum plant, including the stalk, panicle, seed and, most commonly observed on the leaves (Ramasamy *et al.*, 2009; Alemayehu Chala *et al.*, 2010). The disease is especially prevalent in sorghum-producing regions that are warm and humid. Infection of foliar tissues reduces photosynthate accumulation while infection of the stalk leads to stalk rot followed by lodging, a detriment to maximizing harvestable biomass. Yield losses as high as 50% have been reported in susceptible lines when infection is followed by wet and dry cycles during periods of high temperatures (Awika and Rooney, 2004; Liu *et al.*, 2012).

Biological control of diseases and pests of crops using microbial antagonists has been an eco-friendly alternative to the use of chemical pesticides (Benítez *et al.*, 2004) and is being studied extensively with many different plant diseases using a variety of microbial antagonists as part of integrated disease management program. It involves the use of naturally occurring nonpathogenic microorganisms that are able to reduce the activity of plant pathogens and thereby suppress diseases. Antagonistic microorganisms can compete with the pathogen for nutrients, inhibit pathogen multiplication by secreting antibiotics, toxins or reduce pathogen population through hyperparasitisms. The filamentous fungi, *Trichoderma* and *Gliocladium*, are well studied and have shown efficiency in the biocontrol of different phytopathogens such as *Alternaria*, *Botrytis*, *Colletotrichum*, *Diaporthe*, *Fusarium, Monilinia, Phytophthora*, *Pythium, Rhizoctonia*, *Sclerotinia*, and *Verticillium* (Beyene Berihun, 2018; Bunbury-Blanchette and Walker, 2019; Chet *et al.*, 1997). Many strains of *Trichoderma* are strong opportunistic invaders, fast growing, prolific producers of spores and powerful antibiotic producers (Djonović *et al.*, 2006; Druzhinina *et al.*, 2011). Therefore, the present study has imitated to isolate, identify and characterize *Colletotrichum* species and evaluate the biological control potential of *Trichoderma* isolates against sorghum anthracnose under *in vitro* condition.

1.2 Objectives

1.2.1 General objective

- To isolate, identify and characterize *Colletotrichum* species and evaluate the biological control potential of *Trichoderma* isolates against sorghum anthracnose under *in vitro* condition.

1.2.2 Specific objectives

- To isolate, identify and examine cultural and morphological characteristics of *Colletotrichum* species isolated from infected sorghum samples collected from Welkait district
- To test the pathogenicity of *Colletotrichum* species on sorghum leaves and seeds
- To evaluate the *in-vitro* competition bio-assay between *Trichoderma* species and pathogenic fungi of *Colletotrichum* species using a dual culture test
- To evaluate the effect of volatile and non-volatile *Trichoderma* metabolites on the growth of *Colletotrichum* species
- To determine the biomass production and the spore yield of *Trichoderma* isolates at different growth parameters

2. LITERATURE REVIEW

2.1 Global sorghum production

Globally, sorghum is the most important economic crop in the area of production next to wheat (*Triticum* spp.), rice (*Oryza* spp.), maize (*Zea mays*), and barley (*Hordeum vulgare*). In sub-Saharan African countries, sorghum remains the third important cereal crop after maize and rice accounting about 22% of the cereal production area. Ethiopia is the sixth largest sorghum producer next to the USA, Mexico, Nigeria, Sudan and India (Hadebe *et al.*, 2017). During 2014, the highest sorghum productivity was recorded by France (6.33 t /ha) followed by Egypt (5.42 t /ha). In Ethiopia sorghum is the third largest cereal crop in area coverage and total production preceded by teff and maize. It is produced by 5 million smallholder farmers with an estimated total grain production of 4.34 million tons from an estimated area of 1.8 million hectares of land. This provides a national average grain yield of around 2.37 t/ha (Taylor, 2016). Sorghum covers 14.85% of the total area allocated to grain crop production (cereals, pulses, and oil crops) and 18.6% of the area covered by cereals in Ethiopia (CSA, 2014).

There is an increasing trend of area allotment for sorghum production in Ethiopia. Besides, its productivity increased during the last 20 years due to considerable use of agricultural inputs. For instance, the area coverage, total production and yield of sorghum increased by 9.37, 13.33 and 3.62%, respectively, during 2013 to 2014. The crop is highly valued, especially in the drier environments of the country owing to its considerable drought-tolerance. Sorghum is recognized as a food security crop in Ethiopia. In recent years, the crop has been considered as a strategic food security crop by the government and thus due emphasis is given to the genetic improvement and technology development of the crop to boost its productivity under the small-scale farming systems. However, several constraints are hindering sorghum production and productivity in the country and globally (Hariprasanna and Rakshit, 2016)

2.2 Taxonomy of sorghum

Sorghum was first described by Linnaeus in 1753 under the name of *Holcus. Moench* later separated the genus sorghum from the *Holcus* and gave it the binomial of Sorghum bicolor. The current formal taxonomic concept of the sorghum genus and species agrees with the one established by Moench. All the different names given by the various taxonomists are hence taken as synonymous to (*Sorghum bicolor* (L.) Moench) (Alagawadi and Gaur, 1992; Zamora, 2010).

The greatest diversity in both cultivated and wild types of sorghum is found in north-eastern tropical Africa. The crop may have been domesticated in that region, possibly Ethiopia (Brouwer *et al.,* 1993).

2.3 Ecology and Botanical Description of Sorghum

Sorghum belongs to the *Poaceae* family and is an herbaceous annual grass of tropical origin that grows from seed, stores an appreciable amount of sugar with modest water requirements (Firew Mekbib *et al.*, 2016). Sorghum is tolerant to arid and saline growing conditions, and reaches maturity in 90 to 180 days. It is considered a crop with universal value because it can be grown in tropical, subtropical, temperate and semi-arid regions of the world. It is adaptable to existing cropping systems which can serve as a secondary crop and is used as a source of forage and silage for livestock production systems (Hadebe *et al.*, 2017). It is an annual and predominantly self-pollinated cereal with the degree of spontaneous cross pollination, in some cases, reaching up to 30%, depending on panicle types. Sorghum, can tolerate a wide range of soil conditions, from heavy clay soils to light sand, with a pH ranging from 5.0 to 8.5 (Cardoso *et al.*, 2013).

Sorghum has culms that may stand 0.6-4.5 m tall, depending on the type and variety. It may produce two or more tillers. The stalk is solid and the center of the stem can be dry or juicy, insipid or sweet to taste. A dry stalked variety has leaves with a white or yellow midrib, while a juicy stalked variety has a dull green midrib because of the presence of the juice instead of air spaces in the pithy tissues. The number of leaves on the plant varies between 7 and 24 depending on the variety. The sorghum inflorescence is a panicle that may be loose or dense. It is usually erect but may curve to form a “gooseneck”. The panicle has a central rachis, with short or long primary, secondary, and mature tertiary branches, which bear groups of the spikelet. The length and closeness of the panicle branches determine the shape of the panicle, which varies from densely packed conical or oval to spreading and lax. A fully developed panicle may contain 2,000 grains, each one usually partly covered by glumes (Hariprasanna and Rakshit, 2016). Sorghum adapts to grow in diverse agro-ecologies, including in the dry lowlands, intermediate and high altitude. Sorghum is tolerant to heat and drought stress, making it an ideal crop under limited rainfall and high temperature conditions in arid and semi-arid regions (Upadhyaya *et al.*, 2013)

2.4 Sorghum species

The genus *Sorghum* has been variously species described by several taxonomists. There are about 25 species in the genus *Sorghum*, that have been classified into five subgenera, two thirds of which are wild Sorghum species of sections *Chaetosorghum, Heterosorghum*, *Parasorghum and Stiposorghum* ((Firew Mekbib *et al.*, 2016). Harlan and De Wet (1972)also recognized five basic botanical races (A) bicolor (B), caudatum (C), durra (D), kafir (K) and guinea (G).Durra sorghums developed primarily in Ethiopia and the Horn of Africa, from where they spread to Nigeria and the savanna region of West Africa. Kafir types originated in eastern and southern Africa. Guinea sorghums are grown mainly in West and Central Africa, while bicolor types are the least important to African production, and occur in East Africa. Caudatum varieties originated from Kenya or Ethiopia (Awika and Rooney, 2004). On the contrary, in Eastern Ethiopia, five basic races, the typical Durra race are found in the lowlands; durra, durra-caudatum, and caudatum are found in the intermediate altitudes and bicolor-caudatum, guinea-caudatum, and bicolor races are found in the highlands (Firew Mekbib *et al.*, 2016). Teshome *et al.* (1999) also showed, four races (bicolor, caudatum, durra and guinea) are found in Ethiopia.

2.5 Genetic Diversity in Sorghum

Genetic divergence is the occurrence of difference among individuals due to differences in their genetic composition and the environment in which they are raised that serves as an important role in evolution by allowing a species to adapt to a new environment (Felix *et al.*, 2015). The ultimate source of genetic diversity is gene mutation, it is a permanent change in the DNA sequence, molded and shaped by selection, recombination, gene flow, genetic drift, and migration in heterogeneous environments in space and time. A great extent of variability exists in quantitative and qualitative traits among sorghum local landmarks, such as maturity, yield, plant height, plant pigments, midrib color, panicle length and width, panicle compactness and shape, glume color, grain color, size and weight and disease reaction (Harlan and De Wet, 1972).

An intensive study of genetic diversity in sorghum local landraces based on race, latitude of origin, photoperiod-sensitivity, grain and nutritional quality, agro-morphological traits and DNA markers, has provided evidence that sorghum has an appreciable genetic variation that has been poorly used in terms of crop improvement program (Dillon *et al.*, 2007).

Several studies showed that, the high potential of sorghum is grown under a wide range of environmental conditions; thus, which indicate the high genetic variability of the crop in terms of grain color, quality, plant height, and disease and pest resistance, adaptable to a wide array of temperature and moisture regime. Such highly variable genetic resources are very useful for sorghum improvement program (Firew Mekbib *et al.*, 2016). Natural selection chooses the best fit among and within a population; there can be no adaptive evolution without genetic variation. Genetic diversity is an essential raw material for evolution, which enables populations of the crop species to survive, adapted to new circumstances, and evolves to produce new genetic variants. Thus, quantitative assessment of genetic diversity is significantly important to determine the extent of genetic differences between and within crop species. The local landraces and newly developed improved cultivars provide raw materials for crop improvement worldwide, for present and future generations (Wang *et al.,* 2016).

2.6 Economic Importance of Sorghum

Sorghum is the fifth most important cereal crop in the world, after wheat, maize, rice and barley. It is cultivated in wide geographic areas in the Americas, Africa, Asia and the Pacific. Even though sorghum has become important in the agricultural production of developed countries, it is still primarily for a developing country with 90% of the world's average found in Africa and Asia. Sorghum is crucially important to food security in Africa as it is uniquely drought resistant among cereals and can withstand periods of high temperature. In dry land areas of Ethiopia, it ranks third in the country following teff and maize in the total production and second to teff in its injera (national bread) making quality (Hariprasanna and Rakshit, 2016; Taylor, 2016).

The production of biofuel from plant-based biomass is becoming an attractive alternative to nonrenewable fossil energy sources. The advantage of plant-based biomass material lies in its photosynthetic ability. Sorghum cultivars are studied intensively as potential biofuel sources due to their high biomass yield and sugar production. The sugars sorghums produce give it an economic advantage over starch-based crops for biofuel use. Sorghum cultivars possess readily available fermentable sugars within the culm; therefore, enzymatic conversion of starch to sugar is not necessary which gives sorghum an economical advantage over starch-based crops. Other desirable characteristics of sorghum that make it an attractive biofuel crop for use on marginal lands include its wide range of adaptation, drought resistance, and salinity tolerance (Harlan and De Wet, 1972; Rao *et al.*, 2014; Hariprasanna and Rakshit, 2016).

2.7 Nutritional composition of sorghum

Sorghum is one of the cereals that constitute a major source of proteins, calories, minerals for millions of people in Africa and Asia. This cereal is mainly considered as a subsistence crop because of its unique tolerance to drought and adaptation to dry tropical and subtropical ecosystems throughout the world. The crop is rich in minerals, but with bioavailability varies from less than 1% for some forms of iron to greater than 90% of sodium and potassium. The reasons for this are varied and complex, since many factors interact to determine the ultimate bioavailability of a nutrient. Like other grains, sorghum protein is generally low in the essential amino acids such as lysine and threonine. Grain sorghum is gluten free and is a good substitute for cereal grains such as wheat, barley, and rye for individuals with celiac disease (Awika and Rooney, 2004).

2.8 Sorghum Production Constraints

Though sorghum is a crop with unique adaptability to a wide range of environmental conditions and with an efficient growth rate, its production is constrained by different abiotic and biotic factors worldwide. Among the abiotic factors are low soil fertility (nutrient deficiency) and drought. Important biotic constraints include the parasitic weed striga (principally *S. hermonthica* and *S. asiatica*), foliar and panicle diseases, stem borers, and shoot fly (Awika and Rooney, 2004; Alemayehu Chala *et al.*, 2010; Hariprasanna and Rakshit, 2016; Hadebe *et al.*, 2017). These biotic constraints are responsible for causing more than 70% of total yield loss in sorghum. This problem is exacerbated by the inability of subsistence farmers in developing countries to apply pesticides owing to their higher costs.

2.9 Major diseases of sorghum

The major diseases of sorghum include anthracnose, leaf blight, rust, smut, mold and downy mildew. Anthracnose disease of sorghum is caused by *Colletotrichum sublineolum* Hann. Kabát et Bub. (syn. *Colletotrichum graminicola* (Ces.) G.W. Wils). Leaf blight disease is caused by *Exserohilum turcicum* (Pass) Leonard and Suggs. Leaf blight infection occurs before flowering, leading to yield loss reaching up to 50%. When leaf blight appears late, yield loss can be minimal (Doggett, 1988; Ngugi *et al*., 2000). On the other hand, rust (*Puccinia purpurea* Cooke) is prevalent in most sorghum producing areas affecting grain yield and forage quality (HullukaMengstu and Esele, 1992). Head smut (*Sporisorium reilianum*) and Downy mildew (*Peronosclerospora sorghi*) are important disease of sorghum (DeVries and Toenniessen, 2001; Alemayehu Chala *et al.*, 2010; Peng *et al.*, 2013; Hariprasanna and Rakshit, 2016). One of the diseases of great economic significance is anthracnose caused by *Colletotrichum sublineolum*.

Anthracnose is associated with black, sunken lenticular symptoms on the infected organs of host species (Freeman and Katan, 1997; Alemayehu Chala *et al.,* 2010; Ranjitham *et al.,* 2011).

2.10 Sorghum Anthracnose

Anthracnose is considered as one of the most destructive diseases for sorghum production due to the rapid development of the disease on susceptible cultivars. *Colletotrichum sublineolum* (P. Henn. in Kabat and Bubák) is the fungal pathogen responsible for sorghum anthracnose (Liu *et al.*, 2012; Upadhyaya *et al.*, 2013). Sorghum anthracnose occurs in all sorghum growing regions of the globe, including Ethiopia. Sorghum anthracnose happens worldwide, but is more typically observed in tropical and subtropical regions where frequent rainfalls and high humidity contribute to the development and spread of the disease. In Ethiopia anthracnose is considered to be one of the major factors limiting sorghum production (Alemayehu Chala *et al.*, 2010)

2.10.1 Economic importance of anthracnose disease

Sorghum anthracnose is one of the most important diseases which cause significant yield and quality losses (Adaskaveg and Hartin, 1997; Freeman and Katan, 1997; Alemayehu Chala *et al.*, 2010). It was believed that anthracnose was caused by *Colletotrichum graminicola* affecting both sorghum and maize. However, recent reports account that the fungi affecting the two crops are different. *Colletotrichum graminicola* is identified to be a fungal pathogen causing anthracnose of maize (*Z. mays*), while *Colletotrichum sublineola* causes anthracnose disease of sorghum. Anthracnose disease is observed in all sorghum growing areas of the world. The effects of sorghum anthracnose disease are pronounced in warmer areas where temperatures and relative humidity are the highest (Munaut *et al.*, 2001; Zamora-Melendez, 2010; Taylor, 2016). Anthracnose disease development occurs on the susceptible sorghum host and conducive weather conditions. Anthracnose affects grain yield and yield components directly or indirectly. It affects directly the seed weight, seed density and exacerbates early abortion of seeds. On the other hand, premature drying and defoliation of leaves due to foliar anthracnose is indirect causes that reduced sorghum grain yield.

2.10.1 Symptoms of anthracnose disease

Anthracnose disease symptoms are observed on all above ground parts of the sorghum plant, but it is most conspicuous on the leaf. The typical symptoms of sorghum anthracnose diseases are leaf blight and stem rot (Felderhoff *et al.*, 2016). It was reported that foliar infection of anthracnose disease occurred at any time of plant growth; however, symptoms are detected 40 days after

emergence (Erpelding and Prom, 2004). Anthracnose disease symptoms are observed as small circular to elliptical (<5mm) spots or elongated red lesions with tan centers on susceptible sorghum cultivars. The fungus sporulates and fruiting bodies (acervuli) are observed as black spots in the center of the lesions and coalescence resulting leaf senescence (Erpelding and Prom, 2004; Crouch and Beirn, 2009).

2.11 *Colletotrichum* species of Sorghum

The genus *Colletotrichum* is one of the most common and destructive fungal genera, comprising a number of devastating pathogens on economically important crops (Damm *et al*., 2010; Cannon *et al*., 2012). The genus was voted the eighth most important group of plant pathogenic fungi in the world, based on perceived scientific and economic importance (Dean *et al*., 2012). Species of the genus are mainly associated with anthracnose diseases on a wide range of woody and herbaceous plant hosts growing in tropical, subtropical, and temperate climates (Damm *et al*., 2010). Members of this genus mainly affect fruit production of economically important crops such as banana, mango, citrus, strawberries, avocado, cassava, coffee, and many other crops including cereals (example, maize, sugarcane, and sorghum), vegetables, and ornamentals (Cannon *et al*., 2012).

The genus *Colletotrichum* belongs to the group *Coelomycetes*, fungi that produce asexual spores (conidia) inside pycnidia or in a bag of hyphae (acervuli; stromata). As noted by Sutton (1992), the first description of fungi that belong to the present day *Colletotrichum* was given by Tode (1790) under the genus *Vermicularia*. The genus name *Colletotrichum* was introduced by Corda (1837) and both names were used interchangeably during the 19th and early 20th centuries (Sutton, 1992). Species designation and diversity studies within *Colletotrichum* make use of various taxonomic tools, including phenotypic traits i.e. size and shape of conidia and appresoria; colony color; growth rate; and presence or absence of setae, sexual stage and formation of sclerotia (Photita *et al*., 2005; Nguyen *et al*., 2009b), host range or pathogenicity (Liu *et al*., 2007; Nguyen *et al*., 2009b), sensitivity to fungicides (Kaboré *et al*., 2002), biochemical markers (isozymes) and various molecular markers including random amplified polymorphic DNAs (RAPD), restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) and microsatellites (Abang *et al*., 2002; Martínez-Culebras *et al*., 2002; Heilmann *et al*., 2006; Liu *et al*., 2007; Bridge *et al*., 2008). In addition, sequencing of various regions in the genome and studies of mating type genes has also improved characterization of the genus (Johnston and Jones, 1997; Moriwaki *et al*., 2002; Toalhinhas *et al*., 2002; Crouch *et al*., 2006; Zanette *et al*., 2009).

However, the taxonomy of *Colletotrichum* still remains controversial, mainly because of the shortcomings associated with each taxonomic tool. Characterization based on morphological features is cheap cost wise and relatively easy to conduct, but such features are inadequate and prone to environmental impacts. Besides, some *Colletotrichum* species are genetically unstable (Fávaro*et al.*, 2007) and hence their phenotypic features may change with time (Crouch *et al*., 2006; Rivera-Vargas *et al*., 2006)

2.11.1 *Colletotrichum* species as plant pathogens

Filamentous fungi of the genus *Colletotrichum (anamorph)* and its teleomorph *Glomerella* are among the most important plant pathogens world-wide. High yield losses due to both pre-harvest and post-harvest diseases caused by various species of *Colletotrichum* have been reported (Jeffries *et al*., 1990; Bailey and Jeger, 1992). Sixty-six species of *Colletotrichum* recently described by Hyde *et al*. (2009) can cause plant diseases.

2.11.2 Diseases caused by *Colletotrichum* species

Diseases caused by *Colletotrichum s*pecies occur in a broad range of crops and are predominantly found in tropical and subtropical regions. The warm and moist environmental conditions in the tropics and subtropics are suitable for the development of anthracnose disease caused by *Colletotrichum*, in particular on perennial crops (Waller, 1992). Species of *Colletotrichum* cause tremendous losses by damaging the fruits, blossoms or other parts of the plants. Reductions in the quantity and quality of the harvested produce are often due to disease problems caused by *Colletotrichum* (Jeffries *et al.*, 1990).

2.11.3 Host range of *Colletotrichum* species

Colletotrichum species are among the most important plant pathogen worldwide, causing the economically important disease (Anthracnose or dieback and fruit rot) in a wide range of hosts, including cereals, legumes, vegetables and tree fruits (Bailey and Jegger, 1992). *Colletotrichum* species are genetically highly variable and this has probably enabled adaptation to a wide range of hosts and environmental conditions (Afanador-Kafuri *et al.*, 2003). *Colletotrichum* species exhibit diverse nutritional styles. Many species are hemibiotrophic and/or necrotrophic invaders of plant hosts, while some are endophytes, living inside healthy plants without harming their host. In rare cases, some species have been reported as the causal agents of diseases of humans (keratitis and

subcutaneous infections) or other animals (e.g. mycotic infection of a sea turtle) (Manire *et al*., 2002; Shiraishi *et al*. 2011 and Shivaprakash *et al.*, 2011).

2.11.4 Variability of *Colletotrichum* species

Traditionally, *Colletotrichum* species have been identified and delimited on morphological characters. Several features have been utilized by taxonomists including size and shape of conidia and appressoria, presence or absence of setae, sclerotia, acervuli and teleomorph state and cultural characters such as colony growth rate and texture (Photita *et al*., 2005; Than *et al*., 2008a; Thaung, 2008).

2.12 Lifestyles of *Colletotrichum* species

The life style patterns found in *Colletotrichum* species can be broadly categorized as necrotrophic, hemibiotrophic, latent or quiescent and endophytic, of which hemibiotrophic is the most common (Peres *et al.,* 2005; Munch *et al.*, 2008; Auyong *et al.*, 2012; Barimani *et al*., 2013). Many *Colletotrichum* species belong to different species complexes containing mostly cryptic species that are closely related to each other and have similar infection and colonization behavior (Sanders and Korsten, 2003; Damm *et al*., 2012a; Jayawardena *et al*., 2016).

2.12.1 Necrotrophic life style

Necrotrophic pathogens are those that actively infect and colonize plant cells, leading to cell death (van Kan, 2006). Necrotrophs typically secrete lytic enzymes to degrade plant components or toxins, which kill the plant tissues. The pathogen subsequently survives on the contents of dead or dying cells to complete its life cycle (Stone, 2001; Kleemann *et al.*, 2012; Gan *et al*., 2013). A necrotrophic lifestyle contrasts with that of biotrophic pathogens which derive nutrients from living cells and therefore must maintain host viability (Kan, 2005). Nearly all *Colletotrichum* species develop a necrotrophic stage at some point in their life cycles except those few that exist entirely as endophytes (Prusky *et al*., 2013; Chang *et al*., 2014).

2.12.2 Biotrophic and hemibiotrophic life styles

A biotrophic life style in the strict sense is where the pathogen remains inside the living plant tissue and actively absorbs plant metabolites for its growth without killing the plant's cells (Mendgen and Hahn, 2002). Fungal biotrophs produce specialized fungal structures, haustoria, that are highly differentiated infection structures necessary for pathogenesis and which facilitate the parasitic relationships with the living host plants to absorb carbon and nitrogen (Agrios, 2004; De Silva *et al*., 2016b). True obligate, biotrophic fungi form haustoria and engage in long-term

suppression of host defense responses (Voegele and Mendgen, 2011). Although the host plants remain alive, often without obvious disease symptoms, plant growth can be affected. To establish biotrophy in host plants and to successfully colonize plant cells, pathogens need to secrete a variety of effector proteins that manipulate their host's physiological and biochemical environment, mainly by suppressing plant defense responses (Dou and Zhou, 2012; O'Connell *et al.*, 2012; Gan *et al.*, 2013; Guyon *et al.*, 2014).

Colletotrichum species generally do not show true biotrophy. Biotrophy is more common in groups such as the rust and powdery mildew fungi, in which the fungi must complete their life cycles within the living host tissue (obligate biotrophy) (Voegele and Mendgen, 2011). However, many *Colletotrichum* species can have a biotrophic stage early in their lifestyle, followed by a switch to a necrotrophic lifestyle, and thus are referred to as hemibiotrophs. For these species, primary infection vesicles are formed during initial infection of the epidermal cells without killing the cells. This is followed by a necrotrophic stage in which secondary infection hyphae invade and kill adjacent cells (Perfect *et al.*, 1999; Barimani *et al.*, 2013). The degree of hemibiotrophic varies among different *Colletotrichum* species according to their typical lifestyle pattern and the timing of the switch from biotrophy to necrotrophs depends on host development stage and environmental conditions (Arroyo *et al.,* 2005; Peres *et al.*, 2005; Crouch and Beirn, 2009; Ranathunge *et al.*, 2012).

2.12.3 Quiescent life style

Quiescence (latency) defines an extended period of time in the fungal life cycle in which the pathogen exists dormant within the host before it switches to an active phase (Prusky *et al.*, 2013). This cannot be taken as a major lifestyle phase, however; it is a period of transition between the other phases of the life cycle. During quiescence, pathogen activity appears to be suspended and almost no growth occurs. This life style phase is common in pathogens causing postharvest disease of fresh fruit and vegetables, especially ascomycetes (*Alternaria, Botrytis, Botryosphaeria, Diaporthe, Monilinia, Phyllosticta and Sclerotinia*) including *Colletotrichum* species. In these cases the pathogens remain dormant inside the plant tissue before disease symptoms develop, often after harvest, storage, transportation and sale of produce (Wikee *et al.*, 2011; Gomes *et al.*, 2013; Prusky *et al.*, 2013; Kan *et al.*, 2014; Shaw *et al.*, 2016).

2.13 Strategies to control anthracnose diseases

In order to improve and/or maintain yields, different control strategies are needed to be employed. The anthracnose is managed by the use of fungicides, cultural practices and resistant cultivars (Singh *et al*., 1989).

2.13.1 Cultural control

The cultural practices include cleaning fields at the end of the harvest and the beginning of the season significantly reduces the incidence and severity of anthracnose compared to the practices of incorporating crop residues into the soil for enhancement of soil fertility. The management of anthracnose using proper field sanitation as a cultural control measure remains highly sustainable in many regions. A change in planting dates can serve as an alternative means of managing anthracnose in farmer's fields (Park *et al.*, 2005). However, for planting dates to be used as an effective control method in disease management, one has to know the cycle of the disease and find out the optimum time when the diseases reached its optimum/peak levels. Altering of planting dates (Ngugi *et al*., 2000; Marley, 2004), planting disease free seeds, and crop rotation can serve as important methods for controlling sorghum anthracnose disease severity (Cardwell *et al*., 1989; Casela and Frederiksen, 1993; Somda *et al.*, 2007). These methods are inexpensive and are environmentally friendly, but may be ineffective, especially if they are to be implemented in large fields.

2.13.2 Chemical control

The other control strategy of anthracnose disease was the use of fungicides. Chemicals such as Apron-plus for seed treatment alongside foliar fungicides such as carbendazim + maneb and mancozeb were applied. These chemicals have been reported to be effective in controlling anthracnose in Nigeria (Akpa *et al.*, 1992). Other fungicides were reported to be effective in controlling anthracnose disease (Marley *et al*. 2004). Sorghum seed treatment with vitavax (carboxin) followed by a spray with zined was also the most effective anthracnose control (Michereff *et al*., 1994). The use of chemicals for control of anthracnose is not economically and sustainable. It is usually costly for farmers who produce sorghum in large quantities globally.

2.13.3 Biological Control

Biological control of disease/ pathogen is the application of natural enemies in the control/ eradication of the pathogen population. Biological control is an environmentally friendly, scientifically proven and effective means of mitigating pathogens or pests through the use of

natural enemies. A world estimated loss due to crop diseases was up to 12%, while a loss due to post-harvest food spoilage was between 10 and 50%. Effective control of crop losses due to pests (microorganisms, insect and weed) therefore holds the keys for steady and stable food supply of the world. Amongst all effective and recommended controls of the crop pests, biological control holds a great promise for the future. Basically, biological control has the advantages of being environmentally friendly and not hazardous to the health of human beings, livestock and wildlife; especially now that the whole world is clamoring for IPM methods of pest control (Lorito *et al.*, 2006; Woo *et al*., 2006; Olabiyi, 2009).

Throughout their lifecycle, plants and pathogens interact with a wide variety of organisms. These interactions can significantly affect plant health in various ways. In order to understand the mechanisms of biological control, it is helpful to appreciate the different ways that organisms interact. Note, too, that in order to interact, organisms must have some form of direct or indirect contact. Odum (1953) proposed that, the interactions of two populations be defined by the outcomes for each. The types of interactions were referred to as mutualism, protocooperation, commensalism, neutralism, competition, amensalism, parasitism, and predation. While the terminology was developed for macro ecology, examples of all of these types of interactions can be found in the natural world at both the macroscopic and microscopic level. And, because the development of plant diseases involves both plants and microbes, the interactions that lead to biological control take place at multiple levels of scale.

2.13.4 *Trichoderma* species as biocontrol agent

Trichoderma is one of the most common fungal biocontrol agents in agriculture for the management of plant diseases caused by an extensive spectrum of fungal pathogens (Elad, 2000). They are typical anaerobic, facultative and cosmopolitan fungi that can be found in large numbers in agricultural soils and in other substrates such as decaying wood (Irina & Christian, 2004). A major mechanism involved in the biocontrol activity of *Trichoderma* species is the ability of competition for space and nutrients in the rhizosphere flora, production of diffusible and/or volatile antibiotics, prevention of abiotic stresses such as salt, heat and drought and finally mycoparasitism (Geiser, 1998).

2.14 The genus *Trichoderma*

The genus *Trichoderma* is among the most prominent and commonly used organisms for plant growth promotion and biological control of plant pathogens (Tronsmo and Hjeljord, 1998). They belong to the subdivision *Deuteromycetes*, members of which do not have or do not exhibit a determinate sexual state as most strains are adapted to an asexual life cycle (Harman *et al.*, 2004). Most species of the genus are photosensitive and sporulate easily with a range of natural and artificial media (Papavizas, 1985). Members of the *Trichoderma* genus are known as imperfect fungi, fast growing in culture and produce numerous green spores. These occur worldwide and are commonly associated with root, soil and plant debris (Howell *et al*, 2003). The genus has attracted considerable scientific attention and gained immense importance since last few decades due to its biological control ability. The role of *Trichoderma* species is not only to control the growth of pathogenic microbes, but there are various other uses for *Trichoderma* such as (i) stimulate colonization of rhizospheres, (ii) stimulates plant growth, root growth, and (iii) enhances plant defense responses (Harman *et al*., 2004a).

Their dominance in soil may be attributed to their diverse metabolic capability and aggressive competitive nature (Elad, 2000). These characteristics make them significant decomposers of woody and herbaceous material. *Trichoderma* species are also able to degrade domestic waste relatively quickly without emitting bad odors (Ilias *et al.*, 2005). Among the various global environmental hazards, municipal solid waste (MSW) is one of the greatest modern problems. Every day large quantities of domestic, kitchen, and municipal wastes are generated in developed and developing countries (Bartone, 2000). Proper management and safe disposal of MSW is a major, complex problem that can affect the air, land, and water. Therefore, attention has been focused on nonhazardous, environmentally friendly, sustainable techniques involving bioconversion or biologically based treatment of domestic waste to overcome this serious problem (Molla *et al*, 2002). Microorganisms from relevant environments possess greater degradation capabilities on related wastes in the biodegradation process (Leahy and Colwell, 1990). Thus, bioconversion or biodegradation might be a potentially effective measure for proper management of municipal solid waste.

Fungi of the genus *Trichoderma* have a track record of being antagonistic to quite a number of agriculturally important pests. It had been most effective bio-pesticides applied for crop protection since the era of traditional farming and nascent organic agriculture. *Trichoderma* has some unique

characteristics that make it scientifically proven and suitable biocontrol agents against a variety of pathogenic organisms infecting economic food crops. These are: non-toxic to human beings, livestock and wildlife; non-pathogenic organism on crops; compatible with other control methods (physical, chemical, cultural and planting of resistance varieties); effective at low concentrations; easy and cheap to culture or produce; could be bottled or prepared in another easily distributable pack; *Trichoderma* is ubiquitous (Lorito, 1998). *Trichoderma* is capable of producing secondary metabolites with antibiotic activity. Of recent, *Trichoderma* composted hardwood bark isolates, was reported to produce a metabolite (Harzianic acid) with antifungal and plant growth promoting activity. *Trichoderma* species have been formulated and used as bio-pesticides, bioprotectants, bio-stimulants and bio-fertilizer on a large variety of crops (Reino *et al.*, 2008; Vinale *et al.*, 2009).

2.14.1 Application of *Trichoderma* species

Various formulations and applications have been used in various reports for applying *Trichoderma* species for plant growth promotion and biological control studies. Seed coating/treatment, root-dip in colloidal suspension, soil additives either in peat-bran, wheat bran, or peat-sand formulations are some of the methods that have been used in plant growth promotion and biological control studies (Koch, 1999; McLean and Stewart, 2000). Other formulations such as processed manure pellets (Kok *et al.*, 1996), impregnated clay granules (Kelly, 1976) and foliar sprays (Huang *et al*., 2000) have also been investigated.

Trichoderma strains have been reported to control a wide range of soil-borne plant pathogens (Huang *et al*., 2000; McLean and Stewart, 2000). So far, a number of *Trichoderma* products have successfully been commercialised and available on the world market. These include SUPRESIVIT® (*T. harzianum*), TRI002 (*T. harzianum*), ECOFIT® (*T. viride*) (Koch, 1999) and TRICHODEX® (*T. harzianum*) (Paulitz and Bélanger, 2001).

2.14.2 *Trichoderma* Mechanisms of Action as Biocontrol

Trichoderma species possess multiple mechanisms of action by which they control plant pathogens (Paulitz and Bélanger, 2001). Among these mechanisms include mycoparasitism, which involves parasitism of a pathogenic fungus (Zhang *et al*., 1999; Ortiz and Orduz, 2000). The destructive stage of the mycoparasitic process, which involves the degradation of the host cell wall, is mediated by the production of lytic enzymes such as glucosidases and chitinases (Chérif and Benhamou, 1990). The production of these enzymes, which is very important in the mycoparasitic

process, is elucidated (Elad *et al.,* 1983; Lima *et al*., 1997; Menendez and Goddess, 1998). Lectin has also been suggested to play a role in the mycoparasitic process (Elad *et al*., 1983).

Competition for nutrients and space is one of the likely mechanisms involved in the biological control of plant pathogens (Tronsmo and Hjeljord, 1998). Usually the biological control agent grows and out competes the pathogen for nutrients and space.The pathogen is suppressed in the process leading to a population reduction, which no longer becomes a problem (Anonymous, 2001). Antibiotic production has been well documented as one of the mechanisms employed by *Trichoderma* species in the suppression of plant pathogens (Graeme-Cook and Faull, 1991; Mischke, 1997). Other mechanisms reported by other workers include production of volatile organic compounds (Wheatley *et al*., 1997), induced systemic resistance (De Meyer *et al*., 1998) and siderophore production (Weller, 1988). A large variety of volatile secondary metabolites could be produced by *Trichoderma* species such as ethylene, hydrogen cyanide, aldehydes and ketones, which play an important role in controlling various plant pathogens (Siddique *et al*., 2012; Chen *et al*., 2015). Many *Trichoderma* species possess one or more of the above-mentioned mechanisms. However, the relative importance of these mechanisms may depend on the specific *Trichoderma* isolate and the pathogen involved. Environmental conditions also play a major role in the expression of these mechanisms (Tronsmo and Hjeljord, 1998). Native *Trichoderma* can have significant biocontrol potential as they are adapted to the environment while exotic *Trichoderma* introduced to a new ecological niche can have problems of climatic adaptability and colonization in soils (Kamala and Davy, 2012; Ommati and Zaker, 2012)

Because biological control can result from many different types of interactions between organisms, researchers have focused on characterizing the mechanisms operating in different experimental situations. In all cases, pathogens are antagonized by the presence and activities of other organisms that they encounter. Here, we assert that the different mechanisms of antagonism occur across a spectrum of directionality related to the amount of interspecies contact and specificity of the interactions (Table 1). Direct antagonism results from physical contact and/or a high-degree of selectivity for the pathogen by the mechanism expressed by the BCA. In such a scheme, hyper parasitism by obligate parasites of a plant pathogen would be considered the most direct type of antagonism because the activities of no other organism would be required to exert a suppressive effect. In contrast, indirect antagonisms result from activities that do not involve sensing or targeting a pathogen by the BCA (Raaijmakers and Weller, 2001).

Stimulation of plant host defense pathways by non-pathogenic BCAs is the most indirect form of antagonism. However, in the context of the natural environment, most described mechanisms of pathogen suppression will be modulated by the relative occurrence of other organisms in addition to the pathogen. While many investigations have attempted to establish the importance of specific mechanisms of biocontrol to particular pathosystems, all of the mechanisms described below are likely to be operating to some extent in all natural and managed ecosystems. And, the most effective BCAs studied to date appear to antagonize pathogens using multiple mechanisms. For instance, pseudomonads known to produce the antibiotic 2, 4-diacetylphloroglucinol (DAPG) may also induce host defenses (Iavicoli *et al*., 2003). Additionally, DAPG-producers can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of wheat through competition for organic nutrients (Raaijmakers and Weller, 2001).

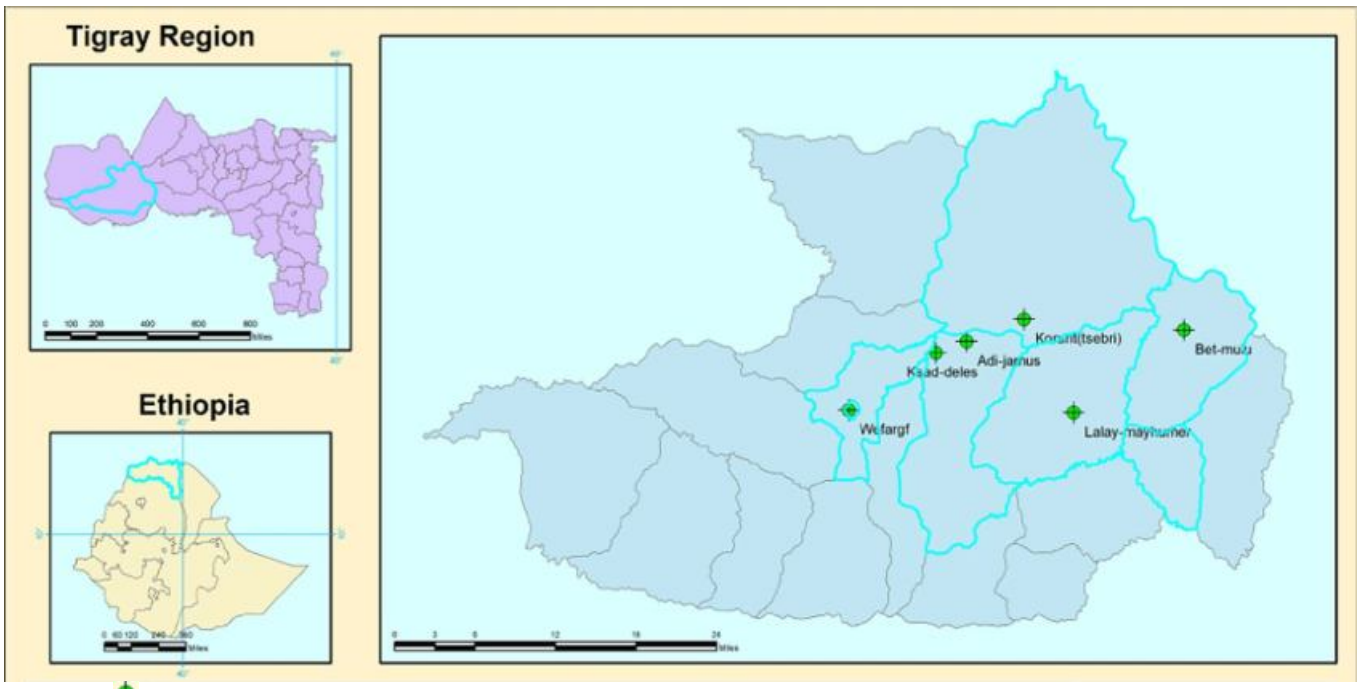
Table 1. Types of interspecies antagonisms, leading to biological control of plant pathogens

Type	**Mechanism**	**Examples**
Direct antagonism	➢ Hyperparasitism/predation	✓ Lytic/some nonlytic mycoviruses ✓ Ampelomycesquisqualis ✓ Lysobacterenzymogenes ✓ Pasteuriapenetrans ✓ Trichoderma virens
Mixed-path antagonism	➢ Antibiotics	✓ 2,4-diacetylphloroglucinol ✓ Phenazines ✓ Cyclic lipopeptides
	➢ Lytic enzymes	✓ Chitinases ✓ Glucanases ✓ Proteases
	➢ Unregulated waste products	✓ Ammonia ✓ Carbon dioxide ✓ Hydrogen cyanide
	➢ Physical/chemical interference	✓ Blockage of soil pores ✓ Germination signals consumption ✓ Molecular crosstalk confused
Indirect antagonism	➢ Competition	✓ Exudates/leachates consumption ✓ Siderophore scavenging ✓ Physical niche occupation
	➢ Induction of host resistance	✓ Contact with fungal cell walls ✓ Detection of pathogen-associated, molecular patterns ✓ Phytohormone-mediated induction

3. MATERIALS AND METHODS

3.1 Description of study area

The study was conducted in Welkait district, Western zone, Tigray region, Ethiopia. Welkait district is located 437 km west and 1220 km northwest from the capital city of Tigray region (Mekelle) and country capital city (Addis Ababa), respectively at 13°30′00″ and 14°07′00″ North latitude, and 36°40′15″ and 37°48′00″ East longitude with an altitude ranges from 677 to 2755 m above sea level. The district has 28 sub-districts of which 14 are with lowland agro-ecology (Fig. 1). The annual temperature and unimodal rainfall of the district are 17.5–25 °C and 700–1800 mm, respectively (Office of Plan and Finance of Welkait district, 2015). Welkait district is known for its fertile alluvial soil, which grows cash crops such as sesame, cotton and sorghum.



Legend: Study sites/kebeles

Fig. 1. Map of the study area, Welkait District, Western Tigray, Ethiopia

3.2 Sample collection and isolation of *Colletotrichum* species

Sorghum leaves, stalks and sheaths showing visible symptoms of anthracnose and soil of each sorghum samples were collected from four different kebeles of Welkait district viz: Adiremets, Korarit, KisadDelesa and Dejena. For each sampling site, sample collections were made from three fields located within 10 to 15 km distance. The top layer of a soil litter and the upper soil horizon (4–6 cm) was discarded, and 100g of soil from approximately 10 cm depth was collected, placed in

polyethylene bags and labeled. Afterwards, similar samples from one Subarea were merged in several larger samples, which were subsequently sieved and dried on sterile paper for 2-3 days. The collection sites represented different geographic locations at varying elevations and with different climatic conditions. The isolation and identification of fungal pathogens were conducted at the Addis Ababa University, College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology, Addis Ababa, Ethiopia.

3.3 Isolation of *Colletotrichum* isolates

Ten grams of soil samples (pulverized by means of a mortar and pestle, and passed through a 0.5 mm soil screen mesh to remove large debris and root fragments) were suspended in 90 ml sterile distilled water and thoroughly mixed. A 1 ml aliquot suspension of soil was then used to suspend in 9 ml of strelize distilled water to prepare a series of dilutions in the range of 10^{-1} to 10^{-3}, and 1ml final suspension was inoculated on PDA supplemented with streptomycin (250 mg/L to prevent bacterial growth). Each soil sample was replicated three times. Plates were incubated, at 25^0C for a period of 7 days and examined daily for colony growth and development.

Samples of infected sorghum leaf, sheath, and stalk specimens showing typical symptoms of anthracnose were washed thoroughly with distilled water; blot dried. The infected tissues of the sorghum samples approximately 2 × 2 mm were cut out with a sterile scalpel. The pieces were surface sterilized by dipping completely in a concentration of 2% sodium hypochlorite solution (NaClO), and or 70% ethanol alcohol for 2 minutes and rinsed in four successive changes of sterile distilled water (SDW). The sections were allowed to dry under a laminar flow hood before plating on Potato Dextrose Agar (PDA). Then they were transferred into PDA plates under aseptic conditions. The Petri dishes were incubated at a temperature of 25°C for 7 days for growth and sporulation of the fungal isolates. After incubation of 7 days fungal growth of *Colletotrichum* species were purified by transferring mycelium discs of 5 mm diameter and incubated for 10 days (Freeman and Katan, 1997; Ranjitham *et al.*, 2011). The identity of the fungal isolates was confirmed based on cultural characteristics, conidial morphology and microscopic examinations. Through frequent sub-culturing, the test pathogen isolates were purified and the pure cultures were maintained on a PDA slant in screw capped glass test tubes, at the 4^oC for further study.

3.4 Morphological characterization and identification of *Colletotrichum* isolates

A 4-mm^3 agar block taken from the advancing edge of a 7days old actively growing culture was transferred onto the PDA and incubated at 25°C with a 12:12 alternating dark and light photoperiod as described by Sutton (1992). Cultures were examined visually and under light in order to identify suitable characters with variations in character status and morphological features of all isolates. The identification of the fungus was confirmed based on microscopic and cultural characteristic.

3.4.1 Slide culture Technique

A clean slide was placed in 9 cm diameter plates and then a small amount of autoclaved melted PDA medium was spread over the slide to make a thin PDA film. Five mm disc of the isolated pathogen was placed on slide. Distilled water was poured in Petri plates to avoid drying (incubated at 28 +2^{o}C for 3 to 5 days. The area was observed microscopically (Olompus Microscope, Germany) by staining with lactophenol cotton blue (Schuster and Schmoll, 2010).

3.4.2 Colony characteristics

The increase in colony diameter was assessed by measuring them every day using a ruler for successive five days. Colony color was described using the degree of pigmentation of the colonies. The appearance of colonies, occurrence of sectors, colony margin, elevation, the vegetative and reproductive structures and several conidial characters were described after 7 days of incubation by multi scale category adopted from Živković *et al.* (2010).

3.4.3 Conidial characters

The conidia were examined under a light microscope and the length and width of 100 conidia per isolate were measured using an optical microscope with camera at 100x magnification (Olumpus Microscope, Germany). In addition, the shape of conidia and presence or absence of visible conidial masses was also recorded (Munaut *et al.*, 2001).

3.5 Pathogenicity test

Under greenhouse conditions sorghum leaves were thoroughly cleaned with sterile distilled water and the leaves were predisposed to humidity for 24 hours. Then after, they were inoculated by spraying with a spore suspension of *Colletotrichum* isolates (10^6spores/ml) on sorghum leaves (Adaskaveg and Hartin, 1997). The leaves were inoculated in a moist chamber, to maintain high humidity; control was maintained by spraying sterile distilled water in a similar manner. Regular

observation was made for the appearance and development of symptoms. Re-isolation was done from the artificially infected lesions on leaves. Based on pathogenicity test, two *Colletotrichum* isolates were further used in this study and designated as Addis Ababa University *Colletotrichum* isolates (AUC-1 and AUC-2).

3.6 *Trichoderma* isolates

Potential *Trichoderma* isolates were obtained from Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU) (based on their potentiality and efficacy of earlier reports). All *Trichoderma* isolates used in this study were previously isolated from soil collected from coffee growing areas, cotton and faba bean farms which represent different agronomic management practices and levels of soil fertility (Table 2). All isolates of *Trichoderma* were assessed for their antagonistic potential against some phytopathogenic fungi. Seven aggressive *Trichoderma* isolates designated as Addis Ababa University *Trichoderma* isolate (AUT-11, AUT-12, AUT-32, AUT-33, AUT-97, AUT-131 and AUT-158); were used in this study. All fungal isolates were preserved, at 4°C on slants of potato dextrose agar (PDA).

Table 2. *Trichoderma* isolates studied, location and substratum

No	***Trichoderma* Isolates**	**Substratum**	**Location**	**Collection year**
1	AUT-11	Faba bean farm, soil	Sheno	2019
2	AUT-12	Faba bean farm, soil	Fiche	2019
3	AUT-32	Coffee rhizosphere	Gera	2017
4	AUT-33	Faba bean, soil	Ambo	2019
5	AUT-97	Coffee rhizosphere	Jimma	2018
6	AUT-131	Cotton farm, soil	Wolkait	2019
7	AUT-158	Coffee rhizosphere	Teppi	2018

3.7 Physiological Characterization of *Trichoderma* Isolates

3.7.1 Growth at different Temperatures

The ability of *Trichoderma* isolates to grow at 4, 25 and 37^{o}C over 8 days was tested on PDA medium (Hermosa *et al.*, 2012).

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

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

3.8 Effect of *Trichoderma* metabolites on sorghum seed germination

The effect of the culture filtrate of *Trichoderma* isolates on the sorghum seed germination was investigated. The inoculum preparation was carried out on potato dextrose broth medium. For inoculation with *Trichoderma* isolate, 10 % of spore suspension at a concentration of 10^5 spore mL^{-1} was used. After incubation under shaking at 150 rpm at 25°C for 7 days, the culture broth was filtered using a Whatman filter paper No 42. The filtrate was centrifuged at 5000 rpm for 15 min, the supernatant was collected and the pellets were discarded. In this respect, sorghum seeds were soaked for 24 h in each of the prepared *Trichoderma* filtrates. Untreated seeds served as a control (Haran *et al.*, 1996; Howell, 2003; Lorito, 2005). The seeds were dried for 1hrunder a Laminar flow cabinet. For each treatment, 24 seeds were planted (8 seed/plate) on wet blotters and then incubated at 22 ± 2°C for two weeks. Three replicates were used for each treatment. After which, the germination percent was calculated.

$$\text{Germination Percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Number of Total seeds}} \times 100$$

3.9 Antagonistic assessment of *Trichoderma* isolates under *in vitro* condition

3.9.1 Dual culture confrontation test

The growth of *Trichoderma* isolates against *Colletotrichum* species isolates (AUC-1 and AUC-2) were evaluated using dual confrontation techniques (Weigle *et al.*, 2005; Verma *et al.*, 2007; Afrasa Mulatu *et al.*, 2013; Syed *et al.*, 2015). A mycelium agar plug (5 mm diameter) of each *Trichoderma* isolates taken from the edge of actively growing 7 days old culture was paired against same sized mycelia discs of the test pathogens at equal distances opposite to each other in 90 mm diameter Petri plates containing 20 ml PDA. The PDA plates inoculated with *Colletotrichum* species isolates were served as control. The dual cultures were incubated for 8 days. The growth of the pathogen in both test and control experiments were recorded according to the method by (Dennis and Webster, 1971; Mukherjee *et al.*, 2013). The percent inhibition of radial growth (PIRG) was computed as follows:

$$PIRG = \frac{(R1-R2)\ X\ 100}{R1}$$

Where R1 = radial growth of pathogen in control. R2 = radial growth of pathogen in dual confrontation experiments with antagonists.

The antagonistic effect of *Trichoderma* isolates were assessed in semi-quantitative means, according to Afrasa Mulatu *et al.* (2013) and Noveriza and Quimio, (2016): >75 PIRG indicating very high antagonistic activity, 61–75 PIRG indicating high antagonistic activity, 51– 61 PIRG indicating moderate antagonistic activity, < 50 PIRG indicating low antagonistic activity, and 0 indicating no activity. A clear zone of inhibition (CZI) was also determined by measuring the clear inhibition between the colony margins of the *Colletotrichum* isolates and *Trichoderma* isolates.

3.9.2 Effect of volatile metabolites

Selected *Trichoderma* isolates based on the mycelium inhibition assay against *Colletotrichum* isolates were evaluated for the production of volatile inhibitory substances under *in vitro* condition following (Dennis and Webster, 1971). Five millimeter disc of *Trichoderma* colony was inoculated centrally in petri plates containing PDA medium in triplicates. The Petri plates were sealed at the edges and incubated, at 28^{o}C. After 5 days, the test pathogen was inoculated on fresh PDA and the lids of the Petri plates inoculated with antagonist were replaced by the pathogen on PDA. The plates were fixed with cellophane-tape and incubated for another 8 days; whereas, control plates were inoculated with pathogen alone (Dubey *et al.*, 2007). The radius of

Colletotrichum species disc was recorded and the percentage inhibition of radial growth (PIRG) was determined after 8 days of incubation by using the same formula as described in dual culture plate testing (section 3.9.1).

3.9.3 Effect of non-volatile metabolites

The production of non-volatile substances by the *Trichoderma* isolates against the test pathogen was studied using the method described by (Dennis and Webster, 1971). *Trichoderma* isolates were inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 28^0C on a rotatory shaker set at 100 rpm for 15 days. The control flasks were not inoculated with any of the culture. The liquid culture was filtered through (Whatman No 42) filter paper for removing mycelia mats and then sterilized by passing through 0.45µm pore biological membrane filter (Aneja, 2005). The filtrate was added to the molten PDA medium at 40°C to obtain a final concentration of 10% (v/v). Heat sterilized filtrate (5 ml) was mixed with 100 ml PDA, poured in Petri plates and 5 mm diameter culture disc of test pathogen was inoculated at the center and incubated, at 28°C for 10 days. There were three replicates of each treatment. The observation was taken and the mycelial growth inhibition percent was recorded and calculated in relation to the growth of the controls.

3.10 Statistical Analysis

The mean values of biological repeats were determined and subjected to either One and two-way ANOVA (SPSS version 25) analysis associated with Tukey's HSD post-hoc test to determine the mean significant differences between mean treatments at the 5 % level of significance.

4. RESULTS

4.1 Isolation and Morphological identification of *Colletotrichum* species

A total of 16 isolates of *Colletotrichum* species, 6 *Fusarium* species and 4 *Alternaria* species were isolated from infected sorghum leaves, sheaths, stalks (Fig. 2 and Table 3) and soils collected from 4 different kebeles of the Welkait district. It is clearly indicated in table 3 that from the 16 *Colletotrichum* isolates that were screened, only 2 isolates namely, AUC-1and AUC-2 were identified as anthracnose of sorghum by pathogenicity test and also selected in this study.

Table 3. Total *Colletotrichum* species isolated, location and substratum

S/No	Sub district	Number of *Colletotrichum* isolates	Substratum
1	Adiremets	6	Leaf and soil
2	Dejena	3	Leaf and soil
3	Korarit	4	Leaf, sheath and soil
4	KisadDelesa	3	Leaf, stalk and soil

Fig. 2. Anthracnose symptoms on different part of the sorghum plant taken from growing field

4.2 Cultural and Morphological characteristics of *Colletotrichum* isolates

Growth of both fungal isolates was observed 7 days after incubation, at 25°C. White mycelial growth was observed at six days after incubation later, turned from white to gray. The maximum colony growth was obtained 7 days after incubation. Then after, the growth of the culture ceased.

The culture was raised fluffy in its growth. The growth of the culture was circular with the mycelia showing a uniform growth pattern. In this study the morphological characteristics of the two isolates (AUC-1 and AUC-2) were made by recording colony growth rate, culture color, colony color, surface mycelium, and the shape and size of the conidia (Table 4 and Fig.3)

Table 4. Colony growth rate, culture color, colony color, surface myelium, and the shape and size of conidia of AUC-1 and AUC-2.

Colletotrichum isolates	Colony growth rate in mm	Culture color	Colony color	Surface mycelium	Conidia shape	Conidial size	
						Length(µm)	Width (µm)
AUC-1	8.33 ± 3.47	White to gray fluffy	White	Concentric ring	Cylindrical	9	5.2
AUC-2	7.80 ± 3.09	White	White	Uniform	Falcate	9.22	4.3

AUC = (Addis Ababa university *Colletotrichum* Isolate)

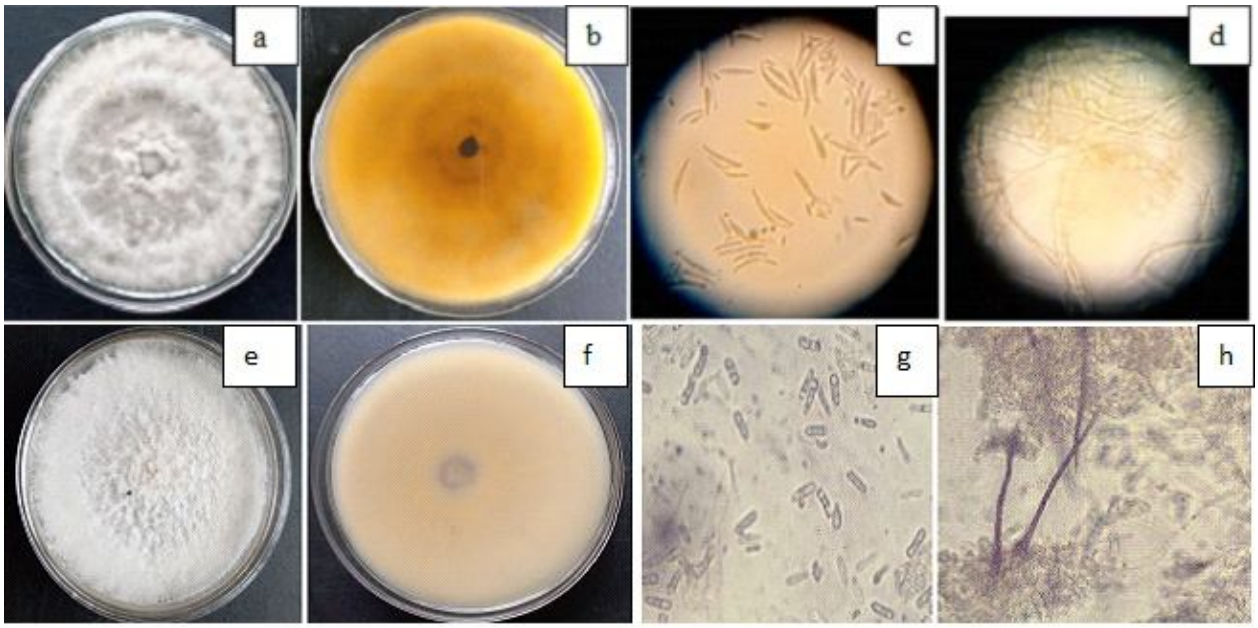


Fig.3. Macroscopically and microscopically morphological identification of *Colletotrichum* isolates (a) culture of AUC-1 (b) inverted culture of AUC-1 (c) conidia of AUC-1 (d) mycelia of AUC-1 (e) culture of AUC-2 (f) inverted culture of AUC-2 (g) conidia of AUC-2 (h) acervuli of AUC-2.

4.3 Pathogenicity test

The pathogenicity test was conducted artificially on 45-day old sorghum plants that were grown under greenhouse condition. After seven days of inoculation with a spore suspension of *Colletotrichum isolates,* typical anthracnose symptoms/spots were observed when each of the two types of conidia was inoculated into the veins of sorghum leaves and stems. Both types of conidia were pathogenic; forming elliptic to elongated reddish lesion (Fig. 4).The pathogen was re-isolated from the infected tissue of sorghum. The re-isolated culture was compared with the original culture and was found to be similar.

Fig. 4. Pathogenicity test of *Colletotrichum* on sorghum (a) infected leaf (b) control leaf (c) infected sorghum plant and (d) control sorghum plant

The pathogenicity test was conducted artificially on surface sterilized seeds and 45 days old leaves of sorghum in a Petri dish under the laboratory condition. Conidial suspension of *Colletotrichum* was applied by a spray inoculation method. After an incubation period of 8 days, anthracnose spots were appearing on the inoculated seeds and leaves. The inoculated detached leaves of sorghum showed anthracnose disease symptoms (Fig. 5).

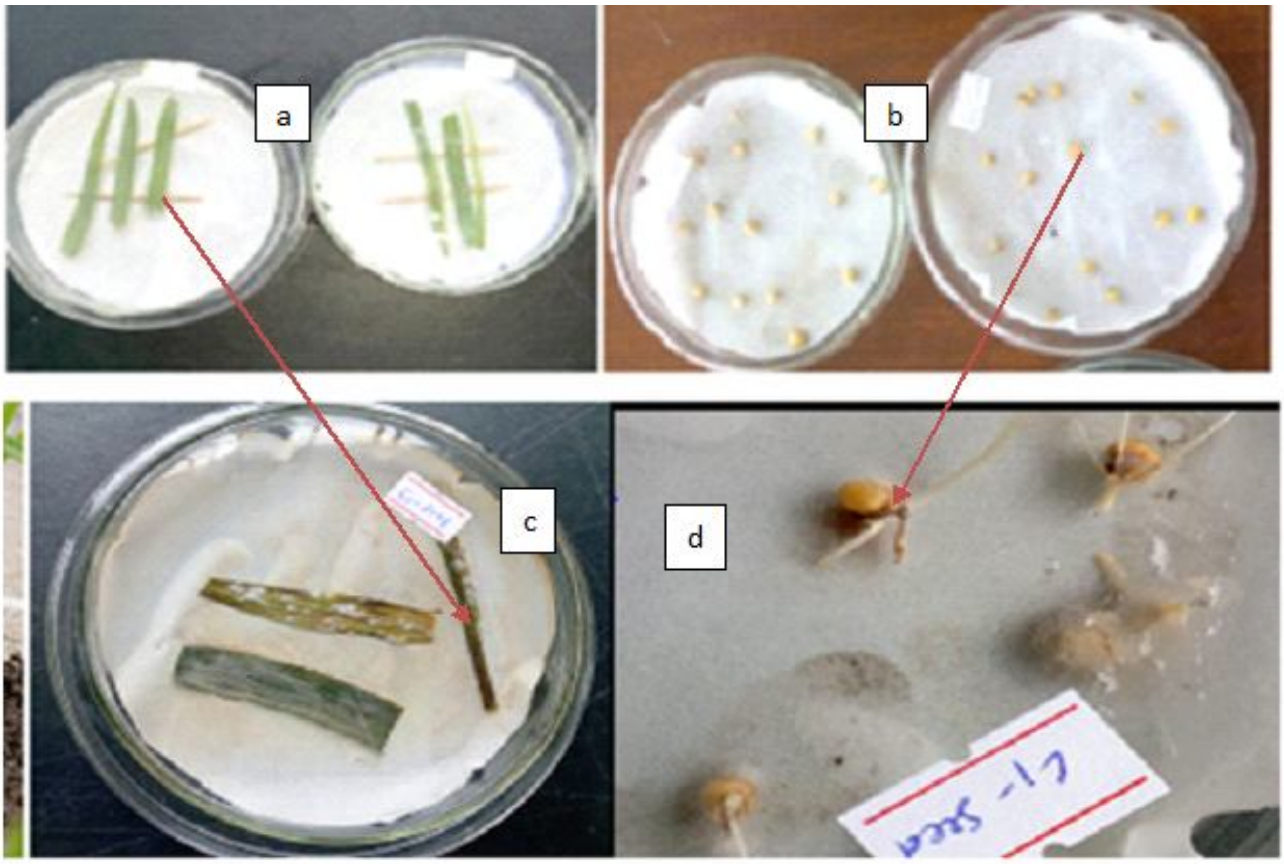


Fig.5. Pathogenicity test was conducted under the laboratory on sorghum leaves and seeds (a) inoculated leaf with conidia suspension of *Colletotrichum* isolates (b) inoculated seeds with conidia suspensions of *Colletotrichum* isolates (c) anthracnose symptoms of leaf (d) anthracnose symptoms on germinated seeds.

4.4 Morphological and Cultural characterization of *Trichoderma* isolates

Trichoderma isolates were examined macroscopically and microscopically. They were found to form colonies with white mycelia, becoming green when forming conidia and conidiophores. There were conidia formed densely over the center and undulating concentric rings toward the edge. Observation through the bottom of Petri dishes showed production of yellowish/cream-white pigmentations by some fungal isolates at an early age. These colors either remained with time or changed into purple or black. Distinct cultural differences were observed in 7 days old cultures of test antagonistic isolates grown on PDA and are summarized in (Table 5 and Fig. 6)

Table 5. Morphological and cultural characterization of *Trichoderma* isolates

Isolates	Cultural characteristics of colony			
	Spore size (µm)	Colony color	Conidia shape	Mycelial appearance
AUT-11	4	Bright green	Oval	Raised, no rings
AUT-12	4	Bright green	Grape	Raised with rings
AUT-32	6	Yellow	Oval	Effused & light
AUT-33	6	Yellow	Oval	Raised with rings
AUT-97	8	Green	Oval	Raised, no rings
AUT-131	8	Yellowish green	Oval	Raised, no rings
AUT-158	4	Deep green	Oval small and numerous	Effused, no rings

AUT = (Addis Ababa University *Trichoderma* isolate)

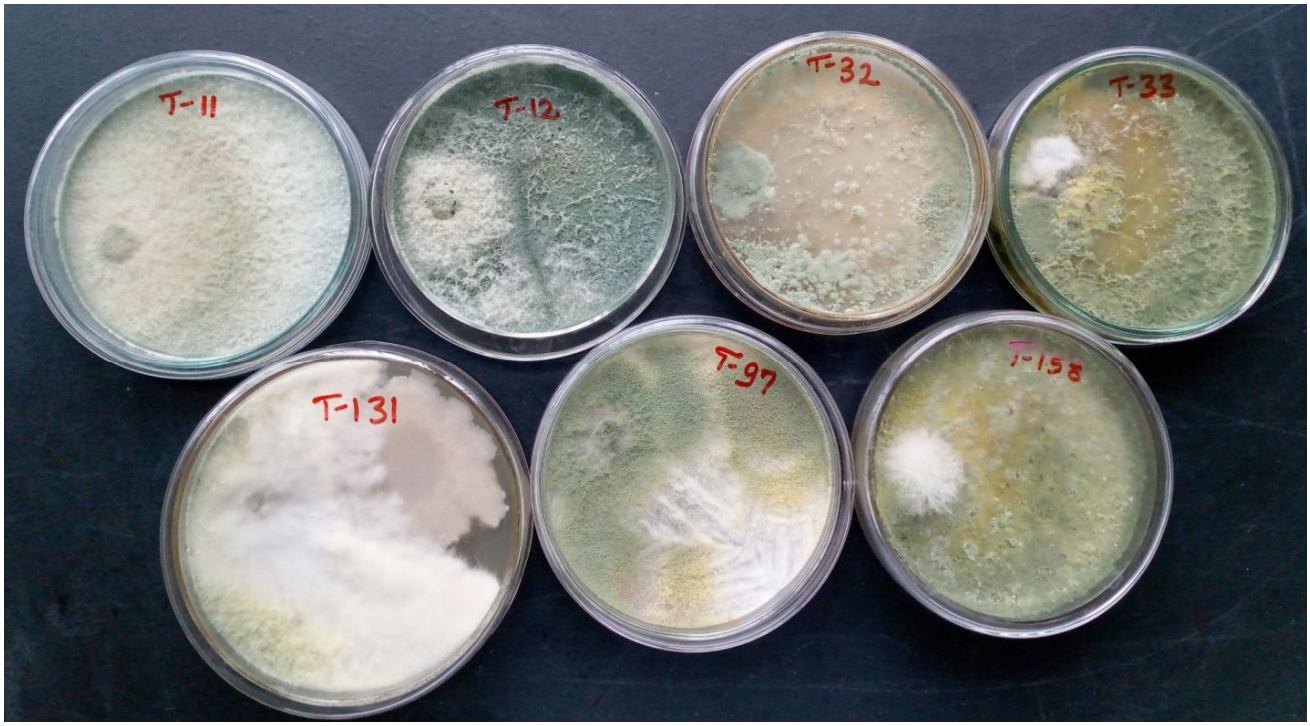


Fig. 6. Pure culture of *Trichoderma* isolates

4.5 The effect of temperature on the growth of *Trichoderma* isolates

Almost all *Trichoderma* isolates grown on PDA plates at 25^{o}C showed confluent growth within 4 days because of their higher growth rates (Table 6). But there were slight variations among some of the isolates. Among them, isolates AUT-97, AUT-131 and AUT-158 were expressed the highest growth. Meanwhile, the slowest growth rates were expressed by isolate AUT-11. Analysis revealed that 25^{o}C temperature is the optimum temperature for mycelial growth in broth culture. In contrast to the above-mentioned temperature, isolate AUT-32 and AUT-97 grown on PDA plates showed confluent growth within 7 days at 37^{o}C whereas the other isolates grow up to 1 cm on the Petri dish (9 cm). On the other hand, all isolates couldn't grow, at 4^{O}C.

Table 6. The average colony diameters of each *Trichoderma* isolate grown on PDA plates, at 25^{o}C

S. No	**Isolate code**	**Average colony diameter (cm)**			
		Day 1	**Day 2**	**Day 3**	**Day 4**
1	AUT-11	2.8 ± 0.15	4.5 ± 0.07	6.8 ± 0.12	8.5 ± 0.08
2	AUT-12	3.3 ± 0.01	6.2 ± 0.09	8.6 ± 0.03	9.00
3	AUT-32	2.9 ± 0.15	6.3 ± 0.14	8.5 ± 0.12	9.00
4	AUT-33	3.2 ± 0.05	7.2 ± 0.05	8.2 ± 0.13	9.00
5	AUT-97	3.6 ± 0.15	8.1 ± 0.05	9.00	9.00
6	AUT-131	3.5 ± 0.12	7.4 ± 0.06	9.00	9.00
7	AUT-158	4.4 ± 0.06	8.0 ± 0.03	9.00	9.00

AUT = (Addis Ababa University *Trichoderma* isolates)

4.6 Biomass production and spore yield determination of *Trichoderma* isolates at different pH value

The effect of pH on the mycelia growth by *Trichoderma* isolates after 10 days in broth culture were shown in (Table 7). Analysis revealed that pH 7.5 supported the maximum mycelial growth of 0.32, 0.28 and 0.27 g/ml produced by *Trichoderm* isolates AUT-97, AUT-32 and AUT-131 in potato dextrose broth culture, respectively, while the lowest mycelial growth 0.08g/ml was recorded at pH 4.5. The optimum pH for the mycelial growth of *Trichoderma* isolates was 7.5 in broth culture. The biomass production after 10 days, i.e., at the end of the experiment ranged from 0.08-0.32 g in all treatments.

Table 7. Influence of pH on the mycelial growth of *Trichoderma* isolates after 10 days in batch culture

***Trichoderma* isolates**	**Mycelia dry weight (g/ml) of *Trichoderma*isolates at different pH values**		
	pH 4.5	pH 6.5	pH 7.5
AUT-11	0.08	0.09	0.25
AUT-12	0.15	0.15	0.23
AUT-32	0.12	0.16	0.28
AUT-33	0.17	0.17	0.17
AUT-97	0.13	0.12	0.32
AUT-131	0.29	0.15	0.27
AUT-158	0.28	0.18	0.23

AUT = (Addis Ababa University *Trichoderma* isolates)

The effect of pH on the spore yield by *Trichoderma* isolates after 10 days in broth culture were shown in (Table 8). The optimum pH recorded in this study for spore yield in broth culture was by AUT-158 at pH 4.5. While, the least spore yield was recorded by AUT-11 at pH 6.5.

Table 8.The effect of pH on the spore yield of *Trichoderma* isolates

***Trichoderma* isolates**	**Spore yield (10^6 spores/ml) of *Trichoderma* isolates at different pH value**		
	pH 4.5	pH 6.5	pH 7.5
AUT-11	2.3	0.3	2.2
AUT-12	1	1.7	1
AUT-32	0.4	0.5	0.8
AUT-33	1	1.3	2.2
AUT-97	1.9	2	2.3
AUT-131	2.1	1.5	2.1
AUT-158	2.6	2.6	1

AUT = (Addis Ababa University *Trichoderma* isolates)

4.7 Induction of sorghum seed germination

The impact of the bioactive metabolites produced by *Trichoderma* isolates on the germination of sorghum seeds were illustrated in (Table 9). Seeds treated with T-33 recorded the highest seed germination enhancement percentage (91.67%) which was followed by AUT11 (87.5%), AUT32 (83.33%), AUT97 (83.33%) significantly at $p<0.05$ respectively. But, AUT131 had recorded the least seed germination percentage (66.67%).

Table 9. Percentage of seed germination treated with *Trichoderma* culture filtrates

Trichoderma isolates	Percentage of seed germination
AUT-11	87.5%
AUT-12	75%
AUT-32	83.3%
AUT-33	91.6%
AUT-97	83.3%
AUT-131	66.6%
AUT-158	79.1%
Control	75%

AUT = (Addis Ababa University *Trichoderma* isolates)

4.8 The antagonistic potential of the tested isolates of *Trichoderma*

4.8.1 Dual Culture Test

The mycelial growth of two *Colletotrichum* isolates and isolates of *Trichoderma* increased with the time increment, but the growths of Trichoderma isolates were more rapid than the test pathogen isolates (Fig 7). After 8 days of incubation of *Trichoderma* isolates against the test pathogens, the assay showed a pronounced reduction in the mycelial growth of the pathogen up to 90.29%. Three days after incubation, mycelium of most isolates of *Trichoderma* overgrew the test pathogens. All the *Trichoderma* isolates tested were exhibited very high antagonistic activity on *Colletotrichum* species isolate (AUC-1). In the case of *Colletotrichum* isolate (AUC-2), *Trichoderma* isolates namely AUT-97, AUT-131, AUT-11, and AUT-12 had recorded very high antagonist activity. Whereas, the remaining *Trichoderma* isolates such as, AUT-158, AUT-33 and AUT-32 had shown high antagonist activity. AUT-11 exhibited the highest growth inhibition (90.29%), while AUT-32

exhibited the least growth inhibition (58.92%) in a dual culture against *Colletotrichum* isolates (Table 10). However, all of the *Trichoderma* isolates exhibited significant inhibition of the two pathogens as compared to control at ($p<0.05$). However, the tested *Trichoderma* isolates differed in their abilities to suppress the two *Colletotricum* isolates.

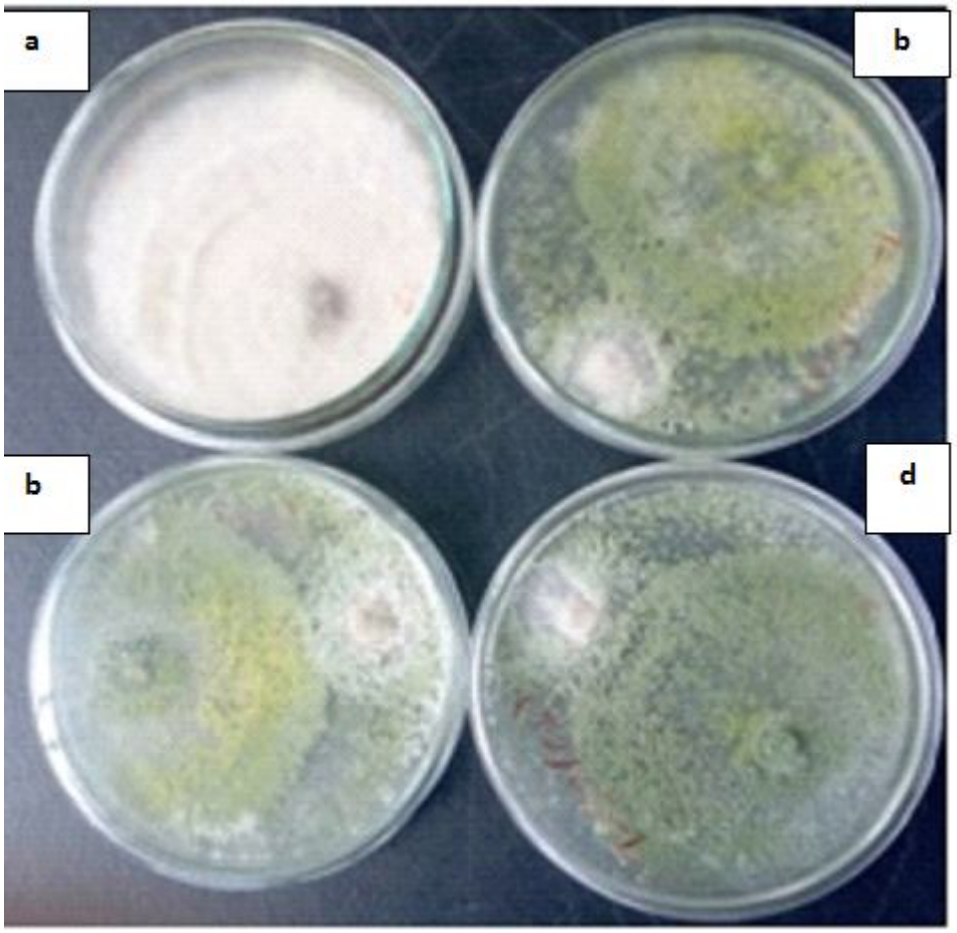


Fig.7. The antagonism of *Trichoderma* isolates against *Colletotrichum* isolates (a) The *Colletotrichum* species alone as a control (b, c and d) contains AUC-1 grown side by side with AUT-11.

Table 10. *In vitro* evaluation of *Trichoderma* isolates in terms of percentage (%) inhibition against *Colletotrichum* isolates AUC-1 and AUC-2 by dual culture, volatile inhibitors and non volatile inhibitors methods.

***Trichoderma* isolates**	**AUC-1**			**AUC-2**			**Scale of antagonistic activity**
	Dual culture	**Volatile compound**	**Non-volatile compound**	**Dual culture**	**Volatile compound**	**Non-volatile compound**	
AUT-11	90.29^{a} ± 1.07	55.81^{b} ± 12.34	72.97 ^{a} ± 3.32	79.67^{ab} ±5 .80	64.96 ^{a} ± 8.94	62.26^{a} ± 3.64	+ + +
AUT-12	84.12^{ab} ± 3.7	75.23^{ab} ± 9.48	63.00 ^{a} ± 7.70	77.00^{ab} ± 8.70	60.71 ^{a} ± 11.91	70.59^{a} ± 5.56	+ + +
AUT-32	71.59 ^{c} ± 5.95	64.68 ^{b} ± 17.96	36.45 ^{b} ± 9.33	58.92 ^{c} ± 10.61	62.67 ^{a} ± 9.87	44.02^{bc} ± 5.56	+ +
AUT-33	76.95^{bc} ± 3.03	63.29 ^{b} ± 14.85	34.56 ^{b} ± 7.16	61.42 ^{c} ± 10.15	57.91 ^{a} ± 10.00	48.21^{bc} ± 6.27	+ +
AUT-97	80.78^{abc} ± 7.0	77.20^{ab} ± 19.47	62.53 ^{a} ± 7.136	81.09 ^{a} ± 5.29	61.79 ^{a} ± 12.96	50.88^{b} ± 10.41	+ +
AUT-131	80.7^{abc} ± 9.07	86.37 ^{a} ± 3.59	62.45 ^{a} ± 12.93	80.5^{ab} ± 5.89	62.91 ^{a} ± 12.16	51.36^{b} ± 4.81	+ +
AUT-158	83.60^{abc} ± 5.1	88.32 ^{a} ± 6.72	38.65 ^{b} ± 3.55	67.92^{bc} ± 8.27	49.36 ^{a} ± 19.43	40.03^{c} ± 4.01	+
Total Mean ± Sd	77.47 ± 18.59	69.67 ± 22.56	50.54 ± 19.61	69.07 ± 19.00	57.32 ± 17.84	50.09 ± 15.70	-

AUC-1= Addis Ababa University *Colletotrichum* isolate 1, **AUC-2**= Addis Ababa University *Colletotrichum* isolate 2 and **AUT**= Addis Ababa University *Trichoderma* isolates

Descriptive assessment of the antagonistic activity as previously described by Soytong (1988) was scaled as follows:

++++ = very high antagonistic activity (> 75 PIRG). +++ = high antagonistic activity (61–75 PIRG).

++ = moderate antagonistic activity (51–60 PIRG). + = low antagonistic activity (< 50 PIRG) and - = no antagonistic activity.

Different alphabets depicted in superscript in the columns indicate mean treatments that are significantly different according to Tukey's HSD post-hoc test at $P < 0.05$. Each value is an average of 9 replicate samples ± Standard error.

4.8.2 Effect of volatile metabolites

Preliminary antagonistic activity under *in vitro* screening results showed that all isolates of *Trichoderma* had a moderate inhibitory activity against the test pathogens. The results obtained showed that AUT-158 inhibited AUC-1 by (88.32%) while AUT-11 inhibited AUC-2 by (64.96%) respectively. The results showed the inhibitory activities of the biocontrol agents are presented in (Table 10 and Fig 8). The result of the volatile metabolites showed that the growth rate of *Colletotrichum* species in the control plates and the treated plates were significantly different ($p<0.05$). In all tests, *Trichoderma* isolates completely grew over the plate containing the colony of *Colletotrichum* species in the 10th day of incubation. Of the seven *Trichoderma* isolates used in this study, AUT-158 showed the highest radial growth inhibition (88.32%), while AUT-11 has shown the least radial growth inhibition (55.81%) on *Colletotrichum* isolate (AUC-1). On the other hand, AUT-11 recorded the highest (64.96%) and AUT-158 recorded the least radial growth inhibition (49.36%) on *Colletotrichum* isolate (AUC-2).

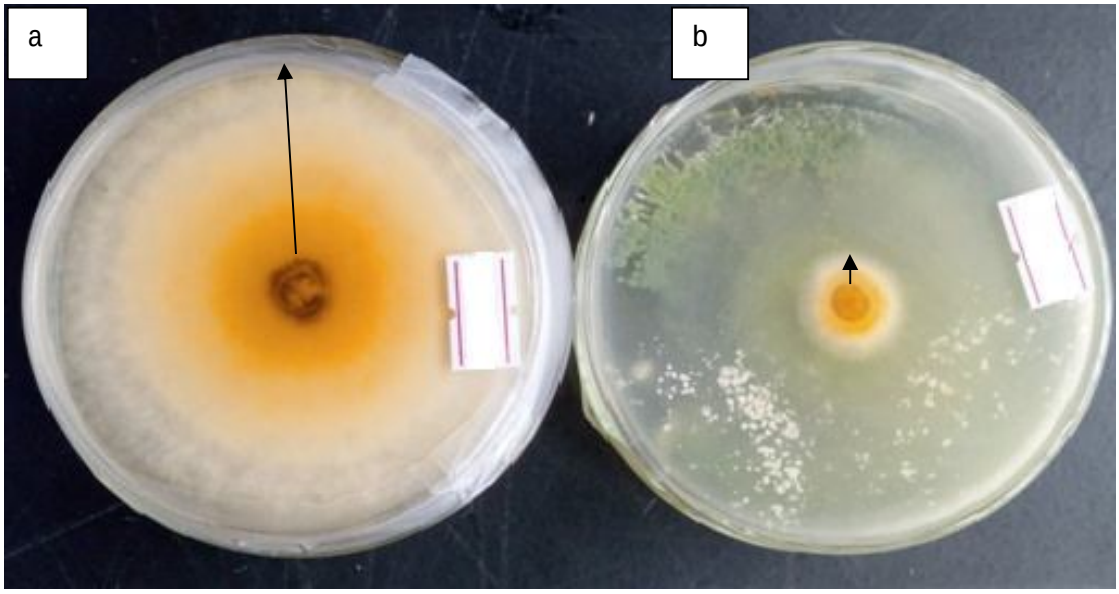


Fig.8. The effect of *Trichoderma* volatile metabolites on *Colletotrichum* species after the 6th day of incubation period (a) control plate AUC-1 (b) treated plate AUC-1+ AUT-12

4.8.3 Effect of non-volatile metabolites of bio agents

The results of the antifungal activity assessment on non-volatile secondary metabolites of *Trichoderma* isolates exhibited an effect on colony diameter and growth inhibition of both *Colletotrichum* isolates (Fig 9 a and b). Based on the results, non-volatile metabolites of AUT-11 and AUT-12 displayed the greatest ability to inhibit the growth of *Colletotrichum* species by

recording (72.97%) and (70.59%) growth inhibition percentage on AUC-1 and AUC-2 isolates respectively (Table 10). The influence of the non-volatile metabolite endophytic fungi *Trichoderma* isolates against *Colletotrichum* isoltes shows that the presence of compounds that can inhibit the mold pathogen. The radial growth of the treated *Colletotrichum* isolates significantly, different from the radial growth of the control *Colletotrichum* isolates at ($p \leq 0.05$) significance level.

Fig.9. The effect of nonvolatile *Trichoderma* metabolites on radial growth of *Colletotrichum* species (a) right control plate and left treated plate, (b) inverted side of a.

5. DISCUSSIONS

It is clearly indicated that from diseased sorghum plant parts isolated the anthracnose causal pathogen (*Colletotrichum* species) of sorghum. Both types of conidia were pathogenic forming elliptic to elongated reddish lesion on the leaves. This is similar to the typical symptoms of anthracnose shown on the infected sorghum in the field but not exactly the same. The slight differences were caused by time and environmental factors. Anthracnose disease symptoms are observed as small circular to elliptical (<5mm) spots or elongated red lesions with tan centers on susceptible sorghum cultivars. The test fungal isolates were sporulated and fruiting bodies (acervuli) are observed as black spots in the center of the lesions and coalescence resulting leaf senescence (Erpelding and Prom, 2004; Crouch and Beirn, 2009).

For exploiting the optimal antagonistic potential of *Trichoderma* which is to be applied as a biocontrol agent (BCA), the effect of temperature on their mycelial growth should be tested. Similarly, high soil temperature is an important factor for the survival of *Trichoderma* species. The result revealed that almost all *Trichoderma* isolates grown on PDA plates at 25^{o}C. In contrast, isolate AUT-32 and AUT-97 grown on PDA plates at 37^{o}C. Among the parameters that could affect biomass production is temperature, generally considered the most important factor. The common incubation temperature for the growth of fungi such as *Aspergillus niger, Trichoderma* species, *Fusarium* species, *Penicillium* species and *Graphium* species is taken to be 30°C (Singh *et al.,* 2014).

The abiotic factors deteriorated the antagonistic properties were temperature and pH that influence the mycelial growth of test fungal isolates as well as biocontrol agents (*Trichoderma* isolates). As in all microorganisms even in *Trichoderma*, the external factors modify its morphological characteristics as well as physiological functions. Among these factors, pH was the most important environmental parameter affecting the mycoparasitic activities of *Trichoderma* sisolates. The result shown that, the optimum pH for the mycelial growth of *Trichoderma* isolates was 7.5 in broth culture while the lowest mycelial growth was recorded at pH 4.5. Bae and Knudsen (2005) have found that optimum biomass production of three *Trichoderma* isolates occurred at pH ranges between 4.6 and 6.8. The studies on the variation of pH revealed that *Trichoderma* isolates showed optimum growth and sporulation rate at different pH values ranging from 2 to 7. In Ethiopia, there is great diversity in soil characteristics,

especially with respect to soil pH. Different *Trichoderma* species are able to grow in a wide range of pH from 2.0 to 8.0 with maximal growth rates at 4.0, the optimum range being 4.5 to 7.5. However, there is a need to have isolates specific for saline soils and acidic soils.

In the present study, the dual culture technique was adopted to identify the potential biocontrol agents. All tested bio-agents decreased the mycelial growth of *Colletotrichum* isolates significantly ($p<0.05$) on PDA plates compared with the control. The results of this study have shown that the highest growth inhibition percentage of *Colletotrichum* isolates was obtained by AUT-11 while the lower one was obtained by AUT-32 being, 90.29% and 58.92%, respectively. Similarly, Begum and Nath (2015) have indicated that, *Trichoderma harzianum* isolate Th-2 was effective in inhibiting the mycelia growth of all isolates of *Colletotrichum capsici* where the highest percent that has been observed was 100% inhibition. *Trichoderma* species possess multiple mechanisms of action by which they control plant pathogens (Paulitz and Bélanger, 2001). Usually the biological control agents grow and out compute the pathogens for nutrients and space. The pathogen is suppressed in the process leading to a reduction, which no longer becomes a problem (Whipps, 2004).

From the results of this study, it is evident that volatile compounds produced by *Trichoderma* isolate reduced the mycelial growth of *Colletotrichum* isolates. All the *Trichoderma* isolates produced toxic volatile metabolites having a significant effect in reducing the radial growth of test pathogens at ($p<0.05$) comparing with the control. *In vitro* studies showed that the volatile compounds produced by AUT-158, exhibited the highest radial growth inhibition percentage (88.32%) on *Colletotrichum* isolates AUC-1. Whereas, AUT-11, exhibited the highest radial growth inhibition percentage (64.96%) on *Colletotrichum* isolates AUC-2. The reduced growth of *Colletotrichum* in the volatile metabolite experiment illustrated that the ability of the *Trichoderma* isolates to produce volatile metabolites. This mechanism has been reported for the *Trichoderma virens* and *Trichoderma harzianum* to control the mycelial growth of *Colletotrichum capsici* by more than 50% (Amin *et al.*, 2010) from this result; it is evident that volatile compounds produced by *Trichoderma* isolate studied reduced the mycelial growth of *Colletotrichum* species. This may be due to releasing of a large variety of volatile secondary metabolites could be produced by *Trichoderma* species such as ethylene, hydrogen cyanide,

aldehydes and ketones, which play an important role in controlling various plant pathogens (Faheem *et al*., 2010; Siddique *et al*., 2012; Chen *et al*., 2015).

The results of the antifungal activity assessment on non-volatile secondary metabolites of endophytic fungi *Trichoderm a*iolates exhibited an effect on colony diameter and growth inhibition of *Colletotrichum* iolates. All the seven *Trichoderma* isolates inhibited the radial growth of the test pathogen iolates by producing non-volatile inhibitors. Based on the results of non-volatile metabolites of AUT-11 and AUT-12 displayed the greatest ability to inhibit the growth of *Colletotrichum* iolates by (72.97%) and (70.95%) on AUC-2 and AUC-1, respectively. The *Trichoderma* species have been reported to produce different antimicrobial compounds like 6-pentyl-α-pyrone, with a strong, coconut-like aroma, was produced by *Trichoderma harzianum* in liquid culture (Bonnarme, 1997). The antifungal compounds production is the most important mechanism of action for the effective biocontrol of soil borne diseases under greenhouse or field conditions as has been reported by Thomashow and Weller (1996).

The present study revealed that the seeds treated with culture filtrates of the antagonists increased the seed germination under *in vitro*. Five of the seven *Trichoderma* isolates were found effective to enhance the germination percentage compared to control. Among the five *Trichoderma* isolates, AUT-33 exhibited high germination percentage (91.67) on sorghum seeds in laboratory conditions. While the control treated with sterilized distilled water were germinated only 75%. The antagonist *Trichoderma* not only suppresses the growth of pathogens and also control the disease, but also has got its growth promoting effect on the sorghum plants.

In general, the use of novel isolates of *Trichoderma* with efficient antagonistic capacity against *Colletotrichum* isolates is a promising alternative strategy to fungicides for sorghum anthracnose disease management.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The optimum pH for maximum mycelial growth and spore yield produced by *Trichoderma* isolates in batch cultures was pH 7.5 while the optimum temperature for mycelial growth and the maximum spore yield was produced at optimum temperature was 25^{o}C. Dual culture confrontation assay analysis revealed that all *Trichoderma* isolates were highly antagonistic against AUC-1 whereas AUT-97, AUT-131, AUT-11 and AUT-12 isolates displayed over 75% inhibition of mycelial growth of AUC-2 isolate. The isolates AUT-11 and AUT-12 showed consistent results in volatile and non-volatile activity under *in vitro* condition against both of the two pathogens tested. The highest mean inhibitory effect on the growth of the test pathogens were achieved by AUT-11 isolate (90.29%) against AUC-1 and AUT-97 isolate (81.1%) against AUC-2 while AUT-32 isolate showed the lowest mean inhibitory effect restricting it almost completely in plates as compared to the control consisting of any of the two test pathogens growing alone. Therefore, *Trichoderma* isolates have the capacity of suppressing *Colletotrichum* isolates causing anthracnose disease of sorghum.

6.2 Recommendations

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

1) Confirm the bioefficacy of *Trichoderma* isolates, AUT-11, AUT-12 and AUT-97 under greenhouse and field conditions.
2) Finally, the germination and growth promoting potential of *Trichoderma* metabolites on sorghum should also be studied broadly under greenhouse condition in the future.

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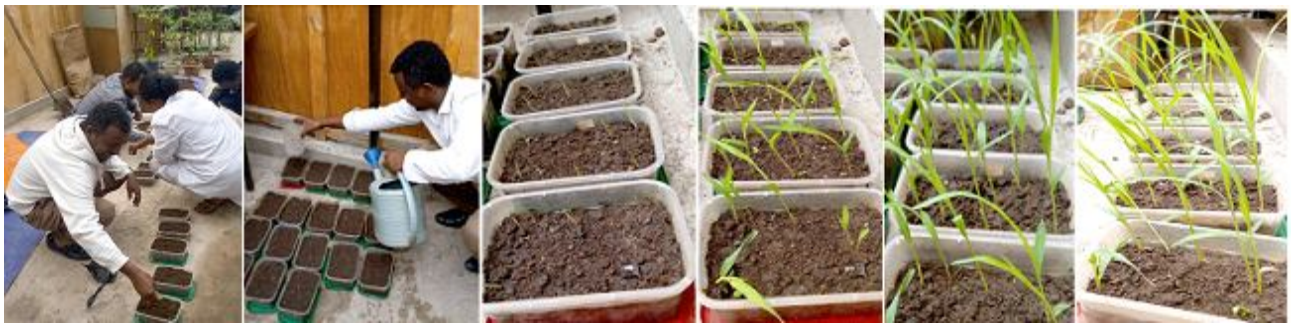
APPENDICES

Appendix 1. Photographs taken from the study area (Welkait district), during sample collection and transportation

Appendix 2. Seedling of sorghum in green house



Appendix.3. Effect of *Trichoderma* filtrates on sorghum germination

Appendix 4. Total isolates with their sampling area, substratum, isolate code and isolate genus.

No	Kebele	Subtratum	Isolated code	Isolated as genus
1	Adiremets	Soil	AUC-6	*Colletotrichum*
2	Adiremets	Leaf	AUC-1	*Colletotrichum*
3	Adiremets	Leaf	AUC-7	*Colletotrichum*
4	Adiremets	Leaf	AUF-1	*Fusarium*
5	Adiremets	Leaf	AUC-8	*Colletotrichum*
6	Adiremets	Leaf	AUC-9	*Colletotrichum*
7	Adiremets	Leaf	AUC-10	*Colletotrichum*
8	Adiremets	Leaf	AUA-1	*Alternaria*
9	Korarit	Sheath	AUC-2	*Colletotrichum*
10	Korarit	Soil	AUF-2	*Fusarium*
11	Korarit	Sheath	AUC-3	*Colletotrichum*
12	Korarit	Leaf	AUC-5	*Colletotrichum*
13	Korarit	Leaf	AUC-4	*Colletotrichum*
14	Korarit	Soil	AUA-2	*Alternaria*
15	Korarit	Soil	AUF-6	*Fusarium*
16	Korarit	Leaf	AUC-4	*Fusarium*
17	k/delesa	Stalk	AUF-3	*Fusarium*
18	k/delesa	Leaf	AUF-5	*Fusarium*
19	k/delesa	Leaf	AUC-11	*Colletotrichum*
20	k/delesa	Leaf	AUC-12	*Colletotrichum*
21	k/delesa	Leaf	AUC-13	*Colletotrichum*
22	k/delesa	Leaf	AUA-3	*Alternaria*
23	k/delesa	Soil	AUA-4	*Alternaria*
24	Dejena	Soil	AUC-14	*Colletotrichum*
25	Degena	Leaf	AUC-16	*Colletotrichum*
26	Degena	Leaf	AUC-15	*Colletotrichum*

Appendix 5. Tukey's Post Hoc tests profile plots for AUC-1 isolate under volatile production

Post Hoc Tests

Profile Plots