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RESEARCH ARTICLE

IN VITRO EVALUATION OF *TRICHODERMA* ISOLATES FOR THE CONTROL OF SORGHUM ANTHRACNOSE (*COLLETOTRICHUM* SPECIES)

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ABSTRACT: Sorghum (*Sorghum bicolor* L.) is one of the most important grain crops grown in Ethiopia for food security. However, the production of sorghum is highly affected by anthracnose diseases. The present study aimed to test, evaluate, and characterize potential biocontrol agents of *Trichoderma* species against two pathogenic isolates of *Colletotrichum* species. Sorghum infected leaf, sheath, stalk, and soil samples were collected from Wolkait districts for the isolation of *Colletotrichum* isolates. In this study, seven *Trichoderma* isolates were evaluated against two pathogenic *Colletotrichum* isolates in dual culture techniques and through the production of volatile and non-volatile inhibitors. The study examined the effect of pH and temperature on the mycelia growth and spore yield of *Trichoderma* isolates. *In vitro* screening results showed that the proportion of isolates with antagonistic activities was highest for the AUC-1 isolate followed by AUC-2 isolate. The analysis revealed that all *Trichoderma* isolates were highly antagonistic against AUC-1 whereas AU-97, AU-131, AU-11, and AU-12 isolates displayed over 75% inhibition of mycelial growth on AUC-2 isolate. Under dual culture test, the highest mean inhibitory effect on the growth of the test pathogens was achieved by AU-11 isolate (90.29%) against AUC-1 and AU-97 isolate (81.1%) against AUC-2 while AU-32 isolate showed the lowest mean inhibitory effect in plates as compared to the controls growing alone.

Key words/phrases: Antagonism, *Colletotrichum* isolates, inhibition, metabolites, *Trichoderma* isolates.

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is one of the most important grain crops grown worldwide for food security. It ranks fifth after wheat, maize, rice, and barley globally and second after maize in Sub-Saharan Africa (DeVries and Toenniessen, 2001; Hadebe *et al*., 2017). It grows in the tropical and sub-tropical areas of the world with the four wild and cultivated races differentiated by head type, grain size, yield potential, and adaptation among

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other traits. The cultivated races include bicolor, guinea, kafir, caudatum, and durra (Mathur *et al*., 2017). 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 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 the total production and average yield are 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 third largest sorghum producer in Africa next to Nigeria and Sudan (Asfaw Adugna, 2014). It is among the top four cereal crops after teff, wheat, and maize in Ethiopia. It ranks third in the area cultivated with cereals and fourth in total production. It is cultivated annually on 1.8 million ha of land contributing 4.3 million tons to the 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 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 (Alemayehu Chala *et al*., 2010; Ramasamy *et al*., 2009). The disease is especially prevalent in sorghum-producing regions that are warm and humid. Infection of foliar tissues reduces photosynthetic accumulation while infection of the stalk leads to stalk rot followed by the lodging, a detriment to maximizing harvestable biomass. Yield losses as high as 50% have been reported in susceptible lines when the 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 the integrated disease management program. It involves the use of naturally occurring nonpathogenic microorganisms that can 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 hyperparasitism. 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*, *Phythium*, *Rhizoctonia*, *Sclerotinia*, and *Verticillium* species under *in vitro* and *in vivo* conditions (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).

Depending on the field observation as well as reports from the Central Statistical Agency (CSA), sorghum production and productivity in Wolkait district has been highly reduced due to different fungal diseases (CSA, 2015). The intensive use of fungicides has resulted in the accumulation of toxic compounds potentially hazardous to humans and the environment. Biological control of plant pathogens by microorganisms has been considered as a more natural and environmentally acceptable alternative to the existing chemical methods (Djonović *et al*., 2006; Druzhinina *et al*., 2011). Therefore, the objective of the current study was to assess the efficacy of *Trichoderma* isolates as biological control of anthracnose (*Colletotrichum* species) of sorghum under *in vitro* conditions.

MATERIALS AND METHODS

Description of the study area

The study was conducted in Welkait district, 1,220 km northwest from the capital city Addis Ababa, at 13°30′00″ and 14°07′00″ North latitude, and 36°40′15″ and 37°48′00″ East longitude with an altitude ranging from 677 to 2,755 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 700–1,800 mm, respectively

(Abrha Bsrat *et al.*, 2018). Welkait district is known for its fertile alluvial soil, which grows cash crops such as sesame, cotton, and sorghum.

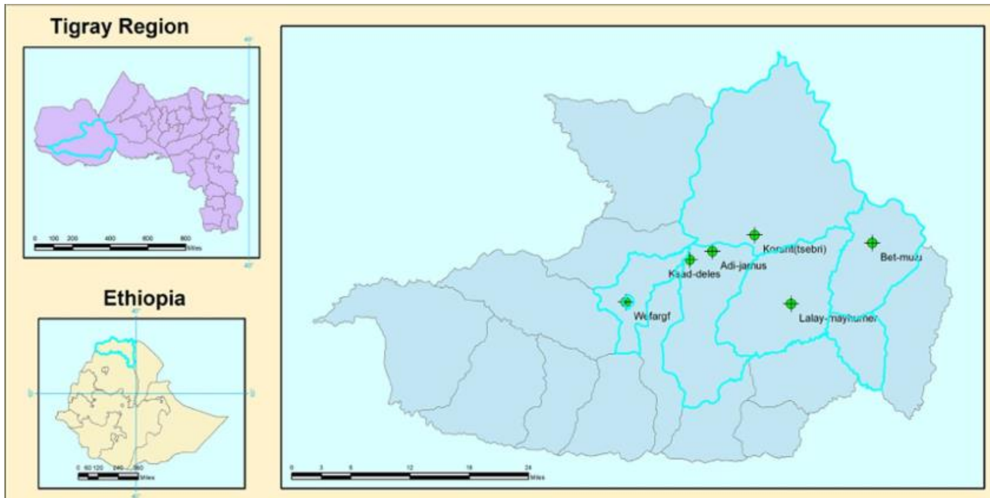

Fig. 1. Map of the study area, Welkait district, Ethiopia.

Sample collection and isolation of *Colletotrichum* species

Sorghum leaves, stalks, and sheaths showing visible symptoms of anthracnose and soil of each sorghum sample were collected from four different kebeles of Wolkait district viz: Adiremets, Korarit, K/Delesa, 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 soiled litter and the upper soil horizon (4–6 cm) was discarded, and 100 g of soil from approximately 10 cm depth was collected, placed in polyethylene bags, and labeled. The isolation and identification of fungal pathogens were conducted at College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia.

Isolation of *Colletotrichum* isolates

Ten grams of soil samples were suspended in 90 ml sterile distilled water and thoroughly mixed. A 10 ml aliquot was then used to prepare a series of dilutions in the range of 10^{-1} to 10^{-3}, and inoculated onto potato dextrose agar (PDA) and supplemented with streptomycin (250 mg/l) to prevent bacterial growth. Three replicates were done for each medium and soil sample. Plates were incubated at 25°C for 10 days and examined daily for colony development. Pathogens were subsequently grown on PDA medium and purification was done using a single spore isolation method (Sidhu *et*

al., 2014).

Samples of infected sorghum leaf, sheath, and stalk specimens showing typical symptoms of anthracnose were washed thoroughly with distilled water and 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 70% ethanol alcohol for 2 minutes and rinsed in four successive changes of sterile distilled water (SDW) (Gangadevi and Muthumary, 2008). The sections were allowed to dry in 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 fungus. After incubation of 7 days, fungal growth of *Colletotrichum* species was purified by transferring mycelium discs of 5 mm diameter and incubated for 10 days (Freeman and Katan, 1997; Thangamani *et al*., 2011). The identity of the fungus was confirmed based on cultural characteristics, conidial morphology, and microscopic examinations. Through frequent sub-culturing, the test pathogens were purified and the pure cultures were maintained on a PDA slant in glass test tubes at the 4°C for further study.

Morphological characterization and identification of *Colletotrichum* isolates

A 4-mm^3 agar block taken from the advancing edge of a 5-day old actively growing culture was transferred to the PDA and incubated at 25°C with a 12:12 alternating dark and light photoperiod as described by Photita *et al*. (2005). Cultures were examined visually and under a light to identify suitable characters with variations in character status, for the phenotypic analysis of all isolates.

Colony characteristics

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

Conidial characters

Suspensions of conidia of each isolate were prepared and the concentration was adjusted to 1×10^7 conidia/ml using a haemocytometer. The conidia were examined under a light microscope and the length and width of 100 conidia per isolate were measured using an eyepiece graticule at 100x magnification. In addition, the shape of conidia and the presence or absence of visible conidial masses were also recorded Munaut *et al*. (2001).

Pathogenicity test

In greenhouse conditions, sorghum leaves were thoroughly cleaned with sterile distilled water and the leaves were predisposed to 95% humidity for 24 hours. Thereafter, they were inoculated by spraying with a spore suspension of *Colletotrichum* isolates (10^6 spores/ml) on sorghum leaves, prepared from 15 days old culture in sterile distilled water (Adaskaveg and Hartin, 1997; Than *et al*., 2008). The inoculated leaves in the treatment and in control were kept in a modified moist chamber to maintain high humidity (95%). Regular observation was made for the appearance and development of symptoms. Re-isolation was done from the artificially infected lesions on leaves. The isolated pathogen was compared with the original culture for conformation in the laboratory (Shivakumar, 2011).

The sorghum plants of 45 days old grown in pots were sprayed first with distilled water. They were covered with polythene bags for 24 hours. The inoculum suspension from 15 days old culture was prepared in sterile distilled water. The spore suspension (10^6 spores/ml) was spray inoculated on leaves using a hand sprayer. Similarly, control plants were sprayed with sterile distilled water for comparison. The plants were covered with polythene bags and were kept for 120 hours to ensure successful penetration and the establishment of the pathogen in the leaf tissue. The polythene bags were removed after six days and plants were kept under greenhouse conditions (Peng *et al*., 2013). Observations were made regularly for the first appearance and development of symptoms. Re-isolation of the pathogen was made from the artificially infected leaves. The re-isolated pathogen obtained was compared with the original culture for confirmation. Based on the pathogenicity test, two *Colletotrichum* isolates were further used in this study and designated as Addis Ababa University *Colletotrichum* isolates (AUC-1 and AUC-2).

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). All *Trichoderma* isolates used in this study were previously isolated from soil collected from coffee growing areas, cotton farms, and faba bean farms which represent different agronomical management practices and levels of soil fertility (Table 1). All isolates of *Trichoderma* were assessed for their antagonistic potential against some phytopathogenic fungi (data not shown). Seven aggressive *Trichoderma* isolates designated as Addis Ababa University *Trichoderma*-11 (AU-11), AU-12, AU-32, AU-33, AU-97, AU-131, and AU-158); were used in this study. All fungi were preserved at 4°C on slants of potato dextrose agar (PDA).

Table 1. *Trichoderma* isolates isolated from plant parts from different locations.

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

Physiological characterization of *Trichoderma* isolates

Growth at different temperatures

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

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 a liquid potato dextrose broth (PDB) medium. For biomass production Erlenmeyer flask (250 ml) containing 50 ml of each PDB medium was inoculated with four mycelial plugs/disc (5 mm in diameter) of the *Trichoderma* isolates taken from seven days old cultures on PDA (Singh and Nautiyal, 2012). The flasks were plugged with cotton in aseptic conditions and placed in an incubator at 25°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°C. The number

of conidia per mg of the biomass was determined by dilution method with the aid of Haemocytometer.

Effect of *Trichoderma* metabolites on sorghum seed germination

The effect of the culture filtrate of each *Trichoderma* isolates on the sesame 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 6 days, the culture broth was filtered using a filter paper. 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 1 h in a Laminar flow cabinet. In this respect, sorghum seeds were soaked for 24 h in each of the prepared *Trichoderma* filtrates. Untreated seeds served as a control. The seeds were dried for 1 h in a Laminar flow cabinet. For each treatment, 24 seeds were plated (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.

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

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

Dual culture confrontation test

The invasive growth of *Trichoderma* isolates against *Colletotrichum* species isolates (AUC-1 and AUC-2) were evaluated using dual confrontation techniques (Afrasa Mulatu *et al*., 2013) with slight modifications. A mycelial agar plug (5 mm diameter) of each *Trichoderm*a isolates taken from the edge of actively growing 7 days-old culture was paired against same sized mycelia disc 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 only with *Colletotrichum* species isolates were served as control. There were six dual cultures (replicates) for each *Trichoderm*a – *Colletotrichum* species isolate combination. The dual-cultures were incubated for 6 to 10 days. The growth of the pathogen in both test and control experiments was recorded according to the method by Dennis and Webster (1971) and Mukherjee *et al*. (2013). The percent inhibition of radial growth (PIRG) was computed as follows:

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

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

The antagonistic effect of *Trichoderma* isolates was 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 clearance between the colony margins of the *Colletotrichum* and *Trichoderma* isolates.

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* conditions following the modified methods of Dennis and Webster (1971). A 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°C. After 5 days, the test pathogen was inoculated on fresh PDA and the lids of the Petri plates inoculated with the antagonist were replaced by the pathogen on PDA. The plates were fixed with cellophane-tape and incubated for another 7 days; whereas, control plates were inoculated with pathogen alone (Dubey *et al*., 2007). The radius of *Colletotrichum* species disc was recorded, and the percentage inhibition of radial growth (PIRG) was determined after 10 days of incubation by using the same formula as described in dual culture plate testing.

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°C on a rotatory shaker set at 100 rpm for 15 days. The control flasks were not inoculated with any of the cultures. The liquid culture was filtered through (Whatman No.1) filter paper for removing mycelia mats and then sterilized by passing through a 0.45 μm pore biological membrane filter (Aneja, 2007). 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.

Statistical data analysis

The mean values of biological repeats were determined and subjected to either one- or two-way ANOVA analysis followed with Tukey's HSD post-hoc test to determine the significance of the mean differences between treatments at $p<0.05$ level of significance.

RESULTS

Isolation and morphological identification of *Colletotrichum* species

A total of 16 isolates of *Colletotrichum* species, six *Fusarium* species, and four *Alternaria* species were isolated from sorghum leaves, sheaths, stalks, and soils collected from four different sub-districts of the Welkait (Table 2). The identity of the fungus was confirmed based on microscopic and cultural characteristics. From the total isolates that were screened, only two isolates namely, AUC-1and AUC-2 were studied in this study (Table 2).

Table 2. Total number of *Colletotrichum* species isolated from different parts of sorghum in Welkait district.

No	Sub district	Number of *Colletotrichum* isolates	Parts of sorghum
1	Adiremets	8	Leaf and soil
2	Dejena	3	Leaf and soil
3	Korarit	8	Leaf, sheath and soil
4	K/Delesa	7	Leaf, stalk and soil

Cultural and morphological characteristics of *Colletotrichum* isolates

Growth of the fungus was observed 6 days after incubation at 25°C in both isolates. White mycelial growth was observed six days after incubation later, turned from white to gray. The maximum colony growth was obtained six days after incubation. The culture was raised fluffy in its growth. The growth of the culture was circular with the mycelia showing a uniform growth pattern. Studies on morphological characteristics of the two isolates (AUC-1 and AUC-2) were made by recording colony growth rate, culture color, colony colour, surface mycelium, and the shape and size of the conidia (Table 3). The pure culture of *Colletotrichum* isolates AUC-1 (Korarit) and AUC-2 (Adiremets) were isolated from sheath and leaf of

sorghum clearly indicated their front and backside, respectively (Fig. 2).

Table 3. Colony growth rate, culture colour, colony colour, surface mycelium, and the shape and size of conidia of the two *Colletotrichum* isolates (AUC-1 and AUC-2).

***Colletotrichum* isolates**	**Colony growth rate (mm)**	**Culture colour**	**Colony colour**	**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

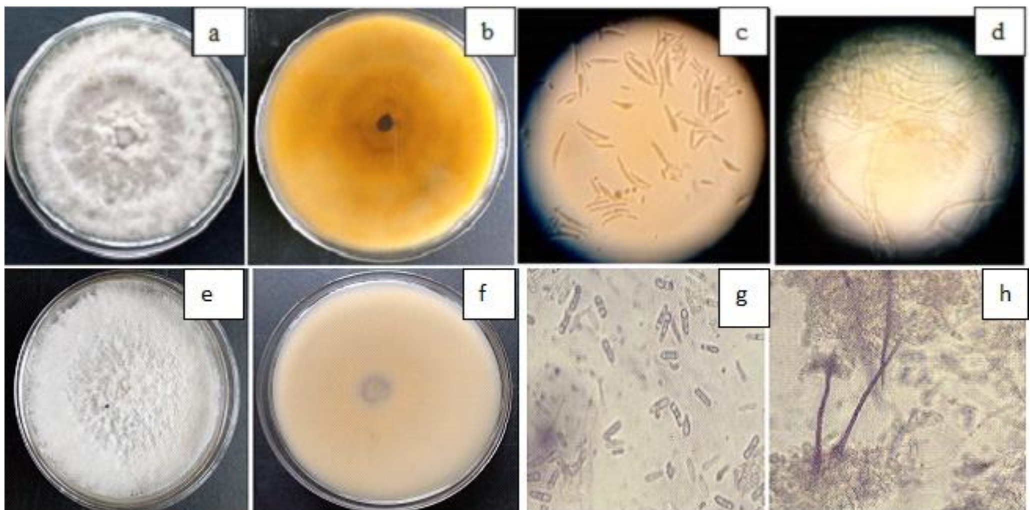

Fig. 2. Microscopically morphological identification of *Colletotrichum* isolates (a) culture of AUC-1, (b) inverted culture of AUC-1, (c) conidia of AUC-1, (d) mycelia of AUC-1, (e) culture of AUC-2, (f) inverted culture of AUC-2, (g) conidia of AUC-2 and, (h) acervuli of AUC-2.

Pathogenicity test

The pathogenicity test was conducted artificially on 45 day old sorghum plant under greenhouse conditions. Seven days after inoculation with a spore suspension of the *Colletotrichum* (1 x 10^6 spores/ml), typical anthracnose symptoms/spots were observed when each of the two types of conidia was injected into the veins of sorghum leaves and stems. Both types of conidia were pathogenic; forming elliptic to the elongated reddish lesion (Fig. 3). From the infected tissue of sorghum, the pathogen was re-isolated. The re-isolated culture was compared with the original culture and was found to be similar.

The pathogenicity test was conducted artificially on surface-sterilized seeds and 45 days old leaves of sorghum in a petri dish under laboratory

condition. Conidial suspension of *Colletotrichum* was applied by a spray inoculation method. After an incubation period of 6 days, anthracnose spots appeared on the inoculated seeds and leaves. The inoculated detached leaves of sorghum showed anthracnose disease symptoms typical of those observed on infected sorghum plants in the field.

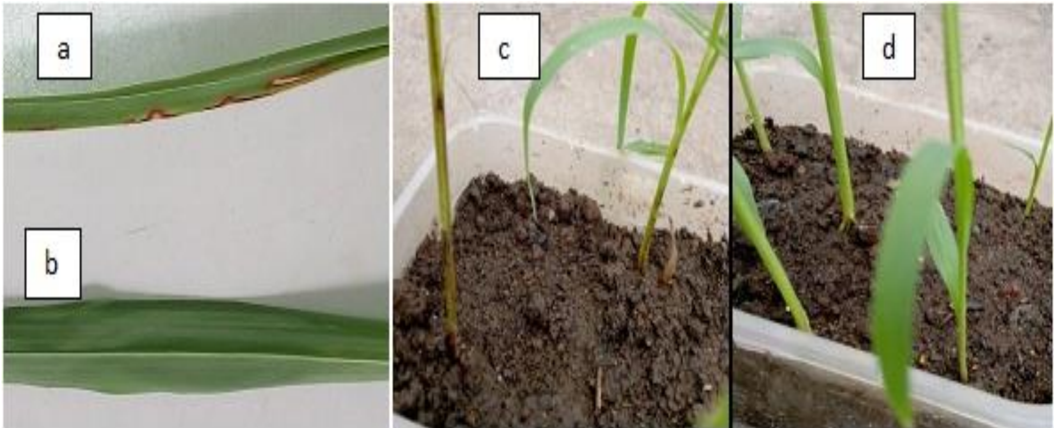

Fig. 3. Pathogenicity test of *Colletotrichum* on sorghum (a) infected leaf, (b) control leaf, (c) treated sorghum plant, and (d) control sorghum plant.

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 towards the edge. It is observed that the bottom of Petri dishes showed production of yellowish/cream-white pigmentations by some isolates at an early age (Table 4). These colours either remained with time or changed into purple or black. Distinct cultural differences were observed in five days old cultures of test antagonistic isolates grown on PDA.

Table 4. Morphological and cultural characterization of *Trichoderma* isolates.

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

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

Almost all *Trichoderma* isolates grown in the PDA plates at 25°C showed confluent growth within 4 days because of their higher growth rates (Fig. 4). But there were slight variations among some of the isolates. Among them, isolates AU-97, AU-131, and AU-158 significantly expressed the highest growth. Meanwhile, the slowest growth rates were expressed by AU-11. Analysis revealed that 25°C temperature is the optimum temperature for mycelial growth in batch culture. In contrast to the above-mentioned temperature, isolate AU-32 and AU-97 grown on PDA plates showed confluent growth within 5 days, at 37°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°C. Therefore, the improvement of stress tolerance in *Trichoderma* isolates could result in increasing their efficacy against plant pathogenic fungi even under unfavourable environmental conditions. So, for exploiting the optimal antagonistic potential of *Trichoderma* which is to be applied as a biocontrol agent (BCA), the effect of pH on their mycelial growth should be tested. Similarly, in major parts of the country, the high soil temperature is an important factor for the survival of *Trichoderma* species.

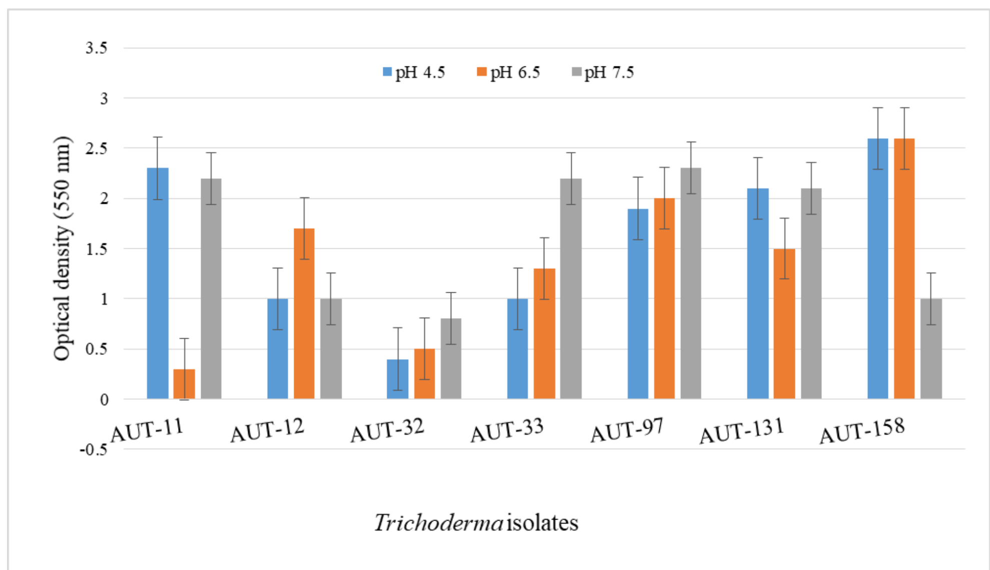

Fig. 2. The effect of temperature on the growth of *Trichoderma* isolates.

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

The effect of pH on the mycelia growth and spore yield by *Trichoderma* isolates after 10 days in batch culture is shown in Fig. 5 and 6. Analysis revealed that pH 7.5 supported the maximum mycelial growth of 0.32, 0.28, and 0.27 g/ml produced by isolates AU-97, AU-32, and AU-131 in batch culture, respectively, while the lowest mycelial growth 0.08 g/ml was recorded at pH 4.5 (Fig. 5). The optimum pH for the mycelial growth of *Trichoderma* isolates was 7.5 in batch culture. The biomass production after 10 days, that is, at the end of the experiment ranged from 0.08–0.32 g in all treatments. With increasing time all isolates showed a significant increase in biomass at all pH levels. A specific value of pH was required to note the maximum growth where these biocontrol agents can be multiplied and pathogen can be controlled. The abiotic factors that deteriorated the antagonistic properties were temperature and pH that influence the mycelial growth of test fungi 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* strains.

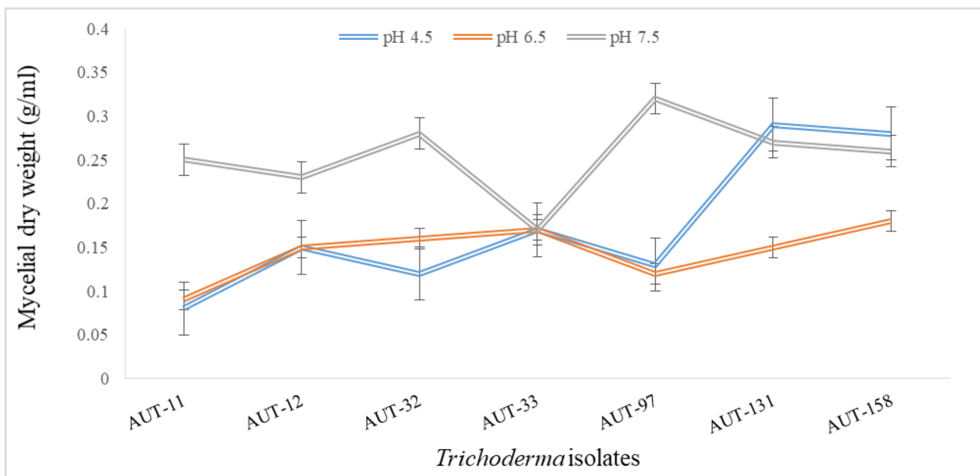

Fig. 5. Influence of pH on the mycelial growth of *Trichoderma* isolates after 10 days in batch culture.

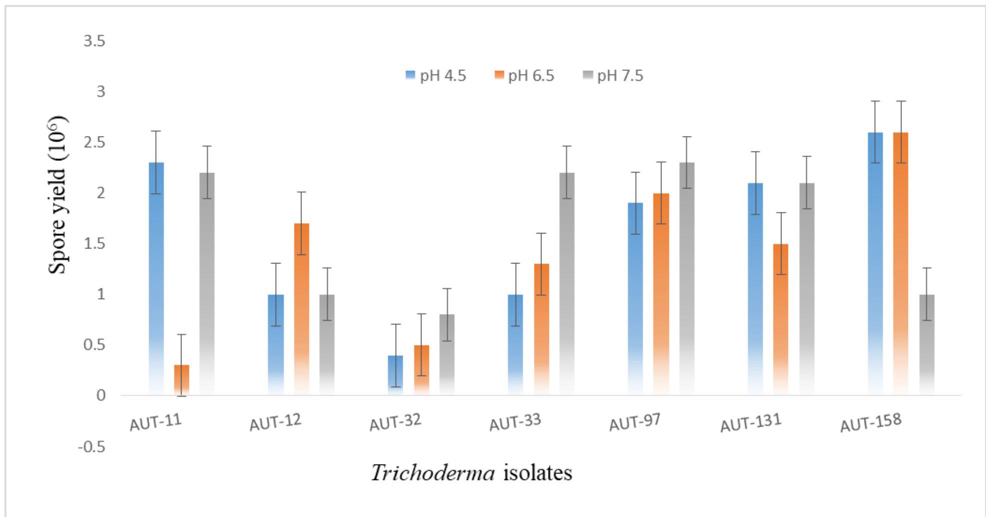

Fig. 6. The effect of pH on the spore yield of *Trichoderma* isolates.

Induction of sorghum seed germination

The impact of the bioactive metabolites produced by the *Trichoderma* isolates on the germination of sorghum seeds is illustrated in Fig. 7. The data showed that the filtrate of AU-33 isolate led to a significant enhancement in the germination of sorghum seeds (91.7%) followed by isolates AU-11 (87.5%), and AU-32 (83.33%). All isolates significantly enhanced the seed germination when compared with the non-treated control (75%).

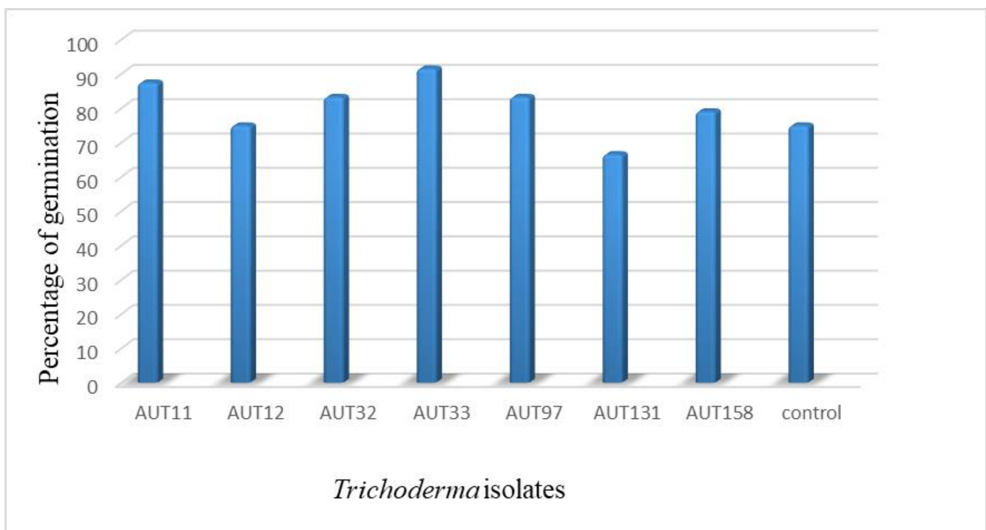

Fig. 7. Percentage of seed germination treated with *Trichoderma* culture filtrates.

The antagonistic potential of the tested isolates of *Trichoderma*

Dual culture test

The mycelial growth of two *Colletotrichum* isolates and the isolates of *Trichoderma* increased with the time increment, but the growth of the *Trichodema* isolates was more rapid than that of the pathogen. After 7 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%, and the inhibition zones were observed (Fig. 8). Three days later after incubation, the mycelia of most isolates of *Trichoderma* overgrew the test pathogens. In the case of *Colletotrichum* isolate (AUC-2), *Trichoderma* isolates namely AU-97, AU-131, AU-11, and AU-12 recorded very high antagonist activity. Whereas, the remaining *Trichoderma* isolates such as AU-158, AU-33, and AU-32 showed high antagonist activity. AU-11 exhibited the highest growth inhibition (90.29%), while AU-32 exhibited the least growth inhibition (58.92%) in a dual culture against *Colletotrichum* isolates (Table 5). However, all the *Trichoderma* isolates exhibited significant inhibition of the two pathogens as compared to control. However, the tested *Trichoderma* isolates differed in their abilities to suppress various pathogens.

Table 5. *In vitro* evaluation of *Trichoderma* isolates in terms of percentage (%) inhibition against fungal *Colletotrichum* isolates AUC-1 and AUC-2 by dual culture, volatile inhibitors, and no volatile inhibitors methods.

***Trichoderma* isolates**	**AUC-1**			**AUC-2**			
	Dual culture	**Volatile compound**	**Non-volatile compound**	**Dual culture**	**Volatile compound**	**Non-volatile compound**	**Scale of antagonistic activity**
AU-11	$90.29^{a} \pm 1.07$	$55.81^{b} \pm 12.34$	$72.97^{a} \pm 3.32$	$79.67^{ab} \pm 5\ .80$	$64.96^{a} \pm 8.94$	$62.26^{a} \pm 3.64$	+ + +
AU-12	$84.12^{ab} \pm 3.7$	$75.23^{ab} \pm 9.48$	$63.00^{a} \pm 7.70$	$77.00^{ab} \pm 8.70$	$60.71^{a} \pm 11.91$	$70.59^{a} \pm 5.56$	+ + +
AU-32	$71.59^{c} \pm 5.95$	$64.68^{b} \pm 17.96$	$36.45^{b} \pm 9.33$	$58.92^{c} \pm 10.61$	$62.67^{a} \pm 9.87$	$44.02^{bc} \pm 5.56$	+ +
AU-33	$76.95^{bc} \pm 3.03$	$63.29^{b} \pm 14.85$	$34.56^{b} \pm 7.16$	$61.42^{c} \pm 10.15$	$57.91^{a} \pm 10.00$	$48.21^{bc} \pm 6.27$	+ +
AU-97	$80.78^{abc} \pm 7.0$	$77.20^{ab} \pm 19.47$	$62.53^{a} \pm 7.13$	$81.09^{a} \pm 5.29$	$61.79^{a} \pm 12.96$	$50.88^{b} \pm 10.41$	+ +
AU-131	$80.7^{abc} \pm 9.07$	$86.37^{a} \pm 3.59$	$62.45^{a} \pm 12.93$	$80.5^{ab} \pm 5.89$	$62.91^{a} \pm 12.16$	$51.36^{b} \pm 4.81$	+ +
AU-158	$83.60^{abc} \pm 5.1$	$88.32^{a} \pm 6.72$	$38.65^{b} \pm 3.55$	$67.92^{bc} \pm 8.27$	$49.36^{a} \pm 19.43$	$40.03^{c} \pm 4.01$	+
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	−

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.

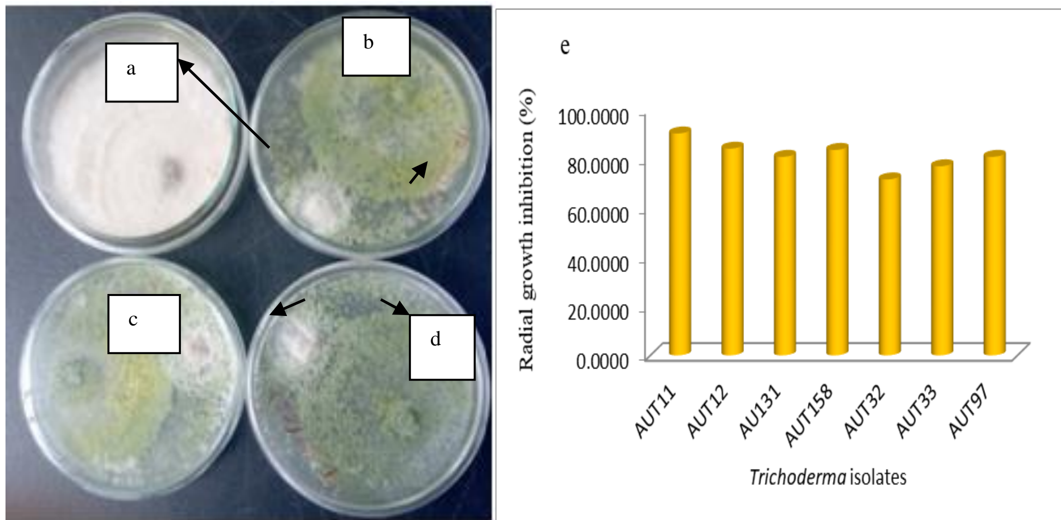

Fig. 8. The antagonism of *Trichoderma* species against *Colletotrichum* species (a) The *Colletotrichum* species alone as control while (b, c, and d) contains *Colletotrichum* species grown side by side with *Trichoderma* and (e) radial growth inhibition percentage of the *Trichoderma* on *Colletotrichum* isolates (AUC-1).

Effect of volatile metabolites

Preliminary antagonistic *in vitro* screening results showed that all isolates of *Trichoderma* had moderate inhibitory activity against the test pathogens. The results obtained showed that AU-158 inhibited AUC-1 by 88.32% while AU-11 inhibited AUC-2 by (64.96%), respectively. The results showing the inhibitory activities of the biocontrol agents are presented in both Table 5 and Fig. 9, respectively. 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 at ($p \leq 0.05$). In all tests, *Trichoderma* isolates completely grew over onto the plate containing the colony of *Colletotrichum* species on the 10th day of incubation. The results of the study showed that volatile secondary metabolites of endophytic fungi *Trichoderma* species are able to inhibit *Colletotrichum* species growth. Of the seven *Trichoderma* isolates used in this study, AU-158 showed the highest radial growth inhibition (88.32%), while AU-11 had shown the least radial growth inhibition (55.81%) on *Colletotrichum* isolate (AUC-1). On the other hand, AU-11 recorded the highest (64.96%) and AU-158 recorded the least radial growth inhibition (49.36%) on *Colletotrichum* isolate (AUC-2) (Table 5).

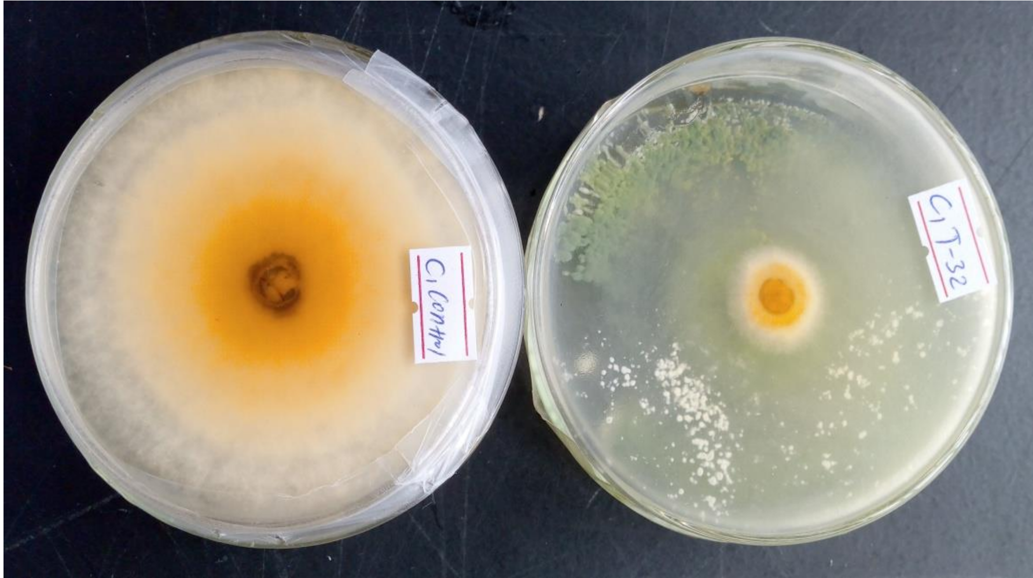

Fig. 9. The effect of *Trichoderma* volatile metabolites on *Colletotrichum* species after the sixth day of the incubation period (a) control plate and (b) treated plate.

Effect of non-volatile metabolites of bioagents

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. 10a, b). Based on the results, non-volatile metabolites of AU-11 and AU-12 displayed the greatest ability to inhibit the growth of *Colletotrichum* species by recording (72.97%) and (70.59%) growth inhibition percentages on AUC-1 and AUC-1 isolates, respectively (Table 5). The influence of the non-volatile metabolite endophytic fungi *Trichoderma* species against *Colletotrichum* species shows the presence of compounds that can inhibit the mold pathogen. The radial growth of the treated *Colletotrichum* isolates is significant, different from the radial growth of the control *Colletotrichum* isolates, at ($p \leq 0.05$) significance level. The *Colletotrichum* species, treated with non-volatile *Trichoderma* metabolites grown vertically rather than horizontally to escape from the effect of the metabolites, whereas, the *Colletotrichum* species, in the control plate grew horizontally (Fig. 10c).

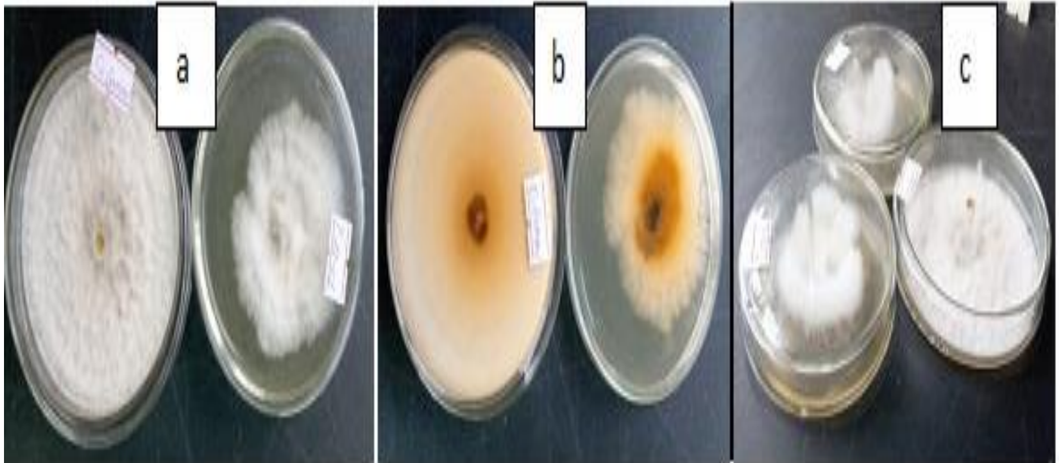

Fig. 10. The effect of non-volatile *Trichoderma* metabolites on radial growth of *Colletotrichum* species (a) right control plate and left treated plate, (b) inverted side of a and, (c) left control plate, right and top treated plates.

DISCUSSION

The use of biological control methods to reduce disease incidence caused by plant pathogens is continually being developed and is being used in a variety of crops (Soytong *et al*., 2005). In this study, it was indicated that the fungi with antagonistic properties reduced the anthracnose disease (*Colletotrichum* sp) incidence in sorghum. Tesfaye Alemu and Kapoor. (2007) and Negash Hailu and Tesfaye Alemu (2010) have also demonstrated that *Trichoderma* species can be used as a biocontrol agent in controlling a number of plant diseases with high efficacy. The antagonistic potentials of different species of *Trichoderma* against plant pathogenic fungi have been reported. The proposed antifungal mechanisms included; competition for space and/or nutrients, mycoparasitism, and antibiosis (Saber *et al*., 2017). In this study, the pronounced reduction in the mycelial growth of both *Colletotrichum* isolates can be attributed to the competition and antibiosis mechanisms exerted by the tested *Trichoderma* isolates. The competition mechanism was recognized by the fast growth rate of the *Trichoderma* isolates compared to that of the test pathogens. On the other hand, the antibiosis was recognized by the inhibition zones around the pathogen colony resulting from the two *Trichoderma* isolates. Mycoparasitism may also have contributed to their antagonistic nature. Different antibiotics have been reported to be produced by various isolates of *Trichoderma* including; trichodermin, harzianolide pyrones, harzianum A, koninginins, trichodermol, viridian, gliovirin, gliotoxin, and peptaibols (Patil *et al*.,

2016).

Among the parameters that could affect biomass production, temperature is 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 was reported by Singh *et al*. (2014), who also emphasized that the media, temperature, and pH had a profound effect on the growth of fungi. They also reported that none of the *Trichoderma* species grew at or above 40°C. The optimum temperature for growth differs among the *Trichoderma* species. Most *Trichoderma* strains are mesophilic, and cannot protect germinating seeds from soil-borne diseases caused by cold-tolerant strains of plant pathogenic fungi during cold autumn and spring conditions.

The studies on the variation of pH by different researchers 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 (Steyaert *et al*., 2010). However, there is a need to have isolates specific for saline and acidic soils.

In several species of *Trichoderma*, low pH seems to be a determinant for conidiation. Conidiation induced by light and mechanical injury is strictly low pH-dependent, with maximum response values below pH 4.4 in *T. atroviride*, *T. hamatum*, and *T. pleuroticola* (Steyaert *et al*., 2010) whereas, in *T. harzianum*, conidiation is higher at pH 5.5 (Moreno-Mateos *et al*., 2007); hence, the quality and quantity of the response seem to be species-specific. It is proposed that photo conidiation is dependent on a low intracellular pH, achieved by the low pH in the environment and the acidification that occurs when mycelia are exposed to light (Steyaert *et al*., 2010). Bae and Knudsen (2005) have found that optimum biomass production of three *Trichoderm*a isolates occurred at pH ranges between 4.6 and 6.8. Similarly, our result revealed that the optimum pH range that favoured the high spore yield of *Trichoderma* isolates in fed-batch culture was at pH 4.5–7.5.

The dual culture technique was adopted to identify the potential biocontrol agents. All tested bio-agents decreased the mycelial growth of *Colletotrichum* isolates markedly to a high extent on PDA plates compared with the control. Results show that the highest growth inhibition percentage

of *Colletotrichum* isolates was obtained by AU-11 while the lowest one was obtained by AU32 being, 90.29%, and 58.92%, respectively. *Trichoderma* species possess multiple mechanisms of action by which they control plant pathogens (Paulitz and Bélanger, 2001). Usually, the biological control agents grow and outcompute the pathogen for nutrients and space. The pathogen is suppressed in the process leading to a population reduction, after which it no longer becomes a problem (Whipps, 2004).

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). From our results, it is evident that volatile compounds produced by *Trichoderma* isolate studied decreased the mycelial growth of *Colletotrichum* species. The effects of volatiles produced by the *Trichoderma* isolates studied over the 10 days incubation period were different for *Colletotrichum* isolates AUC-1 and AUC-2. *In vitro* studies showed that the volatile compounds produced by AU-158, exhibited the highest radial growth inhibition percentage (88.32%) on *Colletotrichum* isolates. This investigation suggests that metabolites released by these *Trichoderma* species are toxic and fungistatic to *Colletotrichum* isolates.

The results of the antifungal activity assessment on non-volatile secondary metabolites of endophytic fungi *Trichoderma* species exhibited an effect on colony diameter and growth inhibition of *Colletotrichum* species. All the seven *Trichoderma* isolates inhibited the radial growth of the test pathogens by producing non-volatile inhibitors. Based on the results, non-volatile metabolites of AU-11 and AU-12 displayed the greatest ability to inhibit the growth of *Colletotrichum* species by 72.97% and 70.95% on AUC2- 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, which was produced by *T. harzianum in* liquid culture. 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 Fotoohiyan *et al*. (2017).

The antagonist *Trichoderma* not only suppresses the growth of pathogens and controls the disease, but also has got its growth-promoting effect on the plants. The present study revealed that the seeds treated with culture filtrates of the antagonists increased the seed germination *in vitro*. Five of the seven

Trichoderma isolates were found effective to enhance the germination rate compared to the control. Among the five *Trichoderma* isolates, AU-33 exhibited high germination percentage (91.67) on sorghum seeds in laboratory conditions, while the control treated with sterilized distilled water, only 75% were germinated. In general, 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.

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 25°C. Dual culture confrontation assay analysis revealed that all *Trichoderma* isolates were highly antagonistic against AUC-1 whereas AU-97, AU-131, AU-11, and AU-12 isolates displayed over 75% inhibition of mycelial growth of AUC-2 isolate. The isolates AU-11 and AU-12 showed consistent results in volatile and non-volatile activity under *in vitro* conditions against any of the two pathogens tested. The highest mean inhibitory effect on the growth of the test pathogens were achieved by AU-11 isolate (90.29%) against AUC-1 and AU-97 isolate (81.1%) against AUC-2 while AU-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* species causing anthracnose disease of sorghum. Biological control agents should, therefore, be employed as alternative ways of controlling sorghum anthracnose compared with synthetic fungicides since they are effective, biodegradable, eco-friendly, less expensive, and target specific.

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