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RESEARCH ARTICLE

ANTAGONISTIC EFFECT OF *TRICHODERMA* ISOLATES AGAINST FUSARIUM WILT DISEASE (*FUSARIUM OXYSPORUM VASINFECTUM*) OF COTTON PLANT (*GOSSYPIUM HERBACEUM*) UNDER *IN-VITRO* CONDITION

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ABSTRACT: Cotton plant (*Gossypium herbaceum* L.) is one of the most important cash crops that is widely grown in Ethiopia. The production of cotton has been declining over the years, due to *Fusarium* wilt disease (*Fusarium oxysporum vasinfectum*). Thus, the objective of this study was to evaluate local *Trichoderma* isolates for their growth inhibiting potential against cotton wilt pathogen (*Fusarium* isolates). The antagonistic potentials of *Trichoderma* isolates against the test pathogen was evaluated using dual culture assays, volatile metabolite and non-volatile metabolite assays using paired plate methods. *Trichoderma* isolates; AUT131 (48.14%) and AUT136 (52.89%) showed the highest inhibitory effect against *Fusarium* wilt pathogen. Moreover, AUT131 showed the highest (78.89%) inhibitory effect against the pathogen based on dual culture assay. Use of volatile and non-volatile metabolites produced by the *Trichoderma* isolates also confirmed that there was a production of inhibitory substances. On the other hand, *Trichoderma* isolates sporulated at different pH values and the pH change was not significant ($p \leq 0.05$) except for *Trichoderma* isolate AUT7. This study concludes that AUT131 (48.14%) and AUT136 (52.89%) isolates have high antagonistic activity against *Fusarium* wilt disease of cotton plant.

Key words/phrases: Bioassay, Biological control, Cotton plant, Dual culture, *In vitro*.

INTRODUCTION

Cotton (*Gossypium herbaceum* L.) crop provides the world's premier source for natural fibers, which is mainly used in the manufacture of a large number of textiles. In addition, the crop has a range of applications in the chemical industry as well (Kim *et al*., 2013). Nutritionally, its seeds are used to obtain edible oil and high-protein cake flour (Hinze *et al*., 2015). *Fusarium* wilt is a destructive disease of cotton in many countries of the world, including Australia, USA, Egypt, Tanzania, China and Ethiopia

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(Cianchetta and Davis, 2015). *Fusarium oxysporum* f. sp. *vasinfectum* is a cosmopolitan wilting agent attacking several species of the genus *G. herbaceum*. Fungicide application is one of the most common methods used to control this pathogen. However, their repeated use may result in the development of fungicide resistance in the pathogen population and may not also be economically justified in the control of this disease. Biological control using microbial antagonists has been shown to be a suitable and ecologically friendly candidate for the replacement of chemical pesticides. Hence, the use of integrated pest management (IPM) is very important to tackle this devastating disease (Jimenez-Díaz and Jimenez-Gasco, 2011). Therefore, this study was initiated to evaluate the endophytic *Trichoderma* isolates to manage *Fusarium* wilt pathogen of cotton plants under *in vitro* conditions.

MATERIALS AND METHODS

Description of the study area and sample collection

The study was conducted in northern Ethiopia between 13°14′21″ North and 36°27′44″ East. Samples of diseased local varieties of cotton plant parts (roots, stems and leaves) were collected from the study areas. These samples were collected in paper envelopes and transported to the Mycology Laboratory, Department of Microbial Cellular and Molecular Biology, Addis Ababa University, for isolation and characterization of the fungal pathogens.

Isolation of *Fusarium* wilt pathogens

Diseased plant parts of cotton (roots, leaves and stems) were thoroughly washed under running tap water. The washed samples were allowed to air dry under laminar air flow cabinet. The air dried roots and leaves were cut into 5 mm and sized slices using sterilized scissors. The slices were then surface disinfected using 2% sodium hypochlorite (NaOCl) and 70% ethanol for 1–2 minutes. The slices were washed three times with sterile distilled water to remove the disinfectant. Then, all cotton plant parts were placed on potato dextrose agar (PDA) and the isolated fungal pathogens were subcultured. All the inoculated plates were kept in an incubator at 25°C for 5 to 7 days (Li *et al*., 2014). From the PDA cultures, a portion of the pure isolate was subcultured onto fresh PDA plates. Once pure culture is fully grown in Petri plates, it was transferred to a slant and stored in refrigerator at 4°C. The isolates were designated as AAUFcot, which stand for Addis Ababa University *Fusarium* isolated from cotton (AAUFCot1 to 6).

Morphological identification of *Fusarium* isolates

The *Fusarium* isolates were grown on PDA plates at 25°C for 7 days. The observation was made in colony pigmentation, presence or absence of macroconidia, microconidia and chlamydospores. Slide cultures of the pathogenic *Fusarium* isolates were prepared and morphological identification was done by referring to the illustrative literature (Summerell *et al.*, 2010). Observation of septation, macroconidia and microconidia measurement were made using compound microscope, at 400x magnification power.

Pathogenicity test

Inoculum preparation

The conidia of *Fusarium* isolates (AAUcot 1, to AAUcot 6) were prepared by scraping mycelia from 7 days old cultures, mixed in 30 ml of sterile distilled water and stirred vigorously for 2 to 3 minutes and then filtered through two-layer cheese cloth. The concentration of spore suspension was adjusted to 1 x 10^5 spores/ml by using haemocytometer before inoculation (Aguiar *et al*., 2013).

Inoculation to detached leaves and greenhouse seedlings

For pathogenicity test, apparently healthy leaves were collected from cotton plants growing in pot culture under greenhouse, washed and surface-sterilized using 2% sodium hypochlorite solution for 1–2 minutes and rinsed three times with sterile distilled water. The leaves were cut and placed in Petri plates lined with 4 layers of sterilized and moisten tissue papers. The leaves were sprayed with spore suspensions of each *Fusarium* isolate and incubated at 25°C until typical symptoms of *Fusarium* wilt were observed (Miller *et al*., 2011). The seedlings were also inoculated by creating slight injury and spraying the isolates to facilitate the entrance of the pathogen. For the control seedlings, sterilized distilled water is used in the same protocol. The relative humidity was adjusted to 80 to 90% for one week until disease symptoms were observed.

Re-isolation of fungal pathogens

The causative agent in the diseased leaf parts was re-isolated on PDA. The characteristics of the re-isolated fungal isolates were compared with that of the original parent culture pathogen.

Source of *Trichoderma* isolates

The potential antagonistic *Trichoderma* isolates were obtained from Mycology Laboratory, Addis Ababa University, which were previously identified from healthy coffee rhizosphere (Afrasa Mulatu *et al*., 2022). The isolates were screened based on their effective antifungal activity against coffee wilt disease (CWD). They were properly stored in Mycology Laboratory and were recovered onto PDA medium before use. The *Trichoderma* isolates were designated as AUT131, AUT136, AUT14, and AUT7.

Antagonistic effects of *Trichoderma* isolates against *Fusarium* wilt isolates

Dual culture method

Four potential *Trichoderma* isolates (AUT131, AUT136, AUT14 and AUT7) were evaluated against *Fusarium* wilt pathogen of cotton. A plug of 5 mm diameters from the edge of an actively growing culture was placed at the periphery of the plate and incubated for 4 days at 25°C. The plate were then inoculated with a 5 mm diameter mycelial discs of the *Fusarium* isolates, placed 6 cm away from the pathogen at the opposite side and incubated at 25°C. A plate inoculated with *Fusarium* isolate alone served as a control. Each treatment had three replicates. The *Fusarium* mycelial growth (mm) was recorded at 2 day intervals. The percentage of growth inhibition (PI) of the *Fusarium* isolates was calculated using the following formula (Raza *et al*., 2013).

$$PI = \frac{R1 - R2}{R1} \times 100$$

Where: R1 = radial growth of the pathogen without *Trichoderma* isolates, R2 = radial growth of the pathogen with antagonistic *Trichoderma* isolates.

Volatile organic compounds (VOCs) production

The effect of volatile antibiosis of antagonistic *Trichoderma* isolates on *Fusarium* isolate were tested following the method described by Naraghi *et al*. (2010). A 5 mm disc of *Fusarium* isolates were placed in Petri plate containing PDA medium and incubated at 25°C for 4 days. Then, 5 mm discs of *Trichoderma* isolates were also cultured in Petri plate containing PDA medium. Two Petri plate bottoms (paired plate method) containing *Fusarium* isolates and antagonistic *Trichoderma* isolates were placed face to face and then sealed with parafilm. The control Petri plates were not inoculated with antagonistic *Trichoderma* isolates. The Petri plates were incubated at 25°C for 10 days and each treatment was replicated three times.

Non-volatile organic compounds (nVOCs) production

The effect of non-volatile antibiotics produced by *Trichoderma* isolates (AUT131, AUT136, AUT14 and AUT7) against *Fusarium* isolates growth was examined using the culture filtrate (Raza *et al*., 2013). Culture filtrates of the antagonistic *Trichoderma* isolates were mixed properly to the Petri plates containing PDA medium by volume-volume ratio of 4 ml:16 ml, respectively and centrally inoculated with 5 mm discs of *Fusarium* isolate. Each treatment was replicated three times. The plates were incubated for 10 days at 25°C and radial growth was measured at two days interval.

Statistical analyses

Statistical analysis was performed with analyses of variances (ANOVA) using SPSS version 25 and mean value replica were separated by Tukey's Honestly Significance Difference (HSD). The significance of effects of *Trichoderma* on growth inhibition characteristics of the pathogen was determined by the magnitude of the F-value ($p<0.05$) (Yuan *et al*., 2017).

RESULTS

Isolation and cultural identification of *Fusarium* isolates

From the diseased cotton plant specimens of leaves, stems and roots, a total of six *Fusarium* isolates were culturally identified. The culturally identified isolates were identified as *Fusarium* isolates: AAUFcot01, to AAUFcot06. Based on the mycelial growth and pigmentation, the isolates showed different colony colours and aerial mycelial growth patterns. Specifically, the colony colour of the isolates on PDA medium varied from creamy white, fluffy white, grey, black, and brown. All isolates displayed noticeable cultural difference being grown on PDA (Fig. 1).

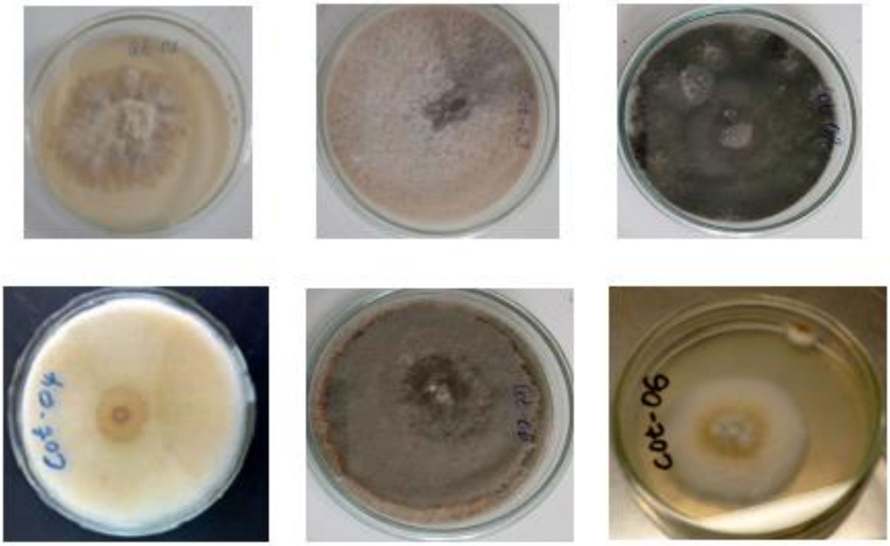

Fig. 1. Some of the isolates from diseased cotton samples: A (AAUFcot01), B (AAUFcot02), C (AAUFcot03), D (AAUFcot04), E (AAUFcot05), and F (AAUFcot06).

Microscopic characteristics of *Fusarium* isolates

The results of slide culture and microscopic observation showed various features like microcroconidia, macroconidia and septation (Fig. 2). The average size of the microcroconidia and macroconidia were measured as 8 x 2.40 and 17 x 2.90 µm, respectively. Similarly, the shape was observed to be oval microconidia and sickle shaped macroconidia with septation (Fig. 2).

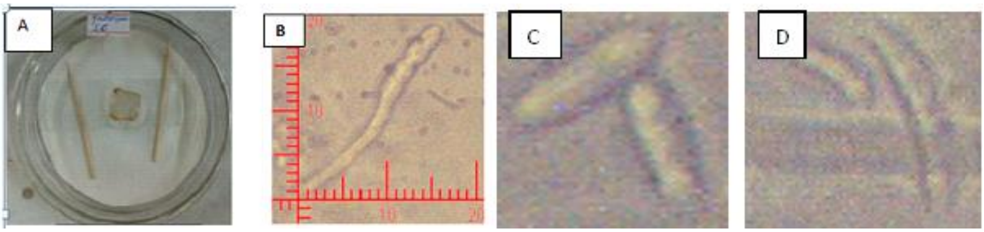

Fig. 2. Microscopic features of *Fusarium* isolates (A) Slide culture preparation, (B) Size of macroconidia, (C) Oval-shaped microconidia, and (D) Sickle-shaped macroconidia.

Pathogenicity test on cotton seedlings and detached leaves

Based on the results, yellow coloration and wilting symptoms were observed on the inoculated leaves but no symptom was shown on the control leaves (Fig. 3). White hyphae growth on the petiole of detached leaves were also seen after 10 days inoculation with the test pathogen AAUFcot04 (Fig.

3).

Fig. 3. Pathogenicity test on detached leaves. (1) Source of cotton leaves, (2) Disinfection and inoculation process in the biosafety, (3) Inoculated detached leaf on a plate, (4) Diseased leaf after incubation, (5) Control pot and, (6) Diseased cotton seedling in the greenhouse.

In vitro antagonistic assays of *Trichoderma* isolates against the *Fusarium* isolates

The cultural confrontation showed good antagonistic activity of *Trichoderma* isolates (Table 1). *Trichoderma* isolates; AUT131 and AUT136 showed fast growth and overgrew on the mycelium of the test pathogen within five days (Fig. 4). Accordingly, *Trichoderma* AUT131 showed the highest (78.99%) growth inhibition against the test pathogens followed by AUT136 (76.14%). On the other hand, the least growth inhibition (52.29%) was shown by the *Trichoderma* isolate AUT7 (Table1). In general, all *Trichoderma* isolates significantly ($p<0.05$) inhibited the mycelial growth of the test pathogens.

Table 1. Effects of *Trichoderma* isolates on mycelial growth of the isolated *Fusarium* wilt pathogen of cotton (AAUFcot04) in dual culture test.

***Trichoderma* isolates**	**Radial growth inhibition of *Fusarium* wilt pathogen (AAUFcot04)**		
	Day 6	**Day 8**	**Day 10**
AUT14	$62.16^{b} \pm 1.68$	$67.41^{b} \pm 1.94$	$73.39^{a} \pm 1.58$
AUT7	$29.27^{c} \pm 1.19$	$41.57^{b} \pm 1.1$	$52.29^{b} \pm 1.2$
AUT136	$64.86^{b} \pm 0.68$	$70.78^{ab} \pm 2.89$	$76.14^{a} \pm 2.17$
AUT131	$68.91^{b} \pm 0.34$	$74.15^{a} \pm 1.94$	$78.99^{a} \pm 1.38$
Control (AAUFcot04)	00 ± 00	00 ± 00	00 ± 00

Mean ± standard deviation. Different alphabets with in the column shows significant values.

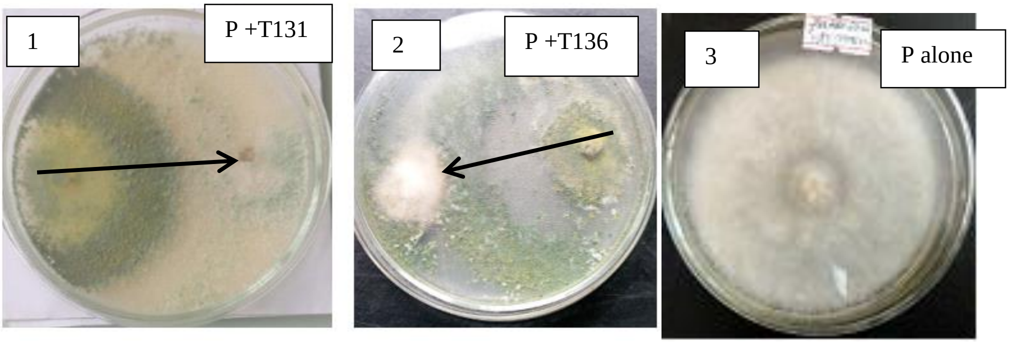

Fig. 4. Dual culture test plates. (1) *Fusarium* isolate + *Trichoderma* (AUT131), (2) *Fusarium* isolate + *Trichoderma* (AUT136), (3) Control (*Fusarium* isolate alone).

Effect of volatile compounds from *Trichoderma* isolates

Trichoderma isolates produced volatile metabolites that reasonably inhibited the mycelial growth of the test pathogens (Fig. 5). The volatile metabolites from these *Trichoderma* isolates showed significant ($p<0.05$) differences in the inhibition against the mycelial growth of the *Fusarium* isolate. The *Trichoderma* isolate (AUT131) gave the highest mycelial growth inhibition 48.14%, followed by isolates AUT136 and AUT14 both at 47.40%, whereas the isolate AUT7 gave the least growth inhibition (34.07%) after 10 days of incubation (Fig. 5 and 6).

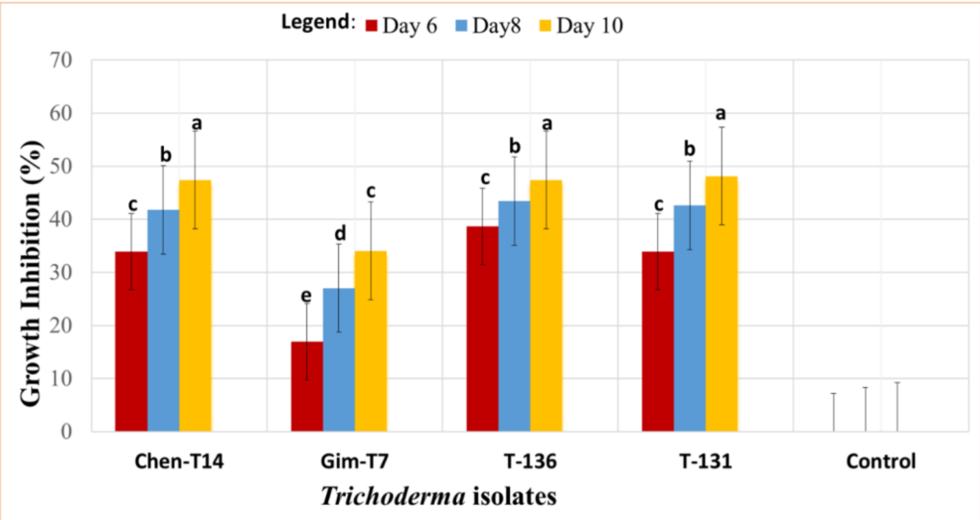

Fig. 5. Effects of volatile compounds from *Trichoderma* isolates on mycelial growth of *Fusarium* wilt pathogen (AAUFcot04). Different alphabets depicted in superscript indicate mean treatments that are significantly different according to Tukey's HSD posthoc test at $p<0.05$, each value is an average of 3 replicate samples ± standard error.

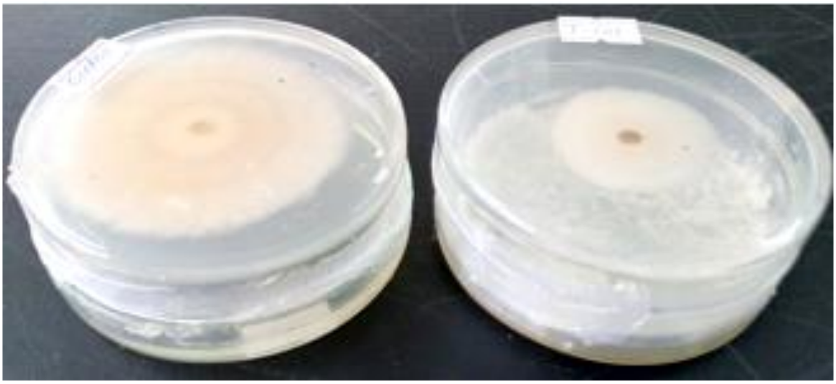

Fig. 6. Antagonistic effect of volatile metabolic compounds extracted from *Trichoderma* isolates on the mycelial growth of *Fusarium* isolate (1) Control (*Fusarium* wilt pathogen (AAUFcot04) alone, (2) *Trichoderma* isolate (AUT131), and *Fusarium* isolate (AAUFcot04).

Effect of non-volatile compounds from *Trichoderma* isolates

The results obtained from the production of non-volatile metabolic compounds showed that the pathogen was significantly suppressed with the culture filtrates of all the antagonistic *Trichoderma* isolates tested (Fig. 7). However, the inhibition of radial mycelial growth of the pathogen varied significantly ($p<0.05$) for each *Trichoderma* isolate. Accordingly, AUT136 isolate showed the highest percentage of inhibition (52.89%) followed by AUT131 isolate (49.58%). Among the *Trichoderma* isolates, the least growth inhibition (38.84%) was recorded by AUT14 isolate after 10 days of incubation at 25°C (Fig. 7 and 8).

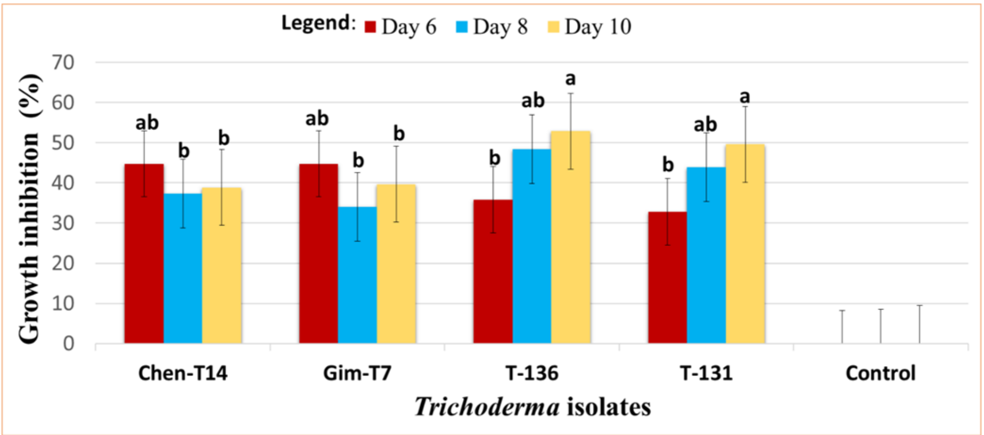

Fig. 7. Effect of non-volatile compounds extracted from *Trichoderma* isolates on mycelial growth of *Fusarium* isolate (AAUFcot04). Different alphabets depicted in superscript indicate mean treatments that are significantly different according to Tukey's HSD posthoc test at $p<0.05$, each value is an average of 3 replicate samples ± standard error.

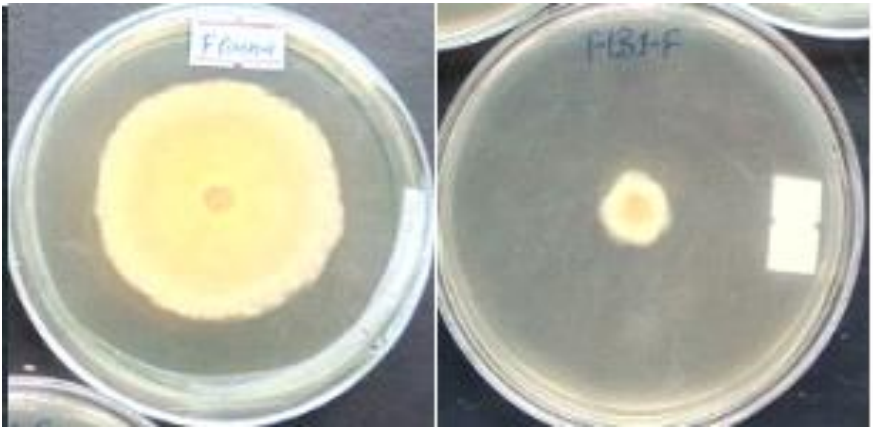

Fig. 8. Effect of non-volatile compounds extracted from *Trichoderma* isolates on mycelial growth of *Fusarium* isolate (AAUFcot04). (1) Control (*Fusarium* isolate alone), (2) Non-volatile culture filtrates of *Trichoderma* isolate (AUT131) + *Fusarium* isolate.

DISCUSSION

Antagonistic effects from the dual culture experiments showed that *Trichoderma* isolates significantly inhibited the mycelial growth of *Fusarium* wilt disease of cotton ranging from 52.29 to 78.89% at 25°C. The results showed that all *Trichoderma* isolates were able to suppress the mycelial growth of the test fungus. In this study, *Trichoderma* isolate AUT131 revealed the highest inhibition percentage value of 78.89%, whereas AUT7 showed the lowest percentage value of 52.29%. Similarly, Consolo *et al*. (2012) earlier reported that *Trichoderma* isolates inhibit the growth of *Bipolaris sorokiniana* and *Pyricularia oryzae* pathogens by more than 85% in dual culture techniques on PDA. In addition, Afrasa Mulatu *et al*. (2022) reported that *T. asperellum* AU131 was found the most inhibiting species of *Fusarium xylarioides* with a value of 82.4%. Unlike the present study, Thanh *et al*. (2014) have documented that *T. harzianum* gave the highest inhibition capacity of 100% in dual cultural test against *Aspergillus flavus*.

Trichoderma species are known to produce both volatile and non-volatile compounds that can suppress the growth of the fungal pathogens (Raza *et al*., 2013). In the present study, the mycelial growth of the *Fusarium* isolates were inhibited when exposed to volatile compounds by *Trichoderma* isolates. Unlike the results of the dual culture test, *Trichoderma* isolates AUT7 inhibited the mycelial growth of the *Fusarium* isolate (AAUFcot04) by 34.07%, which is the least inhibition of all the *Trichoderma* isolates tested. This may suggest that this particular isolate is likely to produce less effective volatile compounds against the test pathogen. Contrary to this, *Trichoderma* isolates AUT131 was able to inhibit the growth of the *Fusarium* isolate by 48.14%. Talla *et al*. (2015) have also reported that secondary metabolites of *T. harzianum* isolates have inhibitory effects on the growth of different plant pathogens. It was also shown that *T. viride* produced large amounts of volatile compounds to affect the hyphal tips of *Lentinus lepidus* and *Coriolus versicolor*. The report by Hermosa *et al*. (2013) demonstrated the ability of *Trichoderma* species to produce volatile and non-volatile antibiotics that can inhibit the growth of plant pathogenic fungi.

The effect of pH on the mycelial growth of the isolates varied slightly among the *Trichoderma* isolates. The mycelial dry weight revealed that the *Trichoderma* isolates grew well in the pH values of 4.5, 5.5, 6.5, and 7.5. *Trichoderma* isolate AUT131 performed better in comparison with other isolates which showed the maximum mycelial dry weight at a pH value of 6.5. This may indicate that this isolate could prefer acidic conditions for its optimum mycelial growth. The optimum pH for maximum biomass production of *Trichoderma* isolate AUT131 was recorded as 0.472 and 0.502 g/ml at pH values 5.5 and 6.5, respectively. Similarly, Mohd *et al*. (2011) have indicated that the most favourable pH ranged between 6.5 to 7.5 in which the total dry weight of mycelium varied between 198.61 to 223.00 mg. Abeyratne and Deshappriya (2018) have also recorded that the biomass of the *Trichoderma* isolates in PDA plates showed the highest weight with pH values of 4, 5, 6 and 7. Similar to the present study, Bagwan (2010) have reported that the most favourable pH for maximum dry weight of *T. viride* against *S. rolfsii* and *R. solani* ranged from 5.5 to 6.5. Mycelial growth and sporulation is another important characteristic of bio-control agents as their efficiency and competence of bio-control is closely associated with the ability to compete with pathogens in the soil. In the present study, the highest mycelial growth (0.27 mm/h) was recorded by the *Trichoderma* isolate (AUT7) at a relatively low pH 4.5 which was comparable to Moretto *et al*. (2001) (0.33 mm/h). The study conducted by Ali *et al*. (2015) and Zehra *et al*. (2014) have confirmed that the optimum pH should be maintained for the mycelial growth and sporulation of different *Trichoderma* isolates under *in vitro* conditions. The suitability of an acidic pH ranges for the survival of *Trichoderma* isolates was also reported by Bhai *et al*. (2010) who recorded that the pH range 4.5–5.5 was more appropriate for the optimum mycelial growth, sporulation, and survival of *Trichoderma* isolates than alkaline conditions.

CONCLUSION

The *in vitro* evaluation of the mycelial growth of the pathogenic fungus (AAUFcot04) showed strong inhibition by the production of volatile and non-volatile compounds of *Trichoderma* isolates as well as under dual culture test. In general, AUT131 (48.14%) and AUT136 (52.89%) isolates showed higher antagonistic activity against *Fusarium* wilt disease of cotton plant.

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