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SHORT COMMUNICATION

IN VITRO EVALUATION OF ANTAGONISTIC POTENTIAL ACTIVITY AND ASSAY OF CULTURE FILTRATES OF *TRICHODERMA HARZIANUM* AND *TRICHODERMA VIRIDE* AGAINST COFFEE WILT PATHOGEN (*GIBBERELLA XYLARIOIDES*)

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ABSTRACT: Isolates of *Fusarium xylarioides,* the causative agent of coffee wilt disease, were obtained from five coffees growing *woredas* in south western Ethiopia. The isolation was made from five Arabica coffee (*Coffea arabica*) trees with severe symptoms of coffee wilt disease. The ability of *Trichoderma harzianum* and *Trichoderma viride* to inhibit mycelial growth of *F. xylarioides* was evaluated *in vitro*. The result showed that use of *Trichoderma* spp caused a radial mycelial growth inhibition of pathogen by 66.2% (Fx.20) and 70.9% (Fx.22), respectively. The minimum percent of mycelial growth inhibition (53.7 %) was observed by *T.viride* on isolate Fx.16. Use of the cell free culture filtrates of *T. harzianum* and *T. viride* with concentrations of 3ml, 4ml and 5ml resulted in radial growth inhibition of the test pathogen. *T. viride* was found to be more efficient than *T. harzianum*. Similarly, *in vitro* evaluation of the culture filtrates showed an average of 63.6% mycelial growth inhibition by *T. viride* over the control on Fx.22. This study showed that *T. harzianum* and *T. viride* have potential to be used as biocontrol agents for inhibition of mycelial growth of *F. xylarioides* isolates. *Trichoderma harzianum* and *T. viride* must be evaluated and tested against coffee wilt disease *in vivo* and in field condition in different agro-ecological zones of coffee growing regions, in order to apply biocontrol agents in large areas to manage *F. xylarioides* in the country.

Key words/phrases: Biocontrol agents, Coffee wilt disease, *Fusarium xylarioides, Trichoderma harzianum, Trichoderma viride.*

INTRODUCTION

The most limiting factor for coffee production in Central and East African countries is tracheomycosis/ vascular wilt disease of coffee that is caused by

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Fusarium xylarioides Steyaert imperfect stage, (*Gibberella xylarioides* Heim and Saccas, perfect stage) (Lewis Ivey *et al*., 2003; Lepoint, *et al*., 2005 ; Leslie, *et al*., 2005; Geiser, *et al*., 2005). The coffee wilt disease kills all affected coffee trees at all stages of development. Coffee wilt disease is found in major coffee growing countries. The disease attacks the vascular systems thereby inhibiting the transport of assimilates and water which finally results in the death of the tree. Tracheomycosis historically was first observed in 1927 in a plantation of *Coffea excelsa* in Central Africa Republic and the causal agent was identified as *F. xylarioides* (Steyaert, 1948; Stewart, 1957). The disease was first recorded in Ethiopia (Kaffa province) in 1957 (Stewart, 1957; Kranz and Mogk, 1973; Nelson, *et al*., 1983). The pathogen also attacks *Coffea arabica* and is endemic in all coffee growing areas of African countries (Flood, 1997; Girma Adugna and Hindorf, 2001; Girma Adugna, 2004; Lepoint *et al*., 2005). In recent years, the distribution of *F. xylarioides* across East Africa has affected 90% and 30% of farms in Uganda and Ethiopia, respectively (CABI, 2003). According to CABI (2003), it has been estimated that income of coffee households is facing a reduction by a third due to coffee wilt disease. In Ethiopia, *F. xylarioides* is becoming severe in coffee farms, at Teppi, Bebeka and Gera; mean prevalence of disease severity was recorded 35.09% (Eshetu *et al*., 2000).

Trichoderma harzianum and *T. viride* are the most studied of all the *Trichoderma* species for biological control and the most effective in reducing diseases caused by soil-borne plant pathogens (Baker and Cook, 1974; Cortes *et al*., 1998; Huang *et al*., 2000; Elad, 2000; Rocco and Perez, 2001; Tesfaye Alemu and Kapoor, 2004). The biocontrol antagonists have a potential to tolerate very extreme environmental conditions and ability to survive in media with high levels of pesticides and other chemicals. The successful use of *Trichoderma* spp. as a biocontrol agent might be attributed to the production of antibiotic and antifungal compounds, secretion of lytic-enzymes, mycoparasitism, hyphal interaction, competition for space and nutrients, and induction of systemic resistance (Cortes *et al*., 1998; Rocco and Perez, 2001). It has been hypothesized that *Trichoderma* species compete with *F. xylarioides in vitro* for the nutrients and space and this is expressed by the suppression or inhibition of the mycelial growth of *F. xylarioides*. Hence, this study was undertaken to evaluate and test *in vitro* antagonistic potential activities and to determine the efficacy of culture filtrates of *T. harzianum* and *T. viride* on mycelial growth inhibition of *F. xylarioides* isolates.

MATERIALS AND METHODS

Isolation of *Fusarium xylarioides* isolates

In 2007, five isolates of *Fusarium xylarioides* were isolated from diseased twigs and stems of Arabica coffee (*Coffea arabica*) trees. The sample trees were selected from five coffee growing districts (Seka- Chekoressa (Fx.3); Bako-from Gazer (Fx.16); Sheko (Fx.20); Gomma (Fx.22) and Godere (Fx. 25) from southwestern Ethiopia. The isolation and identification of the pathogen were carried out in mycology research laboratory of Department of Biology, Addis Ababa University. From each of these plant materials, small pieces were taken from the margin of infected and healthy regions. These pieces were washed in sterilized water for two minutes in separate plates in order to minimize surface contaminants**.** Subsequently, the samples were dipped in 70% ethanol for one minute to sterilize the surface and rinsed three times in sterile distilled water (Dhingra and Sinclair, 1993; Aneja, 2005). After sterilization, samples were then directly transferred into Petri dish containing malt extract agar (MEA) and potato dextrose agar (PDA). The growth media were amended with 60 mg/l streptomycin to suppress the growth of bacteria. Each isolate from symptomatic plant parts were identified as *Fusarium xylarioides* (Fx isolate) and designated as Fx - 3; Fx -16; Fx -20; Fx -22 and Fx -25. The identification of *F. xylarioides* based on the microconidia, macroconidia, chlamydospores, sporodochial formation and sporulation of the pathogen was carried out according to illustrated manual of Nelson (1983), Burgess *et al*. (1994) and Booth (1971).

Source of biocontrol agents

Trichoderma harzianum (accession No. 2895) and *T. viride* (accession No.1433) used as biological control agents were obtained from from Indian Type Culture Collection (ITCC), New Delhi, India. *In* v*itro* evaluation of the antagonistic potential of these two species of *Trichoderma* against the test pathogen (*F. xylarioides*) isolates was carried out in the mycology laboratory, Department of Biology, Addis Abba University.

Inoculum preparation

Mycelial agar plugs of each *F. xylarioides* isolates from 7 days old colony culture margins were placed onto Petri dishes (90 mm in diameter), each containing approximately 20 ml of potato dextrose agar (PDA). The Petri dishes were incubated at 25^0C for 7 days (Tesfaye Alemu and Kapoor, 2004). Spores were washed off by flooding the colony in each Petri dish with 10 ml of sterile distilled water and scraping the colony surface using a

glass spatula. The spore suspensions of similar isolates were pooled and filtered through four layers of cheese cloth. The fungal isolates and biocontrol agent cultures were kept for further study at 4^0C (Dhingra and Sinclair, 1993 and Aneja, 2005).

Antagonistic activity test

Dual culture method (Silvakumar *et al*., 2000) was employed to evaluate the antagonistic potential of *T. harzianum* and *T. viride.* A 5 mm diameter mycelial disc taken from the periphery of 7 day old culture of the pathogen and the bioagents as placed separately at the opposite edge of the Petri plate containing PDA. The *F. xylarioides* were inoculated 12 hours prior to the placement of the *Trichoderma* spp. Each of the 5 isolates of the pathogen was tested in separate Petri plates and each of the combination with biocontrol agent was replicated three times. Additional plates having only the test isolates were used as control. All plates were incubated at 25 ± 1^0C. The radial mycelial growth inhibitions were recorded at the 8^{th} day of inoculation. Percent of radial growth inhibition was calculated according to Montealegre *et al*. (2003) in relation to growth of the control. Percent of radial mycelial growth inhibition was calculated by the following formula.

$$\text{Percent of inhibition} = \frac{(C - T)}{C} \times 100$$

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

Assay of culture filtrates

Trichoderma harzianum and *T. viride* were grown on potato dextrose broth (PDB) to evaluate the ability of the two species to produce antifungal and inhibitory substances. Two hundred fifty ml flasks containing 100 ml of PDB were used to culture *T. harzianum* and *T. viride* separately. Each flask was inoculated with 5 mm diameter of mycelial disk. The inoculated flasks were incubated at 20^0C on shaker at 120 rpm for 20 days. After 20 days of incubation, the broth was filtered through Whatman number 42 filter paper and subsequently the filtrate of each isolate was centrifuged at 10000 rpm for ten minutes (Tesfaye Alemu and Kapoor, 2004; Aneja, 2005) to make it cell free. The influence of cell free culture filtrates of the *T. harzianum* and *T. viride* was examined on the mycelial growth of *F. xylarioides* isolates (Fx.3, Fx.16, Fx.20, Fx.22 and Fx.25). The determination of the amount culture filtrate level that inhibits the mycelial growth of the test pathogen was used in this study as followed by Tesfaye Alemu and Kapoor (2004).

For this purpose, 3ml, 4ml and 5ml of culture filtrates of *T. harzianum* and *T. viride* were used. These filtrates were then separately mixed with sterilized and cooled growth media that contained 20 ml PDA. 23, 24 and 25 ml of these mixtures were aseptically poured into separate Petri plates with three replications each. The prepared media were allowed to solidify for 24 hours. A 5 mm disk taken from the periphery of a 7- day old culture of *F. xylarioides* isolates was placed at the center of each plate. *Trichoderma* spp were replicated three times and a control contained PDA without culture filtrates. All the Petri plates were incubated, at 25^0C $\pm$1 for 8 days. The mycelial growth measurements were made after 8 days of inoculation. Percent mycelial growth was calculated according to Montealegre *et al*. (2003).

Statistical analysis

Mean comparisons of *Trichoderma harzianum* and *T. viride* and *F. xylarioides* isolates with controls and percent of mycelial growth inhibition of the test isolates by bioagents were analyzed using the GLM (General Linear Model, multivariate) and SPSS statistical analysis software (SPSS institute Inc., Cary, NC) version 13. The experiment used the randomized complete design and the least square mean comparisons test with $P<0.05$.

RESULTS

Antagonistic Test

In vitro evaluation of the antagonistic activity of *Trichoderma harzianum* and *T. viride*, indicated that the two biocontrol agents caused mycelial growth inhibition on the test pathogens. The results also indicated that there were differences between the biological control agents in inhibiting the mycelial growth rate of the pathogens. *T. viride* showed the highest growth inhibition (70.9 % and 67.8 %) on *F. xylarioides* isolates, Fx.22 and Fx.3, respectively (Fig.1). The minimum growth inhibition (50%) was recorded on *F. xylarioides* isolate Fx.25. Similarly, *T. harzianum* showed highest percent of mycelial growth inhibition (66.2%) on isolate Fx.20 (Fig.1). Simultaneously, the minimum percent of mycelia growth inhibition by *T. harzianum* (55%) was observed on isolate Fx.16 (Fig.1). Therefore, the result indicated that the mechanism of action between *F. xylarioides* and *T. harzianum* and *T. viride* were due to competition for space, nutrients and intermingling that resulted in inhibition of the mycelium growth of the test pathogen.

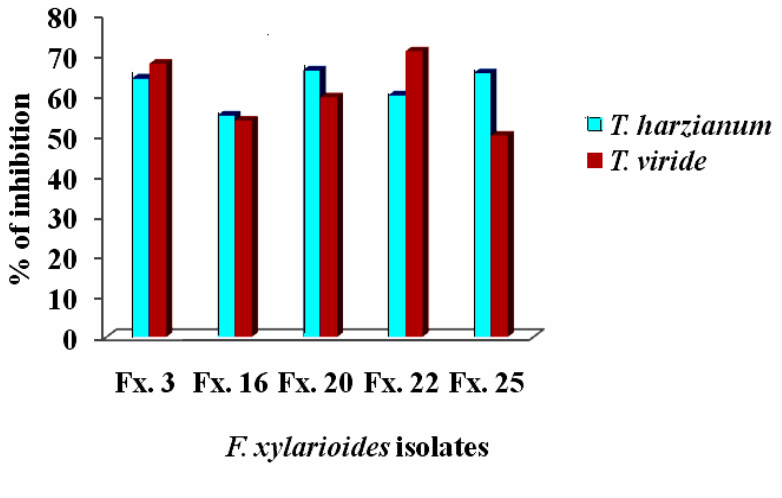
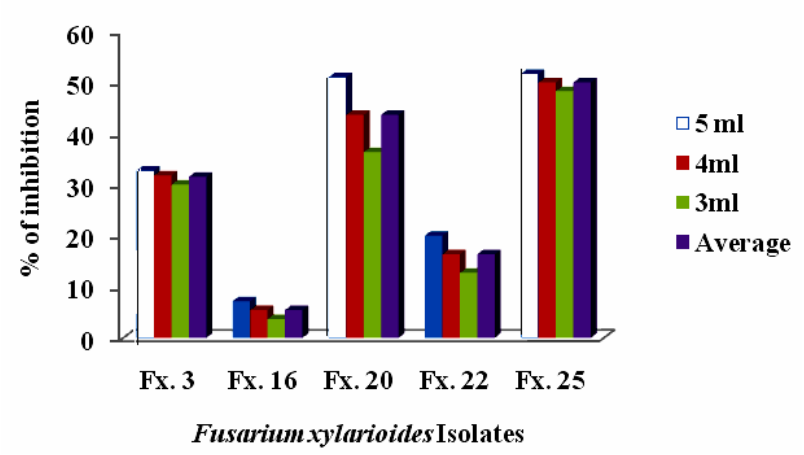


Fig. 1. *In vitro* evaluation of percent of mycelial growth inhibition of *F. xylarioides* isolates by *Trichoderma harzianum* and *T. viride* over control.

Effect of culture filtrates of *T. harzianum* and *T. viride* on mycelial growth of *F. xylarioides* isolates

The results from application of culture filtrate of *T. harzianum* and *T. viride* on PDA growth medium resulted in mycelial growth inhibition of *F. xylarioides* isolates (Fig. 2a and 2b). The highest inhibition was recorded for 5ml of culture filtrates application, whereas minimum inhibition was recorded for 3ml culture filtrates application of both *T. harzianum* and *T. viride*. The average mean value of culture filtrates of *T. viride* showed highest percent of mycelial growth inhibition (63.6%) on the *F. xylarioides* isolate Fx.22 (Fig. 2b); whereas *T. harzianum* produced minimum (5.4%) percent of mycelial inhibition on isolate Fx.16 at an average mean value of concentration of culture filtrates (Fig 2a). In general, the highest percent (70.2%) of mycelial growth inhibition was recorded in plates that contained 5ml culture filtrates of *T. viride* on isolate Fx.20 and also the highest percent (51.7%) of mycelial growth inhibition in Petri plates which contained 5ml of culture filtrates of *T. harzianum* on isolate Fx.25 (Fig. 2a and 2b). Minimum percent of inhibition (35.4%) was observed in Petri plates which contained 3ml of culture filtrates of *T. viride* on isolate Fx. 16 (Fig. 2b). Similarly, the minimum percent of inhibition (3.7%) was recorded in Petri plates which contained 3ml of culture filtrates of *T. harzianum* on isolate Fx.16 (Fig. 2a).

a)



b)

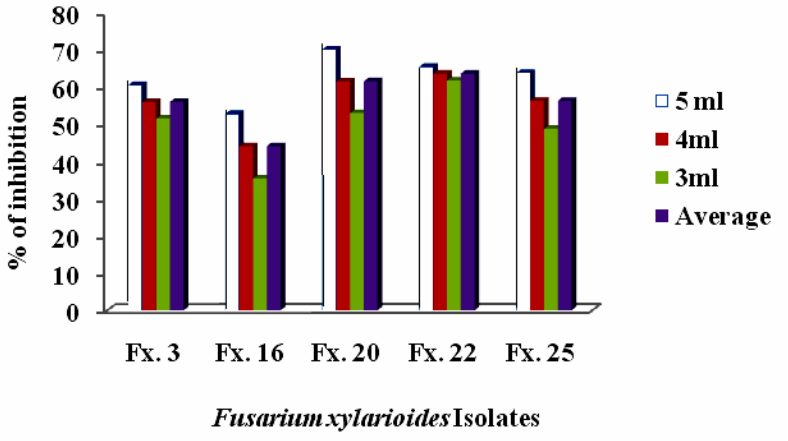


Fig. 2a) Percent of mycelial growth inhibition of concentration of culture filtrates of *T. harzianum* over control. 2b) Percent of mycelial growth inhibition of concentration of culture filtrates of *T. viride* over control

DISCUSSION

In vitro evaluations of *T. harzianum* and *T. viride* have shown inhibition of mycelial growth of *F. xylarioides* isolates. This might have been due to competition between the pathogens and biocontrol agent for space and

nutrient. The isolates Fx-16, and Fx-22 had higher mycelial growth or lower percent of inhibition by *T. harzianum*, whereas the isolates Fx-20 and Fx-22 showed lower mycelial growth or higher percent of inhibition. *T. viride* and *T. harzianum* showed maximum percent of mycelial growth inhibition of 70.9% and 66.2% against the isolates Fx-22 and Fx-20, respectively (Fig 1). *T. harzianum* caused mycelial growth inhibition of 55%, 60% and 65.5% on Fx-16, Fx-22 and Fx-25, respectively (Fig.1). *T. viride* exhibited relatively higher percentage of inhibition compared to *T. harzianum* against the test isolates (Fig. 1). *T. harzianum* and *T. viride* are the most effective biocontrol agents applied to control plant diseases caused by soil-borne plant pathogens (Baker and Cook, 1974; Hermosa, 2000 and Weller, 2002). This result is in agreement with previous reports by Tesfaye and Kapoor (2004) where *Trichoderma viride* caused 57.4% mycelial growth inhibition of *Botrytis gladiolorum*. Macroscopic observation of dual cultures revealed that inhibition of mycelial growth of *F. xylarioides* occurred soon after contact was made with the antagonists; *T. harzianum* and *T. viride*, however, continued to grow over the colony of *Rhizoctonia solani* (Gao *et al*., 2005). The culture filtrates of *T. harzianum* and *T. viride* hindered the mycelial growth of *F. xylarioides* isolates. Different concentration of culture filtrates of *T. harzianum* and *T. viride* inhibited mycelia growth of *F. xylarioides*, varying between 3.7% and 70.2% (Fig. 2a and 2b). The highest mycelia growth inhibition (70.2%) was produced by *T. viride* when using 5ml culture filtrate of *T. viride*. As indicated in figures 2a and 2b, the percentage of inhibition increased with increasing concentration of culture filtrates of *T. viride* and *T. harzianum*. Similar result (68%) was reported by Tesfaye and Kapoor (2004) that *in vitro* evaluation of the mycelial growth inhibition of *Botrytis gladiolorum* was obtained by *T. viride* at highest concentration of the culture filtrate (5ml) whereas *T. harzianum* produced minimum percent of 51.2% inhibition at all the concentrations of the culture filtrates. From these results, it is possible to conclude that *T. viride* produces larger amount of inhibitory substances that can inhibit the mycelial growth of *F. xylarioides*, which coincided with the results reported by Barbosa *et al*. (2001) and Tesfaye Alemu and Kapoor (2004). *Trichoderma harzianum* is effective against *Botrytis* grey mould on green house cucumber and tomato (Elad *et al*., 1995; O'Neill *et al*., 1996; Zimand *et al*., 1996) on apple (Tronsmo, 1991; Elad, 1994) on chick pea foliage in controlled environments (Mukherjee and Haware, 1993).

The present result of *in vitro* evaluation may suggest that lower mycelial growth inhibition levels of *F. xylarioides* isolates were due to high

competition for nutrients and space by *T. harzianum* and *T. viride* when compared with growth of uninoculated control of the test pathogen isolates. The study of Barbosa *et al*. (2001) on the *in vitro* antagonism of *Trichoderma* species on *Cladosporium herbarum* also revealed that colonies of *Trichoderma* species grew faster than *C. herbarum* in single or mixed culture. Papavizas (1985) and Elad (2000) also indicated that *Trichoderma* species are potential biocontrol agents for the control of plant pathogens. Biocontrol efficacy of microscopic examination of cultures of *Trichoderma* species and *Drechslera tritici-repents* in close proximity showed differences in hyphal morphology of pathogen among treatments and control (Perello *et al*., 2003). Subsequently, *in vitro* evaluation and testing of biological control agents in this finding have indicated that *T. harzianum* and *T. viride* do produce and release antifungal compounds that can inhibit the isolates of *F. xylarioides* to some extent.

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