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Biopotential of Some *Trichoderma* spp. against Growth of *Fusarium oxysporum* f. sp. *Vasinfectum* Causal Agent of Cotton Wilt

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KTH and BBBA designed the study, performed the experiments and wrote the manuscript. Author SDF carried out the experimental process. Author KKL was also responsible for data interpretation and statistical analysis. Author CS helped in experiments and preparing the manuscript. Authors KKL, KT and KD participated in experiments, data collection, managed the analyses of the study and literature searches. Author KTH also wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Evaluation of the antagonistic activity of some *Trichoderma* species by direct confrontation method was used in this study. The mycoparasitism inhibitory effects of *Trichoderma* species on the growth of the causal agent of cotton Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*) were investigated by dual culture under *in vitro* condition. Seventeen isolates of *Trichoderma* and one

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strain of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) were used. These different isolates of *Trichoderma* were *in vitro* confronted to FOV. The invasion ability of antagonist colonies was measured during 14 days. Thus, at the end of these control trials, isolates of *Trichoderma h*ave controlled the growth of FOV. Indeed, FOV is confined to a small area and is unable to extend its growth after the third day of incubation in the culture room at 30℃. Beyond this period and after then days, they never sporulated, revealing the inhibitory action of *Trichoderma* against FOV. Consequently, the *Trichoderma* isolates were found able to biologically control of FOV growth.

Keywords: Cotton; biological control; Fusarium wilt; mycoparasitism; Trichoderma spp.

1. INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is not only the most important fibber crop in the world, but also the second best potential source for plant proteins after soybean and the fifth best oil-producing plant after soybean, palm-tree, colza and sunflower [1]. Cotton is infected by a number of pathogens. But *Fusarium oxysporum* and *Rhizoctonia solani* are recognize as the main pathogens in cotton seedling disease. Moreover, *F. oxysporum* f. sp. *vasinfectum* (FOV) cause *Fusarium* wilt of cotton disease. It occurs in most major cotton production regions of the world [2]. Wilt of cotton is a vascular disease caused by the soil borne pathogen FOV. The disease is widespread and causes substantial crop losses. Control of FOV may be achieved by applying tremendous volume of fungicides during the cotton plant culture. However, the continued use of these products has created serious problems for the environment and human health.

Biological control of phytopathogens by microorganisms is a method of plant disease management. It has been used as an alternative in the fight against pathogens in plants [3].

In addition, biological control using microbial pesticides or microorganisms is accepted as an efficient mean of reducing environmental pollution and poisoning of consumers [4-7].

The genus *Trichoderma* includes the most common saprophytic fungi in the rhizosphere and is found in almost any soil. The harmful ability of *Trichoderma* species against pathogens for induce resistance at plants allows the development of different bio-control strategies [8,9]. Several *Trichoderma* spp. reduce the incidence of soil borne plant pathogenic fungi under natural conditions [10]. Recently there have been numerous attempts to use *Trichoderma* spp. against soil borne pathogens such as *Fusarium, Phytophtora, Pythium* and *Rhizoctonia* species [11]. *Trichoderma* is often used to prevent development of several pathogenic fungi in biological controls. Moreover, John et al. [12] reported that *Trichoderma* can be considered as a biological control agent against *Fusarium oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes* and as a growth promoter of soybean.

Different isolates of *Trichoderma* have various strategies for fungus antagonism as well as several indirect effects on plant health. Possible mechanisms of antagonism applied by *Trichoderma* spp. includes nutrient and niche competition, antibiosis and myco-parasitism [13] that act as inhibitors against many soil-borne fungi, and last parasitism [14]. Mobilization and withdrawal of soil nutrients were more important within plants colonized by Trichoderma than in other organisms. Tjamos et al. [15] showed that *Trichoderma* uses the competition to colonize the rhizosphere and the nutrients to the detriment of *Fusarium*. It thus succeeded to controlling germination of spores and mycelia growth of *Fusarium oxysporum*. These researchers have finally established a positive correlation between biological control and decrease concentration of the nutrient in soil and this can be used in direct biocontrol. They parasitize a variety of fungi and they are able to detect other fungi, and growing towards them for prevents from developing. Perception is due to degradation of the cell wall and in particular to certain enzyme, such as chitinases, lucanases and proteases [16]. Volatile and nonvolatile toxic metabolites produce by *Trichoderma* strains inhibit micro organisms' colonization. Vey et al. [17] described some of metabolites as tricholin, gliovirin, glisoprenins, alamethicins, viridian, etc. Moreover, the important role played by *Trichoderma* in plant growth has been reported. Plants treated by *Trichoderma* have a fresh size and weight significant as well as a fast flowering [18]. Furthermore, several studies reported that *Trichoderma* is involved in plant defense mechanism by stimulating phytoalexins and PR proteins biosynthesis [10,19]. *Trichoderma*

produces several types of propagules especially conidia whose interest in biological control is well established [20]. However, despite this, this fungus has not conquered the markets struggle against pathogens. Certainly, the rapid effect of synthetic fungicides on pathogens found by farmers would be responsible for the important value of pesticides compared to *Trichoderma* noted by them. Therefore, identification of more aggressive *Trichoderma* strains seems necessary to increase their action on pathogens and thus compete with the pesticides.

The aim of the present investigation is to evaluate the *in vitro* biological potential of the *Trichoderma* species against *Fusarium oxysporum* f. sp. *vasinfectum,* causal agent of Fusarium wilt of cotton.

2. MATERIALS AND METHODS

2.1 Fungal Isolate

Seventeen strains of *Trichoderma* and one strain of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) were used in the present study. The seventeen *Trichoderma* isolates (T1 to T17) were obtained from the Laboratory of Plant Physiology fungus collection of Félix Houphouët Boigny University (Abidjan, Côte d'Ivoire). Antagonism of these fungi was assessed against the pathogen, FOV which was provided by the Phytopathology Laboratory of the Agronomy school of Félix Houphouët Boigny, National Polytechnic Institute (Yamoussoukro, Côte d'Ivoire). FOV were isolated on cotton in zone of Béoumi (center of Côte d'Ivoire, West Africa). Stock cultures of test fungi were maintained on PDA (Potatoes Dextrose Agar) slants and stored at 4℃ in refrigerator.

Pure culture of isolates of *Trichoderma* and FOV were identified using Barnett and Hunter [21] method. The pure culture of the isolates was inoculated on Potatoes Dextrose Agar (PDA). It was fortified with penicillin and streptomycin antibiotics at a concentration of 0.1 ml stock solution (0.5 g of streptomycin and 1.0 UI of penicillin in 20 ml of distilled and sterile water) according method described by Tuite [22], modified and adapted to our biological material.

2.2 Pathogenicity Test

Pathogenicity test was carried out when isolates of antagonists (*Trichoderma*) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) were together inoculated on PDA in Petri dishes. Three replicates samples of each inoculation test were obtained. To achieve this one 5 mm (in diameter) culture disc was obtained from the edge of a seven days old pure isolates each of antagonists are placed 5 cm from each other and incubated at 30℃. FOV and *Trichoderma* were inoculated simultaneously.

2.3 Dual Culture Technique

The antagonistic activity of *Trichoderma* spp against FOV was evaluated by dual culture technique as described by Morton and Strouble [23]. Mycelia disc (5 mm diameter) from the 7-day-old margin of the *Trichoderma* spp culture was placed on a plate. Each pathogen was then placed on the opposite of the plate at equal distance from the periphery. The experimental design used was a completely randomized with three Petri dishes for each isolate. In the control plates (without *Trichoderma* spp.), a sterile agar disc was placed at opposite side of the pathogens inoculated plates. Inoculated plates were incubated at 25±1℃ for 7 days. Twenty days after the incubation period, radial growth of mycelia was measured. PDA plates were used in triplicates and inhibition percentage of average radial growth was calculated in relation to the controls growth as follows:

$$I = [(C - T)/C] \times 100$$

where I is inhibition of radial mycelia growth; C is radial growth measurement of the pathogen in control; T is radial growth of FOV in the presence of *Trichoderma* isolates [24].

2.4 Statistical Analysis

Experiments were performed using a completely randomized design. Data were subjected to analysis of variance (ANOVA) using STATISTICA software (release 7.0) and differences between means were compared using Newman-Keuls test. Differences at $P \leq 0.05$ were considered as significant.

3. RESULTS

3.1 Growth Competition between *Fusarium* and *Trichoderma* on Dual Culture Plate

Trichoderma strains have almost completely encircled *Fusarium oxysporum* f. sp. *vasinfectum*

(FOV) on dual culture plate. The distal side of the disc colonized by FOV was curved by *Trichoderma* metabolites. Later, *Trichoderma* was trapped by *Fusarium* on all sides (Fig. 1).

3.2 Effect of *Trichoderma* on *Fusarium* Growth

Growth of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) on dual culture plate and on control plate (FOV alone) was compared against inhibition's zone. The actual impact of *Trichoderma* on FOV is demonstrated by an irregular pattern between the zone of inhibition and growth of FOV. Indeed, this figure shows that the seventeen *Trichoderma* strains tested have an antagonistic relationship with FOV. Each isolate *Trichoderma* expressed a specific behavior with respect to FOV with different degree of stress (Fig. 2).

Isolate 12 of *Trichoderma* (T12) showed highest degree of antibiosis on *Fusarium* as depicted by Fig. 3. Some differences were observed in this study, but, their inferences remain similar. These differences may result from the fact that the metabolites of *Trichoderma* act distance. T12-*Fusarium* interaction is the best example of long distance influence of metabolites released by both the organisms on each other.

3.3 Analysis of Inhibition Zone Related to Growth of *Trichoderma* and FOV

Fig. 4 shows in control that *Trichoderma* (C0) greatly colonized the medium compared to zone of inhibition (C1). Indeed, *Trichoderma* slowed down in presence of FOV. This shown that FOV can sometimes exerts inhibition effect on the growth of *Trichoderma*. No correspondence wasn't found between the growth *Trichoderma* under influence of *Fusarium* and conversely.

3.4 Analysis of Inhibition Zones

Majority of *Trichoderma*'s isolates have inhibitory effect on *Fusarium*. Isolate of *Trichoderma* such as T3, T5, T7, T10 and T13 suffered strong opposition from *Fusarium* (Fig. 4). Exceptionally intense and almost equal opposite inhibitory counter action was observed between T8 and *Fusarium*. It is similar for isolates T13 and T17 but the inhibition counter was weaker. Moreover, isolate T2 showed average intensity. The plots indicate that antibiosis caused by each isolate of *Trichoderma* on *Fusarium* and *Fusarium* reactions were specific. Therefore, stress occasioned by confrontation between *Trichoderma* and *Fusarium* revealed a special character between the interaction of these two fungi for each Trichoderma isolate.

3.5 Relationship between *Trichoderma* and *Fusarium oxysporum* f. sp *vasinfectum*

In vitro biological control of fungi in plate was started with the phase of antibiosis and followed process of inhibition zone and actuates the mechanism of parasitism. Capacity of *Trichoderma* to cross the inhibition zone posed by the pathogen and to exceed was allowed to show the effectiveness of biological control agent such as *Trichoderma*. The best biological control agent is that which takes less time to stop the growth of the antagonist and to destroy the pathogens' chlamydospores. Among the seventeen isolates, eleven such as T1, T2, T7, T8, T9, T10, T11, T12, T13, T14 and T15 showed a high antagonist activity against *Fusarium*. On the other hand, isolate T16 is slowest of all whereas isolates T3, T4, T5, T6 and T17 presented the moderate activities opposite to *Fusarium* and were unable to cross the inhibition zone imposed by *Fusarium* (data not show).

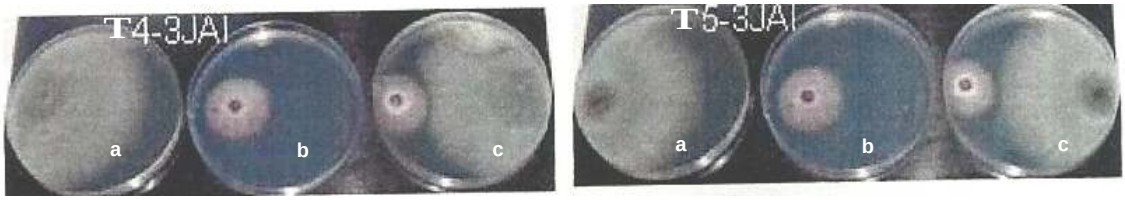

Fig. 1. *Trichoderma* isolates 4 and 5 with *Fusarium oxysporum* f. sp. *vasinfectum* on a dual culture plate

Strong antibiosis between Trichoderma and Fusarium oxysporum f. sp. vasinfectum (FOV). FOV is confined to a small area and is unable to extend its growth due to the inhibitory action exerted by Trichoderma; JAI, day after infection; a, control, Trichoderma greatly colonized the medium; b, Trichoderma have almost encircled FOV; c, Trichoderma was trapped by Fusarium on all sides; T, Trichoderma; 4 and 5 represent the Trichoderma isolates

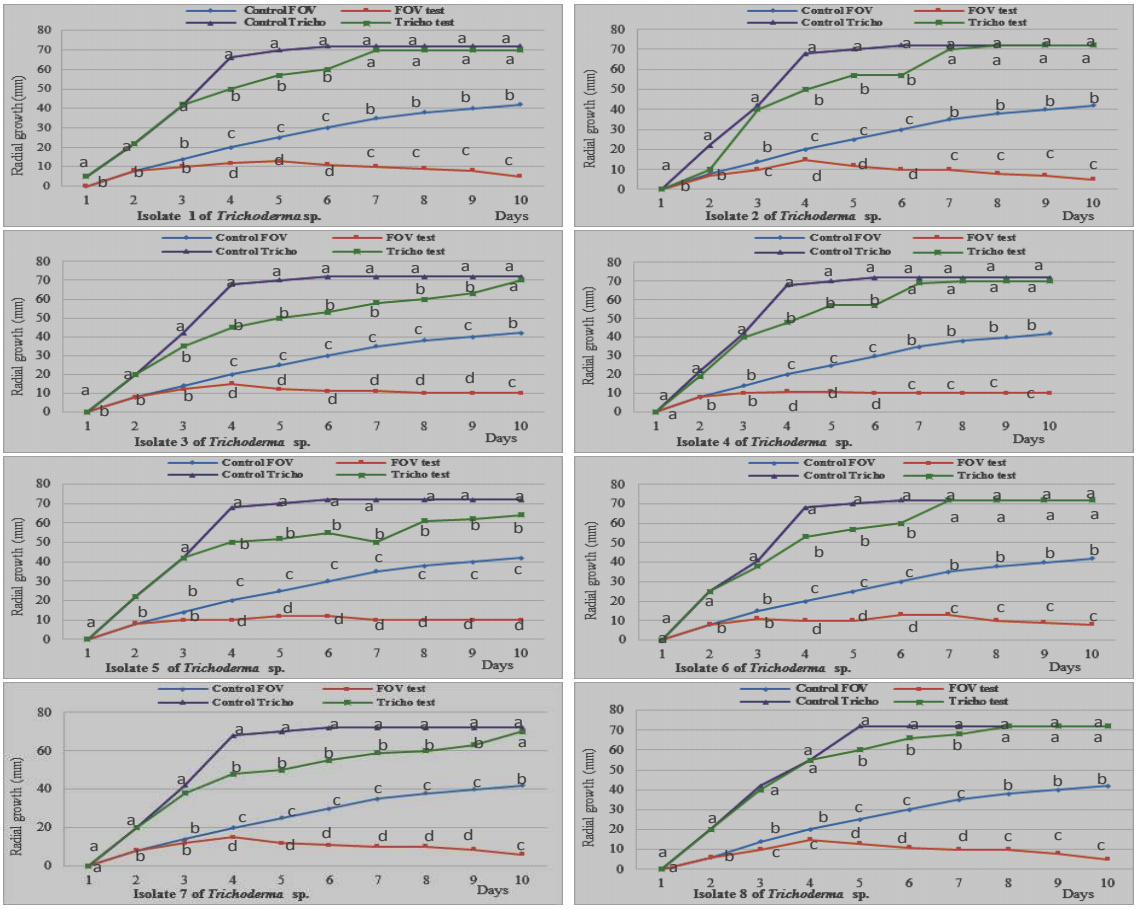

Fig. 2. Zone of inhibition analyzed in relation to growth of *Fusarium oxysporum* f. sp. *vasinfectum* influence *Trichoderma* isolates 1 to 8

Data are expressed as mean of three replicates; for a given day, values followed by a different letter are significantly different according Newman-Keuls test at 5%. The experiments were run for 10 days

4. DISCUSSION

Biocontrol agents like *Trichoderma* exercise antibiosis and mycoparasitism that are mechanisms well known to control pathogens. Competition for nutrition and space are s also use strategies to stop the action of the antagonists in the middle. The interaction between *Trichoderma* and *Fusarium* can be divided into three phases. The first was marked by the contactless long distance interaction with the mycelium. In this phase, metabolites from both organisms determine the interaction importance. These results are similar with ones of Fuji et al. who demonstrated that volatile antibiotics such as 6-pentyl-∝-pyrone (6pp) have a quite long distance range of influence on the host. The intermediate phase, in which *Trichoderma* may be inhibited or not by *Fusarium* In this intermediate phase, some chemo-attractive mechanisms may be activated. The final phase is characterized by the inhibition of *Fusarium* by *Trichoderma*. Biological control with seventeen *Trichoderma* isolates in confrontation with *Fusarium* was tested in this study. The results showed that the interaction between these two fungi appears to increase defensive and offensive mechanisms of each. Indeed, the reason for the inhibition zone between the two organizations made it clear that *Trichoderma* and *Fusarium* are secreted metabolites. However, it suffered of stress caused by metabolites released by *Trichoderma*.

Morphological changes like waxy moist appearance, scanty or no mycelia and most pigmentation were observed with *Fusarium*. This result corroborate that of Lorito et al. [25] which reported that modifications of the tests plates are a significant characteristic of *Fusarium* and that

is under the effect of *Trichoderma.* The presence of this fungus and its metabolites in the vicinity could be the response of the high production of pigments. On the other hand, metabolites secreted in the medium by *Fusarium* seem to inhibit *Trichoderma* sporulation. Indeed, often T*richoderma* sporulation was initially hindered. However, in later stages, exceedingly fast growth rate and increasing series of *Trichoderma* isolates' metabolites reinforced itself after the initial halt. Further in the course of interaction, *Trichoderma* extended its mycelia towards *Fusarium* to complete its colonization.

These characteristics were observed in most of strains T1, T2, T7, T8, T9, T10, T11, T12, T13, T14 and T15 of *Trichoderma* tested against *Fusarium*. Thereafter the meeting zone, competent strains of *Trichoderma* reached and overwhelmed *Fusarium* in later stage of interaction. Thereafter the meeting zone, competent strains of *Trichoderma* reached and overwhelmed *Fusarium* in later stage of interaction. Except T16, all *Trichoderma* strains encroached the inhibition zone of *Fusarium* and extended its mycelia towards this pathogen. This result is in agreement with ones of Brunner et al. [26] where *Trichoderma* encroached *Fusarium* inhibition zone, extended its mycelia and sporuled heavily on the *Fusarium* colony. In addition, this activity is regarded as a response of the change in the vicinity of the fungus. Thus, many signals are activated in fungi. It then modifies the expression of genes [27-28]. Furthermore, according Carlile [29], the purple to red color observed in Fusarium pigmentation after confrontation with *Trichoderma* is the result of a mixture of carotenoids and dihydroxynaphthoquinones. Moreover, the production of these metabolites is C/N dependent and light conditions as well as variations in pH of the culture medium [30]. Other studies have also reported that nutritional and physiological variations as a reason of *Fusarium* pigmentation but their defence role in antagonistic confrontation would be often evoked [31-32]. Thus, after the fight phase against *Fusarium, Trichoderma* was able to overcome the obstacle, but could attract *Fusarium* towards itself. This is a remarkable characteristic developed by *Trichoderma*. Moreover, Omann and Zeilinger [33] revealed that the importance of proteins-G in *Trichoderma* myco-parasite activity. In *Trichoderma* isolates T3, T4, T5, T6 and T17, a strong pigmentation of *Fusarium* as well as a significant sporulation of *Trichoderma* around the colony of *Fusarium* was observed. In the same time, the growth of *Trichoderma* was seen, specifically, on the colony of *Fusarium* and showed great sporulation With Trichoderma isolates T7 to T15 colony of *Fusarium* was completely overwhelmed and covered by *Trichoderma*. Precisely, *Trichoderma* covered the zone where *Fusarium* was growing, leaving the peripheral zone of the colony. Later, this peripheral zone is colonized. This behavior of *Trichoderma* suggests that *Fusarium* was the target toward which *Trichoderma* was especially attracted. However, the isolates T3, T4, T5, T6, T16 and T17 of *Trichoderma* were unable to cross the infested zone by Fusarium. These results corroborate with those of Di Pietro et al. [34] who reported that *Trichoderma* enzymes and antibiotics showed effectiveness 95% inhibition of pathogen spore germination when they acted in synergism but singly they were able to show only 20% inhibition. In this study, *Trichoderma* isolate T12 was the fastest crossing first the inhibition zone and interfere *Fusarium*. Trichoderma parasitism seems to be a powerful strategy in pathogens inhibition. But *Trichoderma* must overwhelm the pathogen before it proliferates and infects seeds and inhibit germination. Therefore, the biological agent reacts responsiveness to control pathogen is very important when looking for an aggressive strain. Thus, the Trichoderma isolate T12 would be a serious candidate for the development an effective biological control against *Fusarium oxysporum* f. sp. *vasinfectum*, the causal agent of *Fusarium* wilt of cotton. It could be used to limit the use of pesticides that would be responsible for many health and environmental degradation beyond.

Trichoderma inhibits the growth of *Fusarium* but it also has an inhibitory activity of *Trichoderma* (antibiosis). T*richoderma* is then able to overcome the inhibition exercised by Fusarium parasite and the latter to the point of regress the growth (parasitism). Moreover, the time taken for each *Trichoderma* isolate to overcome the inhibition exerted by *Fusarium* determines its effectiveness as a biological control agent. Other studies have shown that when *Trichoderma, Fusarium* interaction, metabolites are synthesized by *Trichoderma* wall to degrade or inhibit the radial growth of the host [35]. These compounds are volatile antibiotics such as 6-pentyl-α-pyrone (6pp) and isocyanide derivatives, or compounds which are soluble in water as the koningic acid, heptelidic acid and finally peptaibols which are peptides [36-41]. It was also reported that these metabolites have a

quite long distance range of influence on the host. However, production of these metabolites is strain dependent [33]. These observations seem justify the different behavior of each *Trichoderma* isolate in this study. In the myco-parasitism exerted by *Trichoderma*, it can detect its remote host (positive chemotropism); it can also ramify with the approach of its target host to choke and prevent its growth [42]. However, the most significant stage is the molecular recognition between the parasite and of the host who implies the interaction between the molecules complementary, likely to be according to Elad et al. [43] the lectin and carbohydrate. Lu et al. [44] reported those metabolites released by the walls of the host cell are responsible for the induction of myco-parasitism genes in *Trichoderma*. Similarly, host molecules are recognized by *Trichoderma* which activate the transcription of genes related to myco-parasitism [45]. Significant changes as the retraction of the plasma membrane, cytoplasm aggregation, partitions training and the initiation of the degradation of the cell wall are in the host cell [46-49]. Finally, a lytic activity of enzymes triggers a cascade (chitinases, glucanases and proteases) to degrade the fungal cell wall [50].

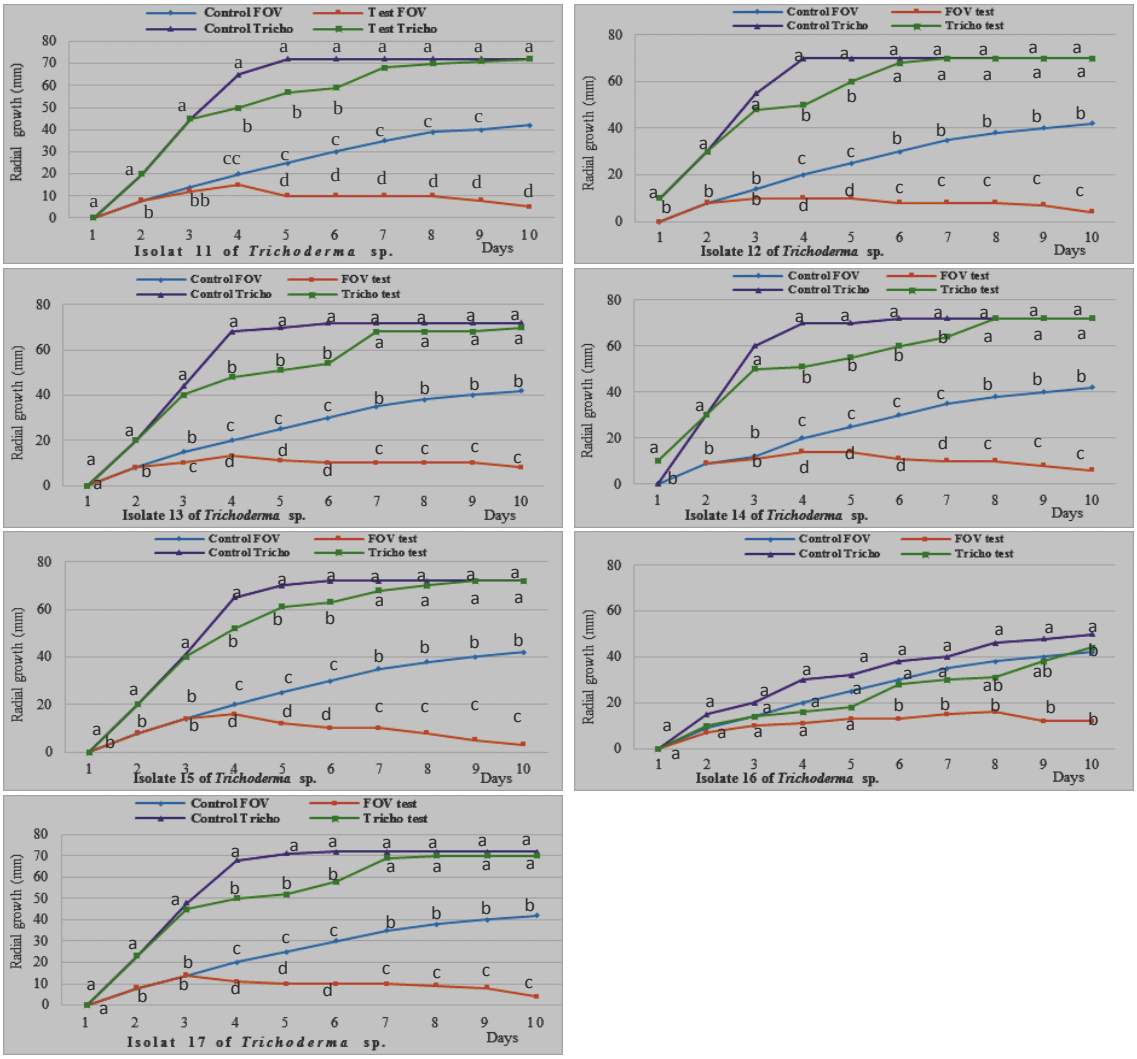

Fig. 3. Zone of inhibition analyzed in relation to *Fusarium oxysporum* f. sp. *vasinfectum* growth under the influence of *Trichoderma* isolates 11 to 17

Data are expressed as mean of three replicates; for a given day, values followed by a different letter are significantly different according Newman-Keuls test at 5%. The experiments were run for 10 days

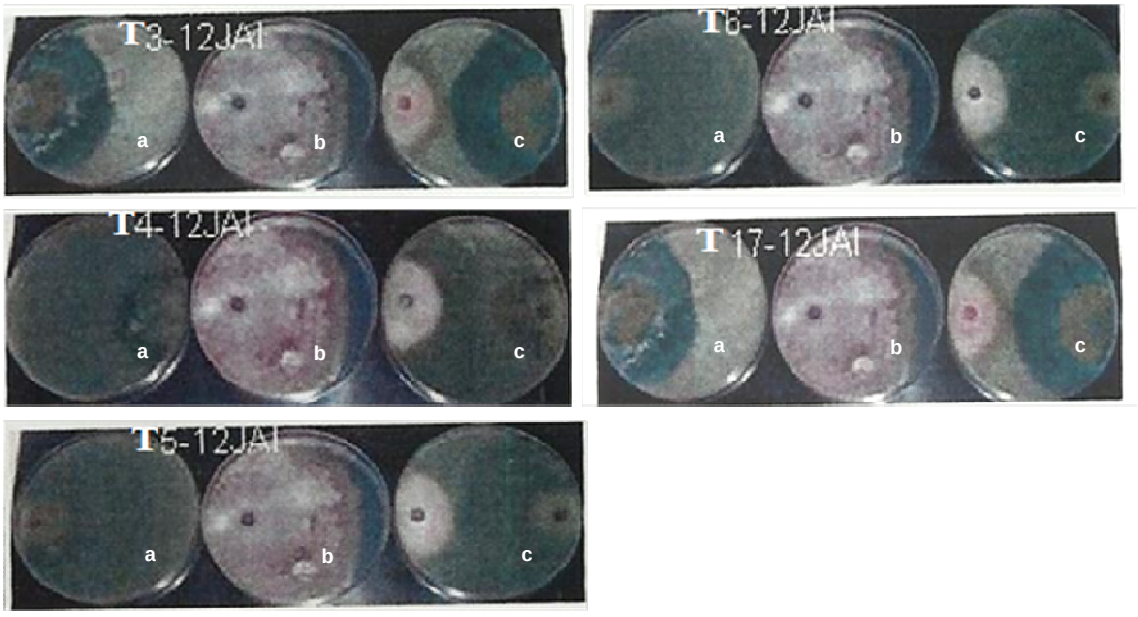

Fig. 4. Isolates of *Trichoderma* greatly affected by *Fusarium oxysporum* after 12 days of infection

FOV is occupies only one small zone under the inhibiting action of Trichoderma and is unable to extend its growth; JAI, day after infection; a, control, Trichoderma greatly colonized the medium; b, Trichoderma slowed down in presence of FOV and unable to cross the inhibition zone imposed by Fusarium; c, Trichoderma cannot cross the inhibition zone set up by Fusarium; Trichoderma is confined to a small area and is unable to extend its growth due to the inhibitory action exerted by Fusarium; T, Trichoderma; 3-6 and 17 represent the Trichoderma isolates

During the recognition of the host, acetylglucosaminidase is among the first enzymes induced by *Trichoderma* as reported Inbar and Chet [51]. Similarly, chitinases produced by *Trichoderma* subsequently also play an important role in the antagonism against pathogens. In addition, chitinolytic and cellulolytic enzymes showed an inhibitory effect on the germination and growth of the host as Fusarium [45-46]. Swelling, branching, vacuolization and necrosis were observed in pathogen by study of Harman and Kubicek [46]. From the above, it is to emphasize that the final effect of *Trichoderma* on pathogens including *Fusarium* depends on the ability to produce enzymes by strains. Studies showed that the enzymes and the antibiotics of *Trichoderma* causes 95% of inhibition of the germination of the pathogenic spores when they acted as synergy, but only 20% of inhibition when each one acts [40,52-54]. However to protect its cellular wall from the lytic enzymes, *Trichoderma* would stimulate the activation of the proteins Qid3 according several authors [55-56]. *Fusarium* would exert a noxious effect on *Trichoderma* by producing toxins like the fusaric acid which are able to harm the antagonistic activity of *Trichoderma* by the negative regulation of genes related to the myco-parasitism of *Trichoderma* [57-58]. It would be implied in the inhibition of mycelium growth and conidies production in *Trichoderma*. However, 6pp produces by *Trichoderma* would remove the fusaric acid biosynthesis. This can explain the initial inhibition of *Trichoderma* by *Fusarium* and thereafter the parasitism of *Fusarium* by *Trichoderma* observed in this study. The excessive pigmentation of *Fusarium* was also observed during interaction with *Trichoderma*. Many signals are then activated in fungi such as modification of gene expression [27], and metabolites production [30].

5. CONCLUSION

This study showed that the isolate T12 of *Trichoderma* has the most rapid action to overcome the inhibition caused by *Fusarium* and parasitize it in a lapse of time. Thus, it can be considered as an effective and ideal biological control agent in the fight against *Fusarium* wilt in cotton. It would be desirable to extend the study to other strains of *Trichoderma* in order to find a

better biocontrol agent. This could limit the use of pesticides in cotton cultivation which is a major consumer of fungicides and thus preserve the environment and the health of farmers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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