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MYCOPARASITISM OF *Trichoderma harzianum* AND *Trichoderma longibrachiatum* ON *Fusarium oxysporum* f. sp. *phaseoli* THE CAUSAL OF BEAN WILT DISEASE

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ABSTRACT

Soil-borne plant pathogen *Fusarium oxysporum* f. sp. *phaseoli* totally encireled by hyphae of both *Trichoderma harzianum* and *Trichoderma longibrachiatum* after 10 days from inoculation. Abundant sporulation of the antagonists was observed over the growth of the pathogen colony on Petri dishes of dual cultures. Examination of semi thin sections in interaction region of *F. oxysporum* f. sp. *phaseoli* and both bioagents with light microscope showed that conidia of *F. oxysporum* f. sp. *phaseoli* were markedly damaged as evidenced by the occurrence of numerous cells reduced to empty shells and features of pathogen penetration. SEM observations evidenced that both bioagent isolates have the ability to attack the examined pathogen; both of the antagonists have close contact with the plant pathogen. Hyphae of *T. harzianum* are easly recognizable by their smaller diameter than the pathogen. Average diameters were 0.5 & 2.7 µm for *T. harzianum* and the pathogen, respectively, while the average of hyphae of both *T. longibrachiatum* and the same pathogen was highly similar (2-3µm), but the two fungi could be differentiated on the basis of conidial morphology. SEM investigations demonstrated that there was variation in coiling behaviour and mode of penetration among the two isolates under study. In the case of hyphal interaction of the antagonist *T. harzianum*

against *F. oxysporum* f. sp. *phaseoli*. it was noticed that the antagonistic hyphae tightly encircled the pathogen by apparent hyphal coiling, penetrating pathogen cells by forming hooks and haustoria like structures which led to cell disruption.Hooks, clamp like structures formed by *T. longibrachiatum* seriously damaged the pathogen. Bursting, pronounced collapse, loss of turgor, wrinkled and shrinking appearance were observed after invading the pathogen with both the tested antagonists.

Key words: *biological control, scanning electron microscope (sem), Trichoderma harzianum, T. longibrachiatum, Fusarium oxysporium f. sp. phaseoli.*

1.INTRODUCTION

Trichoderma spp. are fungi present in substantial numbers in nearly all agricultural soils and in other environments such as decaying wood. Among their other activities they grow tropically toward hyphae of other fungi, coil about them in a lectin- mediated reaction and degrade cell walls of the target fungi. This process (mycoparasitism) limits the growth activity of plant pathogenic fungi (Chet, 1987 & Harman,1996). it is effective against a wide range of plant pathogenic fungi including *Pythium* sp., *Rhizoctonia solani*, *Fusarium* sp., *Botrytis cinerea*, *Sclerotium rolfsii* and *Sclerotinia homoeocarpa* (Elad *et al.* 1983 &1984; Sivann and Chet 1986 & 1989; Cherif and Benhamou 1990; Sreenivasprasad and Manibhushanrao 1990; Devaki *et al.*,1992; Benhamou and Chet 1993 &1996; Belanger *et al.*,1995; Haran *et al.* 1996; Harman. 1996; Younis and Ahmed 2003& 2004). The major challenge now is how to select a biocontrol agent on the basis of its specific potential to control pathogen populations and to reduce plant disease incidence (Benhamou *et al.*, 1999).

Hyphal interaction between *Trichoderma* spp. and some plant pathogenic fungi have been studied under the light and electron microscope levels (Elad *et al.*, 1983; Ridout *et al.* 1988; Cherif and Benhamou 1990; Benhamou and Chet 1993 & 1996; Kumar *et al.* 1998 and Gupta *et al.* 1999). SEM observations in the interaction region of dual cultures for *Trichoderma* sp. and *F. oxysporum* f. sp.

radicis- lycoperisici were carried out by Cherif and Benhamou (1990). They demonstrated that it was difficult to differentiate both fungi during the first hours after the inoculation, although apparent hyphal coiling was sometimes observed, as early as 3 days after inoculation. Significant alterations of conidia and hyphae of the pathogen were readily discernible, pronounced collapse and loss of turgor of the host mycelium were observed frequently together with severe cell wall disintegration. They also added that hyphae of the pathogen that were not affected by the antagonist retained a normal elongated shape with an average diameter of 2µm. from 5-7 days after inoculation of the mycelium of the pathogen consisting highly distorted and collapsed filaments that ultimately were entirely covered by hyphae and conidia of the mycoparasite.

Benhamou and Chet 1993 demonstrated the macroscopic observations of fungal growth in dual cultures of *T. harzianum* against *R.solani* and revealed that pathogen growth inhibition occurred soon after contact with the antagonist. SEM investigations demonstrated that coiling of the antagonist around its pathogen was an early event preceding hyphal damage. Kumar *et al.*, 1998 observed in SEM investigation that *T. harzianum* hyperparasitized *F. solani* the causal of root rot disease in mulberry by forming appressoria- like structure over the pathogenic hyphae, and by tightly coiling around it. Within 96 hr. of contact and within 6 days, the pathogenic fungus was completely inhibited, while *T. harzianum* multiplied extensively by conidiogenesis.

Gupta *et al.*, (1999) studied the mycoparasitic activities of various isolates of *T.viridae, T. harzianum, T. hamatum, T.longibrachiatum, T. koningii, T.pseudokoningii, Gliocladium virens* and *Laetisaria arvalis* against *Botryodiplodia theobromae* by SEM. They observed that most isolates made hyphal contact with the pathogen within 2 days after inoculation in dual cultures leading to an inhibition in pathogen growth, however *T.viridae* Tv-4, *T. hamatum* and *T.pseudokoningii* inhibited pathogen growth before hyphal contact and exhibited an inhibition zone between the colonies of both fungi. SEM investigations demonstrated that in the case of hyphal interaction the firm binding of antagonists (*T.viridae* Tv-1 & Tv-3, *T. harzianum* Th-1 & Th-2, *T.longibrachiatum* and *L. arvalis*) to *Botryodiplodia theobromae* hyphae established either by coiling

around its hyphae, or by penetrating its hyphal cells by forming hooks, haustoria and appressoria- like structures which invariably led to cell disruption. Although *T. koningii*, and *Gliocladium virens* Gv-2 & Gv-3 did not interact physically by way of coiling and penetration, they produced lytic enzymes of antifungal substances after coming in contact with *B.theobromae* which caused wrinkling, bursting and collapsing of pathogen mycelium. The authors suggested the outcome of the interaction of antagonist hyphal contact that triggered a series of events in pathogen degradation.

In a previous work (Younis &Ahmed, 2004) found that culture filtrate of *T. harzianum* and *T. longibrachiatum* at 80% concentration suppressed completely the growth of certain root infecting fungi. *i.e. F.moniliforme, F.oxysporum, F.semitectum, F.solani, F.tabacinum, Phytophthora infestans, R.solani,* and *Verticillium* sp. Both isolates were able to produce extracellular-chitinase when allowed to grow on both potato dextrose and Czapek's Dox broth media, and factors affecting the secretion of this antifungal were studied. Consequently, partial purification and some properties of this enzyme by both isolates were investigated (Younis & Ahmed 2003). Complementary to a previous study and to confirm it, the present investigation was carried out to provide a picture about the mycoparasitic process between the two mentioned antagonists and one from the previously tested pathogenic fungi (*Fusarium oxysporum* f. sp. *phaseoli*) using SEM and examination of semi thin section of dual cultures.

2.MATERIALS AND METHODS

2.1.Fungal isolates and growth conditions: The isolates of *Trichoderma harzianum* and *Trichoderma longibrachiatum* used in this study were isolated from phylloplane of different plants (*Duranta* sp. and *Rosa* sp.) and also from different rotten materials (e.g. cotton and old books). *Fusarium oxysporum* f. sp. *phaseoli* was isolated from infected roots of Bean (*Phaseolus vulgaris* L.). Both the antagonists and the pathogen isolates were grown on potato dextrose agar medium (PDA) at 22°C.Fungi isolates were identified to the generic level according to Gilman (1957). However confirmation of the identification of the fungal isolates to species was carried out at the Botany Dept., Fac. of Sci., Aussiut Univ.

2.2.Dual culture: Mycelial disks (5mm in diameter) cut from actively growing colonies of both *T. harzianum* and *Fusarium oxysporum f.* sp. *phaseoli* were placed 3 cm apart on the surface of PDA medium at 22°C. The same was carried out with *T. longibrachiatum* and the pathogen. Mycelial samples from the interaction zone were taken 10 days after inoculation for SEM examination and semithin sections were cut with a thickness of 1 µm. and stained by toluidine blue dye, then examined by light microscope.

2.3.Scanning electron microscopy (SEM): The process was carried out by the Regional Center Mycology and Biotechnology- Al-Azhar University, Cairo, Egypt as follows: mycelial samples from the interaction region were vapor-fixed with 2% (w/v) osmiumtetroxide in distilled water for 20 hr. at room temperature, air dried and sputter-coated with gold palladium in a polaron E 500 sputter coater. Samples were kept in a desicator until examination with the scanning electron microscope (JEOL JSM-5500 LV).

3.RESULTS AND DISCUSSION

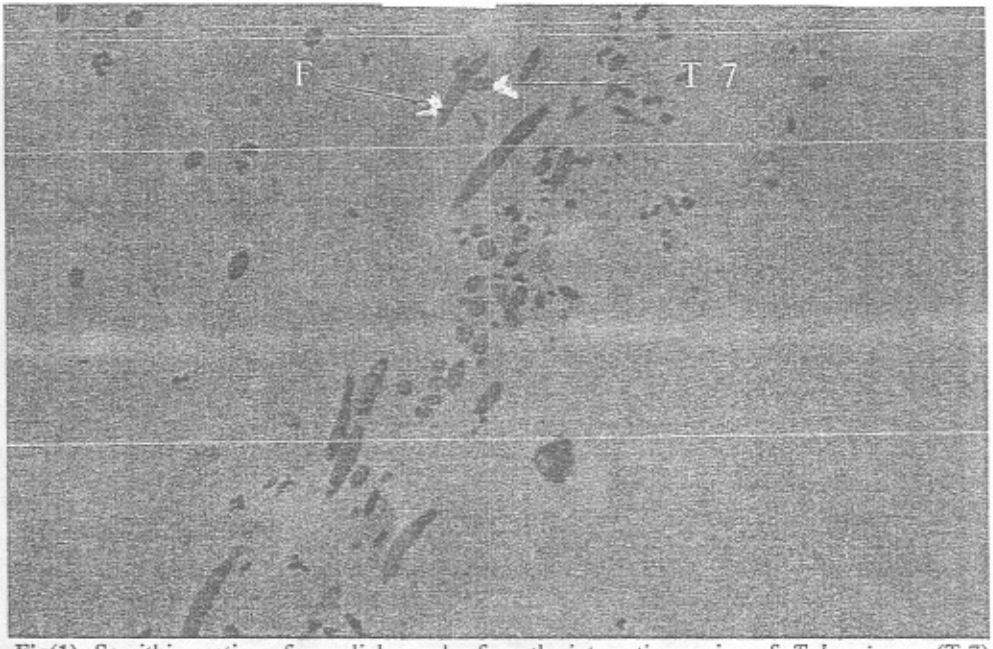
Since *T. harzianum* and *T. longibrachiatum* were capable of producing extracellular chitinase when allowed to grow on both potato dextrose and Czapek's Dox broth media, they are a valuable source of biocontrol of chitinous plant pathogens. Percentage of reduction for *F. oxysporum* f. sp. *phaseoli* at 50% concentration of culture filtrate of both bioagents were 100 & 72.14 %, respectively and were 100% with both at 80% concentration (Younis & Ahmed 2004). Metabolites may affect the pathogen in various ways depending on the biocontrol strain. This includes extracellular enzyme system that lyses the pathogen and prevents growth(Fravel,1988; Cherif and Benhamou 1990 and Mischke 1997). Complementary to a previous study, the present investigation was undertaken to obtain sight about mycoparasitic activity of both bioagents against the mentioned pathogen.

Fusarium wilt caused by *F. oxysporum* is a severe disease of many plant species. Researches to generate environmentally sound ways to control this pathogen have been conducted worldwide (Leeman *et al.* 1995; Toyoda *et al.*, 1993; Yang & Kim 1996 and

Singh *et al.*, 1999). *Fusarium oxysporum* Schlect. emend. Snyd. and Hans. f. sp. *phaseoli* the causal of been yellow or wilt disease in *Phaseolus vulgaris* L. has been reported in different growing regions of the world (Woo *et al.*, 1996).

Visual fungal development in dual cultures showed that growth inhibition of the host mycelium occurred after contact with both *Trichoderma* isolates hyphae. Soil born plant pathogen *F. oxysporum* f. sp. *phaseoli* (colony) totally encircled by both hyphae of *T. harzianum* and *T. longibrachiatum* 10 days after inoculation. Abundant sporulation of the antagonists was observed over the growth of *Fusarium* colony on Petri dishes of the dual cultures. These results confirmed the work of Benhamou and Chet (1993). Semithin section mycelial samples taken 10 days after inoculation in the interaction region of plant pathogen and both of the two bioagents under experiment were examined by light microscope, showed that *F. oxysporum* f. sp. *phaseoli* conidia were markedly damaged as evidenced by the occurrence of numerous cells reduced to empty shells and features of host penetration(Figs.1&2). Benhamou *et al.*, (1999) reported that *Pythium. ultimum* appeared highly vacuolated and numerous cells of *F. oxysporum* f. sp. *radicis- lycopersici* were reduced to empty shells under light micrographs of sectioned mycelial samples examined 2 to 3 days after inoculation in the interaction region between mycoparasite *P. oligandrum* and the previously mentioned plant pathogens stained with toluidine blue.

SEM observations evidenced that both the examined antagonists were closely contacted with *F. oxysporum* f. sp. *phaseoli.* Hyphae of *T. harzianum* isolate were easly recognizable by their smaller diameter than the pathogen. Average diameters were 0.5 and 2.7 µm for *T. harzianum* and *F. oxysporum* f. sp. *phaseoli.* respectivelly (Fig 3). Benhamou and Chet 1993 found that the antagonist *T. harzianum* was most distinguished from *R .solani* by hyphal diameter the average diameter of the antagonist was estimated to be 2 µm, whereas those of the pathogen hyphae ranged between 3 and 6 µm. The average of hyphae of both *T. longibrachiatum* and the same pathogen under this study was highly similar (2-3µm), but the two fungi could be differentiated on the basis of conidial morohology (Figs. 11 & 12). In view of other workrs, Cherif and Benhamou (1990) reported that, the average diameter of hyphae of



Fig(1): Semithin section of mycelial samples from the interaction region of *T. harzianum* (T 7) (subglobose to obovoid phialospores) against *F. oxysporum* f. sp. *phaseoli* (F). Signs of alteration characterized by increased vacuolation, features of pathogen penetration (Light microscope, X400)

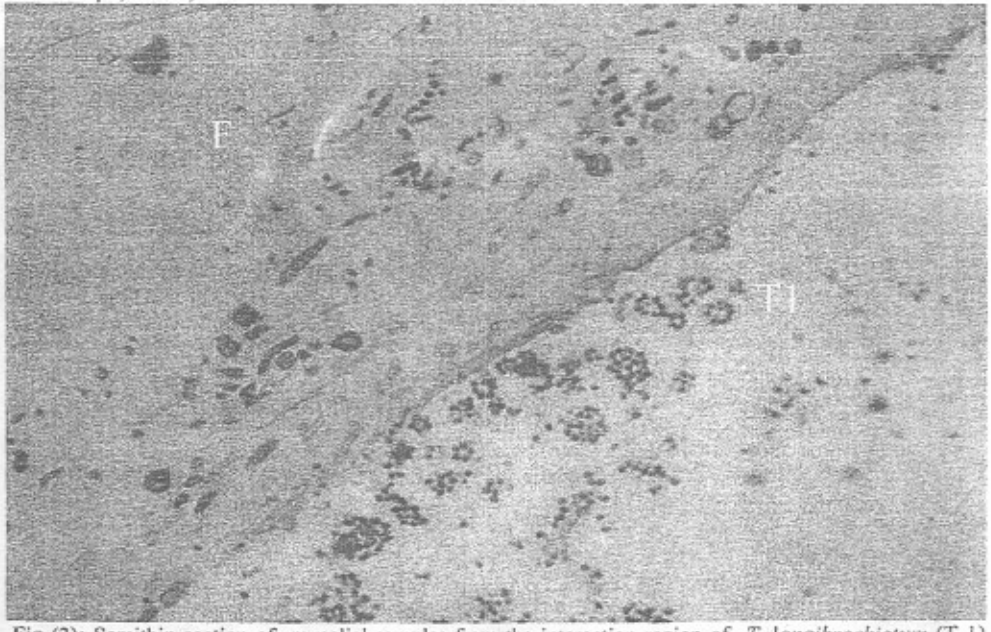
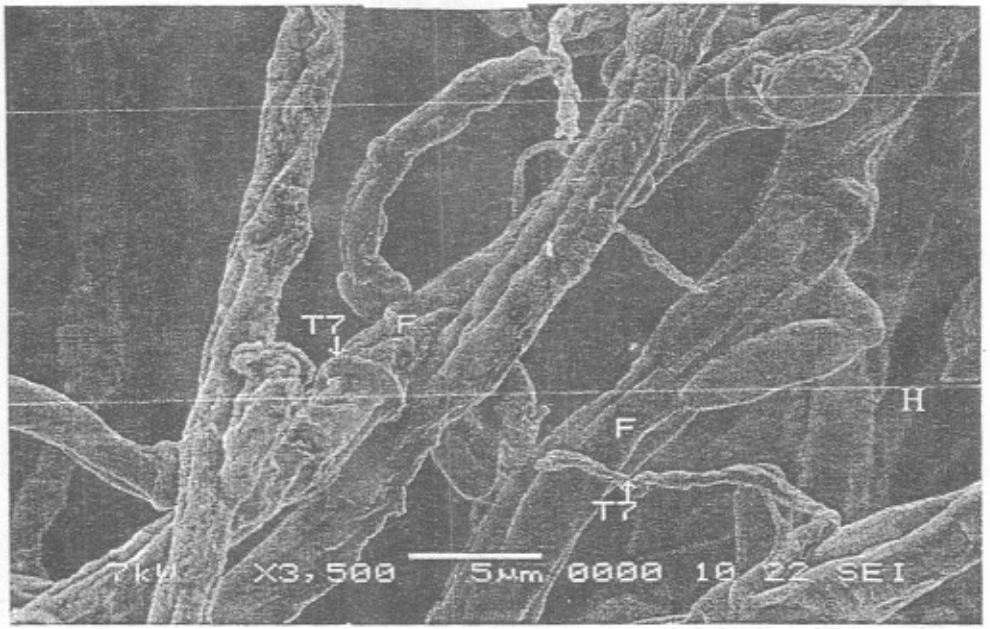


Fig (2): Semithin section of mycelial samples from the interaction region of *T. longibrachiatum* (T 1) against *F. oxysporum* f. sp. *phaseoli* (F). Conidia of the pathogen are markedly damaged as evidenced by the occurrence of numerous cells reduced to empty shells, features of pathogen penetration (Light microscope, X 400).



Fig(3): *T. harzianum* (T7) *hyphae* form dense coils and tightly encircle hyphae of *F. oxysporum* f. sp. *phaseoli* (F), active growth of the antagonist is associated with marked collapse and loss of turgor of the pathogen. Signs of shrinking and wrinkled appearance of the pathogen cell surface. Haustorium (H) like structure formed by the antagonist, (SEM).

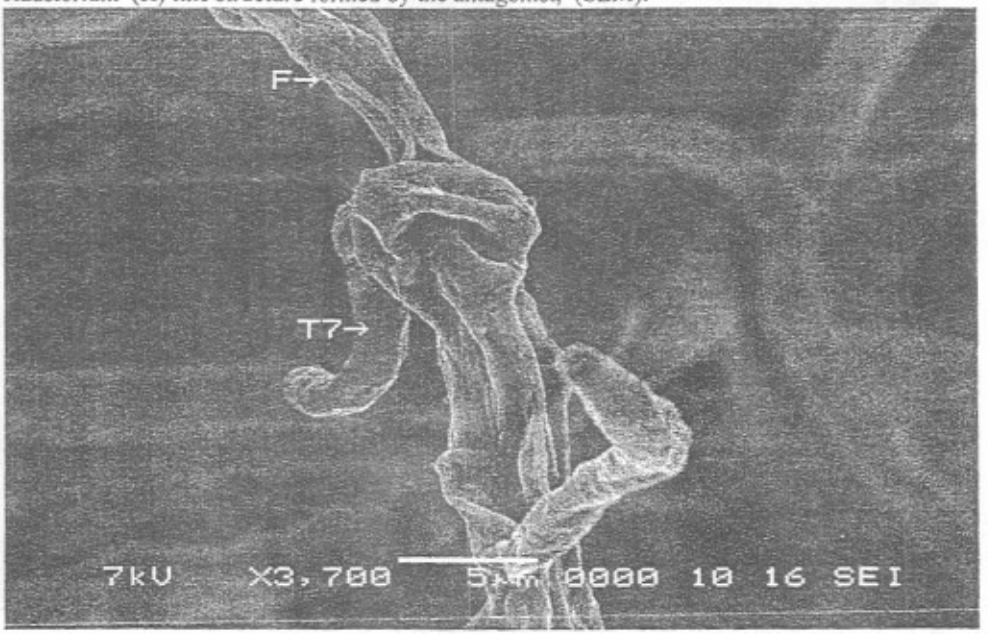


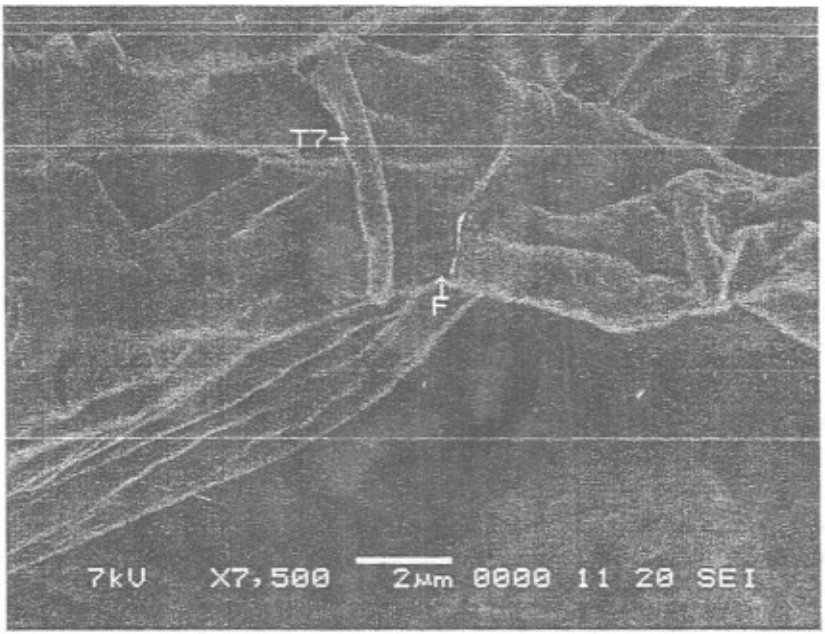
Fig. (4): *T.harzianum* (T7) hypha intensively coil around the pathogen (F) forming a loop, the pathogen suffers from damage characterized by a marked loos of turgor and collapse, (SEM).

both *F. oxysporum* f. sp. *radicis-lycopersici* and *Trichoderma* sp. was highly similar (2-3 µm).

SEM micrographs of *T. harzianum* and *F. oxysporum* f. sp. *phaseoli* in this investigation revealed that the antagonist established by tightly coiling around pathogen's hyphae or by pentrating its hyphal cells by forming hooks, haustoria-like structure which led to signs of collapse, loss of turgor, shrinking and wrinkled appearance of pathogen cell surface (Figs.3, 4, 5, 6 &7). *T. harzianum* intensively coiled around the pathogen forming a loop (Fig.4), and the pathogen hypha appeared suffering from damage. Sreenivasprasad and Manibhuushanrao, (1990) observed the formation of similar hooks or loop-like structure by the antagonist fungi (*Gliocladium virens* and *T. longibrachiatum*) around *R solani*, also *T.viridae* Tv-3 hypha intensively coiled around a hypha of *Botryodiplodia theobromae* forming a loop (Gupta *et al.,* 1999). In addition, *T. harzianum* isolate in the present work formed a hook showing bulging at the tip after coming in contact with *F. oxysporum* f. sp. *phaseoli* (Fig.6). The same observation was reported by Gupta *et al.*, (1999) with *T. harzianum* isolate Th-2, against *Botryodiplodia theobromae.*

Mycelial samples taken from the interaction region between *T. longibrachiatum* and the same pathogen in this work, were also examined with SEM and revealed that the hyphae of the antagonist coiled around the pathogen, were an early event coming before hyphal damage. Variations in coiling behaviour and mode of penetration were observed among the two isolates under study as shown from different micrographs. Clamp-like structure (Fig. 8) formed by this isolate, recognizable by the size and shape of conidia, overgrows the highly altered mycelium of *F. oxysporum* f. sp. *phaseoli,* pronounced collapse, features of alteration, cell wall break down and hyphal disintegration were also observed (Figs. 8, 9, 10, 11 & 12). Gupta *et al.*. (1999) observed clamp like structure formed by *T. harzianum* isolate Th-1, squeezing a hypha of *Botryodiplodia theobromae.* They also observed variation in coiling behaviour and mode of penetration even between isolates of the same species group.

Benhamou and Chet 1993 reported that *T. harzianum* established close contact with *R.solani* by coiling around the hyphae. The coils were usually dense and appeared to tightly encircle the hyphae of the pathogen. By 4 days after inoculation, the antagonist multiplied



Fig(5): Wrinkling, shrinking and bursting of *F. oxysporum* f. sp. *phaseoli* (F) after invading by *T. harzianum* (T7) hyphae.

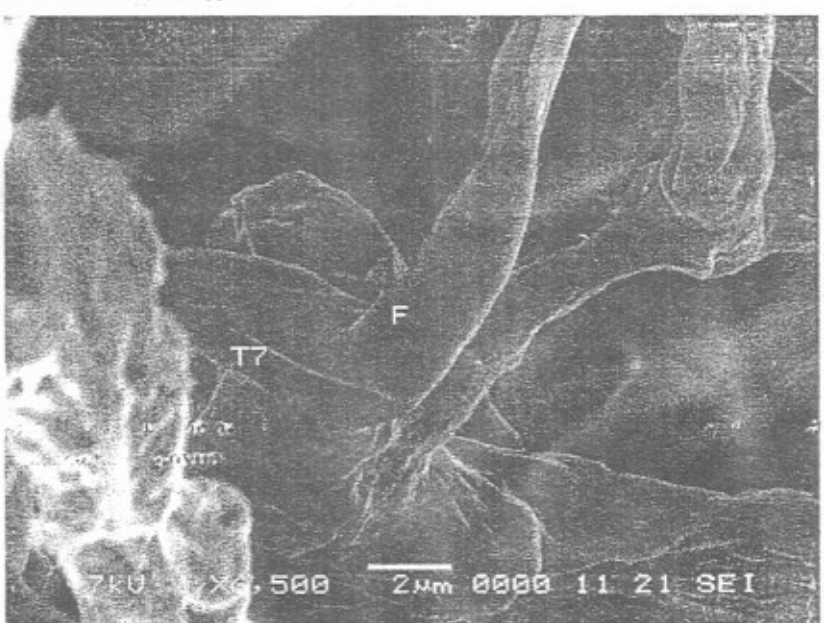
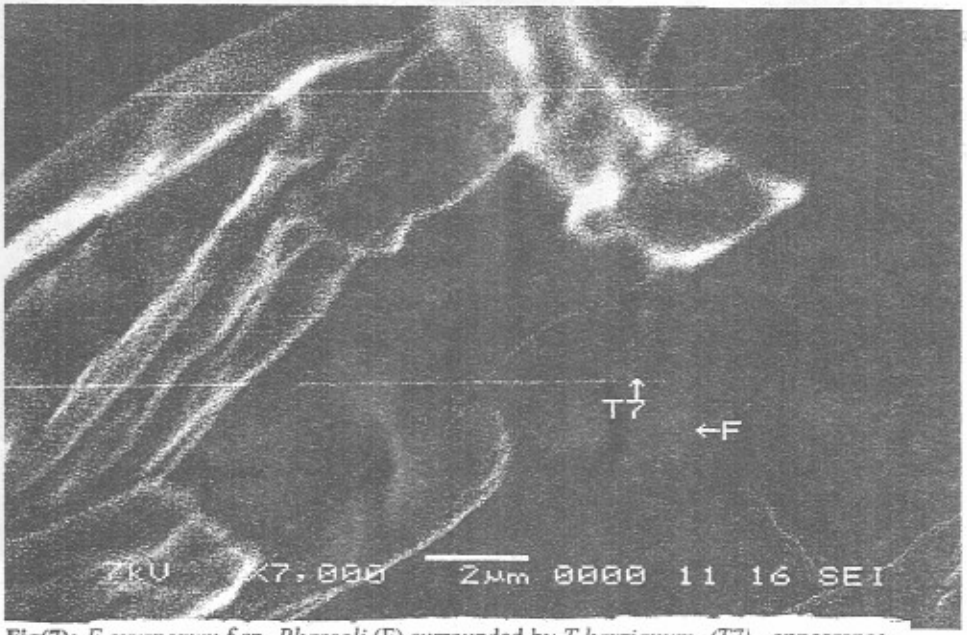
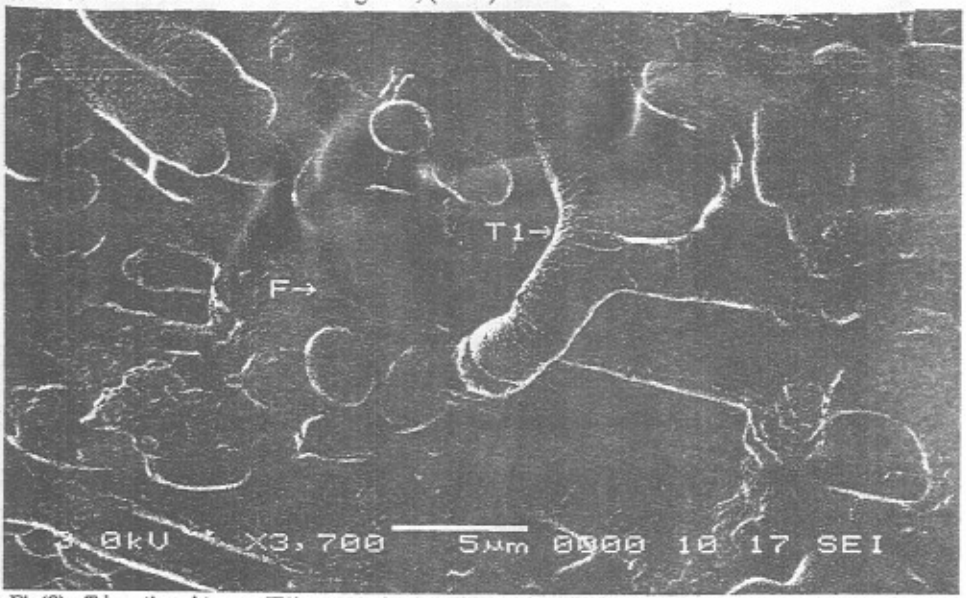


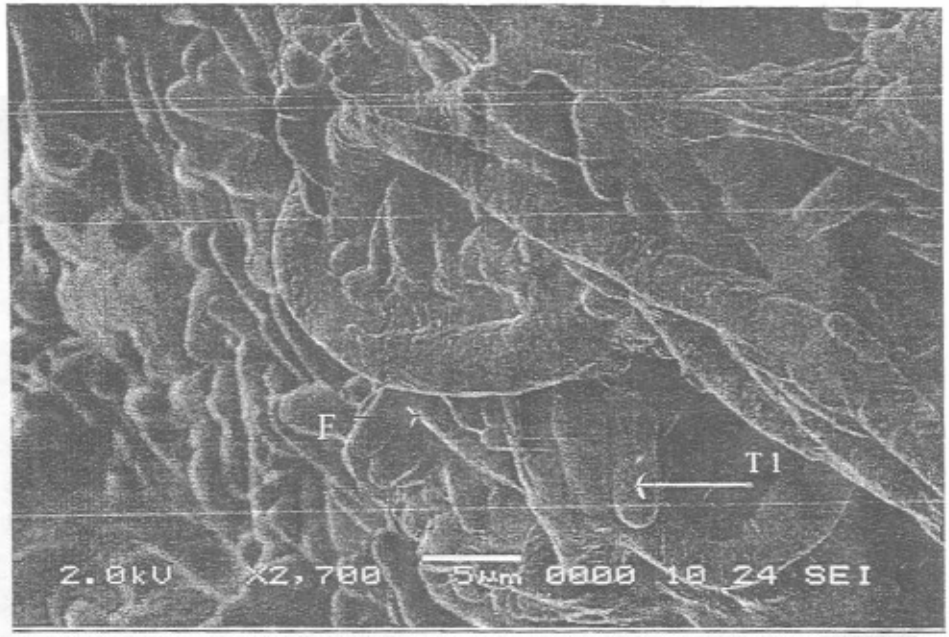
Fig (6): *T. harzianum* (T7) coiled around the pathogen and a hook showing bulging at the tip penetrating a hypha of *F. oxysporum* f. sp. *phaseoli* (F), pronounced collapse and bursting of the pathogen.



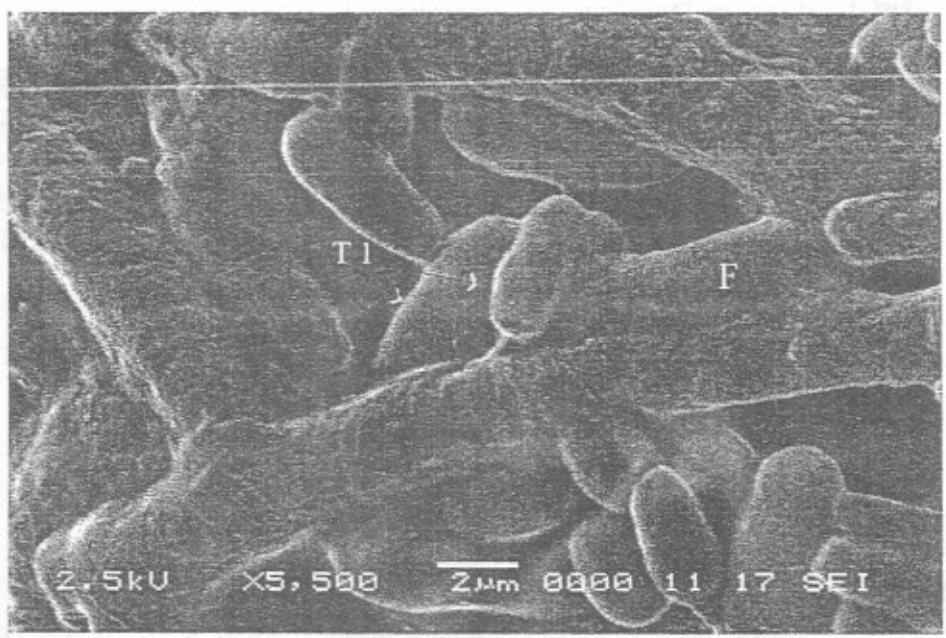
Fig(7): *F.oxysporum* f.sp. *Phaseoli* (F) surrounded by *T.harzianum (T7)* , appearance of a hook like structure of the antagonist, (SEM).



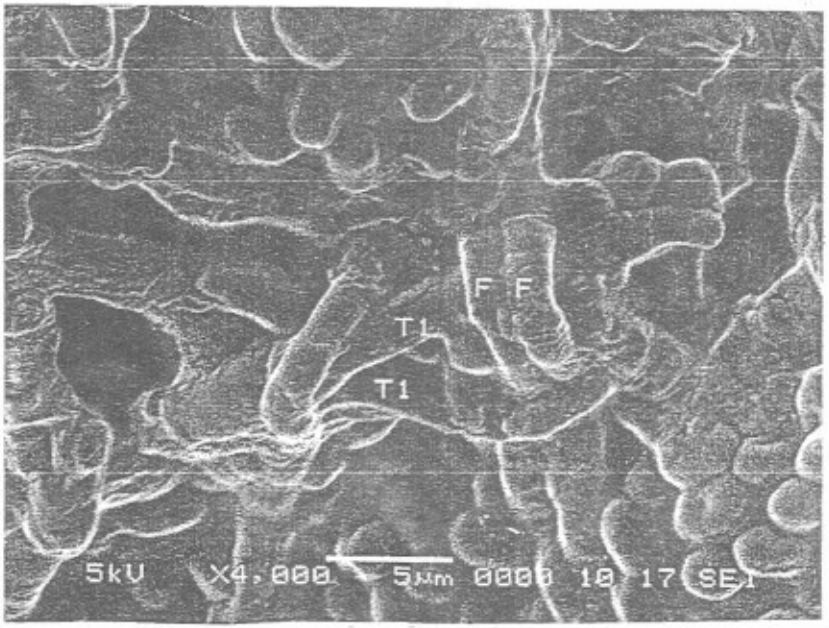
Fig(8): *T.longibrachiatum* (T1) recognizable by the size and shape of conidia (broadly ellipsoidal to subcylindrical), overgrows the highly altered mycelium of the pathogen (F). Clamp- like structure formed by the antagonist squeezing a hypha of the pathogen which appeared seriously damaged and showing bursting appearance, (SEM).



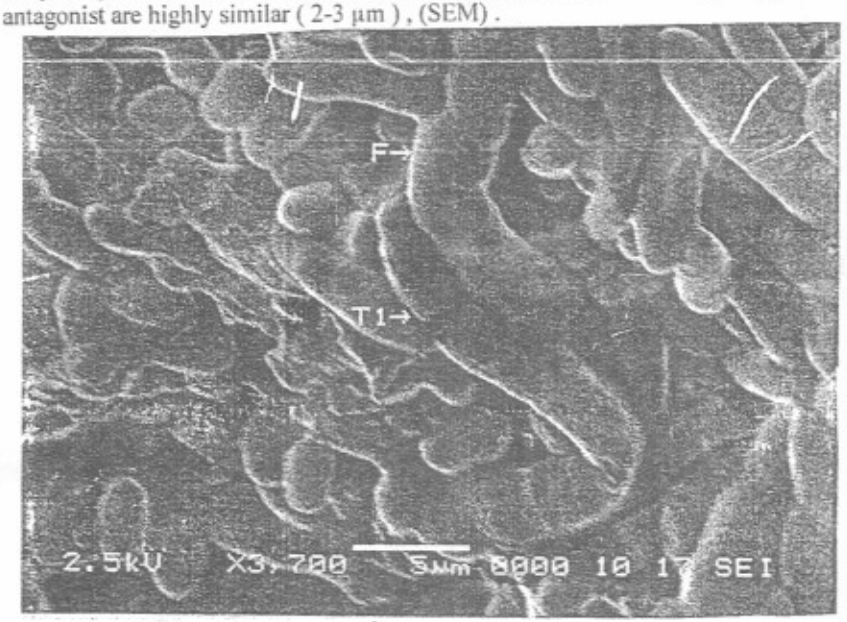
Fig(9): Hook like structure of *T.longibrachiatum* (T1) attached hypha of *F. oxysporum* f. sp. *phaseoli* (F), (SEM).



Fig(10): Hooks of *T.longibrachiatum* (T1) penetrate a hyphae of *F. oxysporum* f. sp. *phaseoli* (F) and coiling apperance of the antagonist, (SEM).



Fig(11): *T.longibrachiatum* (T1) encircle the the pathogen , marked cell collapse for the pathogen (F) . The avererage diameter of both the hyphae of the pothogen and the antagonist are highly similar (2-3 µm) , (SEM) .



Fig(12): Coiling of *T.longibrachiatum* (T1) around the pathogen (F), hook like structure of the antagonist attached to hypha of the pathogen, prounced collapse of the pathogen. Diameter of the hyphae of both the pathogen and the antagonist are highly similar (2-3 µm), (SEM).

abundantly and coiling persisted. Early signs of collapse were visible in *R. solani* hypha surrouned by *T. harzianum* 6 days after inoculation and there was a loss of turgor and marked cell collapse in the pathogen.

The noticeable wall alterations together with rapid collapse and the loss of turgor of the pathogen in areas where *Trichoderma* was not in direct contact with the pathogen mycelium provided an evidence to the concept that extracellular metabolites could be responsible for the degradation events (Cherif and Benhamou, 1990; Benhamou and Chet, 1996; Durman *et al.*, (1999) and Younis and Ahmed, 2004). SEM observations of parasitism of both examined bioagents in this work against *F. oxysporum* f. sp. *phaseoli* confirmed the work of Cherif and Benhamou (1990); Benhamou and Chet (1993 & 1996); Kumar *et al.*, (1998); Benhamou *et al.*, (1999) and Gupta *et al* (1999). It is worth to mention to what reported by Benhamou and Chet. (1993) that chitinase produced by *T. harzianum* may not be the only enzyme responsible for the degradation of *R. solani* cell walls; it is likely that the coordinated action of several hydrolases (*i.e.*, β- 1,3-glucanases, lipases and proteases) are required for a complete dissolution of the cell wall. The observation that marked cellular changes such as retraction of the plasma membrane and cytoplasm aggregation occurred in cells of *R. solani* during mycoparasitic process raises a question as to the involvment of antibiosis, in addition to enzymatic action, in host- cell degradation.

In conclusion, SEM investigation of the present work demonstrated that both bioagent isolates have the ability to attack the examined pathogen, although there was variation in coiling behaviour and mode of penetration among them noticed in micrographs. in the case of hyphal interaction of the antagonist *T. harzianum* against *F. oxysporum* f. sp. *Phaseoli*. It was noticed that the antagonistic hyphae tightly encircled the host with apparent coiling, penetrating pathogen cells by forming hooks and haustoria-like structures which led to cell disruption, while *T. longibrachiatum* formed hooks, clamp-like structures and also coiling which seriously damaged the pathogen. Brusting, pronounced collapse, loss of turgor, wrinkled and shrinking appearance were observed after invading the patbogen with both the examined isolates. Once an effective biocontrol agent has been identified, it should be commercialized, as a biocontrol product. This

work demonstrated that *T. harzianum* and *T. longibrachiatum* indeed have the ability to invade *F oxysporum* f. sp. *phaseoli*.

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التطفل الفطرى لتريكودرما هارزيانم و تريكودرما لونجيبراكياتم على الفطر فيوزاريم اوكسيسبورم المسبب لذبول الفاصوليا

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ملخص

تم دراسة الفعل التضادى لانواع من الجنس تريكودرما (هارزيانم و لونجيبراكياتم) ضد الفطر فيوزاريم اوكسيسبورم المسبب لذبول الفاصوليا وذلك فى اطباق بترى تحتوى على بيئة اجار دكستروز البطاطس. اتضح بعد عشرة ايام من التلقيح ان المضادين اظهرا فاعلية فى تضاد الفطر فيوزاريم حيث احاطا تماما هيفات كل منهما بالفطر فيوزاريم و لوحظ تجرثم وافر لكل من الفطرين المضادين على النمو الفطرى لمستعمرة المسبب المرضى. ظهر بفحص (المقاطع شبة الرفيعة) باستخدام الميكروسكوب الضوئى لمنطقة الإتصال ان كونيديا المسبب المرضى تم اتلافها بشدة حيث ظهر كثير من القشور لخلايا فارغة للمسبب المرضى وظهور اختراق له بواسطة السلالتين المستخدمتين. ظهر باستخدام الميكروسكوب الاكترونى الماسح ان كلا السلالتين المستخدمتين كمضادان بيولوجيان لهما المقدرة التحقيقية على مهاجمة المسبب المرضى تحت الدراسة فكلاهما كان على اتصال مباشر له . هيفات التريكودرما هارزيانم من السهل التعرف عليها بقطرها الاصغر من المسبب المرضى حيث كان القطران ٢,٧ & ٥,٠ ميكرومتر على التوالى- بينما متوسط قطر هيفات تريكودرما لونجيبراكياتم والمسبب المرضى كانت متقاربة الى حد كبير (٢ - ٣ ميكرومتر) ويمكن التفرقة بينهما مورفولوجيا عن طريق الكونيديات. وتشير الدراسة بالميكروسكوب الإلكترونى الماسح ايضا الى وجود اختلاف فى سلوك الالتفاف وطبيعة الاختراق بين السلالتين تحت الدراسة. ففى حالة تفاعل هيفات التريكودرما هارزيانم ضد المسبب المرضى فيوزاريم اوكسيسبورم لوحظ ان هيفات المضاد البيولوجى احاطت المسبب المرضى بالتفاف واضح وكان الاختراق للمسبب المرضى بواسطة خطافات وممصات حيث ادت الى تمزق خلايا المسبب. وفى حالة تريكودرما لونجيبراكياتم كانت هناك الخطافات والممصات والالتفاف وكلها ادت لاتلاف المسبب المرضى. أدى غزو المسبب المرضى بواسطة كل ا من السلالتين المستخدمتين كمضادين بيولوجيين

ادى الـــى حـــدوث تمزق وتجعد وانكماش لهيفات المسبب المرضى. لهذه النتائج اهمية فى المجال التطبيقى فيما لو تم تعميمها تجاريا.

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