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Study the Mechanisms of Parasitism and Antagonism of Different Biocontrol Agents Against *Sclerotinia sclerotiorum,* the Causal Organism of White Rot Disease on Eggplant in the Laboratory

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ABSTRACT

Testing the parasitic ability of different species of the biocontrol agents by the use of dual culture technique revealed high Inhibition of *Trichoderma harzianum1* reached 1 followed by *T.harzinum2* and 3 , *T.viride ,T.ressei,Ulocladium atrum* and *Fusaium laterium*, while *T.longibrachium* appeared to have weak parasitic capability which reached 4. When the pathogen *S.sclerotiorum* was cultured at the center of the petridish ,the isolates 1 and 2 of*T.harzianum* proved to have the best inhibition ability to the pathogen by the ratio of 100% .All the tested isolated of the biocontrol agent gave high ability in producing volatile compounds, which inhibited the mycelial growth of the pathogen ,with the best of them was *F.laterium* (69.9%),there were no significant difference among the three isolates of *T.harzianum* compared to *T.ressei* and *U.atrum* in their production of volatile compounds. HCN production by the biocontrol agents showed high ratio of all the tested antagonists except *U.atrum* and *T.harzianum2* .When two concentrations (30 and 50%) of the culture filterate of the antagonists were added to Potato Dextrose Agar (PDA) , they inhibited the radial growth of the pathogen by the ratio of 100% except *F.laterium* which gave 23.7%. The use of slide culture technique to test the mycoparasitism of the biological agents and the pathogen ,it was noticed coiling of the hyphae around the mycelial growth and penetration of the hyphae of *S.sclerotiorum*.

دراسة اليات التطفل والتضاد لعوامل حيوية مختلفة ضد *Sclerotinia sclerotiorum* المسبب لمرض التعفن الابيض في الباذنجان مختبريا

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الخلاصة

أظهر اختبار القدرة التطفلية في تقنية الزرع المزدوج للعوامل الأحيائية أن العزلة *Trichoderma harzianum1* اثبتت كفاءة عالية في تثبيط نمو الممرض *S. sclerotiorum* اذ اعطت درجة تضاد بلغت 1ضمن مقياس Bell ذو الخمس درجات بينما اعطت العزلتين 2 و3 من *T.harzianum* فضلا عن باقي العوامل الأحيائية المختبرة *T.viride* و*T.ressei* و *Ulocladium atrum* و *Fusarium laterium* درجة تضاد بلغت 2بينما اعطى *T.longibrachium* قدرة تطفلية ضعيفة ضد الفطر الممرض. وعند زراعة الفطر *S.sclerotiorum* في مركز الطبق تفوقت كلا العزلتين *T.harzianum*1 و*T. Harzianum 2* اذ اعطت تثبيطا بلغت نسبته 100% لنمو غزل الفطر الممرض ، وفي اختبار تأثير المواد المتطايرة للعوامل الأحيائية على نمو الفطر الممرض اظهرت جميع الأنواع المختبرة قدرة عالية على انتاج المواد المتطايرة المثبطة لنمو الفطر الممرض وكان اعلاها انتاجا *F.laterium* الذي احدث تثبيطا بنسبة 69.9% ولم تختلف العزلات الثلاثة من*T.harzianum* عن *T.ressei* و*U.atrum* في انتاجها للمواد المتطايرة، كما اوضح اختبار انتاج العوامل الأحيائية لغازسيانيد الهيدروجين HCN انتاجا عاليا لجميع العوامل المختبرة بأستثناء *U.atrum* و*T. harzianum2* وعند اضافة راشح العوامل الأحيائية بتركيزين 30و50% الى الوسط الغذائي اعطت جميع العوامل المختبرة تثبيطا قدره 100%لنمو الفطر *S.sclerotiorum* بأستثناء

الكلمات المفتاحية:
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*F. latrium*الذي بلغت نسبة تثبيطه 23.7% كما اوضح استخدام تقنية الزرع على شريحة الأكار لمنطقة التداخل بين العوامل الأحيائية والفطر الممرض إلتفاف خيوط العوامل الأحيائية حول الفطر الممرض واختراقها بعد تجمع والتصاق ابواغ العوامل الأحيائية على غزل خيوط الفطر *S. sclerotiorum* .

Introduction

Eggplant (*Solanum melongena*L.) is considered one of the food vegetable crops that has gained popularity in the last century in many countries of the world and is concentrated in China, India, Egypt, Iran and Turkey (FAO,2008) Iraq production of eggplant was about 360 thousand tons in 2010 (Directorate of Agriculture Stalistics,2010). Cultivation of eggplant is attacked by many pathogens especially the endemic fungi in the soil , causing serious losses to the farmers , including *Sclerotinia sclerotiorum* which causes white mold disease(Satyendra et al.,2012).

The disease was first recorded in Iraq on the eggplant crop in protected agriculture by El-Bahadli and El-Azawi (1979) and It was spread in ninevah province /Mosul city in 2013.

This fungus Known as one of the destructive pathogens to the plants world -wide ,affecting more than 400 species of the plants and is difficult to control because of Its cabability to from black sclerotia that resists unfavorable conditions ,so,the methods of Its control is varied and differed . One of them is the use eco-friendly orgamisms like species of the fungus *Trichoderma* as apromising alternatives to the chemical fungicides, that cause multiple damage to human, animal, plant and environment (Aziz, 2013).

The object of this study is the use of different species of biocontrol agents for the control of *Sclerotinia sclerotiorum* the causal agent of white rot disease on eggplant invitro and the study of the mechanisms involved in the biological control of this disease.

Material and Methods

Sclerotia of the fungus *Sclerotinia sclerotiorum* and the biocontrol agents were obtained from the Dept. of plant protection college of Agriculture and Forestry and from college of Education for pure sciences – university of Mousel, which was identified by PCR in Macroger Korean center/Thialand. Surface sterilization of the sclerotia was done by 1% sodium hypochlorite solution for three min. ,then washed by sterilized distilled water also for 3 min. twice and transferred by sterile forceps to sterile filter papers . A single sclerotium /petridish was planted on PDA supplemented with chloramphenicol (50 mg / L.) .The plates were incubated at 28±2 C for one week according to Sarlil (1988).

Antagonistic and parasitic test of the Biocontrol Agents (BCA)on the growth and formation of sclerotia of *S. sclerotiorum*

1-Dual culture Technique

Three isolates of *Trichoderma harzianum*(T.h) 1,2 and 3 and *T. viride* , *T. longibracheatum*,*T. ressei* ,along with *Fusarium lateriumand Uloclodium atrum* were cultured in a dual culture with the pathogen *S. sclerotiorum* on PDA in the center of each half of the petridish by putting a 5 mm disc of the BCA/ pathogen is taken by cork borer and planted in the center of half of the medium in an inverted position ,so that the mycelium of the fungus became in contact with the surface of the medium , three replicates from each treatment were made .All the plates were incubated at 28±2 C for one week . Results was taken by measuring two perpendicular diameters of the pathogen according to Bell et al .,(1882) ranking of 5 degrees:

1-The BCA covered all medium on the petridishwith out the growth of the pathogen .
2-The BCA covered 2/3 of the medium and the other 1/3 is covered by the pathogen.
3-the BCA andthe pathogen each covered 1/2 the medium.
4- the growth of the BCA covered 1/3 medium while the pathogen covered 2/3 the medium.
5- No growth of the BCA and the pathogen covered all the medium. .

Percent inhibition of growth of the pathogen by the BCA was calculated as

Inhibition% = Control-treatment / Control × 100

After measuring %inhibition , the plates were again returned to the incubator for the rest of 10 days for the formation of sclerotia.

2- Effect of plating the pathogen at the center of the plate on the diameter of the colony and % inhibition

A disc (5 mm) of the pathogen was planted at the center of medium in the plate ,and four discs of each of the BCA was planted at each of the four sides the plate at a distance of 3cm of the pathogen according to Seyed Ali et al ., (2004).In the control plates ,the pathogen only was planted without a BCA and each treatment was replicated 3 times and incubated as before.

3-Effect of volalile substances of the BCA on the growth of *S. sclerotiorum*

According to Dennis and webster (1971a) A 5mm disc of the BCA was put in the center of PDA plates and incubated for 48 hrs.,then a disc (5mm)of the pathogen was plated also, on the center of each plate containing PDA medium .The pathogen plate was put above the plate of the BCAS then the two plates were folded tightly by a stripe of parafilm control plates included only the pathogen without a BCA plate .Three replicates were used for each treatment ,then incubated as before

4-Production of HCN gas by the BCA:

HCN production of each of the BCA was tested according to Bakker and Shippers (1987).Results was taken by the change of color for the filter papers towards the dark color using the following criteria:

+++ High production
++ moderate production
+ Low production
-No production

5-Effect of Nonvolatile substances of the BCA on the growth of *S.sclerotiorum:*

The method of Dennis and Webster (1971b) was used for this purpose .Cuture filterate of each BCA' s was sterilized by Millipore (45 mm) and added to the medium before solidifying at two concentrations 30% and 50% .After the medium was solidified ,a disc (5mm) of *S. sclerotiorum* was planted at the center of the medium .Control plates do not include any culture filterate .Three replicates was used for each treatment , then all the plates were incubated as before.

Mycoparasitism of the BCA on *S. sclerotiorum*:

The interaction between the BCA and the pathogen was done on agar slide using the method of Larone (1993).Results was taken by detecting adhesion and penetration of the hyphe of the BCA to the pathogen hyphe, as well as observing the extent of abnormal modifications and distortions of the cells of the pathogen due to the excretions of chitinases by the BCA. .Statistical analysis of the data was performed by the use of SAS system and the means of treatments was comparaed using Duncan's Multiple range test (Al –Rawi and Khalaf, 2000). .

Results and Discussion

1-Dual Culture technique:

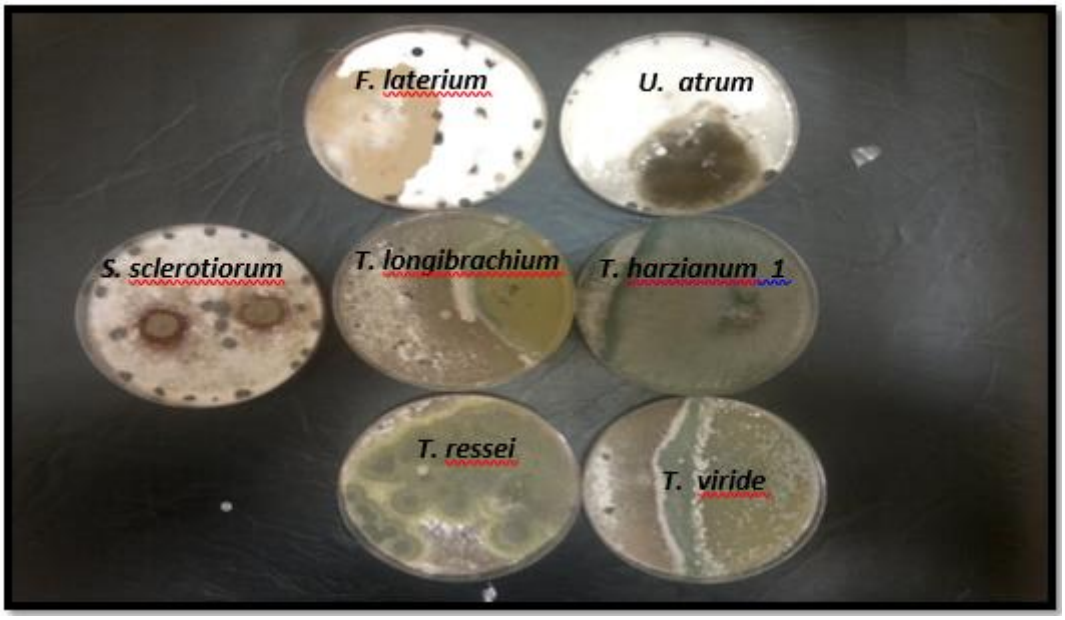
Results of dual culture technique(Table 1) revealed that the 3 isolates of *T. harzianum* (T.h) ,was the best in inhibiting the growth of *S. sclerotiorum* .T.h1 ranked degree 1 in the Bell ladder that includes 5 degres ,followed by T.h2 and T.h3 giving degree 2 as well as *T. viride* and *T.ressei*,*Ulocladium atrum* and *Fusarium laterium*

Table(1) Dual culture technique for testing the antagonistic and parasitic abitiy of the BCA against *S. sclerotiorum*

Treatments	Mean Diameter pathogen colony(mm)	Mean No. sclerotia	% Inhibition No. sclerotia	Degree of Antagonism
Control	6.0 b*	28.0		
T.harzianum 1	2.5 d	0.0	100.0 a	1
T.harzianum 2	3.0 d	0.0	100.0 a	2
T.harzianum 3	2.9 d	0.0	100.0 a	2
T.viride	4.7 d	3.0	89.2 b	2
T.ressei	2.7 d	2.0	92.8 ab	2
T.longibrachium	6.9 a	18.0	35.7 e	4
U.atrum	5.5 b	8.6	69.2 c	2
F.laterium	5. .0 b	15.0	46.4 d	2

*Mean numbers of the same column hare the same letters do not differ significantly at 0.05 level according to Duncan Multiple test.

The three isolates of *T. harzianum* was also efficient in inhibiting the formation of sclerotia completely (100%) ,followed by *T. ressei*and *T. viride* by 92.8% and 89.2% respectively . then *U. atrum* and *F. laterium*with 69.2% and 46.4% inhibition respectively.*T. longibracheatum*had a weak parasitic capapility ranked 4 with sclerotial inhibition of 35.7% (Fig 1).Howell et al., (2000) suggested that the dual culture technique may not reflect all the mechanisms which the BCA exhibited against the fungal pathogens.



Fig(1): Dual culture technique for the parasitic ability of the BCA against *S. sclerotiorum*

2-Pathogen at the center :

Statistical analysis of the results of Table(2) showed that maximum inhibition of *S. sclerotiorum* was by *T. harzianum*1 and 3 by 100% followed by *T. harzianum*2 (84.2%) then *F. laterium*and *U. atrum* (79.5% and 70.5%) respectively . *T. viride* gave the least inhibition by 36.4%(Fig.2) . the antagonistic ability of *T. harzianum* may be due to the secretion of antibiotics that inhibit the growth of the pathogen and formation of sclerotia. Like, Almethacin,Trichozianin,Trichodermine(Mukerje and Gary,1987; Correa et al.,1995),which may make the penetration of hyphae of the pathogen easier, thus prevents it from attacking the host (Horvath et al .,1995;Corley et al.,1994).

The formation of inhibition zone between BCA and the pathogen colonies may be due to the production of volatile metabolites substances and extra-cellular enzymes.

Table(2) : Effect of plating the pathogen at the center of the plate on the diameter of the colony and % inhibition .

Treatments	Mean Diameter pathogen colony (mm)	% Inhibition
Control	8.5 a *	
T.harzianum 1	0.0 f	100 a
T.harzianum 2	1.3 e	84.2 b
T.harzianum 3	0.0 f	100 a
T.viride	6.4 b	36.4 e
T.ressei	2.5 dc	70.5 cd
U.atrum	2.9 c	65.0 d
F.laterium	1.7 de	79.5 cb

*Mean numbers of the same column hare the same letters do not differ significantly at 0.05 level according to Duncan Multiple test

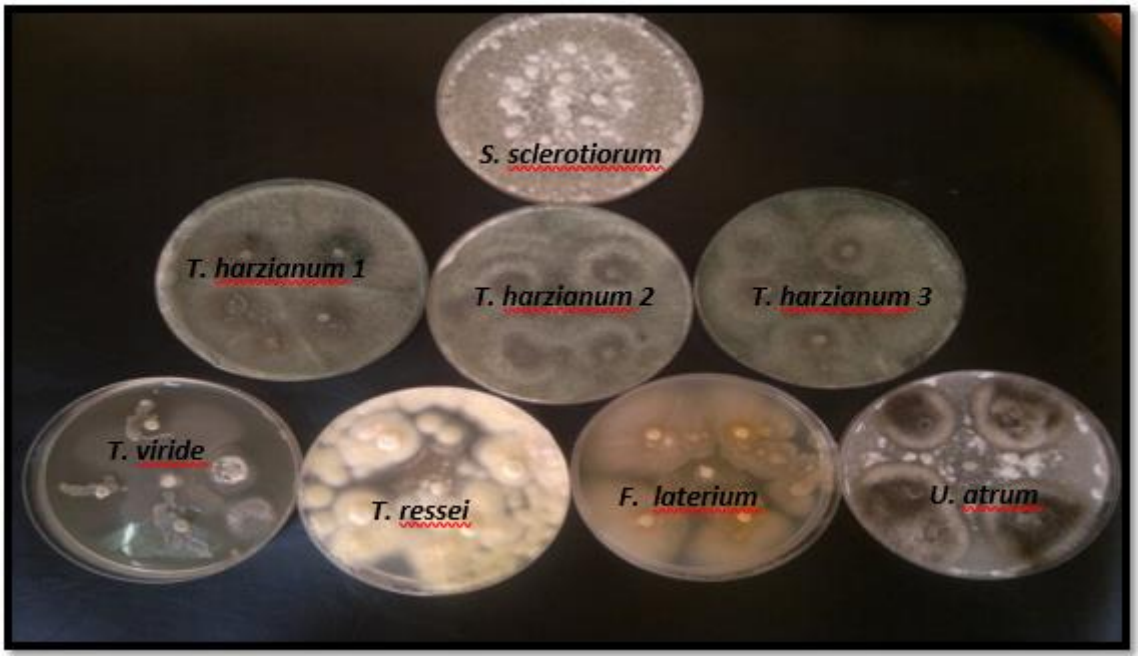
Simon and Sivasithamparam (1988) pointed that *Trichoderma* species including *T. ressei* invade the pathogen cells by the screetion of lysing enzymes. Also,the production of antibiotics inhibits the growth of fungal filaments and the germination of fungal spores. Decreasing the formation of sclerotia, beside scausing disformation of the fungal cells and hyphae and lysing of the cell protoplasm .

On the other side *T. viride* has a superior inhibition in the dual culture technigue than the pathogen at the center of the plate may be because the inhibition process is increased with the increase of inoculum concentration of the BCA (Friedrich,2003).

U. atrum declared a good ability to fungal growth inhibition and decreasing the number of sclerotia of the pathogen , as it is considered as a promising BCA of fungal plant pathogens especially *Botrytis cinerea* (Kohl et al. ,2000 ; Boff et al .,2001)

The effect on the formation of the necessary acids for cell division ,which is not in agree with results of Osbourn et al.,(1996) that *U. atrum* dose not develop any aggressive abilities against the fungal pathogens.Also the capability of *F. laterium*is obviously increased by the use of pathogen at the center technique (Pessi and Haas,2000).

T. longibracheatum gave a weak parasitic ability in inhibiting the radial growth of *S. sclerotiorum* that is also, not in agree with the results of Compant et al .,(2005) who revealed that it played an important role in the competition for nutrients ,besides it has an ability of inhibiting the growth of the pathogen. .



Fig(2):parasitic activity of the BCA's against the pathogen *S. sclerotiorum* through plating the pathogen in the center of the plate

3-Effect of volatile substances of the BCA on the growth of *S. sclerotiorum*

Results of table(3) showed that *F. laterium* was better than other BCA in inhibiting the growth of the pathogen *S. sclerotiorum* by 69.9% followed by *T. ressei* and *T. harzianum* which marked 49%, then the two isolates 2,3 of *T. harzianum* that gave 45% inhibition while the least inhibition caused by volatile substances of BCA was noticed by *U. atrum* and *T. viride* (40.8 and 35.8% respectively) Gehring et al .,(1993) stated that *F. laterium* screets HCN gas as an inhibitor of the growth of the organisms in the competition

In addition to prevents cytochrome oxidase (Larkin et al ., 1999). these results also is in accordance with Behbudi et al .,(2005) in curbing the growth of pathogenic fungi, and also excretion of fusaric acid which playsan important role in controlling pathogenic fungi (Ethur et al ., 2001). *T. harzianum* and *T. ressei* have the ability to screet harzianic acid and tricholin as well as esters and alcohols asatoxic secondary metabolites

Table(3) Effect of volatile substances of BCA on the diameter of the pathogen *S. sclerotiorum* and % Inhibition.

Treatment	Mean Diameter of pathogen colony (mm)	% Inhibition
Control	8 a*	
T.harzianum 1	4 d	49.9 b
T.harzianum 2	4.4 cd	45.0 cb
T.harzianum 3	4.4 cd	45.0 cb
T.viride	5.1 b	35.8 c
T.ressei	4 cd	49.1 b
U.atrum	4.7 cb	40.8 cb
F.laterium	2.4 e	69.9 a

*Mean numbers of the same column hare the same letters do not differ significantly at 0.05 level according to Duncan Multiple test

4-HCN production:

All BCA had high production of HCN gas except *T. harzianum2* and *U. atrum* that demonste little production (Table4). This is in quite in agree with the results of Bakker and Sehippers (1987).HCN production by *F. Laterium* is considered one of it schara ctersstics, although it is proved that *T.harzianum,T. ressei* and *T. viride* are good HCN producers also .Moreover they have the ability to produce some volatile secondary compounsds like ethylene and ketones all which is responsible for inhibiting the growth of fungal pathogens (Bakker and Sehippers ,1987) .

Table (4) : HCN production by the BCA

Biocontrol Agents (BCA)	HCN production
T.harzianum 1	* +++
T.harzianum 2	+
T.harzianum 3	+++
T.viride	+++
T.ressei	+++
U.atrum	+
F.laterium	+++

*+++ Effect High production ++ Effect moderate production + Effect Low production - Effect No production

5- Effect of Non-volatile substances of BCA on the growth of *S. sclerotiorum* :

The use of 50% concentration of culture filterate BCA gave complete inhibition (100%) to the mycelia growth of the pathogen,but 30% conc. vary considerably (Table 5),where as the three isolates of *T. harzianum* inhibited the radial growth of the pathogen by 100%,while *T.ressei* and *U. atrum* had 54% inhibition followed by *T.viride* (49.5%) ,but *F. laterium* had weak effect on the growth of the pathogen at both concs. 30 and 50% by 23.1 and 23.7 respectively. This may be justified for the production of antibiotics by*T. harzianum*in the medium like Pyrone ,Glisporenin ,Glioviridin and other inhibition (Chet ,1987).*T. viride* produced some antimicrobial substances like peplides and pyrone (compant et al .,2005).

Table(5):Effect of non-volatile substances of the BCA's on the growth of *S. sclerotiorum* (mm)

Concentration	30%		50%	
Treatments	Mean Diameter of pathogen colony (mm)	% Inhibition	Mean Diameter of pathogen colong (mm)	% Inhibition
Control	8.2 a *		8.1 a	
T.harzianum 1	0.0 d	100 a	0.0 c	100 a
T.harzianum 2	0.0 d	100 a	0.0 c	100 a
T.harzianum 3	0.0 d	100 a	0.0 c	100 a
T.viride	4.1 c	49.5 b	0.0 c	100 a
T.ressei	3.8 c	54.0 b	0.0 c	100 a
U.atrum	3.7 c	54.0 b	0.0 c	100 a
F.laterium	6.3 b	23.1 c	6.2 b	23.7 b

*Mean numbers of the same column hare the same letters do not differ significantly at 0.05 level according to Duncan Multiple test

Mycoparasitism of the BCA on *S. sclerotiorum*

Testing the interference of BCA with the pathogen on the agar slide ,*Trichoderma* isolates proved high parasitic activity bypocessing one or more of the overal mechanisms involved in the direct parasitism on the hyphae of the pathogen .Microscopic examination of the interference region showed the presenceof coiling of hyphaeof BCA around the hyphae of *S. sclerotiorum*(Fig 3,4,5) and feeding on the living cell contents as well as production of cell wall lysing enzymes like Lipases ,proteases,chitinases and B-1,3 Glucanases.

After gathering and adhesion the BCA spores to the mycelium of the pathogen .While *U.atrum* and *F. laterium* developed weak parasitic activity ,since they produce HCN, that plays an important role in growth inhibition parasitism mechanismis . considered one of the complex procecesses that includes chemotropism (Durzhinia et al ., 2000) and formation of traps and penetration pegs (Etebarian et al ., 2000) and appressoria which penetration the cell walls of the host hyphae accompang to the production of adhesive substances. followed by chitinases (Benhamau and chet 1997) and proteases (Howell ,2003) which lysis the host cell wall and making poro through it (Elad and Kapat ,1999)which made penetration process easier, then absorbing the cell wall constiyuents (Rahman et al.,2012).

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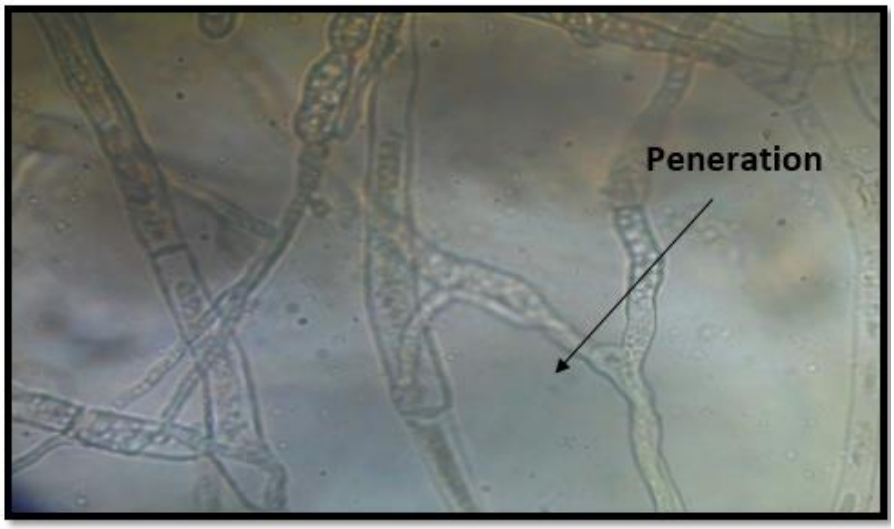
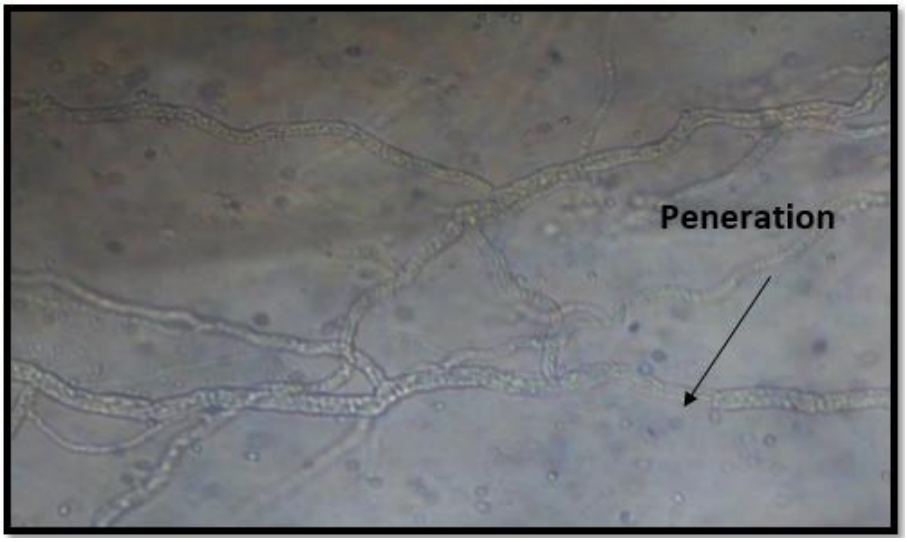
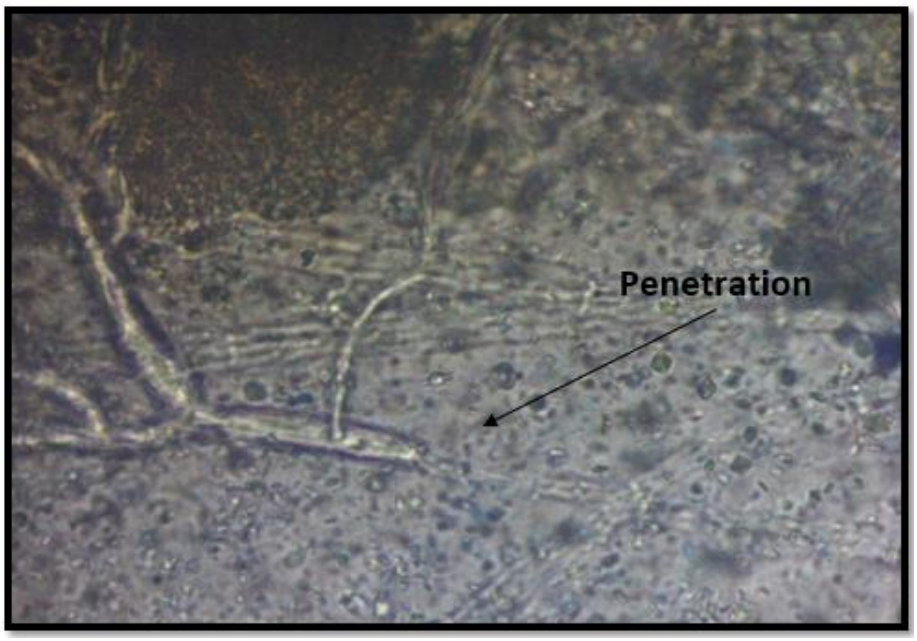


Fig(3):Mycoparasitism of the BCA *T. harzianum*on the hyphae of the pathogen *S. sclerotiorum*



Fig(4): Penetration and growth of *T. viride*hyphae inside the mycelium of the pathogen *S. sclerotiorum*



Fig(5): Penetration of the hyphae of the BCA *T. ressei* to the hyphae of the pathogen *S. sclerotiorum*

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