Biological Control of *Fusarium Oxysporum* and *Verticillium dahliae*By *Trichoderma harzianum* and *Gliocladium virens of Two Mint Species*

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ABSTRACT

Fungal plant diseases are one of the major concerns to agricultural *Verticillium*which cause wilt disease of mint, attack most of the economically important crop plants resulting in loss of billions of dollars. Application of *Trichoderma harzianum* to the infested soil, at the same time, didn't affect plant height; 30 days after transplanting except with *Fusarium oxysporum* which resulted significantly higher plants, however after 60 days from planting, total mint plants were higher than control. The most effect was noticed with spearmint plants treated with *Verticillium dahlia* followed by *Fusarium oxysporum*.*Trichodermahamatum*increased plant height compared withcontrol in most cases of soil infestation, after 30 days fromplanting. The same effect was noticed after 60 days and the bestin both mint species.

Keywords: Biological Control**;** Mint; *Fusarium Oxysporum;Verticillium dahlia;Trichoderma harzianum;Gliocladium virens.*

INTRODUCTION

Mint belongs to the Labiatae family Genus *Mentha* which includes 25 to 30 species that grow in the temperate regions of Eurasia, Australia and South Africa (**Douhan and Johnson, 2001)**. Two types of mint (spearmint and peppermint) were used in this study. Spearmint (*Mentha spicata* or *Mentha viridis*) is known as garden or common mint is the most associated with using fresh or dried in cooking. It has an aromatic and fresh bouquet and is widely used in many European based and North African cuisines.

Peppermint's Latin name, Peppermint *Mentha piperita*, comes from the Greek *Mintha*, the name of a mythical nymph thought to have metamorphosed into the plant, and the Latin *piper*, meaning pepper. It is one of the world's oldest medicinal herbs, and is used in both Eastern and Western traditions. Ancient Greek, Roman and Egyptian cultures used the herb in cooking and medicine. Peppermint leaf oil is one of the most popular and widely used essential oils, mostly because of its main components menthol and menthone. This oil is used for flavouring, cosmetic and pharmaceutical products throughout the world **(Foster, 1996)**.

Medicinally, the various mints have been used worldwide for centuries as a cure or relief for numerous ailments from flatulence and digestive complaints to fevers (**Bove, 1996 and Hoffman, 1996**).

Peppermint is taken internally as a tea, tincture, oil or extract and applied externally as a rub or liniment. Herbalists consider peppermint as astringent, antiseptic, antipruritic, antispasmodic, antiemetic, carminative, diaphoretic, mild bitter, analgesic, anticatarrhal.

Antimicrobial, rubefacient, stimulant and emmenagogue **(Bove, 1996 and Hoffman, 1996)**. It has traditionally been used to treat a variety of digestive complaints such as colic in infants, flatulence, diarrhea, indigestion, nausea and vomiting, morning sickness and anorexia and as a spasmolytic to reduce gas and cramping. Also, it is currently used to treat irritable bowel syndrome, Crohn's disease, ulcerative colitis, gallbladder and biliary tract disorders and liver complains **(Blumenthal, 1998 and Fleming, 1998)**. Peppermint oil is used to relieve menstrual cramps, and externally for neuralgia, myalgia, headaches, migraines and chicken pox **(Bove 1996 and Blumenthal, 1998).**

MATERIALS AND METHODS

Laboratory Experiments;

Isolation, purification and identification of the causal organisms

Mint plants showed different disease symptoms were collected from different governorates i.e, Behaira, Dakhliya, Gharbiya, Kafrel-Sheikh, Menofiya, Marsa Matroh, Siwa (El-Wadi El-Gidid) and Sharqia. Plant roots were washed thoroughly using running tap water, surface sterilized by immersing into 0.5 % chlorine solution for 3 minutes, rinsed several times with distilled sterilized water and then dried between two sterilized filter papers. Samples were cut into small pieces using sterilized cutter and plated on potato dextrose agar medium (PDA) in Petri dishes. Plates were incubated at 25 ºC and examined every day for any fungal growth.

Emerged hyphal tips were separately transferred to new PDA plates and single spore culture technique, using streak method, was used for purification of spore forming fungi, Obtained isolates were identified at Micro Analytical Center, Cairo University.

Isolation of antagonistic microorganisms.

Soil samples from the rhizosphere of healthy mint plants collected from some governates such as Menofiya, Gharbiya and Dakahliya were used to isolate the antagonistic microorganisms.

Isolation of the pathogenic fungi:

Collection of the infected materials

Infected mint plants showing typical symptoms of wilt, root rot and stolon rot diseases were collected from fields at January, 2006.

Isolation from the roots

To isolate internal pathogen, the infected roots were washed thoroughly with tap water and immersed in ethanol 70% for 1 min. for surface sterilization. The roots were cut into small pieces on sterilized filter papers to dry. The root pieces were transferred on potato dextrose agar PDA medium, incubated at 25ºC±1 and examined every day for fungal growth. Hyphal tips of the edges of the grown cultures were cultured on PDA media.

Some healthy and/or infected roots were cut into small pieces without serialization, cultured on PDA medium and incubated as mentioned above; in order to obtain either pathogenic or biocontrol microorganisms grow on and around the root samples.

Media used.

Nutrient Broth Medium **(**Beef extract 10.0 g, Peptone 10.0 g, NaCl 5.0 g, Distilled water up to 1000 ml). Potato Dextrose Agar (PDA) Medium, The extract of peeled 200 g potatoes after boiling for 20 min. in 300 ml distilled water. After boiling the volume was completed to 1000 ml (Dextrose 20.0 g, Agar 20.0 g, Distilled water up to 1000 ml).

Biocontrol agents used in the experiments

Source of bioagents

Trichoderma harzianum, Trichoderma hamatum and Gliocladium virens were obtained from Agricultural Botany Dept., Faculty of Agriculture, Menufiya University. The abovementioned three fungi beside an observed bacterial isolate (*Bacillus subtlus*11) had double action; biofertilizer and biocontrol agents.

Assay of antagonism, in vitro

The antagonistic effects between different benifecial microorganisms and the pathogens were studied. The selected microorganisms were subjected to the test under laboratory conditions to evaluate their antagonistic effect against the pathogens, Petri plates (9.0 cm in diameter) each contained 15 ml of PDA medium were used to detect the antagonistic effect between the pathogens and biocontrol agents in dual cultures.

Different plats were inoculated with 0.6 cm in diameter disc of each tested fungus obtained from the periphery of 3 days old cultures. Each fungus was cultured at one side of the plate and the opposite side was inoculated with other disc of 0.6 cm in diameter, obtained from 3 days old culture of *Trichoderma spp*. or with streak of the antagonistic bacteria grown in nutrient glucose agar medium for 48 hours. Three plates were used for each particular treatment. Plates inoculated with the pathogenic fungus only served as control treatments.

The inoculated plates were incubated at 25ºC. When mycelial growth cover all the medium surface in control treatment , all plates were then examined and the redial growth of the pathogens were recorded and percentage of reduction in growth were pooled out using the following formula;

% Reduction =- control-treatment/control *100.

Inhibition zones between the pathogenic fungi and biocontrol agents were estimated.

Pathogenicity tests

Rhizocotinia solani (A), Verticillium dahliae, Curvularia lunata, Fusarium oxysporum and Rhizoctonia solani (B) were tested for their pathogenicity to spearmint and peppermint species. Plastic pots (15 cm in diameter) were sterilized by immersing them in 5 % sodium hypochloride for 15 min, left to dry in the open air. Peatmoss and sand mixture (2:1 w:w respectively) were mixed thoroughly and autoclaved for 3h.

Fungi were individually grown on sterilized Barley medium (25 g clean sand + 75 g barley grains+100 ml. water) in flasks (500 ml size). Flasks contained sterilized medium were inoculated with each particular fungus and incubated at 25ºC for two weeks. Inocula were mixed thoroughly with sterilized soil at the rate of 3% of soil weight.

Infested pots were kept moist irrigated regularly for a week to allow fungal spread into the soil. Soil of control pots was a mended with the same amount of sterilized Barley medium. Seedlings nearly equal of spearmint and peppermint species were surface sterilized by immersing in 0.5 % sodium hypochloride for 5 min rinsed several times with sterilized water, left to dry and then planted. Four pots were used as replicates per each treatment. Results were recorded 30 and 60 days after transplanting.

Biological control experiments

According to the data obtained from the laboratory experiments, the most effective antagonists were selected for greenhouse experiments. *Rhizocotinia solani (A), Verticillium dahliae, Curvularia lunata, Fusarium oxysporum and Rhizoctonia solani* (B) were grown on Barley medium as mentioned before in pathogenicity test experiment. Each inoculum was applied to the soil at the rate of 3 % by weight.

Trichoderma harzianum, Trichoderma hamatum and *Gliocladium virens* individually, grown on Barley medium (up to 15 days), was used as biocontrol agents inocula. The growth of the above mentioned biocontrol agents was separately added to the soil, at the same rate andtime of application the pathogens inocula. (1.5 % pathogns with 1.5 % biocontrol agents). However, nutrient broth medium was used for *Bacillus subtilis* which also used as a biocontrol agent. Control pots were amended with sterilized barley medium (blank) and /or any of the pathogenic fungi (infested control). Also, pots had sterilized Barley medium and any of the biocontrol agents were included. All the treatments were irrigated as required. Results were recorded 30 and 60 days after transplanting.

Inoculum of antagonists.

The same technique was followed on biocontrol agents.

Bacterial antagonist.

The selected bacterial antagonist was multiplied in nutrient broth medium. The medium were inoculated by loop full from the antagonist and incubated at 25ºC on a shaker for 72 hours. Heamacytometer was used to evaluate the concentration unites of bacterial suspension called colony forming unit (CFU). Bacterial suspension was added to the pots at the rate of 12x104 cfu/plant (single application) and 8x104 cfu/plant (combined application) pots were then irrigated to permit the bacteria dispersed inside the soil.

RESULTS

Soil borne pathogens of mint.

The isolated fungi from diseased mint plants showed different disease symptoms were sent for identification at Agricultural Botany Dept., Faculty of Agriculture, Menufiya University.

However both *Rhizocatinia solani* isolates *(A)* and *(B)* colonized roots and stem bases of root–rotted plants.

Fusarium oxysporum and Verticillium dahliae associated the roots of wilted plants. *Curvularia lunata* isolate was regularly observed in the diseased plants showed yellow brownish leaves.

The above five fungal isolates had high isolation frequency and so they were used further in this investigation.

Results present in Table (2) clear that mint samples of Siwa and Matrouh had the least number and percentage of the pathogens. Samples from Dakahlyia and Menufiya yielded the highest frequency of isolates. On the other hand; *F. oxysporum* was more frequently observed in all governorates followed by *Rhizocotinia solani (A)* and *Rhizocotinia solani (B) Curvularia lunata* showed the lowest frequency of isolation.

Table (1): Frequency of the isolated fungi from diseased mint plants obtained from eight governorates of Egypt.

Governorate	**Rhizoctonia solani (A)**		***Verticillium dahliae***		***Curvularia lunata***		***Fusarium oxysporum***		***Rhizoctonia solani (B)***		Total
	No.	%	No.	%	No.	%	No.	%	No.	%	
Minufiya	6	19 %	5	16%	3	0.9%	10	32%	7	22%	31
Gharbia	4	18 %	3	13 %	3	13 %	7	31 %	5	22 %	22
Sharqia	5	17%	6	21 %	4	14 %	8	28 %	5	17 %	28
Dakhalyia	7	21 %	7	21 %	5	15 %	7	21 %	6	18 %	32
Bahaira	3	21 %	2	14 %	2	14 %	4	28 %	3	21 %	14
Kafr El-Sheikh	4	28 %	2	14 %	1	0.7%	4	28 %	3	21 %	14
Marsa Matroh	3	23 %	2	15 %	2	15 %	4	30 %	2	15 %	13
El-Wadi El-Gidid	2	2 %	1	1 %	2	2 %	3	3 %	2	20 %	10
Total	**34**		**28**		**22**		**47**		**33**		

Isolation of bioagents.

A bacterial isolates were isolates from rhizosphere of healthy mint plants. This isolate was identified as *Bacillus subtilis11* and used in biocotrol study.

Pathogenicity tests.

Under greenhouse and artificial inoculation conditions, the isolated fungi were tested for their pathogenicity to mint plants (Spearmint; *Mentha spicata* and peppermint, *M. piperita*). Results presented in Table (2) and Figure (1&2) clears that all tested fungi were pathogenic to both species of mint. *Fusarium oxysporum* was the highly pathogenic isolate both after 30 and 60 days of transplanting. The second rate of infection was attributed to *Verticillium dahliae* and *Curvularia lunata*. The highest disease severity was recorded with *Fusarium oxysporum* and *Verticillium dahliae* followed by infection of 60 days after planting. In general; spearmint plants were more susceptible than peppermint ones to all pathogens except *C. lunata.*

Laboratory experiments.

Assay of antagonism.

The antagonistic relationships between the benificial microorganisms and the pathogenic fungi were studied, *in vitro.*

Reduction of mycelial growth.

Results shown in Table (3) and Figures (3-7) indicate that, all tested bioagents significantly reduced the average diameter of growth of the pathogenic fungi as compared with control. *Rhizocotinia solani(A)* fungus was severely affected by *Bacillus subtilis 11* (73 % reduction). *Trichoderma harzianum, T. hamatum and G. virens* reduced the growth of the pathogen by 30.23 and 26 % respectively. *Bacillussubtilis11* was also superior against *V. dahliae* (84 % reduction of the growth). This was followed by *G. virens* (66 % R) and both *Trichoderma spp*. which nearly showed the same effect.

Table (2): Severity of infection (%) with different fungal isolates spearmint and peppermint species (Pathogenicity test).

After 30 days from planting Isolate	After 60 days from planting			
	Spearmint	Peppermint	Spearmint	Peppermint
Rhizoctonia solani (A)	12.50	8.33	16.66	12.50
Verticillium dahliae	12.50	15.00	19.16	22.50
Curvularia lunata	14.16	11.10	20.00	18.60
Fusarium oxysporum	21.10	17.50	30.56	25.00
Rhizoctonia solani (B)	10.26	8.60	19.16	16.40
Control	1.50	2.00	7.16	5.20
L.S.D at 5%	6.93		9.83	
L.S.D at 1 %	9.30		13.16	

Table (3): Average diameter of growth (cm) of the pathogenic fungi as affected by different biocontrol agents.

Biocontrol agents	***Rhizoctonia solani (A)***		***Verticillium dahliae***		***Curvularia lunata***		***Fusarium oxysporum***		***Rhizoctonia solani (B)***	
	AD*	**R*%**	**AD***	**R*%**	**AD***	**R*%**	**AD***	**R*%**	**AD***	**R*%**
T. harzianum	5.67	30	3.57	59	2.20	73	2.02	76	2.43	70
T.hamatum	6.22	23	3.63	58	2.57	68	3.77	56	2.23	72
Gliocladium virens	6.00	26	2.98	66	2.05	75	3.97	53	2.22	73
Bacillus subtlus11	2.15	73	1.42	84	3.30	59	3.70	57	1.82	77
Control	8.13	-	8.67	-	8.13	-	8.50	-	8.10	-
L.S.D at 5%	0.45		0.63		0.58		1.17		0.78	
L.S.D at 1 %	0.64		0.90		0.80		1.66		1.08	

***AD :** Average diameter of growth (cm) .

* **R :** Colony reduction % compared with control.

Figure (1): Pathogenicity test experiment of different isolates (soil infestation) with spearmint plants.

A . *Rhizocotinia solani(A);* B. *Verticillium dahliae;.*

C . *Curvularia lunata;.* D. *Fusarium oxysporum;.* E . *Rhizocotinia solani(B).*

Figure (2): Pathogenicity test experiment of different isolates (soil infestation) with peppermint plants.

A . *Rhizocotinia solani(A);* B. *Verticillium dahliae.;* C . *Curvularia lunata;*

D. *Fusarium oxysporum.;* E . *Rhizocotinia solani(B).*

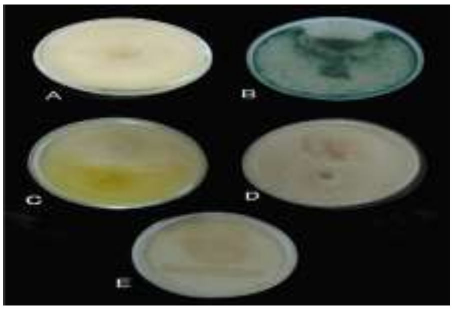


Figure (3): Antagonistic relationships between *Rhizocotiniasolani(A)* and the tested biocontrol agents, (A): control; (B): *Trichoderma harzianum*; (C): *Trichoderm hamatum*; (D): *Gliocladium virens*;*hamatum* (E): *Bacillus subtlus11*.

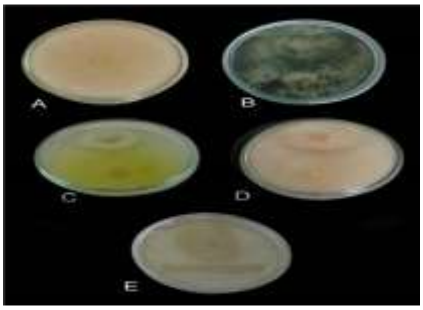


Figure (4): Antagonistic relationships between *Rhizocotiniasolani(A)* and the tested biocontrol agents, (A): control; (B): *Trichoderma harzianum*; (C): *Trichoderm* ; (D): *Gliocladium virens*; (E): *Bacillus subtlus11*.

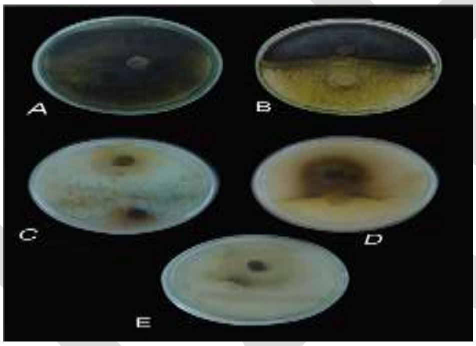
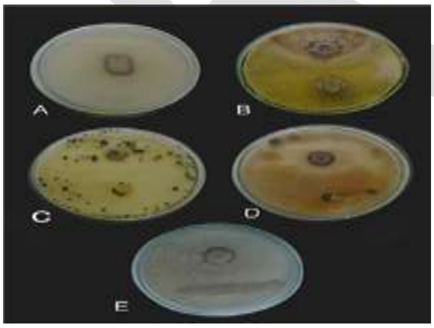
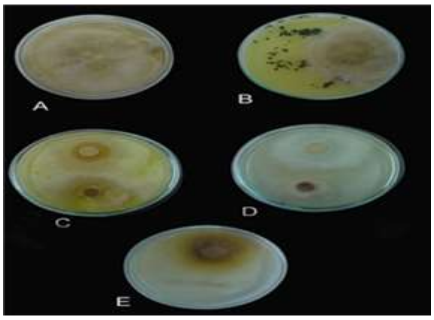


Figure (5): Antagonistic relationships between C*urvularia lunata* and the tested biocontrol agents(A): control;

(B): *Trichoderma harzianum;* (C): *Trichoderm hamatum*

(D): *Gliocladium virens*; (E): *Bacillus subtlus 11.*





Figure(6): Antagonistic relationships**Figure (7):**Antagonistic relationships betweenrelationships between

Rh*izocotiniasol*ani (B); and the testedRh*izocotiniasol*ani (B); and the tested

biocontrol agents (A):control,biocontrol agents(A):control,

(B): *Trichoderma harzianum;* (B): *Trichoderma harzianum;*

(C): *Trichoderm hamatum;* (C): *Trichoderm hamatum;*

(D): *Gliocladium virens;* (D): *Gliocladium virens*

(E): *Bacillus subtlus 11* .(E): *Bacillus subtlus 11.*

Gliocladium virens and T. harzianum were more effective against *Curvularia lunata*. However, *T. harzianum* had more efficacy (76 % reduction), than the other three bioagents (53–57 % reduction); when tested against *F. oxysporum. Rhizoctonia solani(B)* was very sensitive to all the bioagents where they reduced its growth from 70% (*T. hamatum*) to 77% (*B. subtilis11*).

Inhibition zone

Inhibition zones between the biocontrol agents and different pathogens were estimated in Petri dishes Results tabulated in Table (4) indicate that *G. virens* and both *Trichodema spp*. resulted wide inhibition zones, between any of them and the isolate of *Rhizocotiniasolani(A)* as compared with *Bacillus subtilis11*. Significant differences could be noticed between the results of *B.subtilis11* in side and any of the other three bioagents on the other side. Nearly similar results were observed with *V. dahliae* and *F. oxysporum*. While *T. harzianum* showed the least inhibition zone with *C. lunata* in dual culture. Inhibition zone between *R. solani(A)* and the bioagents ranged from 2.07 to 2.76 and had no significant variations among themselves.

Petri dishes were left for another 7 days to evaluate the mode of action between the tested biofertilizers (biocontrol agents) and the pathogens; Table (5) and Figure (8-11). Results of this table indicate that both *Trichoderma spp*. over grew and Microparasitism, however, led to lyses of the pathogen's mycelia and microparasitised the pathogen while *G. virens and B. subtlus11* didn't.

Table (4): Inhibition zone (cm) between the biocontrol agents and pathogenicfungi (7 days after incubation).

Biocontrol agents	*R. solani (A)*	*V. dahliae*	*C. lunata*	*F. oxysporum*	*R. solani (B)*
T. harzianum	4.97	2.79	2.54	2.33	2.57
T. hamatum	5.49	2.80	3.02	2.66	2.76
Gliocladium virens	5.61	2.34	3.38	2.65	2.44
Bacillus subtlus11	2.50	1.43	3.13	1.77	2.07
L.S.D at 5%	0.60	0.51	0.53	0.48	N.S
L.S.D at 1 %	0.88	0.75	0.77	0.69	N.S

Table (5): Summary of recorded mode of action of the tested biocontrol agents against different mint pathogens.

Biocontrol agent	Inhibition zone	Overgrowth	ActiveMycoparasitinm)
Trichoderma harzianum	-	+	+
Trichoderma hamatum	-	+	+
Gliocladium virens	+	-	-
Bacillus subtlus11	+	-	-

The same reactions were observed with all tested pathogens.

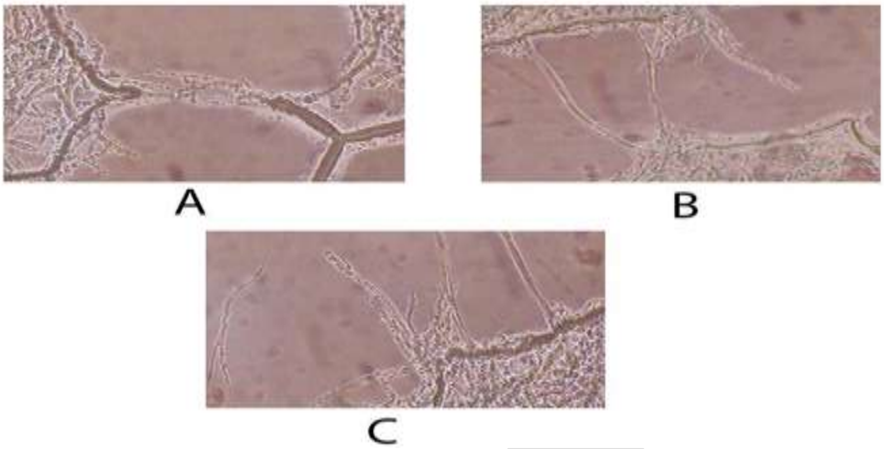


Figure (8): Mycoparasitism of *Trichoderma harzianum* against *Rhizoctonia solani(B)* (A); *Trichoderma hamatum againstRhizoctonia solani(B)* (B); and *Trichoderma harzianum* against *Rhizocotinia solani(A)* (C).

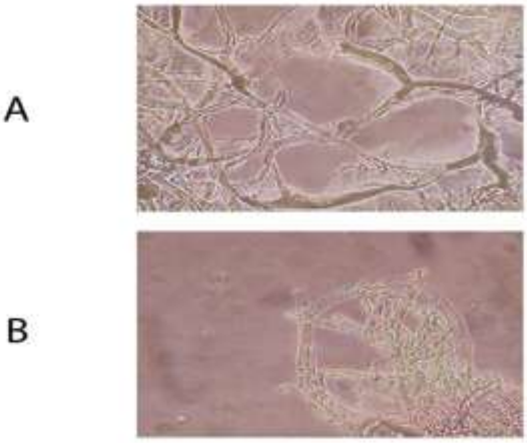


Figure (9): Mycoparasitism of *Trichoderma harzianum* against *Verticillium dahliae* (A); and *Trichoderma hamatum* against*Verticillium dahliae* (B).

__Notice:__ coiling of the antagonisms around the mycelium of *Verticillium dahliae*.

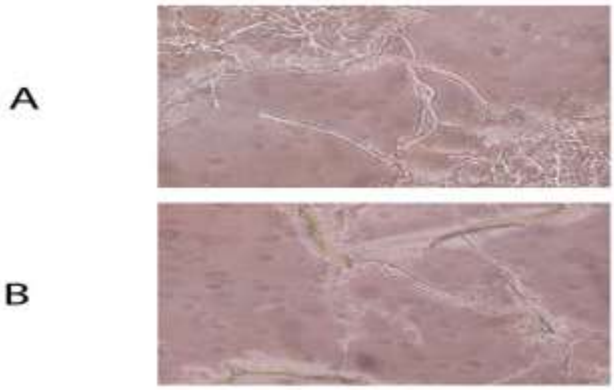


Figure (10): Mycoparasitism of *Trichoderma harzianum* against *Curvularia lunata* (A); and *Trichoderma hamatum against* Curvularia *lunata* (B).

__Notice__*:* coiling of the antagonisms around the mycelium of *Curvularia lunata*

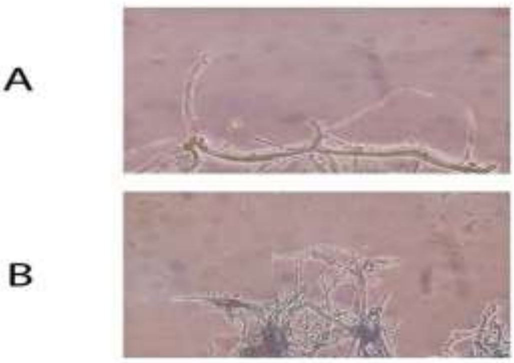


Figure (11): Mycoparasitism of *Trichoderma harzianum* against *Fusarium oxysporum* (A); and *Trichoderma hamatum* against *Fusarium oxysporum* (B).

DISCUSSION

Results of pathogenicity test experiment proved that root and stolon rot disease of mint was caused by either *Rhizoctonia solani(A)* or *Rhizoctonia solani(B*). This result was also observed by **Klara *et al*., (2005).**

Fusarium oxysporum and *Verticillium dahliae* caused wilt symptoms of mint plants. However, **Douhan *et al.,* (2001)**, **Johnson *et al.,* (2001)** and **Wang *et al.,* (2003)** attributed the disease to *V. dahliae*.

While **Sattar and Husain, (1980)**, mentioned that mint root-rot and wilt disease could be due to several pathogens and reported a vascular wilt disease of Japanese mint caused by *Fusarium oxysporum*.

Bacillus subtilis associated healthy mint roots; where positive isolates were obtained. However, many authors expressed the beneficial effect of *B. subtilis* and *B. megaterium* in biological control process i.e.,

Hamed, (1999), **Zheng and Sinclair,(2000)**, and **BrewerMarin & Larkin, (2005)**.

On the other hard; *Trichoderma harzianum*, *T. hamatum* and *Glioclodium virens* which obtained from Agric. Bot. Dept., Fac. Agric., Shebin El-Kom and used in this investigation are well known as biocontrol agents **(Papavizas, 1985).**

In vitro studies demonstrated that all tested biocontrol agents reduced mycelial growth of different tested pathogens. *Bacillus subtilis 11* was superior in the growth reduction of *R. solani(A)* and *V. dahliae*. *Gliocladium virens* had the second rank in suppression the growth of *V. dahliae* followed by both tested *Trichoderma spp*. *Gliocladium virens* and *T. harzianum* were more effective against *Curvularia lunata*.

Generally; the biocontrol agents could secrete toxins and antibiotics which reduce pathogens growth as reported by **Ammar (2003), Daniel *et al.,* (2005)**, **Grosch *et al.,* (2006)**, **Federico *et al.,* (2007)** and **Sunil *et al.,* (2007)**.

Inhibition zones were noticed between the tested biocontrol agent and the pathogens; when a Petri dishwas full with growth. However; whendual cultures were left for seven days incubation, it was noticed that both *Trichoderma spp.* grew over the mycelia of the pathogens.

Examination of the contacted area cleared coiling, microparasitism and lyses of the pathogens mycelia. The penetration of *T .harzianum* Rifai into the cell wall of other fungi is attributed to the production of enzymes lead to the breakdown of chitin, a primary component of fungal cell wall, as reported by **Zeillinger *et al.,* (1999).** However; **Lu *et al.,* (2004) and Zhu *et al.,* (2004)** mentioned that the microparasitic hyphae of *Trichoderma* grew along side the pathogen mycelia (*Rhizoctonia solani(B)*) followed by coiling and formation of specialized structure similar to hooks, appressoria and papillae.

CONCLUSION

Mint is subject to attack by many fungal diseases that cause high lossess in the production. This study was carried out to evaluate the damage due to such diseases and find out safe control methods, mainly biological ones.

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