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Impact of Certain Local Isolated Fungi as Biocontrol Agents against Tomato Wilt Disease

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SEVERAL fungal diseases affect tomato plants and cause severe economic loss worldwide. Locally isolated fungi were examined for biological control of tomato disease. Pathogenic fungi were isolated from infected tomato plants and identified as *Fusarium oxysporum*, *F. oxysporum*, *F. solani*, *F. semitectum*, *F. equsiseti*, *Alternaria solani, and A. alternata*. Further, non-pathogens, such as *Chaetomium globosum*, *Trichoderma harzianum*, *Aspergillus tires*, and *Trichoderma asperelium,* were isolated in the rhizosphere from the roots of healthy plants. Both pathogenic and nonpathogenic fungal strains were isolated from two cultivars in Alexandria and Giza Governorates in Egypt. Genetic similarity was assessed using GenBank and PCR (**Polymerase Chain Reaction)** with ITS1 and ITS4 primers. Efficacy of *T. harzianum* and *C. globosum* as biological control agents were evaluated under laboratory conditions for each fungal pathogen. *T. harzianum* was superior to *C. globosum*. Changes in protein profiles were noted among treatments. A greenhouse experiment compared the efficacy of the biocide, Bio-Ark, as a model of a bacterial bio-pesticide, and *T. harzianum.* Both biological agents were assessed against the chemical pesticide, Uniform 390 SE. The *T. horizon* isolate was most effective based on plant shoot dry weight.

Keywords: *Alternaria* spa*,* Biocides, Fungicides, *Fusarium* spp.,Tomato, *Trichoderma*.

Introduction

The world's most valuable vegetable crop is tomato (*Solanum lycopersicum* L.). This product is ranked first in world commodity prices and second to potato in global production next to potato (FAOSTAT, 2012). The global area of cultivated tomato was 5 million hectares in 2017. Total production was 171 million tons (FAOSTAT, 2017).

Egypt is a top-five tomato producer, accounting for 7.2 to 8.6 million tons annually (FAOSTST, 2017). Unfortunately, several pathogens frequently cause a major reduction in production and quality (De Curtis et al., 2010). Two distinct diseases are vascular wilt caused by *Fusarium oxysporum* f. sp. *lycopersici*, and root and crown rot due to *F. oxysporum f.* sp. *radicis-lycopersici* infection (Benhamou et al., 1997; Radwan et al., 2016). *F. oxysporum* causes extreme root-rot and plant death; infection can cause a 50% decrease in crop yield (De Araujo et al., 2009; Nihorimbere et al., 2010). An interaction between *F. oxysporum* and *F. solani* causes a root-rot disease complex that severely damages tomato production (Klotz, 1973). Controlling such diseases currently depends mainly on chemical fungicide application, which may present hazards to human health and increase environmental pollution (Rauf, 2000). Increasing regulations and restrictions of chemical pesticides or failed attempts at control have increased interest in biological control. Biological control by microorganisms that antagonize pathogens is particularly attractive because diseases caused by soil pathogens are difficult to control with certain fungicides (Moussa et al., 2007). Further, rhizosphere-competent fungi and bacteria can

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regulate pathogens or produce growth-stimulating factors (Akrami & Yousefi, 2015). Microorganisms isolated from specific plant roots or rhizospheres may be better adapted such plants than organisms isolated from other plant species (Cook, 1993).

Culture of *Trichoderma* spp under adverse environmental conditions may identify mycoparasite strains that achieve successful biological control of plant fungal pathogens (Manczinger et al., 2002). For example, *Chaetomium globosum* has reduced the impact of rotten seeds and transmission of pathogens through soil (Aggarwal et al., 2004). The present study isolated pathogenic fungi that commonly cause severe losses in tomato production in Egypt and searched for antagonistic rhizosphere bioagents to control such diseases. Effective biological control using such agents will allow diminishing use of hazardous pesticides.

Materials and Methods

Tomato samples

Twenty samples each of tomato (*S. lycopersicum* L.) plant cultivar (types 448 and 765) were collected from Giza Bain El-Bahrain Island and a private farm in Alexandria, respectively. Sampling occurred during the growing season in 2014. Ten samples each of healthy and infected plants were collected for each cultivar. Healthy intact plants were used for isolating possible control agents, while infected plants displaying crown root-rot, foot rot, black rot, and vascular wilt diseases were used for isolating phytopathogenic fungi.

Isolation and identification of pathogenic fungi and biocontrol agents

Seven representative phytopathogenic fungi were isolated on potato dextrose agar medium plates (PDA) (Difco, 1984 from infected plants (Cultivar type 448 and 765, respectively) showing symptoms as described by Burr et al. (1978)) and incubated at 27°C for 10 days. Individual colonies were selected and transferred to PDA slants, then purified using the hyphal tip method (Dhingra & Sinclair, 1995). Isolated fungi were morphologically and molecularly identified to the species level according to Nelson et al. (1983), Barrett & Hunter (1987), White et al. (1990), Abd-Elsalam et al. (2007), Lievens et al. (2008), and. Radwan et al. (2016). Stock cultures were maintained on PDA slants at 4°C. Four representative fungal biocontrol agents were isolated from plant root rhizospheres and identified morphologically and molecularly as above.

Pathogenic and antagonistic isolates (biocontrol agents) were identified using 18S rDNA sequences. Selected fungal isolates were individually inoculated into 20 mL potato dextrose broth. Mycelia were harvested for DNA extraction according to Abd-Elsalam et al. (2007). Universal ITS1 and ITS4 primers were used to amplify fungal transcribed distance (ITS) fragments. Sequences of the ITS1 and ITS4 primers were5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′, respectively (White et al., 1990). PCR amplification conditions were: 2 min at 94°C, 40 cycles of 60 s at 94°C, 90 s at 52°C and 2 min at 72°C, and final elongation for 7min at 72°C. The PCR products were analyzed and purified using an agarose gel electrophoresis kit (AccuPrep PCR DNA Purification Kit, K-3034-1, Bioneer Corporation). Sequencing was performed by Macrogen Inc. (Seoul, South Korea) using the purified PCR products. Both strands of DNA fragments were assessed to provide inter-transcribed spacing sequences. Sequences were compiled, edited, and synchronized using DNA STAR SeqMan (DNASTAR Inc.) and the CLC sequence viewer. Sequences of PDA isolates were deposited with GenBank, accession numbers MT032354 to MT032358.

Pathogenicity of fungal isolates

Pathogenicity of isolated soil-borne fungi, FoRL2, FoL6, As4, and *F. semi* was examined as described in Whitehead (1957) in the greenhouse of the Central Agriculture Pesticide Laboratory of A.R.C., Giza, Egypt during the growing season of 2014. Fungal inocula were prepared on autoclaved corn sand meal medium (Abd El-Ghany, 2001). Medium was inoculated with 5 mm diameter disks holding aliquots of 7-day old fungal cultures. Isolates were then incubated at 27°C for 14 days. Thirty-day old tomato seedlings (*Solanum lycopersicum* L Cultivar Castle rock) were transplanted into 3 kg sterilized plastic pots filled with sterilized sand infected with 5% by soil weight of single strains of fungal inocula. Percent death of seedlings was calculated as:

$$D = (C - L / C) \times 100$$

where, D is percent death; C is the total count of live seedlings in controls and L is the count of live seedlings in treatments.

Healthy tomato leaves were detached for testing pathogenicity of the foliar isolate, *A. alternata,* according to Browne & Cooke (2004). Disease was assessed by calculating means of standard area diagrams for each treatment using the modified scale described by Granger & Horne (1924) (Table S1).

Antagonism between biocontrol agents and the phytopathogenic fungi

Two mycelium plugs (6mm in diameter) were cut from the margins of active PDA cultures. One plug was obtained from a culture of a possible biocontrol species, and the other was cut from a culture of a pathogenic fungal isolate. The mycelium plugs were placed on PDA medium in 9cm plates at the plate periphery. One plug with pathogenic fungi was maintained as a positive control without bioagent on a separate plate. Three plates were used for each treatment. Dual and control culture plates were incubated for 7 days at 25°C ± 2°C (Mokhtar & Aid, 2013). Percent reduction in mycelial growth of the pathogenic fungus was calculated as:

[Inhibition Percentage (%) = (I − T / C) × 100]

where: I= Inhibition zone of pathogen growth with antagonist, C= Radial growth in control and T= Radial growth in treatment (Deans & Svoboda, 1990).

Chemical and Biopesticides

Two fungicides, Uniform 390 SE and Antracol WP 70% (Table S2), were examined *in vitro* for their ability to eradicate soil- and foliage-borne fungal pathogens. Growth inhibition was expressed using changes in protein profiles in PDA broth in comparison with profiles of fungi grown in the presence of the commercial bio-pesticide, Bio-Ark, at its LC_{25} (Table S3).

Protein profiles of the isolated fungi

Total cellular proteins of five tested pathogens, both untreated and treated with *T. harzianum*, chemical fungicides (Uniform 390 SE, Antracol WP 70%) and the biocide, Bio-Ark, were analyzed. Proteins were prepared as described by Guseva & Gromova (1982). Soluble proteins were assessed as total protein by electrophoresis (Bielenin et al., 1988).

SDS-PAGE

Total cellular proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as adopted by Laemmli (1970). Molecular weight was assessed using a BLUeLf Prestrained Protein Ladder (Jena Biosciencr^{R}). This marker product is a mixture of 13 recombinant, highly purified proteins with molecular weights (MW) ranging from 5 to 245kDa. Gels were stained and photographed to visualize fractionated proteins. MW were determined using Image, version 2.6.

Effect of fungicide, bioagents and biocide on the incidence of Fusarium infection (Greenhouse pot experiment)

T. harzianum was examined *in vivo* as a biocontrol agent for soil-borne pathogenic fungi in the greenhouse of the Central Agriculture Pesticide Laboratory of A.R.C., Giza, Egypt during 2014 season. Plastic pots (20 cm in diameter, 20cm depth) containing sterilized sand (1.25kg/pot) inoculated with 5% (w/w) pathogenic fungi grown on sand cornmeal medium. Pots were watered regularly for seven days before transplanting to ensure growth distribution of inocula. Soil was then inoculated with *T. harzianum* at a rate of 5g/kg soil and pots were watered for 7 days before planting. Both biocide (Bio-Ark) and chemical fungicide (Uniform 390 SE) were added by soil drenching using recommended doses (250g/L and 650mL/ feddan, respectively). Four tomato seedlings (cv. Castle rock) were transplanted in each pot. Treatments were applied in four replicates (Table S4). After 30 days the percentage of wilted plants was recorded and plant height and shoot and root fresh and dry weights were measured.

Statistical analysis

All treatments were performed in triplicate and data were statistically analyzed with an L.S.D. of P 0.05 via comparisons of means (Gomez & Gomez, 1984).

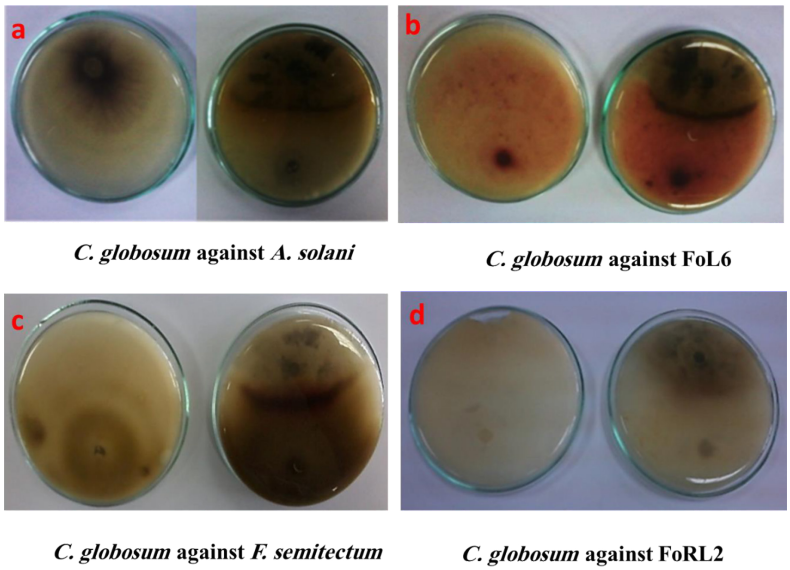
Results

Identification of the fungal isolates

Seven different fungal species were isolated from infected tomato plant parts and identified as *F. oxysporum*1(FoL6), *F. oxysporum* 2 (FoRL2), *F. solani* (*F. s*), *F. semitectum* (*F. semi*), *F. equiseti* (Feq1), *A. alternata* (*A.alt*) and *A. solani* (As4). Also, four fungal species were isolated from healthy plant roots and identified as *T. harzianum* (*T.h*), *T. asperelium* (Tas5), *Chaetomium globosum* (*C.g*), and *Aspergillus terreus* (Ate3) (Table 1, Fig. 1, and Fig. 1S).

TABLE 1. Occurrence of some selective fungi isolated from 2 types cultivar infected and healthy tomato plants in two locations in Egypt (Morphological and molecular identification)

Isolate No.	Source of isolation		Identification	Code	Accession in Gene Bank (Assassin number)
	Plant part	Location			
1	Infected roots	Alex. governorate, Egypt	*Fusarium oxysporum* 1	FoL6	-
2	Infected stem	Alex. governorate, Egypt	*Fusarium oxysporum* 2	FoRL2	MT032355
3	Infected roots	Giza governorate, Egypt	*Fusarium solani*	F. s	-
4	Infected roots	Giza governorate, Egypt	*Fusarium semitectum*	*F. semi*	-
5	Infected stem or leaves	Giza governorate, Egypt	*Alternaria alternate*	*A. alt*	-
6	Healthy roots	Alex. governorate, Egypt	*Trichoderma harzianum*	*T. h*	-
7	Healthy roots	Alex. governorate, Egypt	*Chaetomium globosum*	*C. g*	-
8	Infected roots	Giza governorate, Egypt	*Fusarium equiseti*	Feq1	MT032354
9	Infected stem or leaves	Giza governorate, Egypt	*Alternaria solani*	As4	MT032357
10	Healthy roots	Alex. governorate, Egypt	*Aspergillus terreus* Ate3	Ate3	MT032356
11	Healthy roots	Alex. governorate, Egypt	*Trichoderma asperelium* Tas5	Tas5	MT032358



C. globosum* against *A. solani ***C. globosum* against FoL6**

C. globosum* against *F. semitectum ***C. globosum* against FoRL2**

Fig. 1. Antagonism of *C. globosum* against *Fusarium* spp. at zero time of the pathogen inoculation (S2a), FoL6 (S2b) *F. semitectum*, (S2c) *A. solani,* and (S2d) FoRL2

Pathogenicity of soil-borne fungi

All fungi tested were pathogenic. *A. solani* showed the highest infection rate, 68.8% after 7 days. However, after 45 days, *F. oxysporum* (FoL6) caused a higher death rate (93.8%). The highest contagious effect was recorded by *A. solani* (As4) and lowest for soil was infected with *F. semitectum* after one week. However, *F. oxysporum* (FoL6) caused a high as 93.8% death in 45-day plants. In contrast, *F. oxysporum* (FoRL2) exhibited the lowest death rate (62.5%) after 45 days from transplanting (Table 2).

TABLE 2. Effect of isolated pathogens on tomato plant death.

Time (days)	% Plant death after treatment				
	Control	FoRL2	FoL6	*F. semitectum*	*A. solani*
After one week of transplantation	6.3	43.8	56.3	31.3	68.8
After 45 days of transplantation	6.3	62.5	93.8	75.0	87.5

Pathogenicity of A. alternata

Leaves were affected with an average infection of >25% of the total number of leaflets (Fig. 2S).

In vitro antagonism between C. globosum or T. harizianum and soil-borne fungi

Data show the reduction % in growth of the four examined pathogens on PDA plates when *C. globosum* or *T. harizianum* reduced the growth of pathogenic fungi. Radial growth of FoL6, FoRL2, *F. semitectum* and *A. solani* reached 4.8, 2.2, 4.4, and 2mm in diameter, respectively, compared to 9mm in single culture control plates (Fig. 1). Correctional growth inhibition was calculated as 53, 24.4, 48.9, and 22.2, respectively, using Abbott's formula. When *C. globosum* was inoculated three days before adding pathogens, growth of FoL6, FoRL2, *F. semitectum* and *A. solani* was reduced to 6.2, 4.4, 6.5, and 3.5 mm compared with 9 mm in control plates (Fig. 2). Percent reduction was 68.9, 48.9, 72.2, and 38.9, respectively. *T. harzianum* diminished radial growth of FoL6, FoRL2, *F. semit* and *A. solani* to 6, 6.1, 9, and 5.5mm compared with 9mm in controls (Table 3 and Fig. 3). Accordingly, percent reduction was 66.7, 67.8, 100, and 61.1, respectively. When *T. harzianum* with complete eradication of the pathogens' growth were challenged, promising results were achieved, which meant that *T. harzianum* was chosen as the successful bioagent against *Fusarium spp*. for further study.

Antagonism by T. harzianum and C. globosum against A. alternata

The effect of antagonistic isolates when inoculated along with *A. alternata* at the same time or when inoculated three days before *A. alternata* inoculation showed that *T. harzianum* was more effective than *C. globosum* in reducing pathogen growth (Fig.3S and Fig. 4). Inoculation 3 days before adding the pathogen caused 83.3% reduction in colony diameter. *C. globosum* reduced growth by 34.4%.

Protein profile

a. Total protein

The total protein content of *F. oxysporum* (FoRL2), *F. oxysporum* (FoL6), *F. semitectum*, *A. solani* and *A. alternata* was assessed for individual fungicides, Bio-Ark, Uniform 390 SE and Antracol WP 70% and *T. harzianum.* Uniform 390 SE was used for treating soil-borne pathogens and Antracol WP 70% for the foliar pathogen. Fungicide treatments either increased or decreased total protein content depending on genotype of the examined fungi and treatment. For example, all fungal protein content was lowered by Uniform but other agents induced higher total protein content in *A. solani* and *A. alternata* (Table 4 and Fig. 5).

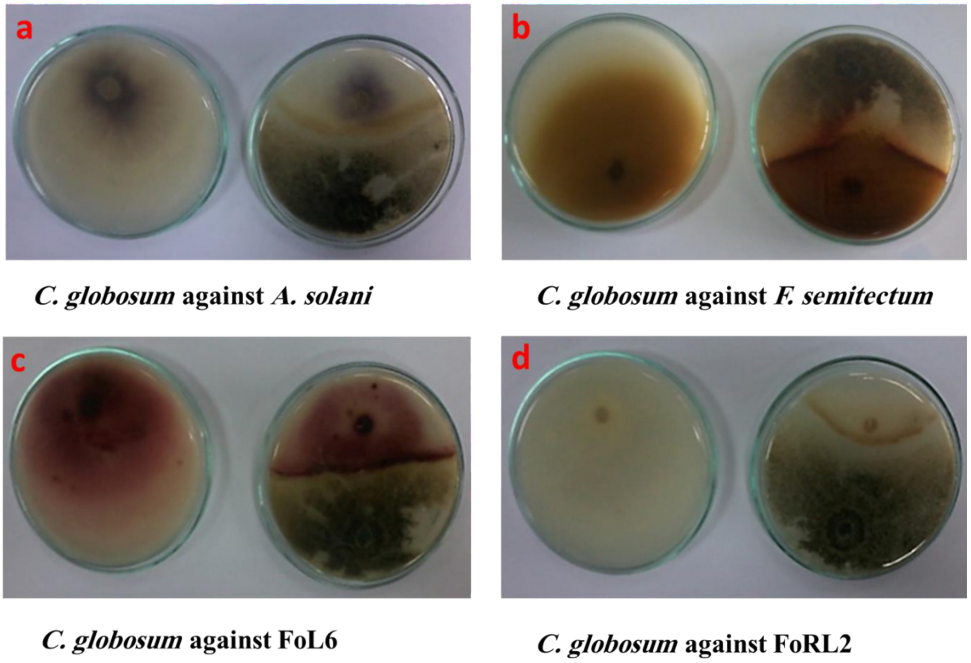
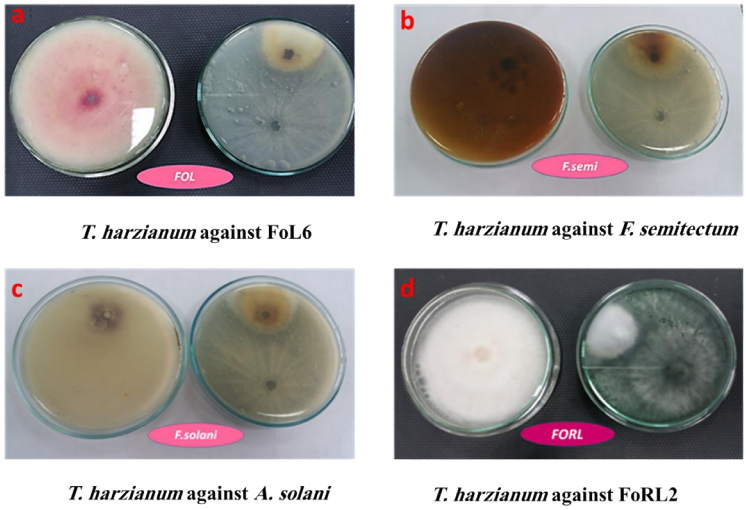


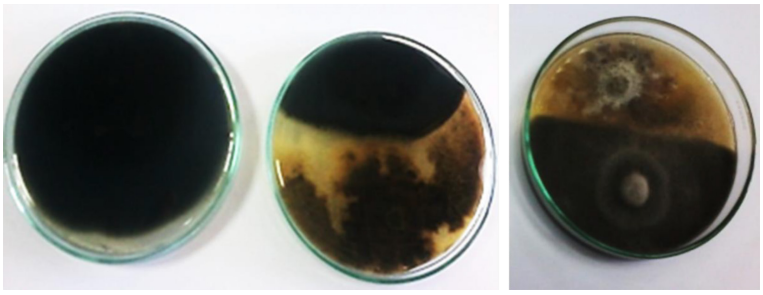
Fig. 2. Antagonism of *C. globosum* against *Fusarium* spp. 3 days before the pathogen inoculation (S3a), *F. semitectum* (S3b), *A. solani* (S3c) FoL6, and (S3d) FoRL2



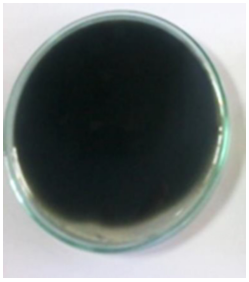
T. harzianum* against FoL6** ***T. harzianum* against *F. semitectum

T. harzianum* against *A. solani ***T. harzianum* against FoRL2**

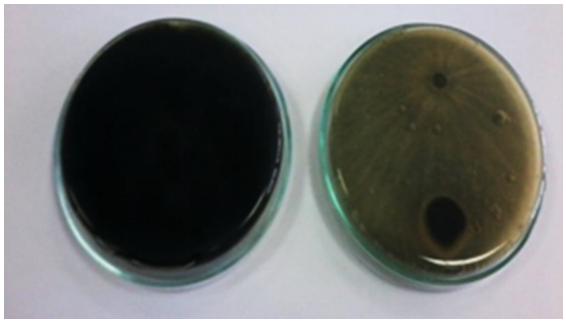
Fig. 3. Antagonism by *T. harzianum* on *Fusarium* spp. (S4a), FoL6 (S4b), *F. semitectum* (S4c), *A. solani*, and (S4d) FoRL2



(a) *C. globosum* (At zero time of the pathogen inoculation)



(b) *C. globosum* (Befor3 days of the pathogen inoculation)



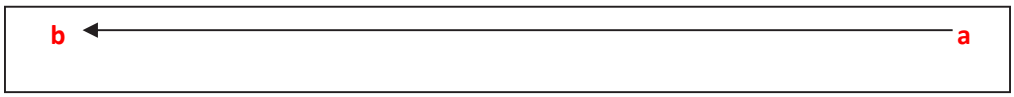
(c)T. harzianum against *A. alternate*

Fig. 4. Antagonistic effect of *C. globosum* (S6a) At zero time and (S6b) Before 3 days of the pathogen inoculation) as well as (S6c) *T. harzianum*, respectively on *A. alternata*

TABLE 3. Antagonistic effect of *C. globosum* (at zero time and before 3 days of the pathogen inoculation) and *T. harzianum*

Bio-agents \ Pathogens		FoL6	FoRL2	*F. semitectum*	*A. solani*	Mean of reduction (%)	F value	L.S.D.
C. globosum (0 time of pathogen inoculation)	M	4.8	2.2	4.4	2	37.12 ^{c}	70.75	0.627
	R%	53 ^{b}	24.4 ^{c}	48.9 ^{c}	22.2 ^{c}			
C. globosum (3days before pathogen inoculation)	M	6.2	4.4	6.5	3.5	57.2 ^{b}		
	R%	68.9 ^{a}	48.9 ^{b}	72.2 ^{b}	38.9 ^{b}			
T. harzianum	M	6	6.1	9	5.5	73.9 ^{a}		
	R%	66.7 ^{ab}	67.8 ^{a}	100 ^{a}	61.1 ^{a}			
Control	M	9	9	9	9			
	R%	0	0	0	0			
F value		3.95	33.44	337.7	131.42			
L.S.D.		1.35	1.13	0.44	0.52			

M: Mean of radial growth (mm) for pathogenic fungi, R %: Reduction percentage in growth of the tested pathogen, means of reduction percentage within a column followed by the same letter are not significantly different (P< 0.05).



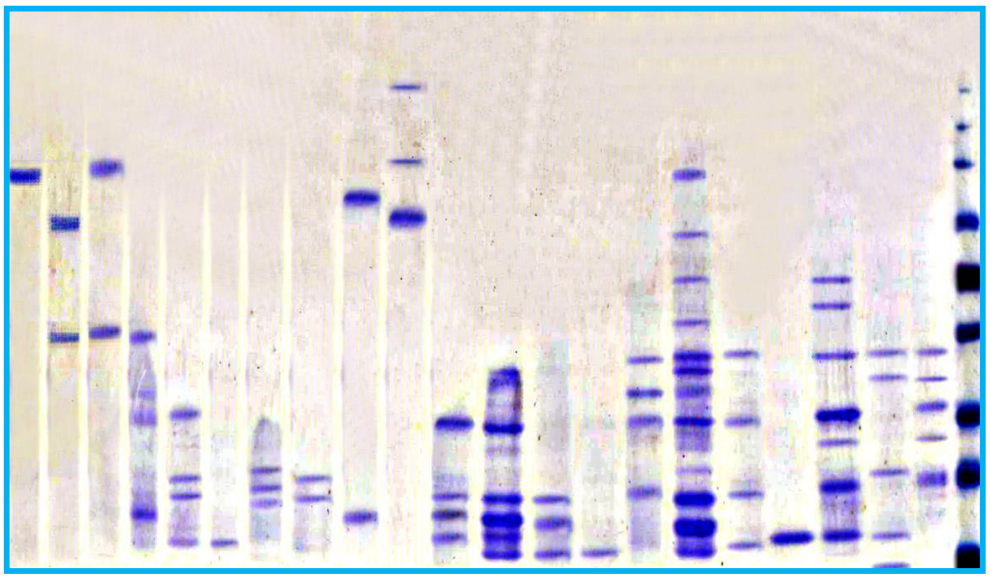


Fig. 5. SDS PAGE demonstrating the relationships between three treatments bio ark, uniform 390 SE and *T. harzianum* on the tested pathogens. While, 2a) Marker, 2b) *T. harzianum*, 2c) *A. solani* (control), 2d) *A. solani* + *T. harzianum,* 2e) *A. solani* +Bio-Ark, 2f) *A. solani*+Uniform 390 SE, 2g) *F. semi* (control), 2h) *F. semi* + *T. harzianum*, 2i) *F. semi*+ Bio-Ark, 2j) *F. semi*+ Uniform 390 SE, 2k) FoL6 (control), 2l) FoL6+ *T. harzianum*, 2m) FoL6+ Bio-Ark, 2n) FoL6+ Uniform 390 SE, 2o) FoRL2 (control), 2p) FoRL2+ *T. harzianum*, 2q) FoRL2+ Bio-Ark, 2r) FoRL2+ Uniform 390 SE, 2s) A. alt (control), 2t) *A. alt* + *T. harzianum*, 2u) A. alt + Bio-Ark, 2v) *A. alt* + Antracol 70% wp.

TABLE 4. Total protein (mg/10mg dry weight) of the isolated fungi treated or not with fungicides or antagonists

Treatments	Tested pathogens				
	FoL6	**FoRL2**	***F. semi***	***A. solani***	***A. alternata***
Control	2.84± 0.16	2.27± 0.12	3.42± 0.11	1.87± 0.09	1.89± 0.13
T. harzianum	2.30± 0.11	2.07± 0.10	1.98± 0.14	2.03± 0.01	2.05± 0.13
Bio-Ark	2.30± 0.09	1.85± 0.09	1.80± 0.03	2.23± 0.13	2.05± 0.11
Uniform 390 SE	2.24± 0.2405	1.91± 0.1	1.97± 0.0997	1.75±0.054	-
Antracol WP 70%	-	-	-	-	1.82± 0.06

SDS-PAGE protein profiles

Fungal proteins were assessed qualitatively on the basis of the molecular weight and reproducibility on SDS-PAGE. Gel bands with the same mobility were regarded same proteins. The final analysis omitted weak bands that represented negligible protein content. The profile SDS-PAGE obtained with three fungicides. Figure 5 showed electrophoretic proteins patterns that differed by pathogenic strain (*F. oxysporum* (FoRL2), *F. oxysporum* (FoL6), *F. semitectum*, *A. solani*, and *A. alternata*) and type of fungicide (Tables S5, S6, S7, S8, and S9). Protein patterns were affected by all treatments. Patterns showed 96 different bands with different relative frequencies (Rf) and MW. Effects of three tested fungicides on *F. oxysporum* f. sp. radicis (FoRL2) and for the control (Table S5). Sixteen bands were detected across control and all treatments. Four bands were recovered from controls (No. 5, 9, 11, and 16). Five bands were detected (No. 3, 4, 7, 10, and 13) after treatment with *T. harzianum*. Only one band with Rf 0.932 and MW 21.661 KDa was observed after treatment with Bio-Ark. Six bands were seen after Uniform 390 SE treatment (No. 1, 2, 6, 8, 12, and 14). Generally, all bands of FoRL2 protein were characteristic for each treatment at LC25 and the control.

Treatment of *F. oxysporum* (FoL6) with Bio-Ark, Uniform 390 SE, and *T. harzianum* showed twenty bands total bands (Table S6). Nine bands (5, 6, 7, 9, 10, 13, 16, 17, and 18) were detected in untreated FoL6. Six bands (8, 11, 12, 14, 17, and 19) were detected after treatment with *T. harzianum.* Three bands were detected after treatment with Bio-Ark (1, 2, and 4), and only two bands (3 and 5) with Rf of 0.307 and 0.886 were detected and treatment with Uniform 390 SE. A total of 19 characteristic bands were identified. Five bands were characteristic of *T. harzianum*, three of Bio-Ark, and two of Uniform 390 SE. MW of these proteins ranged from 19.140 to 251.50kDa. Conversely, seven bands were characteristic of healthy controls with MW ranging from 20.251 to 55.597kDa. Only one band (17) with Rf 0.922 and MW 22.124kDa was detected as a common band between control and *T. harzianum* treatment.

Fourteen bands were observed in controls (1, 2, 3, 4, 5, 7, 8, 11, 12, 14, 15, 18, 21, and 23) with Rf ranging from 0.263 to 0.949 and MW from 126.301 to 20.701kDa (Table S7). Six bands were observed after *T. harzianum* treatment (6, 9, 10, 13, 17 and 22) with Rf ranging from 0.598 to 0.931 and MW from 58.095 to 21.714kDa. Three bands (10, 16 and 24) were seen after treatment with Bio-Ark and three after treatment with Uniform 390 SE (19, 20, and 25). All detected bands were characteristic for *T. harzianum* control, Bio-Ark,.and Uniform 390 SE treatments. The only common band (10) was between *T. harzianum* and Bio-Ark treatments with Rf 0.710 and MW 47.108kDa.

Eight bands appeared in *A. solani* controls (3, 6, 8, 12, 14, 16, 20, and 24) with Rf and MW ranging from 0.587 to 0.976 and 59.238 to 18.358 KDa, respectively (Table S8). Nine bands were detected (1, 2, 5, 7, 9, 13, 17, 19, and 21) after treatment with *T. harzianum* with Rf and MW ranging from 0.455 to 0.920 and 74.613 to 22.225 KDa, respectively. Only one band was detected after Bio-Ark treatment (22) with Rf 0.925 and MW 21.971kDa. Five bands (4, 11, 15, 18, 23) were all that was observed for *A. solani* treated with Uniform 390 SE. No common bands were observed and all bands were considered characteristic for controls and treatments.

Eight protein bands were detected from *A. alternata* without treatment (8, 9, 10, 11, 12, 13, 14, and 15) with Rf ranging from 0.557 to 0.932 and MW from 62.045 to 21.661kDa. Bands 3 and 6 only were observed after treatment with *T. harzianum* with Rf 0.250 and 0.549, respectively. Other bands, 2 and 4, with Rf 0.233 and 0.225,

were the only bands seen after Bio-Ark treatment. Treatment with Uniform 390 SE produced three bands (1, 5, and 7) with MW 143.631, 100.577, and 62.254kDa, respectively. No common bands were seen and all bands are considered characteristic for *A. alternata* controls and treatments.

Effect of fungicide, bioagents and biocide on the incidence of Fusarium infection (Green house pot experiment).

Root and shoot fresh and dry weights were not significantly affected by the application of the chemical fungicide despite the increase in plant height compared with control plants (Table 4). This fungicide was superior in comparison with other treatments in decreasing the disease incidence to as low as 37.5%. The minimum disease incidence was recorded with plants treated with the *T. harizianum* isolate, which supported good growth of roots and shoots as well as increased plant height. Significant differences were also observed between growth parameters in controls and plants treated with Bio-Ark.

Disease was completely eradicated from plants exposed to *F. oxysporium* FoRL2 following treatment with Bio-Ark. Survival was 100% with no improvement to plant growth (Table 5A). The application of Uniform 390 SE decreased disease incidence by 62.5% but had no significant effect on plant growth. In contrast, *T. harzianum* effectively reduced disease incidence to as low as 6.3% and significantly improved shoot and root fresh and dry weight.

TABLE 5A. Disease incidence and plant growth of tomato plants in soil infested with *Fusarium oxysporium* (FoL6) as affected by biocide, bioagent, and chemical fungicide treatments

Fungi	Treatments	Root			Shoot				Death %
		Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	No. branches	
Fusarium oxysporium FoL	Bio Ark	0.49	0.13	7.4	5.9	0.57	34.5	8.0	50
	Uniform 390 SE	0.28	0.07	6.4	2.8	0.29	21.1	8.0	37.5
	T. harzianum	1.32	0.26	16.3	9.3	0.44	22.6	6.2	6.5
	Control	0.21	0. 03	2.8	1.8	0.27	10.7	2	87.5
	L.S.D.	0.19	0.07	0.75	1.66	0.39	2.34	0.45	-

TABLE 5B. Effect of various treatments on Death %, fresh and dry weight (g), length (cm) of roots and shoot and the number of branches of tomato infected by *A. solani*

Fungi	Treatments	Root			Shoot				Death %
		Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	No. branches	
A. solani	Bio Ark	0.30	0.16	6.25	3.60	0.51	29.2	6.8	33.3
	Uniform	0.25	0.06	6.15	3.01	0.24	21.6	6.50	31.3
	T. harizianum	0.80	0.21	17.0	8.12	0.88	35.60	7.75	5.9
	Control (infested)	0.97	0.13	7.27	9.61	0.24	26.5	5.7	33.3
	L.S.D.	0.43	0.26	0.36	1.27	0.22	0.48	0.33	-

TABLE 5C. Effect of various treatments on Death %, fresh and dry weight (g), length (cm) of roots and shoot and the number of branches of tomato infected by *F. semitectum*

Fungi	Treatments	Root			Shoot				Death %
		Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	No. branches	
F. semitectum	Bio-Ark	0.23	0.11	4.68	4.93	0.38	33.1	7.25	0
	Uniform 390 SE	0.22	0.05	6.70	4.88	0.27	30.45	7.75	25
	T. harzianum	1.20	0.24	18.73	7.33	0.72	30.4	7.75	0
	Control	0.19	0.02	3.0	2.11	0.21	17.20	3	87.5
	L.S.D.	0.35	0.11	0.96	0.95	0.28	2.21	0.89	-

Root fresh and dry weights and shoot fresh weight were altered by treatments in the soil inoculated by *A. solani* in comparison with control plants (Table 5B). However, plants inoculated with *T. harizianum* formed roots with 2-fold higher length compared to control plants. Bio-Ark did not significantly affect shoot dry weight compared to infected controls.

Overall, *T. harzianum* was most effective compared to other treatments. A significant effect of Bio-Ark was noticed on shoot length when compared with the infected control. Increases in shoot length after Bio-Ark treatment were greater than after application of Uniform 390 SE. Significant increases over control were recorded in the number of branches after *T. harizianum* and Bio-Ark treatments.

Neither Bio-Ark nor Uniform displayed significant effects on root fresh and dry weights of plants infected with *F. semitectum* (Table 5C); however, treatment with *T. harzianum* induced significant improvement in plant growth compared to untreated controls. This treatment improved shoot fresh weight and length as well as the number of plant branches. Again, *T. harizianum* was most efficacious for control of *F. semitectum*.

Discussion

Tomato is an important economic vegetable crop, ranking 7th in worldwide production. Approximately 171 million tons were produced on a cultivated area of almost 5 million hectares in 2014 (FAOSTAT, 2017). Tomato belongs in the family, Solanaceae, and is susceptible to attack by several pathogens that significantly reduce crop quality and quantity. Tomato is subject to diseases, such as wilt and root rots and early and late blights caused by *Fusarium, Rhizoctonia, Alternaria*, and *Phytophthora,* respectively. Our work aimed to identify common pathogens in Egypt and explore alternative ways to control these pathogens in the field. Biological control by bioagents and biopesticides were the focus of this study to avoid or limit health impacts and environmental pollution caused by chemical pesticides.

Identification of fungal isolates obtained from diseased plants in Giza and Alexandria governorates identified *F. oxysporum* strain two (FoRL2), the causative agent of root-rot, *A. solani,* the causative agent of foot rot, and *F. semitectum,* the causative agent of fruit rot, as soil-borne fungi. Mahovic et al. (2004) also reported that tomato rot was caused by *Fusarium* species.

Another strain was identified as *F. oxysporum* strain two (FoL6), which is responsible for vascular wilt diseases as previously reported by Armstrong & Armstrong (1981). Morphological identification of fungi isolated in this work used characteristics of macro-conidia, phialides, micro-conidia, chlamydos pores, and agree with previous reports (Nelson et al., 1983; Windels, 1992).

The most destructive disease in tomatoes is root-rot caused by *F. oxysporium*, *Rhizoctonia solani* Kuhu, *F. solani* (Mart) Sacc, and *Sclerotium rolfsii* Sacc (El-Mougy 1995; Benhamou et al., 1997). Root and crown rot and wilt diseases are caused by

F. oxysporum f sp *radicis lycopersci* (FoRL2) and *F. oxysporum* f.sp. *lyucopersci* (FoL6), pathogens that survive in soil and are responsible for serious losses in vegetable crop yield (Radwan. & AL-Masri, 2012; Lobo & Silva, 2000). Moreover, severe diseases in open fields or plastic housing are late blights caused by *A. solani* and *Phytophthora infestans* (Khurana, 1998; Cook & Deahi, 1998). One of the most prevalent and serious diseases of tomato is wilt caused by *F. oxysporum* (Schlecht) and *F. lycopersici* (Sacc.) (Reis et al., 2004). *A. alternata* is a model for foliar disease in tomatoes. This species is isolated from infected leaves with brown to black necrotic lesions and identified based on conidiophores, number of septa, colony color, and growth traits (Rayner (1970).

Eleven isolates of pathogens (7) and bioagents (4) were identified by molecular means using PCR techniques, which confirmed morphological and physiological identifications. Species isolated and identified from tomato infected roots and stems in plants from the Alex governorate, Egypt were *F. oxysporum* two (FoRL2) and *F. oxysporum* one (FoL6). Pathogenic fungi isolated and identified from tomato infected roots and stems or leaves from Giza governorate were *F. equiseti* (Feq1) and *Alternaria solani* (As4.), respectively. Conversely, bioagent isolates identified were *Trichoderma asperelium* (Tas5) and *Aspergillus terreus* (Ate3).

Eight *F. oxysporum* isolates were recovered from wilted tomato plants collected from various locations in Middle Egypt, mainly in El-Menia Governorate during the 2012 growing season (Radwan et al., 2016). In addition, Akbar et al. (2018) used morphological and molecular tests to isolate and classify *F. exosporum*, *F. equiseti,* and *F. solani* from infected tomatoes. Several fungi were also isolated and identified from soil and healthy tomatoes for use as antagonists against tomato pathogenic fungi, i.e., *A. terreus* (Melo et al., 2006; Cazar et al., 2005), *Chaetomium globosum* (Fayyadh & Youssif, 2019), *T. harzianum* (Green et al., 1999), and *T. asperellum* (Karuppiah et al., 2019).

Several effective synthetic fungicides are available for control of *Fusarium.* These fungicides may be harmful to human health and the environment, and efforts to find alternative, environmentally safe control methods have been sought (Paul & Sharma, 2002).

Pathogenicity tests were carried out under laboratory conditions using positive controls with an average of more than 50% involvement of leaflet area in comparison with uninfected controls. These results are consistent with Mukesh et al. (2017) who showed higher pathogenicity of *A. alternata* for tomato plants compared to another host, *Lycopersicon esculentum*. Symptoms of disease were characterized by black sunken necrotic lesions with typical concentric rings increasing gradually and covering most leaf area compared to untreated control samples. Finally, the pathogen causes maximum damage by covering 75%–80% of leaves within 4–5 days. An antagonistic study was conducted to assess the ability of two bioagents to control four soil-borne fungi, FoL6, FoRL2, *F. semitectum* and *A. solani* under laboratory conditions and one foliage pathogen, *A. alternata*.

The first tested bioagent, *Chaetomium. globosum,* applied at time 0, was effective against all pathogens. However, the lowest percent reduction was observed against *A. solani* (22.2%). The highest percentage was recorded for FoL6 (53%). Inoculation with *Chaetomium globosum* three days before pathogen inoculation controlled all fungal pathogens, and the highest reduction was recorded with *F. semitectum* (72.2%). The lowest was recorded with *A. solani* (38.9%). These results are consistent with Alabouvette et al. (2006) who studied the antagonistic activity of biocontrol microorganisms as demonstrated by inhibition of growth, infection, or reproduction of pathogen. Sun et al. (2006) and Longoni et al. (2012) also found that pathogen mycelial degradation was possibly a result of lytic enzymes commonly secreted by *Chaetomium spp.* The overall highest percent reduction was recorded for *T. harzianum* tested against *F. semitectum* (100%); however, the lowest was recorded with *A. solani* (61.1%). In contrast, *C. globosum* showed only 22.2% lower growth.

Growth of *T. harzianum* is faster than *C. globosum* and this phenomenon may explain its high efficacy. Results in agreement with Harman et al. (2004), who recommended the use of *Trichoderma* for agriculture. These fungi have several advantages: (1) Plant root and rhizosphere colonization, (2) Plant pathogen regulation by various mechanisms, such as parasitism, antibiosis production, and induction of systemic resistance, (3) Improvement of the plant health by promoting plant growth, and (4) Stimulation of root growth.

The present study also evaluated the relative potency of the commercially available bio-pesticide (Bio-Ark) and *T. harzianum* isolated as a bioagent for control of soil-borne and foliar disease both *in vitro* and in the greenhouse. Uniform 390 SE and Antracol WP 70% were used as reference chemical fungicides. This bioagent was able to eradicate pathogens and improve plant survival and growth.

Molecular biology methods using electrophoretic detection of protein banding patterns showed the highest fungal protein content for untreated *F. semitectum* (0.262mg/g). This content was reduced to 0.174mg/g by treatment with Bio-Ark. The lowest protein content was found for untreated *A. alternata* (0.140mg/g) and was not affected by Antracol WP 70%. Treatment with Bio-Ark induced increased content to 0.154mg/g. Bio-Ark was thus the more effective treatment for either decreasing or increasing fungal protein content.

Under greenhouse conditions, a pot experiment was to compare three treatments (Bio-Ark, Uniform 390 SE, and T. *harzianum*) soil inoculated separately with each of four soil-borne *Fusarium* species. *T. harzianum* induced the greatest reduction in disease incidence in the soil inoculated with *F. semitectum* and improved plant growth as indicated by elevated plant dry and fresh weight and length and number of branches. Bio-Ark and Uniform 390 SE, in that order, were less effective. Consistently, El-Shennawy (2011) concluded that antagonists can offer highly significant protection and reported that the biocontrol, Perpetrate Plant Guard, was the most effective biocide, followed by Bio-zeid, Rhizo-N, and Bio-ARC. The effectiveness of native *Trichorema* isolates to promote the growth and yield of tomatoes and manage *Fusarium* wilt disease was further demonstrated *in vitro* and *in vivo* by Sundramoorthy & Balabasker (2013). Plants treated with *Trichoderma harzianum* (AN R-1) showed important stimulating effects on plant height (by 73.62cm) and dry weight (by 288.38g) compared to other isolates and nontreated controls. In a pot experiment, plant height and fresh and dry weights of plants were found to be significantly increased ($P< 0.05$) in all treatments, except in soil amended with *P. autumnal* (Alwathnani et al., 2012). This finding is attributed to *Trichoderma* defensive mechanisms described by Vinale et al. (2009). These defenses include enzymes and chemical weapons. Thus, *Trichoderma* spp make efficient mycoparasites, antagonists, and biocontrol agents, with features that may prove effective in efforts to control pathogenic fungal plant diseases.

Conclusions

A locally isolated bioagent, *Trichoderma herizianum*, was superior in controlling tomato fungal disease in comparison with chemical and biopesticides. *F. oxysporum* was identified as the most virulent pathogen for tomato wilt disease and *T. harzianum* proved to be the most active bioagent isolated. Protein patterns of fungal pathogens were affected by all treatments, reflecting *T. harzianum* efficacy. Significant improvement in plant growth parameters was recorded after application with *T. harzianum* in comparison with Bio-Ark, Uniform 390 SE, and untreated controls.

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تأثير بعض الفطريات المحلية المعزولة كعوامل تحكم بيولوجي لمرض ذبول الطماطم

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يصاب نبات الطماطم بالعديد من الأمراض الفطرية مثل الأعفان والذبول والتي تسبب فقد في المحصول بدرجة كبيرة. لذلك فان الهدف من الدراسة الحالية هو التعرف على الفطريات الممرضة وتقييم طرق المكافحة البيولوجية والكيماوية الي جانب الحصول على عزلات جديدة يمكن أستخدامها في مجال المكافحة الحيوية. لذلك تم عزل وتعريف بعض فطريات التربة الممرضة وتشمل: من المجموع الخضري *Aternaria.alternata* , *Fusarium.oxysporum, Fusarium.semitectum and Fusarium.solani* وكذلك تم عزل كلا من *Chaetomium globosum* ، *Trichoderma harizianum*من منطقة الريوسفير المحيطة بنبات الطماطم السليم والتي يمكن استخدامها كمواد حيوية فعالة ضد فطريات التربة الممرضة. وقد تم عزل تلك الفطريات من محافظتي الجيزة والأسكندرية. وقد تم أختبارهما معمليا لمعرفة قدرتهما على مقاومة الفطريات الممرضة المعزولة. أظهرت النتائج كفاءة كلا منهما ضد الفطريات الممرضة جميعها تحت ظروف المعمل. ومع ذلك كان فطر *T.harzianum* الأكثر فعالية ضد أمراض الفيوزاريم محل الدراسة مقارنة بفطر *C.globosum* حيث سجل أنخفاض كامل للنمو الميسلومي للفطر *F.semitecum* بنسبة 100 %. كذلك تم تقييم كفاءة المبيدات الحيوية بيو أرك و بيوزيد و بلانت جارد معمليا ضد الفطريات الممرضة المعزولة مقارنة بالمبيدات الكيماوية (يونيفورم و وانتراكول) والفطر المعزول *T.harzianum* وأظهرت النتائج تفوق المبيدات الحيوية سواء المستحضرات التجارية أو الفطريات المعزولة علي حساب المبيدات الكيماوية. وقد تم تقدير المحتوي البروتيني للفطريات المعاملة وتوصلت النتائج إلى ان المبيد البكتيري بيوأرك هو الأكثر تأثيرا علي زيادة أو نقصان المحتوي البروتيني للفطريات الممرضة. تم عمل تطبيق في الصوبة الزجاجية لأستكمال تقييم المبيد الحيوي بيوأرك مقارنة بالمبيد الكيماوي يونيفورم والفطر المعزول *T.harianum* وقد أكدت النتائج تفوق المبيدات الحيوية في مقاومة الأمراض الفطرية.كما أوضحت النتائج أن فطر *T.harzianum* كان الأكثر كفاءة في أختزال القدرة المرضية للفطريات حيث تراوحت تلك النسبة ما بين 81 إلى 100% ويليه المبيد الحيوي بيوأرك بنسبة من صفر إلى 100% ثم المبيد الكيمائي يونيفورم بنسبة تتراوح من 6 إلى 75% وذلك إلى جانب تأثير *T.harizianum* الملحوظ علي كل من طول النبات والوزن الجاف والرطب للجذر والساق بينما لم يلاحظ أي فروق معنوية في عدد الأفرع.