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Biocontrol of pepper wilt disease by antagonistic fungi and their modes of action for the biocontrol

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Abstract. Thirty species of fungi related to 16 genera were isolated from the rhizosphere soils of healthy pepper plantations in different localities in Assiut (13 localities), Behera (2 localities), and Sohag Governorates (2 localities) in Egypt. Seventy–five native isolates related to 10 genera and 17 species were screened *in vitro* against the pathogenic *Fusarium oxysporum*, *Fusarium solani* and, *Macrophomina phaseolina. In vitro*, the highest activity *was* obtained with all isolates of *Trichoderma harzianum* followed by *Penicillium oxalicum* (AUMC 11419, AUMC 11420) and *Clonostachys rosea* (AUMC 11417, AUMC 11442) on the three pathogenic fungal species tested. *In vivo Botryotrichum atrogriseum* AUMC 11415, *Aspergillus nidulans* AUMC 11418, *Albifimbria verrucaria* AUMC 11414, *C. rosea* AUMC 11442, and *T. harzianum* AUMC 11422 were reduced disease severity up to 90% over the control. The results revealed that *Trichoderma harzianum* (3 isolates) showed the highest chitinase activity in a range of 2.5-3.3 IU/ml, and *Penicillium oxalicum* AUMC 11419 presented a maximum lipase activity of 1.01 IU/ml. Also, the volatile metabolites assay revealed that *Trichoderma* strains produced the highest inhibitory effect against the highly pathogenic strain of *Fusarium oxysporum* f. sp. *capsici* AUMC11424 in the range of 31.1-34.4% of the untreated control.

Keywords*: rhizosphere fungi, pepper, wilt, chitinase, volatile metabolites.*

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1. Introduction

Fusarium oxysporum is the cause of the most severe disease of commercial plants in the world. The plant pathogens produce enzymes that degrade the plant cell wall components [1]. Farmers are, in general, familiar with chemical pesticides due to their quick, practical actions [2]. However, using chemical fungicides increases production costs and makes the products more expensive [3]. Also, pesticides could pose potential risks to food safety, the environment, and all living organisms [4]. Another remarkable feature of chemical pesticides is the evolving genetic resistance to some pathogenic fungi [5].

Trichoderma species have displayed physical interaction (root surface colonization) with the plant, resulting in beneficial effects on plant metabolism such as promoted growth, nutrient availability, improvement of crop production [6]. Also, *Trichoderma* species are major mycoparasites that parasitize many plant pathogens; they are also capable of producing extracellular lytic enzymes responsible for their antagonistic activity and producing antibiotics [7-9]. Furthermore, cucumber, bell pepper, strawberry, mustard, and tomato yields were increased significantly by the application of *Trichoderma* spp. [10]. They can control many soil-borne diseases such as damping off of peas [11], *Fusarium* wilt of sweet peppers [12], *Verticilium*, and *Fusarium* wilt of tomato [13].

Gliocladium and *Penicillium* species are essential biocontrol agents due to their hyperparasitism, competition for infection sites, and capacity to produce several antibiotic metabolites [14]. *Gliocladium* sp. also produced different enzyme inhibitors such

as argifin, a potent chitinase inhibitor, which can destroy the chitin of a pathogen [15]. *Myrothecium verrucaria, Penicillium* sp. [16], *Penicillium oxalicum* [17], *Aspergillus* sp. [18], and *T. harzianum* have been used to develop a mycopesticide for the control of plant pathogenic fungi and insect pests due to their capability of producing chitinolytic enzymes [19] which degrade chitin in the protective covers, the cell walls of plant pathogenesis fungi and cuticle of insects, respectively.

Therefore, the objective of the present study was to isolate and identify fungi from the rhizosphere of pepper and used some of them as antagonists on the control of pepper wilt caused by *Fusarium oxysporum*, *Fusarium solani*, and *Macrophomina phaseolina in vitro* and under greenhouse. The efficacy of chitinase and lipase activities and their volatile metabolites was also assessed.

2. Materials and methods

2.1. DNA extraction and sequencing of the most virulent *Fusarium oxysporum* strain

Fusarium oxysporum AUMC 11424, the most virulent strain tested, was inoculated into 100 ml of potato dextrose broth medium and incubated in an orbital shaker at 150 rpm at 25 °C in the dark. After one week of growth, the mycelium was filtered, washed thoroughly twice with distilled water, and transferred into a 1.5 ml Eppendorf tube. The mycelium was freeze-dried for 24 h.

DNA extraction by the CTAB method [20] as follows: Cell walls of fungal mycelia were broken down by grinding in a mortar in the presence of liquid nitrogen; a fine powder is best for extracting DNA. For each 100 mg mycelium powder, 500 µl of CTAB Extraction Buffer (2% CTAB, cetyltrimethylammonium bromide), 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA was added and mixed and then placed in a water bath for 30 min at 60 °C. Following the incubation period, the homogenate was centrifuged for 5 min at 13000 × g. The supernatant was transferred to a new tube, and 5 µl of RNase was added and then incubated at 32 °C for 20 min. Afterward, 500 µl of chloroform was added, and then the supernatant was vortexed for 5 sec and centrifuged for 10 min at 13000 × g to separate the phases. The aqueous upper phase was transferred to a new tube. The DNA was precipitated by adding cold isopropanol, and then 50 µl sodium acetate (3 M) was added and incubated at 20 °C for 15 min. The supernatant was centrifuged at 13000 × g for 1 min, decanted without disturbing the pellet, and subsequently washed with 500 µl ice-cold 70% ethanol. The residual ethanol was removed by drying the pellet long enough in a Speed Vac. for 5 min. The DNA pellet was dissolved in 20 to 50 µl of pure water. The pellet may need warming to dissolve.

2.2. Estimation of the extracted DNA

The quality of the extracted DNA was obtained using sub-marine gel electrophoresis. A 1% agarose in TBE buffer (Tris base, 10.8 g, boric acid 5.5 g, EDTA 0.93 g, distilled water 1000 ml, and pH 8.2-8.4) was prepared and left cool down. Afterward, 5 µl of DNA with 1 µl 5x loading dye [20 mM Tris-HCl (pH 8), 0.03% bromophenol blue, 100 ml glycerol (50%), and 100 mM KCl] were loaded in the 1 % agarose gel using ethidium bromide coloration for staining. The gel was run at 100 V for approximately 30 min. Fluorescent bands exposed to UV light were compared relative to a 2-log DNA ladder [21].

2.3. Polymerase chain reaction (PCR) amplification

PCR was performed in a final volume of 50 µl containing 4 µl of the supernatant, 25 µl of Maxima Hot Start PCR Master Mix, 1µl of forwarding primer (10 µM), FOF1 (5'- CCATCGTCAATCCCGACCAA- 3'), 1 µl of reverse primer (10 µM), FOR2 (5'-ACGACGCACTGATTGAGGTT-3') and 19 µl of DNase/RNase-free water. Primers were synthesized by Primer Synthesis Company, Eurofins Genomics, Eu. PCR was performed in an Eppendorf Master Cycler. The amplification program consisted of an initial denaturation step at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing for 30 sec at 55 °C, and extension for 30 sec at 72 °C. A final extension step at 72 °C for 7 min followed by 4 °C until gel loading. A 5 µl of PCR mixture was loaded on 1% agarose gel against 1 Kb plus ladder (Thermo) to examine the PCR product. The gel was run at 80 volts for approximately 45 min. Fluorescent bands exposed to UV transilluminator were compared relative to a 2-log DNA ladder.

2.4. Sequencing of the PCR products

The sequencing of the PCR products was made at GATC Company through Sigma Scientific Services Company, Cairo, Egypt, using the same primers used for PCR (FOF1: 5'-CCATCGTCAATCCCGACCAA-3'), (FOR2: 5'-ACGACGCACTGATTGAGGTT-3').

2.5. Isolation and identification of rhizosphere soil fungi of healthy pepper plants

Samples of rhizosphere soil were collected from the root system of healthy pepper plants from different localities in Assiut (13 samples), Behera, and Sohag (2 samples each), Egypt. Plant roots were carefully dugout, and the excess soil was gently shaken off, and only the soil which was adherent closely to the root system was used. Fungi were isolated on potato dextrose agar (PDA) by the dilution plate method [22]. Five replications were made for each sample, and the developing fungi were identified based on macroscopic and microscopic features following the keys [23] for *Fusarium* species [24] of fungi.

2.6. In vitro screening of the antagonistic effect of fungi isolated from the rhizosphere of pepper plants against the causal pathogens of wilt disease

The antagonistic capability of representative isolates of the identified fungal species recovered from rhizosphere soil of pepper plants was assessed using dual culture [25]. Petri dishes (9 cm diameter) containing ~ 20 ml PDA medium were inoculated with one disk (4 mm diam.) of the pathogen on one side of the plate and the other side of the plate, a disk of the tested fungal isolates on the opposite position. Plates inoculated with the pathogenic fungi only were used as the control. The radial growth of the test fungus was recorded after 7 days of incubation at 28 ± 1 ºC. Each assay was performed in triplicates, and the data were analyzed using one-way ANOVA and Duncan's multiple range tests (SPSS 18.0 software program). The percent inhibition was obtained using the following formula [26]:

Inhibition (%) = $(1-D_2/D_1) \times 100$

Where D_1 represents the diameter of the pathogen colony in control and D_2 represents the diameter of the pathogen colony in treatment.

2.7. Chitinase enzyme sources

Some highly fungal biocontrol agents that produce chitinases include *Botryotrichum atrogriseum* (AUMC 11415, 11416), *Clonostachys rosea* (AUMC 11442), *Albifimbria verrucaria* (AUMC 11414), *Penicillium oxalicum* (AUMC 11419), and *Trichoderma harzianum* (AUMC 11422, 11440, 11441).

2.8. Colorimetric assay of chitinase activity

Medium described by Anjanikumari and Panda [27]. The medium contains (g/l) $(NH4)_2SO_4$ 1.4, KH_2PO_4 2.0, NaH_2PO_4 6.9, $MgSO_4$. $7H_2O$ 0.3, colloidal chitin 1.0, peptone 10. Colloidal (acid swollen) chitin was prepared by adding: one gram of chitin (crab shell) to 10 ml of 85% orthophosphoric acid. The mixture was stirred to make agelatinous paste and stored at 0°C for 24 h. After that, an excess of tap water was added, and the gelatinous white material formed was separated by filtration through filter paper. The retained material was washed with tap water until the filtrate had a pH of 6.5. The colloidal chitin had a soft, pasty consistency and 90–95% moisture [28].

The medium was autoclaved at 121 °C for 15 min. One agar plug (4 mm diam.) from the growing edges of 3-day-old cultures was used for inoculation 250 ml conical flask containing 100 ml of chitin-containing medium. The cultures were incubated at 28 ºC on a rotary shaker at 120 rpm. After 6 days, the mycelia were removed from the culture medium by filtration, and the culture filtrate was used as a crude enzyme.

Spectrophotometric determination of chitinase activity was estimated according to Monreal and Reese [29]. The reaction mixture contained 1 ml of 1% colloidal chitin in phosphate buffer (pH 5) and 1 ml of culture filtrate. The blank was performed using distilled water instead of culture filtrate. After incubation at 37 °C for 1 h, the reaction arrested by adding 0.5 ml of DNS, then boiled for 15 min in a water bath and cooled to room temperature. The optical density was then measured at 540 nm against the blank, and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of chitinase activity is defined as the amount of enzyme that catalyzed 1.0 µ mol of glucose per minute during the hydrolysis reaction.

2.9. Titration assay of lipase

Lipase producing media consist of (g/l):KH_2PO_4 2.0, $(NH4)_2SO_4$ 1.4, $MgSO_4.7H_2O$ 0.3, $CaCl_2$ 0.3, Urea 0.3, trace element solution 1.0 ml, and supplemented with peptone, 0.25, olive oil, 20 ml and yeast extract, 5 mg. Trace element solution contained (g/l): $MnSO_4.H_2O$, 1.56, $FeSO_4.7H_2O$, 5.00, $ZnSO_4$, 1.67, $CoCl_2$, 2.00. Inoculated flasks were incubated under shaking conditions at 120 rpm at 28 ºC for 6 days. At the end of the incubation period, the culture broth was separated by filtrations, and culture filtrate was used as a crude enzyme [30].

The reaction mixture, consisting of 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, 1 ml of olive oil, and 0.5 ml of culture filtrate, was incubated at 40 ºC for 30 min. The enzyme reaction was terminated by adding 2 ml of acetone ethanol mixture (1:1 v/v). Total contents were titrated against 0.05 N sodium hydroxide using phenolphthalein as an indicator. One unit is defined as the amount of enzyme which liberates 1 µmol of free fatty acid per minute at 40 ºC [31] as follows

Fatty acid + NaOH ⟶ Na-Fatty acid + H_2O

The formula calculates 1 unit of lipase activity:

Lipase Activity (IU/ml) = [5.61x Vol. of NaOH consumed (ml) x Normality of NaOH]/ [Vol. of the crude enzyme (ml) × Reaction time (min)]

Where 5.61= unit constant for identifying an acid value

2.10. The potential of highly biocontrol fungal volatiles against the highly pathogenic strain

Volatile compounds were detected by the method reported by Shivapratap et al. [26]. Antagonistic strains and the pathogen were cultivated in PDA medium in separate plates, and then the plate with pathogen was placed over the plate with the antagonist, avoiding direct contact between the two, sharing only the air. Both plates were sealed from the bottom with Parafilm, and plates were incubated at 29 °C for 5 to 6 d.

2.11. Greenhouse experiments

Evaluation of the efficacy of different approaches of some fungal biocontrol agents against wilt disease of pepper was carried out in pot experiments in the greenhouse of Plant Pathology Department, Faculty of Agriculture, Assiut University. Seedlings of cv. Balady and *F. Oxysporum* f. sp. *capsici* (AUMC 11424) were used. The tested antagonistic fungi were *A. nidulans* (AUMC 11418, 11443), *A. niger* (AUMC 11421), *B. atrogriseum* (AUMC 11415, 11416), *C. rosea* (AUMC 11417, 11442), *Albifimbria verrucaria* (AUMC 11413, 11414), *P. oxalicum* (AUMC 11419, 11420) and *T. harzianum* (AUMC 11422, 11440, 11441). These antagonistic bioagents proved to be highly active against *F. oxysporum*f. sp. *capsici in vitro* conditions. One agar plug (4 mm diam) of the pathogen and the antagonistic fungus each were grown on sterilized barley medium (150 g barley + 50 g clean sand + 4 g glucose + 0.2 g yeast extract + 20 ml water) in 500-ml flasks and incubated at 28 °C for 15 days. Sterilized plastic pots (25 cm diam) were inoculated with the pathogenic fungus at the ratio of 3% of soil weight. After 10 days, pots were re-inoculated individually with inoculation of bioagents at the rate of 1.5% of soil weight.

Another set of soil inoculated only with the pathogen (3% w/w) was kept as a control. Three pepper seedlings were planted in each pot at the time of the planting, and three replicas were used for each particular treatment. The percentage of disease severity was recorded after 8 weeks from the sowing date, and the severity of wilt was determined for each treatment using the following scale. 0 = no disease, 1 = minor symptoms on a few leaves, 2 = minor dwarfing, 3 = significant dwarfing, yellowing, wilt, 4 = some shoots with severity and some shoots dead, and 5 = the death of the whole plant.

3. Results and discussion

3.1. PCR amplifications for *F. oxysporum* AUMC 11424 the most virulent strain tested

PCR amplifications analyses of *F. oxysporum* f. sp. *capsici* (Foc) isolate using the primers (FOF1: 5' CCATCGTCAATCCCGACCAA-3'), (FOR2: 5'- ACGACGCACTGATTGAGGTT-3') showed an amplicon of approximately 350 bp (Figure 1a). The sequence analysis was done blasted with sequence data of EF-1α gene collected from Gene bank (KT 318747.1) and gave 98% similarity [32] (Figure 1b, 1c).

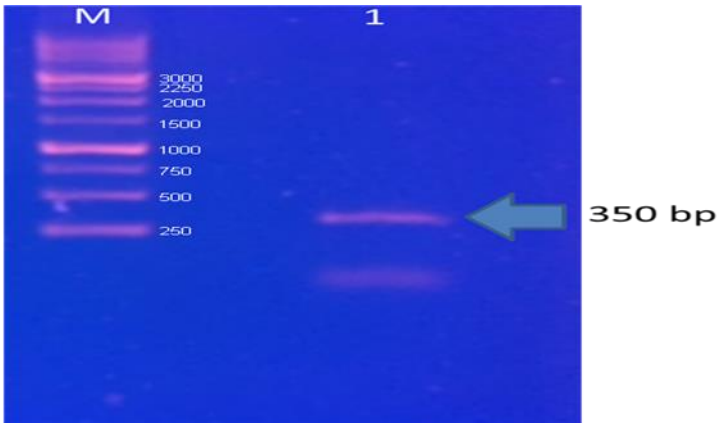


Figure 1a. Lane M- 1 kb DNA ladder, Lane 1 - PCR amplicons of around 350 bp obtained with *F. oxysporum* AUMC 11424

5GCCTAGTGCATTCTCAAGTCACATACTGACATCGTTTCACAGACCAGGTCACTTGATCTACCAGTGCGGTGGTATCG
ATAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATTTCCCCTACG
ACTCGAAACGTGCCCGCTACCCCGCTCGAGACCAAAAATTTTGCAATATGACTGTAATTTTTTTTGGTGGGGCACTTACCCC
GCCACTTGAGCGACGGGAGCGTTTGCCCTCTTAACCATTCTCACAACCTCAATCAGTGCGTCGTAGAGAGATGTGTCGTAG
AGAGATGTAAAAAAGGCGGGAAAAAAGAAAAAGGAAAAAGGAAAAAAGAAAAAAGAGAAAAAAAAGGAAAAAAAACACAAAA
TTAGTGCCCCT-3

Figure 1b. EF 1 alpha sequence analysis of *F. oxysporum* AUMC 11424

3.2. Diversity of rhizosphere fungi of the healthy pepper plant

Thirty species related to 16 genera were isolated from the rhizosphere soil of pepper plant collected from different localities in Assiut (26 species related to 14 genera), Behera (13 species, 7 genera), and Sohag Governorates (6 species, 5 genera). In Assiut Governorate (13 localities), the most comprehensive spectrum of species (12) was recorded from samples collected from Manfalout (Bani-Adi), and the lowest (3) was recorded in El- Fath plantations (Table 1). *Aspergillus* (10 species) gave the highest number of CFU among other genera. It accounted for 65.7% of the total fungi. It was recorded in all localities. *A. niger* (16.8% of total fungi), *A. flavus* (12.4%), followed by *A. terreus* (5.6%) were the most dominant species. The remaining *Aspergillus* species were isolated either from 4 localities: *A. nidulans* (10.2%), *A. candidus* (0.8%), 3 localities: *A. ochraceus* (19.1%), or one locality: *A. fumigatus* (0.1%, El-Koussia) and *A. chevalieri* (0.3%).

Fusarium (3 species) was recorded in eight localities, accounting for 6.7% of total fungi. *F. solani* was the most common species (found in 7 localities), followed by *F. oxysporum* (2 localities) and *F. vertcillioides* (one only). *Penicillium oxalicum* (from 7 localities), *Clonostachys rosea, Trichoderma harzianum* (each from 6 localities), and *Curvularia tsudae* (five localities) accounted for 4.5%, 7.8%, 4.5% and 3.4% of total fungi, respectively. Other fungi were less frequently recorded from either 3 localities: *Albifimbria verrucaria* (1.7%), *Cladosporium cladosporioides* (1%), two localities: *Botryotrichum atrogriseum* (0.9), *Epicoccum nigrum* (0.2), or one locality: *Chaetomium* sp. (0.3), *Humicola grisea* (0.1), *Mucor* sp. (0.2) and *Pleospora tarda* (0.1) (Table 1).

In the current work, 30 species related to 16 genera were isolated from the rhizosphere of pepper plants cultivated in different localities in Assiut, Behera, and Sohag Governorates. The genus *Aspergillus* gave the highest number of CFU among other genera. *A. niger* (20.2%), *A. ochraceus* (15.0%), *A. terreus* (11.1%) and *A. flavus* (11.1%) gave the highest CFU among species. The remaining fungi possessed low percentages of CFU and these were: *Curvularia tsudae* (2.8%), *Albifimbria verrucaria* (1.4%), *Botryotrichum atrogriseum* (0.9%), *Cladosporium cladosporioides* (0.8%), *Macrophomina phaseolina* (0.5%), *Chaetomium* sp. (0.2%), *Mucor* sp. (0.4), *Epicoccum nigrum* (0.2%), *Humicola grisea* (0.1%) and *Pleospora tarda* (0.1%). In this respect, [33] isolated four genera *Penicillium*, *Aspergillus*, *Trichoderma*, and *Candida* with *P. digitatum, P. expansum, T. harzianum, A. nidulans, A. niger, Penicillium* sp., *Candida albicans,* and *T. virens* from rhizosphere of pepper plants. Ajokpaniovo and Oyeyiola, [34] isolated also *Aspergillus flavus, A. candidus, A. niger, A. fumigatus, A. ustus, Rhizopus stolonifer, Penicillium expansum, P. citrinum, Fusarium avenaceum, F. oxysporum, F. poae, F. verticillioides, Mucor racemosus, M. mucedo,*

Verticillium lateritium, Trichophyton mentagrophytes and *Saccharomyces cerevisiae* from the rhizosphere of red pepper (*Capsicum frutescens*). Also, *Aspergillus flavus*, *A. niger*, *Chaetomium globosum*, *P. islandicum,* and *A. nidulans* were encountered most frequently in seeds of Brazilian black pepper [35]. *Penicillium*, *Paecilomyces,* and *Aspergillus* were the most dominant genera in the rhizosphere of black pepper in the Philippines [36].

Table 1. Colony-forming units (CFU/g soil) and frequency of occurrence of fungi recovered from the rhizosphere of pepper plants during the seasons of study in Assiut, Behera and Sohag Governorates.

Fungal taxa	Total CFU	CFU %	Frequency	Frequency %
Albifimbria verrucaria	680	1.4	4	23.5
Alternaria alternata	160	0.3	2	11.76
Aspergillus	29920	68.9	17	100
A. candidus	280	0.6	4	23.5
A. chevalieri	120	0.2	1	5.88
A. flavus	4840	11.1	16	94.1
A. fumigatus	80	0.2	2	11.7
A. nidulans	3760	8.6	6	35.2
A. niger	8800	20.2	14	82.3
A.quadrilineatus	40	0.1	1	5
A. ochraceus	6520	15.0	3	17.6
A. sydowii	240	0.5	2	11.7
A. terreus	5160	11.8	9	52.9
Aspergillus sp.	80	0.2	1	5.88
Botryotrichum atrogriseum	400	0.9	4	23.5
Chaetomium sp.	120	0.2	1	5.88
Cladosporium cladosporioides	360	0.8	3	17.6
Clonostachys rosea	2840	6.5	8	47.5
Curvularia tsudae	1200	2.8	5	29.4
Epicoccum nigrum	80	0.2	2	11.7
Fusarium	2600	6	9	52.9
F. oxysporum	120	0.2	3	17.6
F. solani	2360	5.4	8	47.5
F. vertcillioides	120	0.2	2	11.7
Humicola grisea	40	0.1	1	5
Macrophomina phaseolina	200	0.5	2	11.7
Mucor sp.	160	0.4	2	11.7
Penicillium	2080	4.8	10	58.8
P. oxalicum	2040	4.7	10	58.8
Penicillium sp.	40	0.1	1	5.88
Pleospora tarda	40	0.1	1	5.88
Trichoderma	1640	3.7	7	41.1
T. harzianum	1600	3.6	6	35.2
T. koningii	40	0.1	1	5.88
Sterile mycelium (white)	880	2	3	17.6
Total CFU	43400	100	17	100
Total No. of genera	16			
Total No. of species	30			

The frequency was calculated out of 17 samples

From Behera Governorate (represented by 2 localities). *Aspergillus* (6 species) gave the highest number of CFU among other genera. It accounted for 80.9% of the total fungi. *A. terreus* (63.4%), *A. niger* (9.4%), *A. flavus* (3.1%), and *A. nidulans* (3.1%) were recorded from two localities, while *A. fumigatus* (0.7%) and *A. quadrilineatus* (0.7%) were registered in one locality only. *Penicillium oxalicum* (4.1%), *Macrophomina phaseolina* (3.6%), *Botryotrichum atrogriseum* (1.8%) were recorded from 2 localities, while *F. solani* (4.1% of total fungi), *F. oxysporum* (1%), and *Trichoderma koningii* (0.5%) were registered each from one locality (Table 1).

From Sohag Governorate (2 localities): *Aspergillus* (2 species) gave the highest number of CFU, accounting for 85% of the total fungi. *A. niger* (75%) and *A. flavus* (10%) were recorded each in two localities. The remaining fungi were recorded from one locality, and these were *P. oxalicum* (7.5%), *C. rosea* (2.5%), *Albifimbria verrucaria* (2.5% of total fungi) and *Mucor sp.* (2.5%) (Table 1).

The present results indicated that most of the tested fungi (75 isolates) inhibited the colony growth of the pathogenic species *Macrophomina phaseolina*, *Fusarium oxysporum,* and *Fusarium solani* but with varying degrees, and these fungal

agents could be classified into four different groups according to their inhibition potential. The growth inhibition by antagonist fungi may be due to the non-volatile metabolites and production of antibiotic substances in the culture filtrate [37] or other inhibitory substances produced by the antagonists viridian, gliovirin, geodin, terricin, terric acid, aspergillic acid, and dermadin [38]. Notably, all tested isolates of *T. harzianum* have high antagonistic ability toward the three pathogens. It was also reported previously that *T. harzianum* could be used as potent bio fungicides to reduce the severity of major soil-borne fungal pathogens of colored pepper; moreover, they may also be applied biofertilizers to promote the growth and productivity of this crop [39]. *T. harzianum* and *T. viride* were potent biocontrol agents of many fungal pathogens [40].

3.3. In vitro screening of the antagonistic effect of fungi bioagents against the causal pathogens of wilt disease

Seventy-five isolates related to 10 genera and 17 species from pepper rhizosphere soil were screened on PDA against *Fusarium oxysporum* AUMC 11424 *F. solani* AUMC 11513 and *Macrophominaphaseolina* AUMC 11512 *in vitro* by dual cultural technique (Table 2). The antagonistic fungi are related to the genera *Aspergillus* (18 isolates), *Albifimbria* (19), *Botryotrichum* (2), *Chaetomium* (1), *Cladosporium* (1), *Fusarium* (5), *Clonostachys* (12), *Penicillium* (9), and *Trichoderma* (8) (Table 2).

The current results also showed that the best isolates showing antagonistic ability were related to *B. atrogriseum* AUMC 11415, *A. nidulans* AUMC 11418, *A. verrucaria* AUMC 11414, *C. rosea* AUMC 11442, and *T. harzianum* AUMC 11422 against *F. oxysporium* under greenhouse conditions which reduce the number of symptomatic plants by more than 90% over the control, whereas the lowest one was *P. oxalicum* AUMC11420 which reduce the number of symptomatic plants by 60.9%. Biological control of *Fusarium* wilt has been previously reported using *Penicillium* species (*P.oxalicum*), *Rhizoctonia*, *Trichoderma*, *Gliocladium* (*G. catenulatum*), and non-pathogenic *Fusarium* spp. [41]. Madbouly and Abd El-Backi [39] isolated *T. harzianum* and *T. viride* from the rhizosphere soil of pepper and used them as antagonists to control *Rhizoctonia solani*, *Fusarium solani*, and *Macrophomina phaseolina in vitro* and *in vivo*. Dwivedi and Enespa [42] showed that the antifungal activities of *Aspergillus* species, *Penicillium* and *Trichoderma*, play an essential role in controlling soil-borne fungal pathogens *F. solani* and F. *oxysporum* f. sp. *lycopersici.*

Table 2. Percentage inhibition indicating high, moderate, low or no antagonistic potential of fungal strains against the growth of *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium solani in vitro*.

Fungal strain (AUMC No.)	AUMC No.	*F. oxysporum* AUMC 11424	*F. solani* AUMC 11513	*M. phaseolina* AUMC 11512
			% inhibition	
Highly antagonistic fungi				
Trichoderma *harzianum*	AUMC11945	61.1	50.95	50
T. harzianum	AUMC 11946	45.3	42.9	75
T. harzianum	AUMC 11947	60.2	40.35	75
T. harzianum	AUMC 11422	67.5	74.95	87.5
T. harzianum	AUMC 11440	77.7	74.95	80
T. harzianum	AUMC 11948	67.5	62.35	75
T. harzianum	AUMC 11441	65.1	68.45	75
T. harzianum	AUMC 11949	60.3	57.9	75
Moderately antagonistic fungi				
Penicillium *oxalicum*	AUMC 11953	29.9	25.85	30
P. oxalicum	AUMC 11954	23.6	29.1	21.8
P. oxalicum	AUMC11955	29.15	14.2	27.2
P. oxalicum	AUMC 11956	33.1	10.85	25
P. oxalicum	AUMC 11420	37.4	37.05	37.5
P. oxalicum	AUMC 11419	37.4	37.05	43.75
P. oxalicum	AUMC 11957	35.8	36.2	37.5
P. oxalicum	AUMC 11958	27.9	28.3	30
Albifimbria *verrucaria*	AUMC11413	**18.3**	**15**	**21.7**
A. verrucaria	AUMC 11951	20	14.1	32.3
A. verrucaria	AUMC 11414	**19.15**	**25**	**20**
A. verrucaria	AUMC 11952	20	14.1	32.3
Clonostachys *rosea*	AUMC 11442	**34.2**	**16.3**	**30.8**
C. rosea	AUMC 11417	**32.8**	**28.9**	**35.7**
Aspergillus *nidulans*	AUMC 11418	26.3	25	29.1
A. nidulans	AUMC 11443	10.85	22.1	27.2
Botryotrichum *atrogriseum*	AUMC 11950	26.3	22.1	25.8
Low or no antagonism on one or two of pathogenic fungi				
Aspergillus *candidus*	AUMC11959	0	1.7	1.7
A. fumigatus	AUMC 11960	0	1.7	0

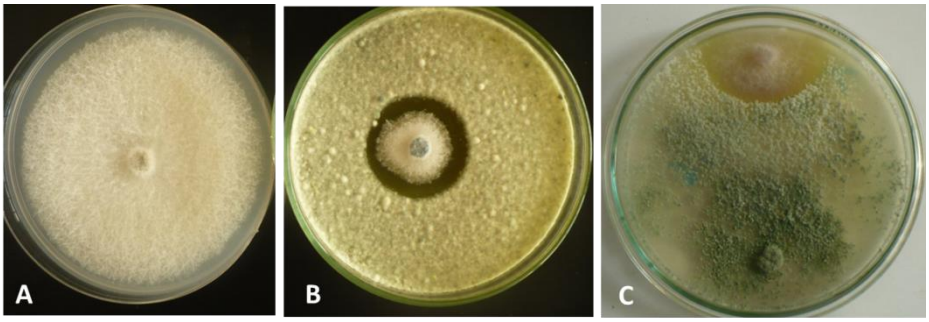
Table 2. Percentage inhibition indicating high, moderate, low or no antagonistic potential of fungal strains against the growth of *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium solani in vitro*.

Fungal strain (AUMC No.)	AUMC No.	*F. oxysporum* AUMC 11424	*F. solani* AUMC 11513	*M. phaseolina* AUMC 11512
			% inhibition	
A. niger	AUMC 11961	9.1	7.1	13.3
A. niger	AUMC 11962	1.4	3.6	7.7
A. nidulans	AUMC 11963	1.5	0	4.6
A. nidulans	AUMC 11964	7.7	1.7	9.2
A. nidulans	AUMC 11965	2.2	0	4.6
A. nidulans	AUMC 11966	0.7	0.85	3.1
A. nidulans	AUMC 11967	0	13.5	3.6
A. sydowii	AUMC 11968	0	1.7	0
A. terreus	AUMC 11969	1.4	4.1	11.1
A. terreus	AUMC 11970	4.25	4.1	9.1
***Botryotrichum** atrogriseum*	AUMC11971	16.1	16.3	10
B. atrogriseum	AUMC 11972	16.1	16.3	10
***Chaetomium** globosum*	AUMC 11973	10.9	7.1	10
***Fusarium** oxysporum*	AUMC 11974	0	1.7	2.3
F. oxysporum	AUMC 11975	3.5	1.7	3.5
F. solani	AUMC 11976	0	0	2.3
Clonostachys rosea	AUMC 11977	14.9	11.7	10.5
C. rosea	AUMC 11978	4.3	19.2	3.5
C. rosea	AUMC 11979	1.3	9.15	5.2
C. rosea	AUMC 11980	12.3	1.6	10.8
C. rosea	AUMC 11981	13.05	12.4	15.4
C. rosea	AUMC 11982	11.5	10.8	15.4
C. rosea	AUMC 11983	2.3	0.85	6.1
C. rosea	AUMC 11984	13.8	8.45	15.3
C. rosea	AUMC 11985	15.4	0.85	20
C. rosea	AUMC 11986	15.4	1.7	20
Albifimbriaverrucaria	AUMC 11987	17.8	7.7	14.3
A. verrucaria	AUMC 11988	17.8	7.7	14.3
A. verrucaria	AUMC 11989	20.8	6.6	20
A. verrucaria	AUMC 11990	12.5	7.4	15
A. verrucaria	AUMC 11991	9.1	6.7	10
A. verrucaria	AUMC 11992	12.5	5	16.7
A. verrucaria	AUMC 11993	10.8	7.5	16.7
A. verrucaria	AUMC 11994	12.5	4.15	13.5
A. verrucaria	AUMC 11995	6.6	1.6	8.3
A. verrucaria	AUMC 11996	4.15	0.8	8.3
A. verrucaria	AUMC 11997	12.5	5.8	10
A. verrucaria	AUMC 11998	5	6.6	8.3
A. verrucaria	AUMC 11999	6.7	19.2	5
A. verrucaria	AUMC 12000	4.1	4.1	3.3
A. verrucaria	AUMC 14201	0	5	1.6
A. verrucaria	AUMC 14203	9.2	10.8	1.6
A. verrucaria	AUMC 14204	11.6	9.15	13.3
Penicillium oxalicum	AUMC14205	4.9	4.15	12

7 Isolates showing no antagonistic effect against the three pathogens were omitted from the Table.
L.S.D 0.05%, A bioagents: 5.6, B isolates: 6.667

The results indicated that most of the tested fungi inhibited the colony growth of *F. oxysporum, F. solani*, and *Macrophomina phaseolina* but with varying degrees. Notably, the 8 tested isolates of *T. harzianum* have high antagonistic ability toward the three pathogens with percentages inhibition ranging from 45.3-77.7% in the case of *F. oxysporum*, 40.35- 74.95% in the case of *F. solani*, and from 50-87.5% in case of *Macrophomina phaseolina*. The moderate inhibition rate was registered for *P. oxalicum* (8 isolates in case of the three pathogens ranging from 10.85-43.75%) followed by *C. rosea* (2 isolates, from 16.3-35.7%), *A. verrucaria* (4 isolates, ranging from 14.1-32.3%), *A. nidulans* (2 isolates, from 10.85-29.1%) and *B. atrogriseum* (1 isolate, from 22.1-26.3%) (Table 2).

The low or no antagonism on one or two of the pathogenic fungi was found in isolates of *A. candidus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. sydowii*, *A. terreus*, *Botryotrichum atrogriseum*, *Chaetomium globosum*, *F. oxysporum*, *F. solani*, *Clonostachys rosea*, *Albifimbria verrucaria* and *Penicillium oxalicum* (Table 2). Some isolates of fungi tested had no antagonistic effect on any of the three pathogens, such as *A. fumigatus*, *A. sydowii*, *C. cladosporioides*, *F. solani*, *F. vertcillioides* (1 isolate each), and *A. ochraceus* (2 isolates).



A. control **B.** Antibiosis **C**. Hyperparasitism

Figure 2. The antagonistic effect of *Trichoderma harzianum* AUMC11440 against *F. oxysporum* f. sp. *capsici* AUMC 11424

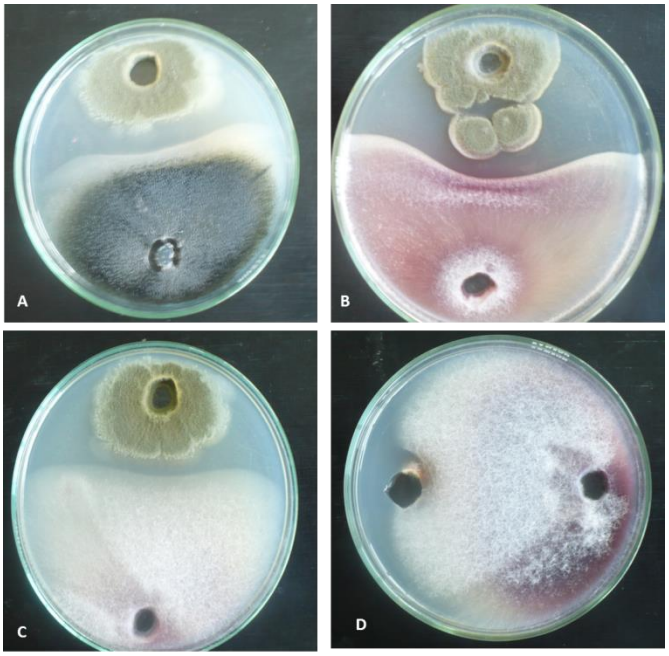


Figure 3. The antagonistic effect of *P. oxalicum* AUMC 11419 on the growth of **A**. *Macrophomina phaseolina* AUMC 11512, **B.** *F. oxysporum* f. sp. *capsici* AUMC 11424, **C**. *F. solani* AUMC 11513, **D**. control.

3.4. Chitinase and lipase activities of some antagonistic isolates isolated from the rhizosphere of pepper plants

Results in Table 3 indicated that *T. harzianum* (3 isolates) showed the highest chitinase activity in the range of 2.5-3.3 IU/ml, followed by *C. rosea* (1.6 IU/ml). The remaining isolates tested produced the low chitinase amounts in the range of 0.2-0.5 IU/ml). On the other hand, *P. oxalicum* AUMC 11419 presented the highest lipase activity of 1.01 IU/ml followed by *B. atrogriseum* (2 isolates, 0.78, 0.84 IU/ml) and *C. rosea* (0.84 IU/ml). In comparison, the remaining 4 fungal isolates were relatively low in their lipase abilities (approximately 0.6 IU/ml).

The results revealed that *T. harzianum* (3 isolates) showed the highest chitinase activity in a range of 2.5-3.3 IU/ml, followed by *Clonostachys rosea* (1.6 IU/ml). The remaining isolates tested produced low chitinase activity in a range of 0.2-0.5 IU/ml. Also, the results showed that *P. oxalicum* AUMC 11419 presented a maximum lipase activity of 1.01 IU/ml followed by

Botrytrichum atrogriseum (2 isolates, 0.78, 0.84 IU/ml) and *Clonostachys rosea* (0.84 IU/ml) while the remaining fungi were relatively low in their lipase abilities (approximately 0.6 IU/ml).

Table 3. Chitinase and lipase production by some fungal bioagents

Fungal taxa	AUMC No.	Chitinase (IU/mL)	Lipase (IU/ml)
Albifimbria verrucaria	11414	0.5	0.61
Botryotrichum atrogriseum	11415	0.4	0.84
B. atrogriseum	11416	0.5	0.78
Clonostachys rosea	11442	1.6	0.84
Penicillium oxalicum	11419	0.2	1.01
Trichoderma harzianum	11422	2.6	0.67
T. harzianum	11440	3.3	0.67
T. harzianum	11441	2.5	0.61

3.5. Effect of volatile compounds of some fungi

The volatile metabolites assay revealed that *Trichoderma* strains produced the highest inhibitory effect on the growth of the highly pathogenic strain of *F. oxysporum* f. sp. *capsici* AUMC11424 in the range of 31.1-34.4%, followed by *Botryotrichum atrogriseum* in the range of 26.6-31.1% while *Albifimbria verrucaria* (2 strains, 15-5.-17.7), *Penicillium oxalicum* (2 strains, 13.3-16.7) and *Clonostachys rosea* (2 strains, 11.1-13.3), had lower inhibitory effects (Table 4 and Figure 4).

Table 4. Radial growth of *F.oxysporum* f. sp. *capsici* AUMC 11424 exposed to volatile compounds from some fungal bioagents.

Fungal Bioagent	AUMC No.	% Growth inhibition of the pathogen
Albifimbria verrucaria	11413	15.5
A. verrucaria	11414	17.7
Botryotrichum atrogriseum	11415	31.1
B. atrogriseum	11416	26.6
Clonostachys rosea	11417	11.1
C. rosea	11442	13.3
Penicillium oxalicum	11419	13.3
P. oxalicum	11420	16.7
Trichoderma harzianum	11422	34.4
T. harzianum	11440	34.4
T. harzianum	11441	31.1

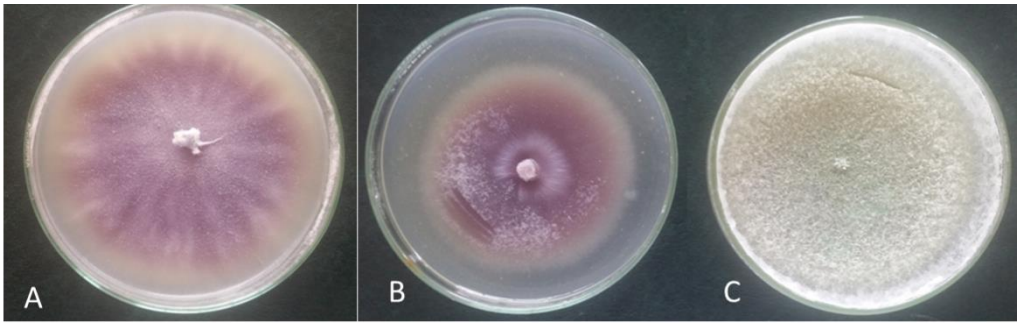


Figure 4. Radial growth of *F. oxysporum* f. sp. *Capsici* **A**. control (Pathogen culture), B. the pathogen exposed to volatile compounds from *T. harzianum* AUMC 11422, and **C**. *T. harzianum* AUMC 11422 from which volatile compounds were originated.

3.6. Antagonistic effect of some fungal bioagents against the causal agent of wilt disease *F. oxysporum* f. sp. *capsici* AUMC 11424 under greenhouse conditions

Fourteen strains of *A. nidulans* (2 isolates), *A. niger* (1 isolate), *Botryotrichum atrogriseum* (2 isolates), *C. rosea* (2 isolates), *A. verrucaria* (2 isolates), *P. oxalicum* (2 isolates), and *T. harzianum* (3 isolates) were tested for their antagonistic

ability to inhibit the pepper wilt pathogen under greenhouse conditions. The isolates *B. atrogriseum* AUMC 11415, *A. nidulans* AUMC 11418, *A. verrucaria* AUMC 11414, *C. rosea* AUMC 11442, and *T. harzianum* AUMC 11422 reduced the number of symptomatic plants by more than 90% over the control. The isolate *P. oxalicum* AUMC 11420 was the lowest in reducing the number of symptomatic plants by only 60.9% (Table 5, Figure 5).

T. harzianum induced chitinase to degrade the cell wall of the tested plant pathogen [43]. Also, it was reported that *T. harzianum* could produce lytic enzymes: β-1, 3-glucanases, chitinases, proteases, xylanases, and lipases [44], antifungal antibiotics [45] and can also be competitors of fungal pathogens [46] and promote plant growth [47]. The aggressive strategies used by *C. rosea* are myco- parasitism, production of chitanase and glucanase enzymes, *Albifimbria verrucaria* [48], *Penicillium oxalicum* [49], and *Gliocladium rosea* [50] were able also to produce lipase.

Table 5. Antagonistic effect of some fungal bioagents against the severity of wilt disease caused by *F. oxysporum* f. sp. *capsici* AUMC 11424 under greenhouse conditions.

Fungal taxa	AUMC No.	Disease Severity	% inhibition rates
Albifimbria verrucaria	11414	6.7 ± 2.8^{ab}	90.1
A. verrucaria	11413	10 ± 0^{abc}	85.3
Aspergillus nidulans	11418	6.7 ± 3^{ab}	90.1
A. nidulans	11443	18.3 ± 2.8^{cde}	73.2
A. niger	11421	18.3 ± 7.6^{cde}	73.2
Botryotrichum atrogriseum	11416	16.7 ± 11.5^{bcd}	75
B. atrogriseum	11415	6.7 ± 3^{ab}	90.1
Clonostachys rosea	11442	3.3 ± 1.9^{a}	95.1
C. rosea	11417	21.7 ± 7.6^{de}	68.5
Penicillium oxalicum	11420	26.7 ± 5.7^{e}	60.9
P. oxalicum	11419	15 ± 5^{bcd}	78
Trichoderma harzianum	11422	8.3 ± 2.8^{abc}	95
T. harzianum	11440	13.3 ± 5.7^{bcd}	80.5
T. harzianum	11441	13.3 ± 5.7^{bcd}	80.5
Control		68.3 ± 2.8^{f}	-

One way ANOVA was performed, with different letters, indicates no significant difference between each other ($p \geq 0.05$) according to the Duncan test

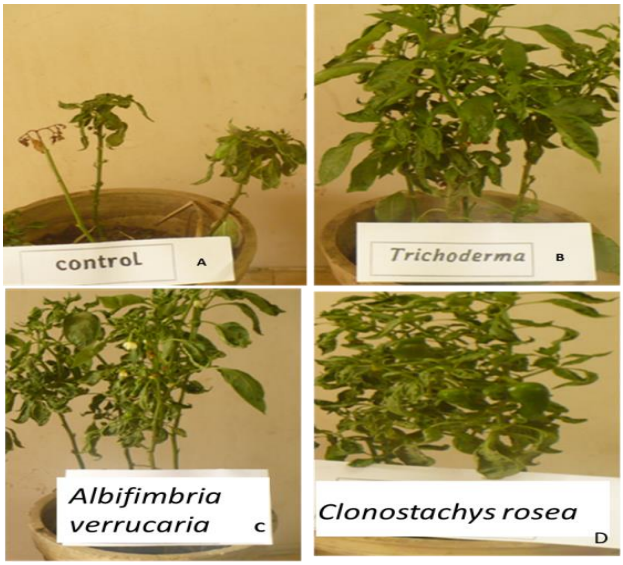


Figure 5. Effect of some bioagents fungi on the wilt disease severity caused by *F. oxysporum* f. sp. *capsici* under greenhouse conditions: Control (the pathogen infesting soil alone) (**A**), soil treated with the bioagent fungus *T. harzianum* (**B**), *Albifimbria verrucaria* (**C**) and *Clonostachys rosea* (**D**).

Conclusion

Results of screening thirty isolates from pepper-rhizospheric fungi related to 16 species against the highest pathogenic fungi *M.phaseolina*, *F. oxysporum*, and *F. solaniin vitro* showed high inhibition to all isolates of *T. harzianum* followed by *P.*

oxalicum AUMC 11419, AUMC 11420, and *C. rosea* AUMC 11417, AUMC 11442 on the three pathogens. The results also showed that the best isolates showing antagonistic ability against *F. oxysporum* under greenhouse conditions were related to *B. atrogriseum* AUMC 11415, *A. nidulans* AUMC 11418, *A. verrucaria* AUMC 11414, *C. rosea* AUMC 11442, and *T. harzianum* AUMC 11422, which reduce the number of symptomatic plants by more than 90% over the control. The results revealed that *T. harzianum* (3 isolates) showed the highest chitinase activity in a range of 2.5-3.3 IU/ml, and *P. oxalicum* AUMC 11419 presented a maximum lipase activity of 1.01 IU/ml. Also, the volatile metabolites assay revealed that *Trichoderma* strains produced the highest inhibitory effect against *F. oxysporum* f. sp. *capsici* AUMC11424 in the range of 31.1-34.4%.

Conflicts of interest. There is no conflict of interest.

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