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In Vitro, Biological Control of *Fusarium Oxysporum* F. Sp. *Lycopersici* and *Rhizoctonia Solani* by Essential Oils and *Trichoderma Harzianum*

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Abstract Biological control of *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani*, the causal agents of wilt and damping-off diseases of tomato, respectively, by using commercial essential oils and *Trichoderma harzianum* was evaluated. *In vitro*, after screening of eighteen commercial essential oils, it was found that clove (*Syzygium aromaticum*) and lemongrass (*Cympopogon citratus*) essential oils gave the highest inhibitory effect on mycelial linear growth and mycelial dry weight of the tested phytopathogens. In addition, *Trichoderma harzianum* when used as an antagonistic fungus showed a significant reduction of mycelial growth of the tested phytopathogens after 6 days of growth.

Introduction

Fusarium oxysporum f. sp. *lycopersici* and *Rhizoctonia solani* are major pathogens of both greenhouse and field grown tomatoes (*Lycopersicon esculentum* Mill.) in Egypt and in the warm vegetable growing areas of the world. Fungicidal application as seed or soil treatment, however, has been found to be ineffective against these pathogens as the propagules are capriciously distributed in the soil and often beyond the reach of chemicals (Campbell., 1989). In addition to that, chemical fungicides are carcinogenic, with high and acute toxicity of the environment, not easily degradable, and have bad effects on useful soil microorganisms. However, alternative methods for the control of phytopathogens are needed. Biological control, therefore, holds promise as a strategy for disease management and it is environment friendly too. Biological control defined as "the use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms and to favor desirable organisms such as crops beneficial insects and microorganisms (Gnanamanickam., 2002).

Recently there has been a considerable concern in discovering plant-derived anti-microbial agents (Sagdic, *et al*., 2003) for alternative application in the strategy of preventing bacterial and fungal growth (Lanciotti, *et al*., 2004). Essential oils used as alternatives for anti-bacterial and anti-fungal treatments (Jenny 2000 and Michael., 2000). Essential oils, which are generally recognized as safe, have been investigated in the last decade as alternative control measures against many preharvest and postharvest plant pathogens (Plaza, *et al*., 2004).

Furthermore, (Juglal, *et al*., 2002) studied the effectiveness of essential oils to

control the growth of mycotoxins producing moulds and observed that clove, cinnamon and oregano essential oils were able to prevent the growth of *Aspergillus parasiticus* and *Fusarium moniliforme*. Lemongrass (*Cympopogon citratus*) essential oil, characterized by high concentrations of citral (3,7-dimethyl-2,6-octadienal), was shown to exhibit *in vitro* antifungal activity against *Colletotrichum coccodes, Botrytis cinerea, Cladosporium herbarum, Rhizopus stolonifer* and *Aspergillus niger* (Tsortzakis and Economakis., 2007).

Antagonistic microorganisms including fungi, bacteria, viruses, insects, or any organism (other than the damaged host or the pathogen causing the disease) may have the potential to be used as agents mediating biological control (Compant, *et al*., 2005). *Trichoderma* spp. have been studied as biological control agents against soil-borne plant pathogenic fungi (Khalaf, 1993; Chet and Inbar, 1994; Padmodaya and Reddy, 1996). Isolates of *Trichoderma harzianum* can produce lytic enzymes (Haran *et al.,* 1996) and antifungal antibiotics (Dennis and Webster, 1971, Brewer *et al.*, 1987 and Almassi *et al*., 1991) and they can also be competitors of fungal pathogens (Whipps., 1987), and promote plant growth (Inbar *et al*., 1994).

The aim of this research was to test *in vitro* the effect of 18 commercial essential oils and *Trichoderma harzianum* as an antagonistic fungus on mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani.*

Materials and Methods

Inoculum preparation

Pathogenic isolates of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and *Rhizoctonia solani* (RS) were isolated from diseased tomato plant with wilt and root rot symptoms from Dakahlia Governorate, Egypt. Isolation of the pathogens was conducted from roots of wilted and root rotted tomato plants at different stages of plant growth. Specimens of diseased tomato plants showing typical symptoms of wilt and damping-off diseases were washed carefully under tap water to remove the adhering soil particles. The washed roots were cut into small pieces and divided into two groups. The first one was surface sterilized by immersing the root pieces in 1% sodium hypochlorite solution for 5 min and then washed several times in sterilized distilled water to remove any residues of sodium hypochlorite. The second group was left without sterilization in order to isolate the surface organisms.

The washed root pieces were dried between two sterilized filter paper, then transferred onto potato dextrose agar (PDA) amended with rose Bengal (0.003 %) and streptomycin sulfate (0.01 %) in Petri dishes and incubated at 25±2^{O}C for 4-7 days. The growing fungi were individually transferred onto PDA medium. Pure cultures of fungi were obtained using single spore or hyphal tip technique suggested by Dhingra and Sinclair (1985). The fungal isolates were then identified according to Clements and Shear, (1957) and Booth, (1977). Pure cultures of the isolated fungi were transferred onto PDA slants and kept in refrigerator at 4^{o}C for further uses.

Screening of different commercial essential oils for their antifungal activities against the selected phytopathogenic fungi

Eighteen essential oils (Table1) *viz*; anise, chamomile, cumin, dill, radish, rosemary, fennel, black seed, marjoram, cinnamon, garlic, rocket, thyme, eucalyptus, lavender, sweet almond, lemon grass and clove essential oil were obtained from Badawyia company, Mansoura, Egypt. These essential oils were examined for their inhibitory activities against the selected phytopathogenic fungi by using agar diffusion technique (Deans and Ritchie., 1987). The most active commercial essential oils were selected for further studies on the fungal growth as mycelial dry weight and mycelial linear growth of the examined phytopathogens.

Effect of essential oils on mycelial linear growth

PDA media, after autoclaving were amended with 0.5% of Tween-80 (v/v) to enhance oil solubility, were prepared as 100 ml aliquots in 250 ml Erlenmeyer flasks with

different concentrations of essential oils (0.25%, 0.50%, 0.75%).The medium was poured in Petri dishes and left for solidification (Hammer *et al* .1999) . Mycelial discs were cut out from the growing edges of the tested fungal colonies and transferred separately to those plates. Control plates without essential oils were used. Triplicate dishes were used for each treatment. All dishes were incubated at 26±2°C. Average of radial growth was recorded after 7 days in case of FOL and after 4 days in case of RS and compared with the control.

Table 1: Common and scientific names of plants produced essential oils used in this study.

No	Scientific name	Common name	Family
1	*Allium sativum* L.	garlic	Alliaceae
2	*Anethum graveolens* L.	dill	Apiaceae
3	*Cinnamomum zeylanicum* Plume.	cinnamon	Lauraceae
4	*Cuminum cyminum* L.	cumin	Apiaceae
5	*Cympopogon citrates* L.	lemongrass	Poaceae
6	*Eruca sativa* Mill.	salad rocket	Brassicaceae
7	*Eucalyptus rostrata* Schlecht.	eucalyptus	Myrtaceae
8	*Foeniculum vulgare* Mill.	fennel	Apiaceae
9	*Lavandula angustifolia* L.	lavender	Lamiaceae
10	*Matricaria chamomilla* L.	chamomile	Asteraceae
11	*Nigella sativa* L.	black cumin	Rununculaceae
12	*Origanum majorana* L.	marjoram	Lamiaceae
13	*Pimpinella anisum* L.	anise	Apiaceae
14	*Prunus amygdalus* L.	sweet almond	Rosaceae
15	*Raphanus sativus* L.	radish	Brassicaceae
16	*Rosmarinus officinalis* L.	rosemary	Lamiaceae
17	*Syzygium aromaticum* L.	clove	Myrtaceae
18	*Thymus vulgaris* L.	thyme	Lamiaceae

Effect of essential oils on mycelial dry weight

Fifty ml of liquid potato dextrose medium in 250 ml Erlenmeyer flasks were amended with different concentrations of commercial essential oils (0.25%, 0.50%, and 0.75%) after autoclaving. Each flask was inoculated using two discs of fungal culture (0.5 cm diameter) and then incubated at 26±2°C for seven days. Control flasks do not contain essential oil concentrations. Triplicate flasks were used for each treatment. At the end of incubation period, the mycelium was filtered off and washed several times with distilled water, then dried in oven at 80°C till constant weight (El-Morsy., 1993).

The antagonistic effect of Trichoderma harzianum against the selected phytopathogenic fungi

The antagonistic effect of *Trichoderma harzianum* (TH) was investigated using dual culture technique (Baker and Cook., 1974). The antagonistic fungus and the two pathogens were grown on PDA for 5-7 days at (25±2°C). The antagonistic effect of the used antagonist on the pathogens was done using one disc (0.5cm in diameter) of the antagonist facing one disc of the pathogen on the PDA surface relatively closed to the periphery of the plates. The untreated control treatment was done on the same medium in Petri dishes by growing one disc of the pathogenic fungus in the same place but without antagonistic disc. Three replicates were used. All plates were incubated at 25±2°C for 7 days for FOL and 4 days for RS. After inoculation, the diameter average of zones of the pathogenic fungus was recorded after 2, 4, and 6 days.

Results

Inhibitory activities of commercial essential oils against the selected phytopathogenic fungi

Eighteen commercial plant essential oils were screened for their inhibitory activities.

The essential oil of clove and lemongrass showed the greatest inhibitory activity against FOL followed by cinnamon then rocket. The rest of essential oils have no inhibitory effect against FOL (Table 2). The essential oil of lemongrass showed the highest inhibitory activity followed by clove oil then eucalyptus oil, cinnamon, black seed, rocket, thyme and lavender oil. The rest of essential oils have no inhibitory effect on RS (Table 3).

Table 2: The inhibitory activities of the commercial essential oils against *Fusarium oxysporum* f. sp. *lycopersici* (FOL)

Essential oil	FOL (inhibition zone, cm)
1. Anis	0 d
2. Chamomile	0 d
3. Cumin	0 d
4. Dill	0 d
5. Radish	0 d
6. Rosemary	0 d
7. Shaman	0 d
8. Black seed	0 d
9. Marjoram	0 d
10. Cinnamon	2 b
11. Garlic	0 d
12. Rocket	0.25 c
13. Thyme	0 d
14. Eucalyptus	0 d
15. Lavender	0 d
16. Sweet almond	0 d
17. Citronella	7.5 a
18. Clove	7.5 a
19. Cont.	0 d
LSD 5%	0.22

The recorded values are the mean values of 3 replicates

Table 3: The inhibitory activities of the commercial essential oils against *Rhizoctonia solani* (RS)

Essential oil	RS (mycelial growth, cm)
1.Anis	7.5 a
3.Chamomile	7.5 a
4.Cumin	7.5 a
5.Dill	7.5 a
6.Radish	7.5 a
7.Rosemary	7.5 a
8.Shaman	7.5 a
9.Black seed	6 c
10. Marjoram	7.5 a
11. Cinnamon	5.5 d
12. Garlic	7.5 a
13. Rocket	7 b
14. Thyme	7 b
15. Eucalyptus	4.5 e
16. Lavender	7 b
17. Sweet almond	7.5 a
18. Citronella	0 g

19. Clove	4 f
20. Cont.	7.5 a
LSD 5%	0.51

The recorded values are the mean values of 3 replicates

Effect of clove and lemongrass essential oils on mycelial linear growth

Antifungal activity of clove and lemongrass essential oils was used against the mycelial linear growth of FOL and RS. In case of clove oil, the mycelial linear growth of FOL was completely inhibited at 0.50% and that of RS reached to the minimum growth at 0.75%. On the other hand, lemongrass oil possessed the highest reduction of mycelial linear growth of FOL at 0.75% and the same concentration, was completely inhibited the mycelial linear growth of RS (Table 4).

Table 4: Effect of clove and lemongrass essential oils on mycelial linear growth of *F. oxysporum* f. sp. *lycopersici* (FOL) and *R. solani*(RS)

Treatments	Lemon grass		Clove	
	F.oxysporum(cm)	*R.solani*(cm)	*F.oxysporum*(cm)	*R.solani*(cm)
Control	7.50 a	8.00 a	8.50a	8.50 a
0.25%	6.78 b	8.00 a	1.1b	0.95 b
0.50%	5.92 c	3.79 b	0.00c	0.95 b
0.75%	5.13 d	0.00 c	0.00c	0.90 b
L.S.D $_{at\ 5\%}$	0.32	0.20	0.19	0.57

Effect of clove and lemongrass essential oils on mycelial dry weight

Both clove and lemongrass essential oils gave the highest reduction of mycelial dry weight of both FOL and RS at the concentration of 0.75% (Table 5).

Table 5: Effect of clove and lemongrass essential oils on mycelial dry weight of *F. oxysporum* f. sp. *lycopersici* (FOL) and *R. solani* (RS)

Treatments	Lemon grass		Clove	
	F.oxysporum(gm)	R.*solani*(gm)	*F.oxysporum*(gm)	*R.solani*(gm)
Control	0.82 a	0.97 a	0.82 a	0.97 a
0.25%	0.70 b	0.57 b	0.62 b	0.56 b
0.50%	0.68 bc	0.51 b	0.63 b	0.56 b
0.75%	0.62 c	0.48 b	0.61 b	0.54 b
L.S.D $_{at\ 5\%}$	0.06	0.10	0.05	0.07

The antagonistic effect of TH against FOL and RS on solid medium (dual culture test)

Data in table 6 showed that, in single culture plates, the mycelial linear growth of TH, FOL and RS increased by the time after inoculation. It was noticed that, the mycelial linear growth of TH was faster than that of FOL and RS. In dual culture plates, a great inhibition of FOL and RS growth was occurred after 6 days from incubation when compared with control and TH overgrow the both tested fungi.

Table 6: Antagonistic effect of *T. harzianum* (TH) against *F. oxysporum* f. sp. *lycopersici* (FOL) and *R. solani* (RS)

Treatments	Mycelial linear growth (cm) after		
	2 days	4 days	6 days
FOL	2.1 e	2.9 e	4.2 d
RS	4.16 b	6.3 b	8 a
TH	5.3 a	7.3 a	8 a
TH x FOL	5.3 a	5.2 c	6.2 c
	1.9 f	2.3 f	0.8 e
TH × RS	4.3 c	4.5 d	6.9 b
	3.1 d	2.8 e	0.3 f
L.S.D $_{at\ 5\%}$	0.11	0.12	0.54

Discussion

Biological control of soil-borne plant pathogens is a potential alternative strategy to agrochemicals that are harmful to human health and the environment **(**Sasirekha, *et al*., 2012; Rivera, *et al*., 2013).). Thus, the present study was planned to replace the undesirable and unsafe chemical control by another effective, inexpensive and safe options for control of *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani*, the causal agents of wilt and root rot diseases of tomato, respectively. The present study showed that which were isolated from infected tomato roots identified as the causal agents of wilt and damping-off diseases of tomato, respectively. These results are in agreement with those obtained by Padmodaya and Reddy, (1996).

Essential oils are volatile aromatic concentrated hydrophobic oily liquids which are obtained from various plant parts such as flowers, buds, seeds, leaves, twigs, bark, woods, fruits and roots. Essential oils are usually terpenoids responsible for the aroma and flavor associated with herbs, spices and perfumes, also called volatile oils because they easily diffuse into the air. The main constituents of essential oils are mono and sesquiterpenes including carbohydrates, phenols, alcohols, ethers, aldehydes and ketones responsible for the biological activity as well as for their fragrance. Phenolic compounds present in essential oils have also been recognized as antimicrobial bioactive components (Sumonrat, *et al.*, 2008).

The screening of antifungal activity of eighteen plant essential oils against FOL and RS Showed that some of these oils exhibited antifungal activities against the tested phytopathogenic fungi. Among the essential oils tested, the clove and lemongrass oils proved to be the most highest growth inhibitors against these fungi. These results are in agreement with those of Wilson,*et al.* (1997) who tested the antifungal activity of 49 plant essential oils against *Botrytis cinerea*, they found that clove oil demonstrated the most antifungal activity against this fungus. Velluti, *et al*. (2003) found that the essential oils of cinnamon, clove, oregano, palmarose, and lemongrass inhibited the growth of *Fusarium proliferatum* but clove oil was the most effective in decreasing growth of *F. proliferatum*. Also, McMaster, *et al*. (2013) tested fourteen essential oils *in vitro* bioassays to determine optimum concentrations which can inhibit growth or kill pathogen mycelium. Three essential oils (thyme, clove bud and origanum) compounds showed broad-spectrum and dose-dependant inhibitory and/or biocidal activity against mycelium of key soil-borne pathogens.

The present results are also in agreement with that of Antonov, *et al.* (1997); Walter *et al.*, (2001) and Elkaffash and Al-Menoufy, (2003) who reported that clove oil *(Eugenia caryophyllus)* suppressed the growth of *F. oxysporum, R. solani*, and *Verticillum dahliae*. This high antimicrobial activity probably related to eugenol as a major compound (Velluti, *et al.*, 2003). Also Riad, *et al*., (2013) tested the antifungal effect of lemongrass oil against some tomato root rot soil-borne pathogenic fungi (*Fusarium oxysporum radicis-lycopersici, F. oxysporum*

lycopersici, *F. solani, Rhizoctonia solani, Sclerotium rolfsii, Macrophomina phaseolinae, Pythium* sp. and *Phytophthora* sp.) *in vitro* conditions. They observed that there was inhibitory activity of lemongrass oil against mycelial growth of tested fungi at all concentrations. The mycelial growth decreased significantly with the increase in concentrations of essential oils and reached minimum mycelia growth at the highest concentration used. A complete inhibition of fungal growth was observed at 1.5% of all tested essential oil. Abdel-Kader, *et al.,* (2013) studied the inhibitory effect of lemongrass oil against the mycelial growth of *Aspergillus niger* under *in vitro* conditions. Results indicated that all essential oils treatments significantly reduced the linear growth of *A. niger*. Complete reduction was obtained with thyme and lemongrass at concentration of 0.5%

Trichoderma harzianum (test organism is used also in this study) is one of the most potent bioagents used for the control of many plant pathogen (Cook and Vesth., 1991 and Harman., 2006).This biocontrol agent has not harmful effects on humans, wild life and other beneficial organisms, safe and effective biocontrol agent in both natural and controlled environments that doesn't accumulate in the food chain and to which it has not been described resistance (Wiest, *et al*., 2002).

The antagonistic effect of *T. harzianum* against *F. oxysporum* and *R. solani* on solid medium was investigated using dual culture technique, and it was observed that, the linear growth of *T. harzianum* was more rapid than that of the tested pathogenic fungi. A great inhibition to the growth of *F. oxysporum* and *R. solani* was clearly observed. An overgrowth of *T. harzianum* on the tested fungi was observed 6 days after inoculation, and dots of conidia are seen on the surface of the phytopathogen. The antagonistic mode of action of the fungus and the ability of conidia to germinate on phytopathogen's surface has been proposed for the production of antibiotics (Schirmbock, *et al.,* 1994). These results are in agreement with Shaikh and Sahera (2013) where they evaluated the antagonistic activity of *Trichoderma viride* and *Trichoderma harzianum* against five different pathogens *in vitro*. Both *Trichoderma* spp. were strongly antagonized with five different pathogenic fungi viz. *Colletotrichum lindemuthianum, Fusarium oxysporum, Rhizoctonia solani, Alternaria solani* and *Fusarium solani* in dual culture assay. *T. viride* gave maximum inhibition of mycelial growth to all pathogenic fungi (67.45%), whereas *T. harzianum* showed (63.89%) inhibition of mycelial growth. Also, Shabir-U-Rehman *et al*. (2013) found that *in vitro*, *T. viride* and *T. harzianum* alone or in combination significantly inhibited the mycelial growth of the *F. oxysporum.* Considering broad-spectrum activities of essential oils, successful development of such compounds as antifungal would not only provide a potent tool for control of wilt and root rot diseases of tomato, but also could promise success in multipurpose biorational alternatives to conventional fungicides for the management of other plant diseases.

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المقاومة البيولوجية لفطري الفيوزاريوم أوكسيسبورم و ريزوكتونيا سولاني باستخدام الزيوت العطرية و فطر تريكودرما هارزيانم معمليا

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تم عزل فطري الفيوزاريوم أوكسيسبورم وريزوكتونيا سولاني المسببين الرئيسين لمرضي الذبول وسقوط البادرات في نبات الطماطم وذلك من جذور النبات التي تظهر أعراض المرضبن.

كما تم دراسة تأثير١٨ زيت عطري تجاري على نمو ميسيليات الفطرين محل الدراسة، اتضح ان زيت نبات القرنفل أعطى أفضل نتيجة يليه زيت حشيشة الليمون ثم زيت القرفة ثم زيت الجرجير و ذلك على فطر الفيوزاريوم اوكسيسبورم .اما بالنسبة لفطر ريزوكتونيا سولاني اعطى زيت حشيشة الليمون افضل نتيجة يليه زيت القرنفل ثم زيت الكافور ثم زيت القرفة ثم زيت حبة البركة ثم زيت الجرجير ثم زيت اللافندر.

تم اختيار أكثر الزيوت تأثيرا على نمو الفطرين وهما زيت القرنفل و زيت حشيشة الليمون وذلك لدراسة تأثيرهما على النمو الخطى و الوزن الجاف للميسليوم الفطرى وذلك بعمل تركيزات مختلفة من هذين الزيتين. أوضحت الدراسة أن زيت القرنفل عند تركيز ٠.٥٠% سبب تثبيط كامل للنمو الخطى لفطر الفيوزاريوم أوكسيسبورم كما أعطى أعلى تثبيط لفطر ريزوكتونيا سولاني عند تركيز ٠.٧٥%. أما بالنسبة لزيت حشيشة الليمون كان أعلى تثبيط للنمو الخطى لفطر الفيوزاريوم أوكسيسبورم عند تركيز ٠.٧٥% وبالنسبة لفطر ريزوكتونيا سولاني كان عند تركيز ٠.٧٥% .

وعند دراسة تأثير هذين الزيتين على الوزن الجاف لكل من الفطرين وجد أنه في حالة زيت القرنفل لا يوجد فروق معنوية بين جميع التركيزات المستخدمة مع الفطرين و كان أقل وزن جاف للفطرين عند تركيز ٠.٧٥%. وفى حالة زيت حشيشة الليمون وجد أن أقل وزن جاف لفطر الفيوزاريوم اوكسيسبورم كان عند تركيز ٠.٧٥%. اما بالنسبة لفطر ريزوكتونيا سولاني وجد انه لا يوجد فروق معنوية بين جميع التركيزات و كان أقل وزن للفطر عند تركيز ٠.٧٥% من الزيت.

ومن ناحية أخري تم دراسة التأثير المضاد لفطر تريكودرما هارزيانم على نمو الفطرين محل الدراسة و ثبت معمليا مقدرة هذا الفطر على الاستحواذ على أكبر مساحة ممكنة من الوسط الغذائى المعرض فى اطباق بترى و ظهور تاثيره المثبط لنمو الفطرين السابقين.