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In Vitro Evaluation of Some Fungicides Alternatives Against *Fusarium Oxysporum* the Causal of Wilt Disease of Pepper (*Capsicum annum* L.)

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Abstract The inhibitory effect of the antagonistic bioagents, chemical plant resistance inducers and some essential oils against the linear growth of two isolates of *F. oxysporum* the wilt pathogen of pepper (*Capsicum annum* L.) was evaluated *in vitro*. The antagonistic microorganisms, *Trichoderma harzianum, T. viride, T. aureiviride, Bacillus subtilis* and *Pseudomonas fluorescens* were tested. Also, the tested chemical inducers were Sodium benzoate, Potassium bicarbonate, Potassium sorbate and Chitosan. Meanwhile, the tested essential oils were Cinnamon, Clove, Thyme, Lemon grass, Lemon, Mint, Pepper mint and Mustard. The obtained results indicate that the antagonistic bioagents, *T. viride, B. subtilis, P. fluorescens* showed superior inhibitory effect against the growth of pathogenic fungi compared with *T. harzianum* and *T. aureiviride*. The fungal mycelial growth reduced gradually by increasing of tested concentrations to reach complete reduction (100%) at the concentrations of 4% for Potassium bicarbonate and Sodium benzoate and at 6% for Potassium sorbate. Data also revealed that the fungicide Topsin-M had superior inhibitor effect on the fungal linear growth than that of tested salts. It cause complete growth reduction at concentration of 300ppm. Chitosan was found to affect the linear growth of the two isolates of *F. oxysporum* that the complete reduction in fungal growth (100%) was observed at concentration of 4.5 g/L. Results also showed that Thyme, Lemon grass, Peppermint, Clove and Mint oils had higher inhibitor effect on fungal mycelial growth than Limon, Cinnamon and Mustard oils. Fungal mycelial growth decreased significantly as the concentrations of essential oils were increased, to reach the fungal growth's minimum at the highest concentration used. Complete reduction (100%) in mycelial growth of two fungal isolates was recorded at concentration of 6% of all tested essential oils. The obtained results in the present study showed the possibility of usage antagonistic bioagents, various plant inducers and essential oils to control plant pathogenic fungi.

Keywords Antagonism, Chemical plant resistance inducers, Essential oils, Linear fungal growth, Pepper wilt

1. Introduction

Hot pepper (*Capsicum annum* L.) is one of the important vegetables in Egyptian diet. Green and red pods are used for fresh meal and food industries. Fusarium wilt of hot pepper induced by *Fusarium* spp. is reported by many investigators to cause great losses in pepper production in different countries in the world[1-7]. *Fusarium oxysporum* f. sp. *lycopersici, corticium solani* and *Pythium debaryanum* are reported to be the causal organisms of soft wilt and damping-off diseases of some solanaceous plants, *e.g.* tomato and pepper[8]. Moreover,[9] reported that different *Fusarium* spp. were isolated from samples of *Capsicum annum* seeds. He added that in soil infestation all transplanted capsicum seedlings were killed within two weeks by *F. equiseti,* 44% were killed by *F. monilifome* after 50 days. *F. oxysporum* and *F. solani* killed 56% and 36% of seedlings, respectively, after 30days. Wilt disease caused by soil borne pathogenic fungi is one of the most serious diseases affected several cultivated plants worldwide. It results in poor production, poor quality, poor milling returns and reduced agriculture income.

Fungal disease control is achieved through the use of fungicides which is hazardous and toxic to both people and domestic animals and leads to environmental pollution. Therefore, a more balanced, cost effective and eco-friendly approach must be implemented and adopted farmers. In order to overcome such hazardous control strategies, scientists, researchers from all over the world paid more attention towards the development of alternative methods which are, by definition, safe in the environment, non-toxic to humans and animals and are rapidly biodegradable. Such strategy is use of Biocontrol agents[10,11] to control fungal plant diseases as well as other fungicides alternatives, *i.e.* plant re-

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sistance inducers[12-16]; essential oils[17-19].

The objective of the present work was to evaluate the inhibitory activity of some fungicides alternatives and growth promoters on the growth of *F. oxysporum* the causal agent of Pepper *in vitro*. The evaluated chemicals were plant inducers, *i.e*. Sodium benzoate, Potassium bicarbonate, Potassium sorbate and Chitosan. Essential oils, *i.e*. Cinnamon, Clove, Thyme, Lemon grass, Lemon, Mint, Pepper mint and Mustard were also evaluated

2. Materials and Methods

2.1. Source of Tested Microorganisms

Two isolates of the fungus *F. oxysporum* which were among sixteen isolates isolated from pepper plants showing wilt disease symptoms, collected from different locations throughout Egypt, and proved to be the highest aggressive ones causing about 90% wilt incidence of pepper under artificial infestation in pot experiment (unpublished data). These two isolates were chosen to be tested in the present study. The antagonistic microorganisms, *Trichoderma harzianum, T. viride, T. aureiviride, Bacillus subtilis* and *Pseudomonas fluorescens* which were isolated from the rhizosphere of healthy pepper plants were also used in the present work.

2.2. Laboratory Tests

The inhibitory effect of antagonistic microorganisms, chemical plant resistance inducers, some essential oils and the fungicide Topsin-M (70%) on the growth of *F. oxysporum* was evaluated using the culture technique[20]. *In vitro* studies of tested microorganisms were performed on PDA medium in 9-cm-diameter Petri dishes. Procedures for growth inhibition measurements in all tests were done as the same followed technique. Tested chemicals were added to conical flasks containing sterilized PDA medium before its solidifying to obtain the proposed concentrations and rotated gently to ensure equal distribution of added chemicals. A separate PDA flask free of tested chemicals used as check (control) treatment. The supplemented media were poured into sterilized Petri-dishes (9cm Ø) approximately 20 ml per each. Mycelial disc (5mm Ø) taken from the periphery of an actively growing PDA culture of tested fungus *F. oxysporum* was placed at the centre of the prepared Petri dishes, then incubated for seven days at 28±2℃. Five replicates were used for each treatment. The average linear growth diameter of colonies was measured and reduction in fungal growth was calculated in relative to check treatment. All experiments were repeated three times.

2.3. Effect of Antagonistic Microorganisms on Fungal Linear Growth

The inhibitory effect of fungal and bacterial antagonistic agents against the linear growth of *F. oxysporum* was evaluated using the modified dual culture technique[21]. 1991). *In vitro* antagonistic studies of biocontrol microorganisms and pathogenic fungus were performed on PDA medium in 9-cm-diameter Petri dishes. The control treatment was inoculated with a culture disk of either a pathogenic or antagonistic culture alone at the same conditions. All inoculated Petri dishes were incubated at 28±1℃ for five days, then the antagonistic effect was measured. This test was repeated five times and the growth inhibition was calculated as the percentage reduction in colony growth diameter of pathogenic fungi in the presence of antagonistic microorganism in relative to their growth in control treatment.

2.4. Effect of Plant Resistance Inducers on Fungal Linear Growth

Different concentrations of Sodium benzoate, Potassium bicarbonate, Potassium sorbate (at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0 and 6.0%, w/v); Chitosan (at concentration of 0.38, 0.75, 1.5, 3.0 and 4.5 g/L); as well as the fungicide Topsin-M (70%) at concentration of 10, 20, 50, 100, 200 and 300ppm were tested. Certain weight or volumes of tested chemicals were added individually to conical flasks containing sterilized PDA medium to obtain the proposed concentrations, then mixed gently and dispensed in sterilized Petri dishes (9-cm-diameter). Another set of conical flasks containing sterilized PDA medium free of tested chemicals was used as check control treatment. Petri dishes were individually inoculated at the centre with equal disks (5-mm) of tested fungus cultures. The average linear growth of each fungus was measured after 7 days of incubation at 25 ±2℃ and reduction in fungal growth was calculated in relative to check treatment.

2.5. Effect of Some Essential Oils on Fungal Linear Growth

Commercial essential oils of Cinnamon (*a.i*. cinnamic, aldehyde, 70-85%), Clove (*a.i*. eugenol, 90–95%) and Thyme (*a.i*. Thymol, 60%), Lemon grass (Citral, 65-85%), Lemon (Limonene, 97%), Mint (menthol, 48%), Pepper mint (Mentone+ menthol, 20%) and Mustard (Allyisothiocynate, 30%) were used in the present work. Essential oils used in the study were obtained from Chemical Industrial Development Company (CID), Egypt. The inhibitory effect of the essential oils was evaluated against the linear growth of the tested *F. oxysporum* fungus *in vitro*. For each of the essential oil, five concentrations, *i.e*. 0.5, 1.0, 2.0, 4.0 and 6.0% were prepared and tested. Fungal inoculation, incubation conditions and growth measurements and calculations were followed as stated before.

2.6. Statistical Analysis

All experiments were set up in a complete randomized design. One-way ANOVA was used to analyse differences between antagonistic inhibitor effect and linear growth of pathogenic fungi *in vitro*. A general linear model option of the analysis system SAS[22] was used to perform the ANOVA. Duncan's multiple range test at $P \leq 0.05$ level was

used for means separation[23].

3. Results and Discussion

The inhibitory effect of antagonistic microorganisms, chemical plant resistance inducers, some essential oils and the fungicide Topsin-M (70%) on the growth of *F. oxysporum* was evaluated under *in vitro* studies. The obtained results are presented as following**.**

3.1. Effect of Antagonistic Microorganisms on Fungal Linear Growth

Antagonistic ability of various tested bio-agents represented in Table (1) revealed that all tested agents could drastically reduce the linear growth of the two tested isolates of *F. oxysporum*. The antagonistic bioagents, *T. viride, B. subtilis, P. fluorescens* showed superior inhibitory effect against the growth of pathogenic fungi compared with *T. harzianum* and *T. aureiviride*. Also, it was observed that the antagonistic fungus *T. viride* had similar inhibitor effect against the mycelial growth of both isolates of *F. oxysporum*. Meanwhile, *F. oxysporum* isolate No. 2 showed significant tolerance against the other tested bioagents revealed in lower reduction in its mycelail comparing with *F. oxysporum* isolate No. 1. These results are also confirmed previously by several researchers[24-26]. Biological control by antagonistic organisms is a potential non-chemical tool for crop protection against phytopathogenic fungi[27].

Table 1. Growth reduction (%) of *F. oxysporm* isolates in response to different bioagents *in vitro*

Bioagent	Linear mycelial growth reduction (%)	
	F. oxysporum (No. 1)	*F. oxysporum* (No. 2)
T. viride	63.00 a	60.77 a
T. harzianum	53.50 b	44.44 c
T. aureiviride	44.64 c	36.80 d
B. subtilis	64.43 a	52.57 b
P. fluorescens	57.14 b	46.29 c

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).

Several strains from the genus Trichoderma have been described as antagonistic fungi able to control a wide range of phytopathogenic fungi. The antifungal activity of Trichoderma involves production of antibiotics, including compounds affecting the integrity of fungal membranes, competition for key nutrients, and production of fungal cell wall-degrading enzymes[28]. Although none of these mechanisms have been convincingly proven, the degradation and further assimilation of fungal structures and contents have been proposed as the major mechanism accounting for the antagonistic process against fungal plant pathogens[29]. Mechanisms of biological control have been the concern of many investigators during the past decades. Superior colonization of substrate, direct predation, production of secondary metabolic compounds such as antibiotics and enzymes were the most known phenomena proven as tools of biocontrol mechanisms[30].

As for antagonistic bacteria, several investigators reported inhibitor effect of antagonistic bacteria against soilborne plant pathogens[31-33]. Seed treatment with *Bacillus* spp. actively controlled three fungal root diseases of wheat[34], and *Pseudomonas cepacia* or *P. fluorescens* applied to pea seeds acted as a biological control agent against Pythium damping-off and Aphanomyces root rot[35].

Table 2. Linear growth of *F. oxysporm* isolates in response to different concentrations of some salts and fungicide Topsin M – 70

Tested chemical	Concentration (%)	Linear fungal growth (mm)	
		F. oxysporm (No. 1)	*F. oxysporm* (No. 2)
Potassium sorbate	0.25%	60.2 b	66.4 b
	0.5%	56.5 c	61.8 b
	1.0%	45.4 d	53.3 c
	2.0%	35.0 e	37.7 de
	4.0%	22.5 f	28.4 ef
	6.0%	0.0 i	0.0 i
Potassium bicarbonate	0.25%	66.5 b	67.8 b
	0.5%	59.8 bc	62.1 b
	1.0%	48.30 cd	44.5 d
	2.0%	23.4 f	28.9 ef
	4.0%	0.0 i	0.0 i
	6.0%	0.0 i	0.0 i
Sodium benzoate	0.25%	51.4 c	61.0 b
	0.5%	36.2 de	55.2 c
	1.0%	21.7 f	41.5 d
	2.0%	6.2 h	24.4 f
	4.0%	0.0 i	0.0 i
	6.0%	0.0 i	0.0 i
Topsin-M 70	10 ppm	59.4 bc	64.4 b
	20 ppm	46.2 d	50.5 c
	50 ppm	33.8 e	38.8 de
	100 ppm	22.7 f	26.6 f
	200 ppm	14.0 g	17.1 g
	300 ppm	0.0 i	0.0 i
Control		90.0 a	90.0 a

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).

3.2. Effect of Plant Resistance Inducers on Fungal Linear Growth

Results in Table (2) and Fig. (1) indicate that all evaluated salts and fungicide concentrations significantly reduced the linear growth of tested two fungal isolates. The fungal mycelial growth reduced gradually by increasing of tested concentrations to reach complete reduction (100%) at the concentrations of 4% for Potassium bicarbonate and Sodium benzoate and at 6% for Potassium sorbate. Data also revealed that the fungicide Topsin-M had superior inhibitor effect on the fungal linear growth than that of tested salts. It cause complete growth reduction at concentration of 300ppm. Also, it is observed that *F. oxysporum* isolate No. 1. showed more sensitivity against different concentrations of salts and fungicide tested comparing with *F. oxysporum* isolate No. 2. In this regards, several types of treatment are reported to be partially effective in removing disease-causing organisms. Sodium benzoate and benzoic acid are employed in a wide range of preservative applications because of their combi-

nation of bactericidal and bacteriostatic action with their properties of being non-toxic and tasteless. They are the most effective preservatives against yeast and mould. Several inorganic salts and organic lipophilic acids and their salts, some of which are used, in the food-processing industry, have antimicrobial properties and could be useful as post-harvest treatment for decay control. The food preservatives potassium sorbate or sodium benzoate, have antifungal activities against post-harvest decaying fungi[36,37]. Sorbic acid and its salt derivatives are the most widely used antimicrobial agents for food preservation worldwide. Using potassium sorbate or sodium benzoate against post-harvest diseases of tomato, apple, carrots and potato was reported [38-40]. Food preservatives potassium sorbate or sodium benzoate when applied to citrus fruits inoculated with *Penicillium digitatum* had similar fungicidal activity and are equivalent to the traditional treatment used as a post-harvest fungicide for controlling citrus fruit decay[41].

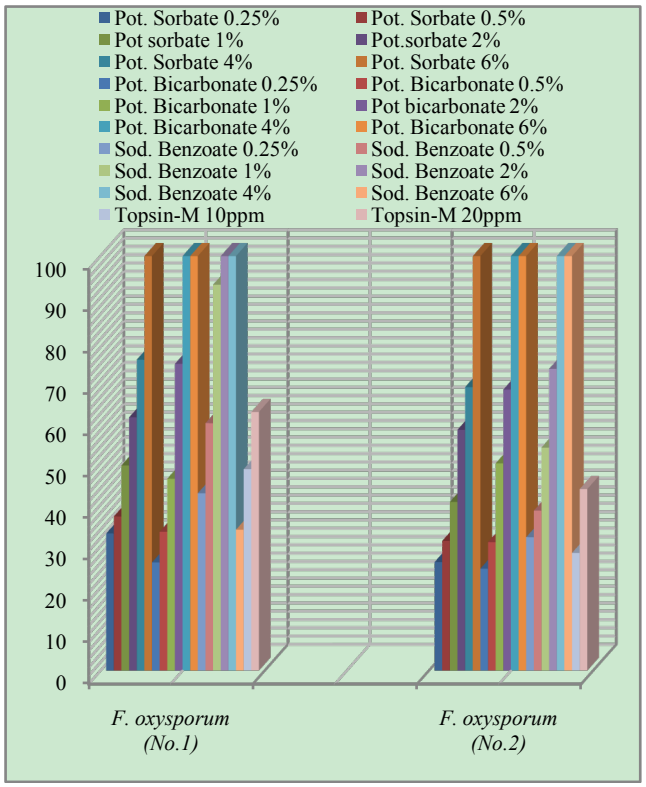

Figure 1. Reduction (%) in linear growth of *F. oxysporm* isolates in response to different concentrations of some salts and fungicide Topsin M-70 *in vitro*

Furthermore,[42] stated that *Aspergillus* was always more sensitive to potassium sorbate than *Penicillium*. *Aspergillus flavus, A. niger* and *Penicillium corylophilum* spoilage of bakery products was prevented by the use of weak acid derivatives such as potassium sorbate, calcium propionate and sodium benzoate.

Many investigations concerning the use of abiotic factors for induction of plant resistance against several diseases have been accumulated. Potassium salts (K_2HPO_4 or KNO_3) as a chemical agent for induction of plant resistance had great attention in many of these reports[43,44]. Moreover, there has been considerable interest in the use of sodium bicarbonate and potassium bicarbonate for controlling various fungal diseases in plants[45-47].

Topsin-M in the present study was found to be more active than salts for inhibiting the linear growth of *F. oxysporum F. oxysporum* isolates *in vitro*. The fungal mycelial growth reduced gradually by increasing the fungicide concentrations in growth medium to reach complete growth reduction (100%) at concentration of 300ppm. Also, the growth of fungal isolate No. 1 showed more sensitivity against the fungicide concentrations than isolate No. 2. Similar results were proved by many investigators, that *F. oxysporum* growth was completely inhibited within a lower range of 50-100 ppm of Topsin-M [48-50].

Table 3. Effect of different chitosan concentrations on the linear growth of *F. oxysporum* isolates *in vitro*

Chitosan concentrations (%)	Linear growth (mm)	
	F. oxysporum (No.1)	*F. oxysporum* (No.2)
0.038 (0.38 g/l)	74.5 b	80.2 ab
0.075 (0.75 g/l)	58.6 c	62.4 bc
0.15 (1.5 g/l)	36.6 d	43.3 cd
0.3 (3 g/l)	16.2 e	21.4 de
0.45 (4.5 g/l)	0 f	0 f
Control	90 a	90 a

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).

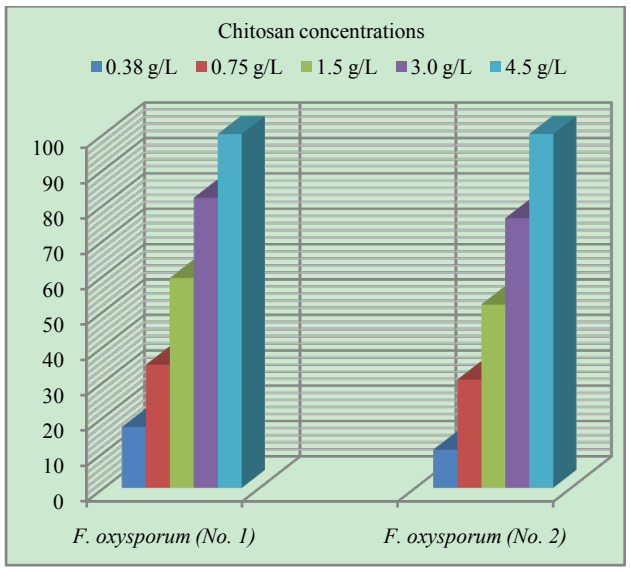

Figure 2. Reduction (%) in linear growth of *F. oxysporm* isolates in response to different concentrations of chitosan *in vitro*

Presented data in Table (3) and Fig. (2) revealed that different tested concentrations of Chitosan affect the linear growth of the two isolates of *F. oxysporum*. Data also showed that complete reduction in fungal growth (100%) was observed at concentration of 4.5 g/L. Similar results were recorded by many investigators with various crops. Chitin and chitosan the safe materials which were reported to induce resistance against soilborne diseases[51-54]. Furthermore, chitosan has different properties, *i.e.* its inhibitory effect against pathogenic fungi[55]. Also, it was reported that chitosan at 6 g/L completely inhibit the linear growth of

all tomato root rot fungi and reduced the total count of pathogenic fungi. In this regards, Under *in vitro* conditions chitin at concentrations of 2, 4 and 6 g/l showed no inhibitory effect against tested fungi, while chitosan at 6 g/l completely inhibit the linear growth of all tested fungi[56]. The inhibitory effect of chitosan against pathogenic fungi was also reported[57]. In this respect, two models have been proposed to explain the antifungal activity of chitosan, the first, its activity is related to the ability to interfere with the plasma membrane function[58]. While the second, the interaction of chitosan with fungal DNA and RNA[59].

Table 4. *In vitro* growth reduction (%) of *F. oxysporum* isolates in response to different concentrations of some essential oils

Essential oil	Concentration (%)	Fungal linear growth reduction (%)	
		F. oxysporum (No.1)	*F. oxysporum* (No. 2)
Thyme	0.5	55.2 e*	38.8 g
	1.0	70.5 c	48.8 f
	2.0	88.8 bc	73.5 c
	4.0	100 a	90.5 b
	6.0	100 a	100 a
Lemon grass	0.5	55.5 e	36.9 g
	1.0	65.2 d	53.3 e
	2.0	79.9 c	68.5 d
	4.0	100 a	90.2 b
	6.0	100 a	100 a
Pepper mint	0.5	52.7 e	39.9 g
	1.0	84.6 bc	55.7 e
	2.0	100 a	90.9 b
	4.0	100 a	100 a
	6.0	100 a	100 a
Clove	0.5	43.3 f	51.5 e
	1.0	73.2 c	67.4 d
	2.0	91.6 b	88.8 bc
	4.0	100 a	100 a
	6.0	100 a	100 a
Mint	0.5	55.5 e	51.1 e
	1.0	68.8 d	58.8 e
	2.0	84.4 bc	66.6 d
	4.0	97.7 b	80.0 bc
	6.0	100 a	100 a
Lemon	0.5	12.2 i	19.3 i
	1.0	27.7 h	24.1 h
	2.0	44.4 f	36.6 g
	4.0	64.2 d	48.1 f
	6.0	100 a	97.7 b
Cinnamon	0.5	18.5 i	14.8 i
	1.0	29.6 h	24.8 h
	2.0	35.1 g	31.1 g
	4.0	64.8 d	61.8 d
	6.0	100 a	100 a
Mustard	0.5	37.4 g	35.9 g
	1.0	47.3 f	46.2 f
	2.0	65.5 d	62.9 d
	4.0	77.7 c	75.1 c
	6.0	100 a	100 a

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).
* Fungal linear growth reduction calculated in relative to control treatment 90mm.

3.3. Effect of Some Essential Oils on Fungal Linear Growth

Results in Table (4) showed that all tested essential oils have been found to have inhibitory effects against the mycelial growth of tested *F. oxysporum* isolates *in vitro*. Thyme, Lemon grass, Peppermint, Clove and Mint oils had higher inhibitor effect on fungal mycelial growth than Limon, Cinnamon and Mustard oils. Fungal mycelial growth decreased significantly as the concentrations of essential oils were increased, to reach the fungal growth's minimum at the highest concentration used. Complete reduction (100%) in mycelial growth of two fungal isolates was recorded at concentration of 6% of all tested essential oils. Mycelial growth of the *F. oxysporum* isolate No. 2 showed more sensitivity to concentrations of all essential oils tested comparing with *F. oxysporum* isolate No. 1. At concentration of 4%, Peppermint and Clove oils showed superior inhibitor effect to cause complete reduction to both fungal isolates.

Meanwhile, Thyme and Lemon grass oils gave the same effect only on *F. oxysporum* isolate No. 2. In this concern, essential oils are promising alternative compounds which have an inhibitory activity on the growth of pathogens. It is possible that essential oils could be used in plant disease control as the main or as adjuvant antimicrobial compounds[60]. It is well established that some plants contain compounds able to inhibit the microbial growth[61]. These plant compounds can be of different structures and different mode of action when compared with antimicrobials conventionally used to control the microbial growth and survival[62]. Potential antimicrobial properties of plants had been related to their ability to synthesize, by the secondary metabolism, several chemical compounds of relatively complex structures with antimicrobial activity, including alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpens, phenylpropannes, organic acids[63]. The aesthetic, medicinal and antimicrobial properties of plant essential oils have been known since ancient times. Numerous studies on the fungicidal and fungistatic activities of essential oils have indicated that many of them have the power to inhibit fungal growth. It is evident from reviews[64,65] that some plant extracts and essential oils exhibited antifungal properties. Also, it was reported that essential oil of *Juniperus communis* may be applicable against a range of damping-off diseases[66]. Furthermore, [67] studied the effectiveness of nine essential oils to control the growth of mycotoxins producing moulds and noted that, clove, cinnamon and oregano were able to prevent the growth of *Aspergillus parasiticus* and *Fusarium moniliforme*. The information was found in the literature concerning mode of action of essential oils on/in the fungal cell in order to promote fungistatic or fungicide effect. In general, inhibitory action of natural products on moulds involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition[68]. Also, it is reported that plant lytic enzymes act in the fungal cell wall causing breakage of *b*-1,3 glycan, *b*-1,6 glycan and chitin polymers[69]. The mode by which microorganisms are inhibited by essential oils and their chemical compounds seem to involve different mechanisms. It has been hypothesized that the inhibition

involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability and unavailability of vital intracellular constituents[70]. Reports indicated that essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had the highest antibacterial performances[71].

The present study demonstrated that antagonistic fungal and bacterial bioagents, some plant resistance inducers and essential oils were found to have an *in vitro* inhibitory effect against the mycelial growth of the two isolates of *Fusarium oxysporum* the causal agent of Pepper wilt. These results may lead to the conclusion that application of these control factors is applicable, safe and cost-effective method for controlling such diseases under nursery and field conditions.

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