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Biological control of Verticillium wilt of greenhouse cucumber by *Talaromyces flavus*

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Summary. *Talaromyces flavus*, a fungal antagonist, was isolated from soil samples collected from cucumber greenhouses in Varamin district, Tehran province, Iran. The antagonistic effects of *T. flavus* isolates against *Verticillium albo-atrum*, the causal agent of greenhouse cucumber wilt were investigated under laboratory and greenhouse conditions. *T. flavus* colonies were recovered after three weeks from soil samples plated on a selective medium. Effects of volatile and non-volatile extracts of *T. flavus* isolates on *V. albo-atrum* growth were investigated in the laboratory, and five isolates that inhibited *V. albo-atrum* more strongly, were selected for greenhouse experiments. The infection index in the greenhouse was compared in a split plot trial with five isolates applied to the soil, the seed, or both seed + soil, arranged in a randomized complete block design with four replications. The greenhouse experiments on the different *T. flavus* treatments indicated that there was no significant difference among them. Of the five *T. flavus* isolates, the most effective was Tf-Cu-V-60. The interactions between the *T. flavus* treatments and the *T. flavus* isolates showed that the lowest infection index was achieved when the soil was treated with Tf-Cu-V-60. The study showed that *T. flavus* may control greenhouse Verticillium wilt of cucumber.

Key words: antagonistic effects, non-volatile extracts, *Verticillium albo-atrum,* volatile extracts.

Introduction

Greenhouse cucumber (*Cucumis sativus* L.) widely grown in many parts of the world. The cultivation of vegetables in greenhouses is an expanding business world-wide. About 83 countries grow greenhouse vegetables commercially, including greenhouse cucumber, tomato and paper commercially, totaling over 400,000 hectares. The average yield of greenhouse cucumber is 60 tons per hectare (Jilani *et al.* , 2009). In Iran, about 2300 hectares are grown with this crop, with an average yield of 200 tons per hectare (Soleimani *et al.*, 2009). Verticillium wilt is one of the most important diseases of greenhouse cucumber and causes serious losses in the field (Al-Rawahi *et al.*, 1998; Sanei *et al.*, 2008). *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke and Berthold are the causal agents of this disease (Correll, 1988; Mansoori and Smith, 2005; Jabnoun-Khiareddine *et al.*, 2006; Roustaee and Baghdadi, 2007).

For controlling Verticillium wilt of greenhouse cucumber, appropriate cultural practices and the use of resistant varieties are the most common strategies recommended but they are not always possible or effective (Rekanovic *et al.*, 2007). Biological control using fungal and bacterial antagonists has been applied in recent years to manage greenhouse cucumber diseases (Singh *et al.*, 1999; Martin and Bull, 2002; Naraghi *et al.*, 2006; Naraghi *et al.*, 2008; Heydari and Pessarakli, 2010).

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Talaromyces flavus (Klocker) Stulk and Samson is an antagonistic fungus that has been used for the biological control of some soil-borne pathogens such as *V. dahliae*, *V. albo-atrum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Marois *et al.*, 1984; Tjamos and Antoniou, 2000; Punja, 2001; Brunner *et al.*, 2005; Gohel *et al.*, 2006). Marois *et al.* (1984) also reported that this fungus grows on the rhizosphere of greenhouse cucumber, cotton, and egg-plant and inhibit germination of the microsclerotia of *V. dahliae*. *T. flavus* decreased the incidence of Verticillium wilt and increased the yield of egg-plant in England (Marois *et al.*, 1982) while Fahima and Henis (1997) found that *T. flavus* decreased Verticillium wilt on egg-plant by 77%.

Talaromyces flavus (the teleomorph of *Penicillium dangeardii*) was also reported as a parasite of sclerotia of *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (McLaren *et al.*, 1982). Tjamos and Fravel (1997) found that *T. flavus* decreased Verticillium wilt on tomato plants. *T. flavus* and *Aspergillus terreus* also inhibit Verticillium wilt of olive trees in Greece (Tjamos *et al.*, 1991). Forty percent of non-volatile extracts (Talaron) of *T. flavus* are thought to affect production of hydrogen peroxide due to the glucose oxidase enzyme, and this gives this fungus antibacterial and antifungal characteristics (Kim and Fravel, 1990).

This present study investigated the antagonistic effects of *T. flavus* isolates, applied with different methods on *V. albo-atrum*, the causal agent of greenhouse cucumber wilt.

Materials and methods

Isolation of *V. albo-atrum* from the soil

During 2008–2009, soil samples were collected from some cucumber greenhouses in the Varamin area, Tehran Province, Iran. Soil samples were air-dried, homogenized using a revolving jar mill and stored at 4°C. *V. albo-atrum* was isolated from the soil by wet sieving (Christen, 1981).

Pathogenicity tests

An isolate of *V. albo-atrum* recovered from cucumber greenhouse soil was used in the study. The isolate was grown on potato dextrose agar (PDA) at 22°C prior to inoculation. Spore suspensions were prepared from 3-week-old cultures by adding 10 mL of sterile distilled water to each Petri dish and scraping off the cultures with a rubber spatula. The inoculum concentration was adjusted to 10^7 conidia per milliliter with a haemocytometer. Greenhouse cucumber seedlings of the susceptible cultivar Negin (Haghani *et al.*, 2006) were inoculated at the 3rd–4th true leaf stage. The cucumber seedlings were uprooted and inoculated using a root-dip technique. The roots were washed under running water and placed for 60 minutes in a conidial suspension. The inoculated seedlings were transplanted to pots containing a mixture of peat moss, vermiculate and perlite (1:1:1, v:v:v). Three replications of five plants were used. The plants were kept on a greenhouse bench at 21–23°C. Daylight was supplemented with fluorescent light to provide a 12 h day length (Kim *et al.*, 2001). Wilt symptoms were recorded at approximately 10 days intervals for about one and a half months after inoculation. Wilt severity was scored on an arbitrary scale where 0, no visual symptoms; 0.5, symptoms on the cotyledon; 1, the first leaf infected; 1.5, the second leaf infected; 2, the third leaf infected and so on (Paplomatas *et al.*, 1999).

Isolation of *T. flavus* from the soil

Since *T. flavus* occupies the rhizosphere of greenhouse cucumber (Marois *et al.*, 1984), soil was collected from some cucumber greenhouses in the Varamin district. A selective medium (TF medium) was adopted for isolation of the fungus from soil (Marois *et al.*, 1984). The TF medium contained 1 L distilled water, 39 g potato dextrose agar (PDA), 2.0 mL of a 50% solution of lactic acid, 100 mg streptomycin sulfate, 50 mg chlorotetracycline, 50 mg chloramphenicol, 4 mg pimaricin, 30 mg nystatine (Mycostatin, 4960 units mg^{-1}) and 0.5 g oxgall (bile, bovine). Lactic acid and the antimicrobial agents were added as aqueous solutions to the autoclaved PDA at about 50°C. One mL of the aliquots was removed from 10^{-2} to 10^{-3} dilutions (soil in water) while stirring with a magnetic stirrer and was spread on the TF medium (five plates per replication). Plates were incubated in the dark at 30°C for 10 days. *T. flavus* isolates were detected and identified on the TF medium based on their colony morphology 10 days after incubation. Using the above described procedure, propagules of *T. flavus* were isolated from the soil samples (Marois *et al.*, 1984).

The antagonistic effects of *T. flavus* extracts on *V. albo-atrum* in laboratory experiments

Volatile extracts

Talaromyces flavus isolates were placed singly on Petri dishes containing PDA and incubated at 30°C for 36 hours, after which time, 9-mm disks of *V. albo-atrum* isolates were cultured on other Petri dishes containing PDA and these Petri dishes (without their lids) were placed face to face with Petri dishes containing *T. flavus* isolates and each pair of Petri dishes was sealed so that the pathogenic and antagonistic fungi could interact. The Petri dishes were incubated at 25°C. After two weeks, the diameter of *V. albo-atrum* colonies that developed was recorded and percent growth inhibition was calculated as follows:

(colony diameter of *V. albo-atrum* in control - colony diameter of *V. albo-atrum* in culture medium affected by volatile extract of *T. flavus* / colony diameter of *V. albo-atrum* in control) × 100.

Non-volatile extracts

For the preparation of non-volatile extracts, *T. flavus* isolates were placed on Czapeck dextrose broth placed on a shaker at 50 rpm for ten days. The broth was then passed through 0.45 μ filters (Eziashi, 2006). The culture filtrates of the fungal isolates were then added to PDA at 20% concentration and poured into Petri dishes. *V. albo-atrum* isolates from greenhouse cucumber soil were cultured on medium amended with the culture filtrates at 25°C. After one week, the diameter of the *V. albo-atrum* colonies was recorded and percentage growth inhibition was calculated as described above.

Five of the *T. flavus* isolates that most strongly inhibited *V. albo-atrum*, were selected for the greenhouse experiments. These isolates were selected because on average they were most effective in reducing *V. albo-atrum* colony growth.

Greenhouse experiments

Plant inoculation with V. albo-atrum

The inoculation procedures described by Paplomatas *et al.* (1999) were followed with some modifications.

Verticillium albo-atrum inoculum was prepared by growing the fungus in 100 mL Czapeck dextrose broth in 250-mL Erlenmeyer flasks at 22°C under continuous shaking at 120 rpm for one week. After one week, the fungal spores and mycelium were separated from the culture medium by suction-filtering through Whatman No.1 filter paper. The fungal biomass was then homogenized in an electric blender to break up the spore clumps and the spore concentration was adjusted to 10^7 spores mL^{-1} using a haemocytometer.

Seedlings at the second true -leaf stage were uprooted, their roots were cleaned as much as possible from the soil mix with tap water and trimmed to create the wounding necessary to ensure the entrance of the pathogen. Seedlings were root-dipped in fungal inoculum for half an hour. Then they were transplanted to a clean soil mix in plastic cups. Control seedlings were also subjected to the above process but in that case water was substituted for the inoculum. Incoculated plants were kept at 22°C in the greenhouse under a 12 h day.

Preparation of *T. flavus* inoculum

Talaromyces flavus inoculum was prepared separately for every isolate as follows: *Talaromyces flavus* isolated from the soil was cultured in test tubes containing TF medium. After 5 days, 20 mL SDW was poured into 50 cm-wide×80-cm-tall sterile plastic bags containing 250 g of peat moss mixed with 10 mL D-lactose monohydrate (20 g L^{-1}). The plastic bags were incubated at 30°C for 30 days. The contents of each plastic bag was evacuated after peat moss was completely covered with *T. flavus* hyphae. The number of ascospores of *T. flavus* in each g of peat moss was then determined using a haemocytometer. In this procedure, one g of peat moss was suspended in 10 mL SDW and the number of ascospores in one mL of this suspension was counted. *Talaromyces flavus* inoculum was added to the soil in the pots at 10^7 ascospores g^{-1} soil (Chet and Baker, 1981). For seed treatment, cucumber seedlings at the second true-leaf stage were floated in this inoculum for half an hour.

The antagonistic effects of *T. flavus* isolates on Verticillium wilt of greenhouse cucumber

The effect of *T. flavus* isolates on the pathogenicity of *V. albo-atrum* was studied experimentally as follows: the experiment was performed as a split plot arranged in a randomized complete

block design with four replications and 3 plants per replication. The main factor was the *T. flavus* treatment: 1, soil; 2, seed; 3, soil+seed, and the sub-factor was the fungal isolates: 1–5, 5 *T. flavus* isolates + *V. a–a.*; 6, *V. a–a.*; and 7, *T. flavus* without *V. a–a*.

Inoculated plants were kept in the greenhouse at 22°C with a 12 h day. Wilt symptoms were recorded at approximately 10 day intervals for about one and a half months after inoculation. Wilt severity was scored on an arbitrary scale in which 0, no visible symptoms; 0.5, symptoms on the cotyledon; 1, the first leaf infected; 1.5, second leaf infected; 2, third leaf infected, and so on (Paplomatas *et al.*, 1999). Data were analyzed by ANOVA using MS TAT C statistical software, while the means were separated by Duncan's multiple range test.

Results

Isolation of *V. albo-atrum* from the soil and pathogenicity tests

The *V. albo-atrum* isolate obtained from cucumber greenhouses soil caused plants to wilt and die, beginning with the older crown leaves. Light-brown streaks were seen inside the lower stems, runners and roots when the plant samples were cut longitudinally.

In the pathogenicity test, the infection index for infected plants based on the number of infected leaves was 4.38.

Isolation of *T. flavus* from the soil

Eight *T. flavus* isolates were obtained from cucumber greenhouses soil samples in Varamin as follows: TF-Cu-V-53, TF-Cu-V-54, TF-Cu-V-55, TF-Cu-V-56, TF-Cu-V-57, TF-Cu-V-58, TF-Cu-V-59 and TF-Cu-V-60.

The antagonistic effects of *T. flavus* extracts on *V. albo-atrum* in laboratory experiments

Volatile extracts

The inhibitory effect of *T. flavus* on *V. albo-atrum* differed between *T. flavus* isolates (Table 1). Isolate Tf-Cu-V-60 had the greatest inhibitory effect, and isolate Tf-Cu-V-54 the least. Inhibition ranged from 28.78 to 55.44.

Non-volatile extracts

Talaromyces flavus isolates differed in the extent to which they inhibited *V. albo-atrum* (Table 1). Inhibition was greatest with isolate Tf-Cu-V-54 and smallest with isolate Tf-Cu-V-56 .

On the basis of the total inhibitory effects of the volatile and non-volatile extracts, the following *T. flavus* isolates were selected for the greenhouse experiments: TF-Cu-V-55, TF-Cu-V-57, TF-Cu-V-58, TF-Cu-V-59 and TF-Cu-V-60.

The antagonistic effects of *T. flavus* isolates on Verticillium wilt of greenhouse cucumber

Treatments affected by the main factor (different types of *T. flavus* treatment) were placed in one statistical group (Table 2). The lowest mean infection index was 2.38 and belonged to the soil+seed treatment. This treatment did not differ statistically from the other two treatments.

The subfactor (the different biocontrol isolates) placed the treatments in six statistical groups. The lowest mean infection index was 1.83 and was caused by Tf-Cu-V-60 + *V. a-a.* by seed or soil (Table 2).

The interaction between the main factor and subfactor, placed all treatments in 10 statistical groups. The lowest infection index (1.62) was found when the soil was treated with Tf-Cu-V-60 (Table 3). This was not statistically different from isolate 60 applied to the seed or to the soil + seed, nor from isolate 59 applied to the soil or to the soil + seed.

Analysis of variance for the data is shown in Table 4.

Discussion

The study shows that Verticillium wilt of greenhouse cucumber is managed effectively by treating the cucumber seed with *T. flavus* isolates. Previous studies found that the volatile and non-volatile extracts of several bacterial and fungal micro organisms inhibited the growth and activity of a variety of some fungal pathogens. For example, volatile extracts of *Fusarium oxysporum* made chickpea (*Cicer arietinum* L.) resistant to a number of pathogenic fungi (Cherif *et al.*, 2007). The non-volatile extracts of some fungi and bacteria, including *Aspergillus flavus*, *A. ochraceus*, *Penicillium aurantiogriseum*, *Trichoderma harzianum* and *Bacillus subtilis* control antracnose disease of *Vigna unguiculata* L. Walp. (Adebanjo and Bankole, 2004).

Table 1. Inhibitory effects of volatile metabolites and culture filtrates from various *Talaromyces flavus* isolates on growth of *Verticillium albo-atrum*.

T. flavus isolate	Mean inhibition (%) [a]					
	Volatile metabolite[b]		Culture filtrates [c]		Average	
Tf-Cu-V-53	22.22	f	35.73	b	28.97	g
Tf-Cu-V-54	11.94	g	45.63	a	28.78	g
Tf-Cu-V-55	65.67	d	27.77	c	46.72	e
Tf-Cu-V-56	51.11	e	9.09	f	30.10	f
Tf-Cu-V-57	82.22	c	13.63	e	47.92	d
Tf-Cu-V-58	82.77	bc	13.42	e	48.09	c
Tf-Cu-V-59	85.00	a	22.22	d	53.61	b
Tf-Cu-V-60	83.33	b	27.55	c	55.44	a
Control	0	h	0	g	0	h

[a]Means followed by a different letter are significantly different ($P \leq 0.01$).
[b] Petri dish containing the pathogen and one containing the antagonist were placed face to face and sealed so that they could interact.
[c] Culture filtrates of *T. flavus* isolates were added to PDA at a 20% concentration used as a growth medium for the pathogen.

Table 2. The effects of the main factor (different *Talaromyces flavus* treatments) and subfactors (different *T. flavus* isolates) on the Verticillium wilt infection index in greenhouse cucumber plants.

Source of variation	Treatment	Mean infection index	Statistical grouping [a]
Main factor	Seed	2.86	a
	Soil	2.45	a
	Soil and seed	2.38	a
Sub factors	*V. a-a.* (positive control)	4.38	a
	Tf-Cu-V-55 + *V. a-a.*	3.46	b
	Tf-Cu-V-57 + *V. a-a.*	3.21	b
	Tf-Cu-V-58 + *V. a-a.*	2.79	bc
	Tf-Cu-V-59 + *V. a-a.*	2.25	cd
	Tf-Cu-V-60 + *V. a-a.*	1.83	d
	Without fungal inoculum (negative control)	0	e

[a] See Table 1.

Table 3. Interactive effects between the main factor and subfactors (all treatments) on the Verticillium wilt infection index in greenhouse cucumber plants.

Treatment	Mean infection index	Statistical grouping [a]
V. a-a. (positive control)	4.38	a
Tf-Cu-V-55 (seed) + *V. a-a.*	4.38	a
Tf-Cu-V-57 (soil) + *V. a-a.*	3.38	b
Tf-Cu-V-55 (soil) + *V. a-a.*	3.25	bc
Tf-Cu-V-57 (seed) + *V. a-a.*	3.13	bcd
Tf-Cu-V-57 (soil and seed) + *V. a-a.*	3.13	bcd
Tf-Cu-V-59 (seed) + *V. a-a.*	3.13	bcd
Tf-Cu-V-58 (seed) + *V. a-a.*	2.88	cd
Tf-Cu-V-55 (soil and seed) + *V. a-a.*	2.75	d
Tf-Cu-V-58 (soil and seed) + *V. a-a.*	2.75	d
Tf-Cu-V-58 (soil) + *V. a-a.*	2.75	d
Tf-Cu-V-60 (seed) + *V. a-a.*	2.13	e
Tf-Cu-V-59 (soil and seed) + *V. a-a.*	1.88	ef
Tf-Cu-V-59 (soil) + *V. a-a.*	1.75	ef
Tf-Cu-V-60 (soil and seed) + *V. a-a.*	1.75	ef
Tf-Cu-V-60 (soil) + *V. a-a.*	1.62	f
Without pathogen inoculum (negative control)	0	g

[a] See Table 1.

Table 4. Analysis of variance for data of the greenhouse experiment.

Source of variation	Degree of freedom	Sum of squares	Mean Squares	F_{value}	Prob
Replication	3	0.631	0.210	0.8762^{ns}	_
Main factor (A): Application method	2	3.792	1.896	5.7238^{**}	0.0161
Error	6	1.280	0.213		
Subfactor (B): Antagonist isolates	6	141.036	23.506	47.0320^{**}	0.0000
AB: Interaction between A and B	12	7.125	0.594	1.3625^{*}	0.0396
Error	54	15.839	0.293		
Total	83	169.703	26.712		
Coefficient of variation			21.16%		

[ns] No significant differences between treatments.
[**], Significant differences between treatments ($P \leq 0.01$).
[*], Significant differences between treatments ($P \leq 0.05$).

Other studies, have reported that these extracts are also effective against various soil-borne fungal pathogens of greenhouse cucumber (Murrary *et al*., 1997; Nagtzaam and Bollen, 1997; Whipps, 2001; Soytong and Ratanacherdchai, 2005; Kulikov *et al*., 2006). Another study, found that in laboratory and greenhouse experiments volatile and non-volatile extracts of *T. flavus* controlled root rot of lettuce (*Lactuca sativa*) caused by *Sclerotinia minor* (El-Tarabily *et al*., 2000). Proksa *et al*. (1992) also reported that *Penicillium vermiculatum* produced the antifungal volatile metabolite 2-methylsorbic acid.

Madi *et al*. (1997) showed that the enzymatic activity of *T. flavus* varied considerably between isolates, with the greatest differences in chitinase (25-fold), followed by glucanase (16-fold), cellulose (11-fold) and glucose oxidase (7-fold). Other studies reported that non-volatile extracts such as chitinase produced by *T. harzianum* and *T. flavus* controlled soybean stem white rot disease caused by *S. sclerotiorum* and bean stem rot caused by *S. rolfsii* respectively (Menendez and Godeas, 1998).

Other fungal metabolites such as glucanase secreted by *Zygorrhynchus moelleri* and glucose oxidase produced by *T. flavus* were also effective against some soil- borne plant pathogenic fungi (Brown, 1987; Murrary *et al*., 1997).

Studies on using *T. flavus* for the biocontrol of pathogenic fungi have found that *T. flavus* is an important antagonist to *V. dahliae* and *V. albo-atrum* (Tjamos and Paplomatas, 1987; Wikins *et al*., 2000; Vidhyasekaren, 2004). Kim and Fravel (1990) reported that glucose oxidase produced by *T. flavus* prevented the formation of microsclerotia of *V. dahliae*, and Proksa *et al*. (1992) showed that 2-methyl sorbic acid secreted by *T. flavus* had an inhibitory effect on *V. albo-atrum*.

Differences in the results obtained by the present laboratory experiments and those from previous experiments could be due to the concentration of the extracts employed. The effectiveness of *T. flavus* isolates applied to the seed in decreasing the Verticillium infection index in greenhouse conditions was consistent with some previous studies (Soytong and Ratanacherdchai, 2005; Kulikov *et al*., 2006). The minor differences between the present and earlier studies could be due to differences in the way, the seed was treated with the *T. flavus* isolates, or to the interval allowed before sowing (Nagtzaam and Bollen, 1997).

Other field studies have found that Verticillium wilt decreased when plants were given treatments containing *T. flavus* (Madi *et al*., 1997; Nagtzaam and Bollen, 1997; Klosterman *et al*., 2009).

This study has shown that *T. flavus* isolates may control Verticillium wilt, which is one of the most important diseases of greenhouse cucumber and of several other major crops. These findings may have a practical application when devising disease management strategies in the field, and may decrease losses from plant diseases generally while minimizing the application of chemical fungicides, which would protect the environment and preserve biological resources in a sustainable agricultural system.

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