

BIOLOGICAL CONTROL OF TOMATO VERTICILLIUM WILT DISEASE BY *TALAROMYCES FLAVUS*

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Abstract: In this study, *Talaromyces flavus* a fungal antagonist, was isolated from soil samples collected from tomato fields in Tehran and the Western Azarbaijan provinces of Iran. Antagonistic effects of *T. flavus* isolates against *Verticillium albo-atrum* (*V. a.-a.*), the causal agent of tomato wilt disease were investigated under laboratory and greenhouse conditions. Soil samples from tomato fields in the Varamin and Uremia regions of Tehran and the Western Azarbaijan provinces respectively, were cultured on selective medium. *T. flavus* colonies were recovered after three weeks. In the laboratory experiments, antagonistic effects of volatile and non-volatile extracts of *T. flavus* isolates on *V. a.-a.* growth were investigated. Among isolates, five that caused higher growth inhibition of *V. a.-a.* were selected for greenhouse experiments. In the greenhouse, first inoculum of *V. a.-a.* and treatments affected by *T. flavus* isolates were prepared. For comparison of the infection index in treatments, the greenhouse experiment was performed with a split plot arranged in a randomized complete block design with four replications. Result of greenhouse experiments on different types of *T. flavus* treatments indicated that there was no significant difference among them. However, among five *T. flavus* isolates, the most effective one was Tf-To-V-24 and Tf-To-U-36. In the experiment on the interaction between different *T. flavus* treatments and *T. flavus* isolates, a minimum infection index was observed when both soil and seed were treated with Tf-To-V-31. The overall results of this study show that it may be possible to manage tomato *Verticillium* wilt disease effectively by *T. flavus*.

Key words: tomato, *Verticillium albo-atrum*, antagonistic effects, *Talaromyces flavus*

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop next to potato. The present world production is about 100 million tons of fresh fruit produced on 3.7 million hectares. Tomato production was reported in 144 countries (Bal and Abak 2007).

Iran is ranked seventh in the world as a tomato producer. In Iran tomato is planted on about 140 000 hectares with an average yield of 37 tons per hectare (Alemzadeh Ansari and Mamghani 2008). *Verticillium* wilt is one of the most main diseases of tomato, which causes serious losses in the field (Paternotte and Van Kasteren 1993; Matta and Garibaldi 1997; Aminaee *et al.* 2006). *Verticillium dahliae* Kleb. and *Verticillium albo-atrum* Reinke and Berthold (*V. a.-a.*) are the causal agents of this disease (Pegg and Young 1982; Hutson and Smith 1982; Paternotte and Van Kasteren 1993; Kim *et al.* 2001; Mansoori and Smith 2005; Aminaee *et al.* 2006).

For controlling *Verticillium* wilt of tomato, cultural practices and the use of resistant cultivars are the most common strategies but they are either not available or not

effective (Jones *et al.* 1995; Burbos and Skoudridakis 1996; Giotis *et al.* 2009). In recent years, biological control using fungal and bacterial antagonists were applied to control tomato diseases (Hanafi 2003; Giotis *et al.* 2009).

Talaromyces flavus (Klockner) Stulk and Samson is a fungal antagonist that has been used in biological control of some soil-borne pathogens such as *V. dahliae*, *V. a.-a.*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Marois *et al.* 1984). Marois *et al.* (1982) showed that *T. flavus* decreased incidence of *Verticillium* wilt and increased yield of eggplant in England. Fahima and Henis (1997) showed that *T. flavus* decreased *Verticillium* wilt disease on eggplant at a 77% rate. The ability of this fungus occupying rhizosphere of tomato, cotton, and eggplant, and decreasing germination of microsclerotia of *V. dahliae* was reported by Marois *et al.* (1984).

The fungus (sexual form of *Penicillium dangeardii*) also was reported as a parasite of sclerotia of *R. solani* and *S. sclerotiorum* (McLaren *et al.* 1982). Tjamos and Fravel (1997) showed that *T. flavus* decreased *Verticillium* wilt on tomato plants. *T. flavus* and *Aspergillus terreus* were

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also reported to be the inhibitory agents of *Verticillium* wilt of olive trees in Greece (Tjamos 1991). Forty percent of non-volatile extracts (Talaron) of *T. flavus* are considered to affect the production of hydrogen peroxide due to the glucose oxidase enzyme and this gives antibacterial and antifungal characteristics to this fungus (Kim and Fravel 1990).

The objectives of this study were to investigate the antagonistic effects of *T. Flavus* isolates on *V. a.-a.* the causal agent of tomato wilt disease and to compare different application methods.

MATERIALS AND METHODS

The following experiments were conducted and executed in 2009.

Isolation of *V. a.-a.* from the soil

During 2008–2009, field soil samples were collected from tomato fields the Varamin area in the Tehran Province of Iran, and carried to the laboratory in plastic bags. Samples were air-dried, homogenized using a revolving jar mill and stored at 4°C. For isolation of *V. albo-atrum* from soil, the wet sieving procedure was used (Christen 1981).

Pathogenicity test

An isolate of *V. a.-a.*, obtained from tomato field soil was used in the study. This isolate was grown on tomato 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 plate and scraping the cultures with a rubber spatula. Using a haemocytometer, the inoculum concentration was adjusted to 10⁷ conidia per milliliter. Tomato seedlings of 'Early Urbuna Y' as a susceptible cultivar (Sahebani and Hadavi 2009) were inoculated at the 3rd–4th true leaf stage. These tomato seedlings were uprooted and inoculated by using the root-dip technique. Roots were washed with running water and placed for 60 minutes in conidia suspension. The inoculated seedlings were transplanted to pots containing a mixture of peatmoss, vermiculate and perlite (1 : 1 : 1, v/v/v). Three replications of five plants were used. The plants were kept on the bench at 21–23°C. Daylight was supplemented by fluorescent lights to provide a 12 h day length (Kim *et al.* 2001).

Disease symptoms were recorded approximately every 10 days for about one and a half months after inoculation. Disease severity was based on an arbitrary scale where 0 was assigned to a plant with no visual symptoms, 0.5 to a plant with symptoms on the cotyledon, 1 to a plant with one leaf infected, 1.5 when the second leaf was infected, 2 when the third leaf was infected and so on (Paplomatas *et al.* 1999).

Isolation of *T. flavus* from soil

Since *T. flavus* occupies rhizosphere of tomato (Marois *et al.* 1984), tomato fields in the Varamin and Uremia area in Tehran and the Western Azarbayjan provinces of Iran were selected for soil collection. Selective medium (TF medium) was adopted for isolation of fungus from the soil. This 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 pimarinic, 30 mg nystatine (Mycostatin, 4 960 units/mg) and 0.5 g oxgall (Bile, bovine). Lactic acid and the antimicrobial agents were added as aqueous solutions to autoclaved PDA at about 50°C. The medium was poured into Petri dishes (18 ml/dish). One ml of aliquots was removed from 10⁻² to 10⁻³ dilutions (soil in water) during agitation 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 7–8 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).

Investigation of the antagonistic effects of volatile extracts of *T. flavus* on *V. a.-a.* in laboratory experiments

In this step, *T. flavus* isolates were separately cultured on Petri dishes containing PDA medium and incubated at 30°C for 36 hours. After this time, 9-mm disks of *V. a.-a.* isolate, which were isolated from tomato field soil, were cultured on other Petri dishes containing PDA medium. These Petri dishes (without lids) were placed inversely on Petri dishes containing *T. flavus* isolates so that the pathogenic and antagonistic fungi affect each other. These Petri dishes were incubated at 25°C. After two weeks, the diameter of *V. a.-a.* colonies were recorded and growth inhibition percents were evaluated as follows:

$$\text{Growth inhibition percents} = \frac{\text{Colony diameter of } V. a.-a. \text{ in the control} - \text{colony diameter of } V. a.-a. \text{ in culture medium affected by volatile extract of } T. flavus}{\text{Colony diameter of } V. a.-a. \text{ in the control}} \times 100$$

Investigation of the antagonistic effects of non-volatile extracts of *T. flavus* on *V. a.-a.* in laboratory experiments

For preparation of non-volatile extracts, *T. flavus* isolates were separately cultured on Czapek-Dox medium and placed on a shaker at 50 rpm for ten days. In the next step, the culture media was filtered through filter paper. The filter paper had pores which were 0.45 μ in diameter (Eziashi 2006). The culture filtrates of fungal isolates

were then mixed with PDA medium at a 20% concentration separately. Culture filtrates mixed with PDA medium were transferred into Petri dishes. *V. a.-a.* which was isolated from tomato field soil was cultured on medium surface in every Petri dish when medium temperature attained 25°C. After one week, the diameter of *V. a.-a.* colonies was recorded and growth inhibition percent was evaluated as described previously.

Five *T. flavus* isolates that caused greater growth inhibition of *V. a.-a.* than the other isolates, were selected for the greenhouse experiments.

Greenhouse experiments

Plant inoculation with *V. a.-a.*

Preparation of *V. a.-a.* inoculum and the inoculation of the tomato plants were carried out according to the procedures described by Paplomatas *et al.* (1999).

Preparation of *T. flavus* inoculum

Preparation of *T. flavus* inoculum for every isolate was carried out separately as follows:

T. flavus isolated from soil was cultured in 1.6-cm-wide x 15-cm-high test tubes containing TF medium. After 5 days, 20 ml SDW was poured into 50 cm-wide x 80-cm-tall sterile plastic bag containing 250 g of peatmoss mixed with 10 ml D-lactose monohydrate (20 g/lit). Plastic bags were incubated at 30°C for 30 days. The content of each plastic bag was emptied after completely covering the surface of rice bran with *T. flavus* hyphae. The number of *T. flavus* ascospores in each g of rice bran was then determined using a hemocytometer. In this procedure, one gram of rice bran was suspended in 10 ml SDW and the number of ascospores in one g of rice bran was determined by counting ascospores in one ml of this prepared suspension. *T. flavus* inoculum was added to the soil in the pots at 10⁷ ascospores per g soil (Chet and Baker 1981). For seed treatment preparation, seed tomato tubers were floated in this inoculum.

Study of the antagonistic effects of *T. flavus* isolates on tomato *Verticillium* wilt disease

The experiment for the study on the effect of *T. flavus* isolates on the pathogenicity of *V. a.-a.* was carried out as follows:

The experiment was performed as a split plot arranged in a randomized complete block design with four replications. The main-factor was using different types

of *T. flavus* treatments at three levels (1 – soil, 2 – seed, 3 – both soil and seed) and the sub-factor was different fungal isolates at seven levels (from 1 to 5: one of five *T. flavus* isolates + *V. a.-a.*, 6: *V. a.-a.*, 7: without fungal inoculum).

Inoculated plants were kept at 22°C in the greenhouse under a 12 h light period. Disease symptoms were recorded approximately every 10 days for about one and a half months after inoculation. Disease severity was based on an arbitrary scale where 0 was assigned to a plant with no visual symptoms, 0.5 to a plant with symptoms on the cotyledon, 1 to a plant with one leaf infected, 1.5 when the second leaf was infected, 2 when the third leaf was infected and so on (Paplomatas *et al.* 1999). Data were analyzed by ANOVA (Analysis of Variance) using the MS TAT C statistical software, while means were separated by Duncan's multiple range test.

RESULTS

Isolation of *V. a.-a.* from soil and the pathogenicity test

After four weeks, inoculated plants exhibited stunting, chlorosis, and defoliation of lower leaves. The leaves were yellow at the edges and between veins. This discoloration occurred on both sides of the leaf. Leaflets showed a characteristic V-shaped or fann shaped yellowing, with the widest part of the V on the leaf edge. Browning of the vascular system was seen by cutting the stem open with a knife. This brown discoloration inside the stem extended from the roots of the plant to the top (Pohronezny 1991).

In this pathogenicity test, infection index for infected plant was 4.25.

Isolation of *T. flavus* from soil

As table 1 shows, fifteen *T. flavus* isolates were obtained from the tomato field soil of the Varamin and Uremia regions of Tehran and the Western Azarbayjan provinces.

Table 1. Inhibitory effects on *V. a.-a.* growth mediated by volatile and non-volatile extracts of *T. flavus* isolates

Code of <i>T. flavus</i> isolate	Collection region	Percentage inhibition of <i>V. a.-a.</i> mycelium growth	
		volatile extracts	non-volatile extracts
Tf-To-V-24	Varamin	54.72	73.00
Tf-To-V-25	Varamin	45.94	74.22
Tf-To-V-26	Varamin	46.62	72.77
Tf-To-V-27	Varamin	45.94	73.33
Tf-To-V-28	Varamin	45.94	72.77
Tf-To-V-29	Varamin	33.33	77.66
Tf-To-V-30	Varamin	50.00	80.88
Tf-To-V-31	Varamin	85.00	80.77
Tf-To-V-32	Varamin	35.00	78.77
Tf-To-V-33	Varamin	31.81	83.66
Tf-To-U-34	Uremia	31.66	73.11
Tf-To-U-35	Uremia	18.33	94.66
Tf-To-U-36	Uremia	33.33	95.22
Tf-To-U-37	Uremia	18.18	75.22
Tf-To-U-38	Uremia	48.33	92.55

Investigation of the antagonistic effects of volatile extracts of *T. flavus* on *V. a.-a.* in laboratory experiments

According to table 1, variable inhibitory effects on *V. a.-a.* growth were induced by different *T. flavus* isolates. Maximum inhibitory effect was mediated by isolate Tf-To-V-31 and minimum inhibitory effect by isolate Tf-To-U-37.

Investigation of the antagonistic effects of non-volatile extracts of *T. flavus* on *V. a.-a.* in laboratory experiments

As table 1 indicates different inhibitory effects on *V. a.-a.* growth were induced by *T. flavus* isolates. Maximum inhibitory effect was mediated by isolate Tf-To-U-36 and minimum inhibitory effect was caused by isolate Tf-To-V-26 and Tf-To-V-28.

Based on total inhibitory effects mediated by volatile and non-volatile extracts, the following *T. flavus* isolates

were selected for the greenhouse experiments: Tf-To-V-31, Tf-To-U-38, Tf-To-V-30, Tf-To-U-36 and Tf-To-V-24.

Evaluation of the antagonistic effects of *T. flavus* isolates on tomato *Verticillium* wilt disease

As table 2 shows, treatments affected by the main factor (different types of *T. flavus* treatments) were placed in one statistical group. The mean minimum infection index was 1.64 when soil and seed treatment were applied.

Treatments affected by the sub-main factor (different fungal isolates) were placed in five statistical groups. The minimum infection index mean was 1.25 in the case of Tf-To-V-24 + *V. a.-a.* and Tf-To-U-36 + *V. a.-a.*

In the experiment of interaction between main factor and sub-main factor, all treatments were placed in 10 statistical groups. Minimum infection index (0.62) was observed when both soil and seed were treated with Tf-To-V-31 (Table 3).

Table 2. The effects of main factor (different types of *T. flavus* treatments) and sub-main factor (different fungal isolates) on *Verticillium* wilt infection index on tomato plants

Variable source	Treatment	Infection index mean	Statistical grouping*
Main factor	seed	1.83	a
	soil	1.71	a
	soil and seed	1.64	a
Sub-main factor	<i>V. a.-a.</i> (control +)	4.25	a
	Tf-To-U-38 + <i>V. a.-a.</i>	2.21	b
	Tf-To-V-30 + <i>V. a.-a.</i>	1.71	bc
	Tf-To-V-31 + <i>V. a.-a.</i>	1.46	c
	Tf-To-U-36 + <i>V. a.-a.</i>	1.25	c
	Tf-To-V-24 + <i>V. a.-a.</i>	1.25	c
	without fungal inoculum (the control -)	0	d

*treatments marked by the same letter (s) are not significantly different (p > 0.01)
V. a.-a. – *Verticillium albo-atrum*

Table 3. The interactive effects between main factor and sub-main factor (all treatments) on *Verticillium* wilt infection index on tomato plants

Treatments	Infection index mean	Statistical grouping*
<i>V. a.-a.</i> (control +)	4.25	a
Tf-To-U-38 (seed) + <i>V. a.-a.</i>	2.75	b
Tf-To-U-38 (soil & seed) + <i>V. a.-a.</i>	2.37	c
Tf-To-V-30 (soil) + <i>V. a.-a.</i>	1.87	d
Tf-To-V-31 (soil) + <i>V. a.-a.</i>	1.87	d
Tf-To-V-31 (seed) + <i>V. a.-a.</i>	1.87	d
Tf-To-V-30 (seed) + <i>V. a.-a.</i>	1.75	de
Tf-To-U-38 (soil) + <i>V. a.-a.</i>	1.50	def
Tf-To-V-30 (soil & seed) + <i>V. a.-a.</i>	1.50	def
Tf-To-U-36 (soil & seed) + <i>V. a.-a.</i>	1.37	ef
Tf-To-V-24 (soil & seed) + <i>V. a.-a.</i>	1.37	ef
Tf-To-U-36 (soil) + <i>V. a.-a.</i>	1.25	f
Tf-To-V-24 (soil) + <i>V. a.-a.</i>	1.25	f
Tf-To-V-24 (seed) + <i>V. a.-a.</i>	1.12	f
Tf-To-U-36 (seed) + <i>V. a.-a.</i>	1.12	f
Tf-To-V-31 (soil & seed) + <i>V. a.-a.</i>	0.62	g
Without fungal inoculum (the control -)	0	h

*treatments marked by the same letter (s) are not significantly different (p > 0.01)
V. a.-a. – *Verticillium albo-atrum*

DISCUSSION

The overall results of this study show that it may be possible to manage tomato *Verticillium* wilt disease efficiently by seed treatment with effective *T. flavus* isolates. The inhibitory effect of volatile and non-volatile extracts of several bacterial and fungal microorganisms on growth and activity of some fungal pathogens were demonstrated in previous studies. For example, the volatile extracts of *F. oxysporum* resulted in induction of resistance to chickpea (*Cicer arietinum* L.) against fungal pathogens (Cherif *et al.* 2007). The efficiency of non-volatile extracts of some bacterial and fungi including *A. flavus*, *A. ochraceus*, *Penicillium aurantiogriseum*, *Bacillus subtilis* and *Trichoderma harzianum* for controlling antracnose of cowpea (*Vigna unguiculata* L. Walp.) were reported (Adebanjo and Bankole 2004).

In other studies, the effect of these extracts on different soil-borne fungal diseases of tomato were also shown (Josh *et al.* 2009). In another study, the effect of volatile and non-volatile extracts of *T. flavus* on root-rot disease of lettuce (*Lactuca sativa* L.) caused by *Sclerotinia minor* was shown in the laboratory and greenhouse experiments. Results of this study confirmed that the inhibitory effect of variable components of extracts on pathogenic agent growth was different (El-Tarabily *et al.* 2000). Results of another study on the use of non-volatile extracts such as chitinase produced by *T. harzianum* and *T. flavus* were effective for controlling soybean stem white rot disease caused by *S. sclerotiorum* and bean stem rot caused by *S. Rolfsii*, respectively (Madi *et al.* 1997; Menendez and Godeas 1998).

Other examples of fungal metabolites such as glucanase secreted by *Zygorrhynchus moelleri* and glucose oxidase produced by *T. flavus* have also been effective against some soil borne plant pathogenic fungi (Brown 1987; Murraray *et al.* 1997).

Results of studies on the use of *T. flavus* in biocontrol of pathogenic fungi showed that *T. flavus* was an important antagonist on *V. dahliae* and *V. a.-a.* (Tjamos and Paplomatas 1987; Wikins *et al.* 2000; Vidhyasekaren 2004). Kim and Fravel (1990) showed that glucose oxidase produced by *T. flavus* prevented formation of microsclerotia of *V. dahliae*. However, Proksa *et al.* (1992) showed that 2-methyl sorbic acid secreted by *T. flavus* showed an inhibitory effect on the growth of *V. a.-a.*

The differences between the results of the laboratory experiments in the present study and the previous ones could have been due to the extract concentration. It was reported that there was a minimum concentration (0.1 µg/ml) for every effective component of non-volatile extracts of *F. oxysporum* (Cyclosporin) used for growth inhibition of *S. sclerotiorum* (Rodriguez *et al.* 2006). In another part of this study, using effective *T. flavus* isolates as seed treatment decreased *Verticillium* wilt infection index in greenhouse conditions. These results agree with those of some previous studies (Soytong and Ratanacherdchai 2005; Kulikov *et al.* 2006). The minor differences between the results of this study and those of the previous ones could be due to method of seed treatment with *T. flavus* isolates and to the intervals before sowing (Nagtzaam and Bollen 1997).

Overall results of our study indicate that the use of effective *T. flavus* isolates could be an effective strategy

for controlling *Verticillium* wilt disease of tomato. The present findings may have a practical application in the formulation of disease management strategies in the field. The promising results of this study may be used for reduction of losses caused by plant diseases, to minimize the application of chemical fungicides, protect the environment and preserve biological resources in a sustainable agricultural system.

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POLISH SUMMARY

BIOLOGICZNE ZWALCZANIE UWIAŁDU POMIDORA PRZY POMOCY GRZYBA *TALAROMYCES FLAVUS*

W pracy przedstawiono wyniki badań nad antagonizującym grzybem *Talaromyces flavus*, wyizolowanym z próbek gleby zebranych z polowych upraw pomidora w Teheranie i zachodnim Azarbayjanie, prowincjach Iranu. W warunkach laboratoryjnych i szklarniowych, badano antagonizujący wpływ izolatów *T. flavus* przeciwko czynnikowi sprawczemu uwiądu pomidorów – *Verticillium albo-atrum* (*V. a.-a.*). Próbkę gleby zebrane w regionach Varamin i Uremia (Teheran) oraz zachodnich prowincjach Azarbayjanu, hodowano na pożywcę selektywnej. Trzy tygodniowe kultury grzyba *T. flavus* wykorzystano do dalszych badań. W doświadczeniach laboratoryjnych badano antagonizujący wpływ lotnych i nielotnych ekstraktów pochodzących z izolatów grzyba *T. flavus* na wzrost *V. a.-a.* Do badań szklarniowych wybrano pięć izolatów *T. flavus* znacznie ograniczających wzrost *V. a.-a.* Przed przystąpieniem do doświadczeń szklarniowych, przygotowano inokulat *V. a.-a.* oraz różne ekspozycje grzyba *T. flavus*. W celu porównania indeksu porażenia poszczególnych wariantów, doświadczenia szklarniowe przeprowadzono na pojedynczo rozsiewionych poletkach w układzie bloków losowanych w czterech powtórzeniach. Wyniki badań szklarniowych, nie wykazały istotnej różnicy pomiędzy kombinacjami z wykorzystaniem grzyba *T. flavus*, choć spośród pięciu badanych izolatów *T. flavus*, najskuteczniejsze okazały się kombinacje Tf-To-V-24 i Tf-To-U-36. Badając interakcję w różnych kombinacjach z grzybem *T. flavus*, najniższy indeks porażenia stwierdzono zarówno w glebie, jak i nasionach traktowanych Tf-To-V-31. Wyniki badań wskazują na możliwość skutecznego ograniczania występowania uwiądu pomidora przy pomocy grzyba *T. flavus*.