



Asian Journal of Plant Sciences

ISSN 1682-3974

science
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Study on Antagonistic Effects of Non-Volatile Extracts of *Talaromyces flavus* on Cotton Verticillium Wilt Disease

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Abstract: In this study, *Talaromyces flavus*, a fungal antagonist, was isolated from soil samples that were collected from cotton fields in Golestan province of Iran. Antagonistic effects of non-volatile extracts of *T. flavus* isolates on *Verticillium dahliae* growth were investigated separately in laboratory experiments. Different isolates of *T. flavus* caused variable percents of growth inhibition of *V. dahliae*. Among isolates, ten of them that caused more growth inhibition of *V. dahliae* than other isolates, were selected for greenhouse experiments. In this step, first inoculum of microsclerotia of *V. dahliae* and seed treatments affected by non-volatile extracts and ascospores suspension of ten isolates of *T. flavus* were prepared. For comparison of infection index in seed treatment, greenhouse experiment was performed as a completely randomized design with forty-three treatments and three replications. Results of disease evaluation showed that among seed treatments affected by ascospores suspension of *T. flavus* isolates, seed treatments of isolates TF-2, Tf-12 and TF-17 caused maximum decrease in Verticillium wilt index. However, among seed treatments affected by non-volatile extracts of *T. flavus* isolates, seed treatments of isolates TF-1, TF-17 and TF-13 resulted in maximum decrease in disease index.

Key words: Cotton, Verticillium wilt disease, seed treatment, non-volatile extracts, *Talaromyces flavus*

INTRODUCTION

Cotton is an important cash crop around the world including Iran. Cotton is cultivated in about 20 provinces of Iran (Heydari *et al.*, 2005; Naraghi *et al.*, 2007). Harmful pests (insects, weeds and pathogens) are among the most important yield reducing agents in cotton fields (Niederhauser and Niederhauser, 2000; Mi *et al.*, 1998; Heydari *et al.*, 2005; Heydari and Misaghi, 1998; Naraghi *et al.*, 2007; Abd-Elsalam *et al.*, 2007).

Verticillium wilt is considered the most important disease of cotton in Iran (Heydari *et al.*, 2007; Naraghi *et al.*, 2007). For controlling Verticillium wilt of cotton, cultural practices and the use of resistant varieties are the most common strategies which are not either available or effective. Biological control using fungal and bacterial antagonists in recent years, have been applied to control cotton diseases (Heydari *et al.*, 1997, 2007; Heydari and Misaghi, 1998, 2003; Spink and Rowe, 1989; Aziz *et al.*, 1997).

Talaromyces flavus (Klockner) Stulk and Samson is a fungal antagonist that has been used in biological control

of Verticillium wilt of several plants (Marois *et al.*, 1984; Tjamos and Fravel, 1995; Naraghi *et al.*, 2004). Marois *et al.* (1982) showed that *T. flavus* decreased the incidence of Verticillium wilt and increased yield of egg-plant in England. Fahima and Henis (1997) showed that *T. flavus* decreased Verticillium wilt disease on egg-plant at the rate of 77%. The ability of this fungus for occupying rhizosphere of potato, cotton, egg-plant and decreasing germination of microsclerotia of *Verticillium dahliae* have been reported by Marois *et al.* (1984).

This fungus (sexual form of *Penicillium dangerdii*) also was reported as parasite of sclerotia of *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (McLaren *et al.*, 1982). Tjamos and Fravel (1997) showed that *T. flavus* decreased Verticillium wilt on tomato plants. *T. flavus* and *Aspergillus terreus* have also been 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* belongs to Glucose oxidase enzyme and the production of Hydrogen peroxide by this enzyme gives antibacterial and antifungal characteristics to this fungus (Kim and Fravel, 1990).

The objective of this study was to compare two methods of cotton seed treatments with *T. flavus* ascospore suspension in biological control of Verticillium wilt disease on cotton.

MATERIALS AND METHODS

The following experiments were conducted during year 2005.

Isolation of *Talaromyces flavus* from soil: Since *T. flavus* is a thermophilic fungus (Guarro *et al.*, 1999), therefore cotton fields in Karkandeh Research Station in Golestan province of Iran was selected for soil collection. Selective medium (TF medium) was adopted for isolation of these isolates from 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 HCl, 50 mg chloramphenicol, 4 mg pimarinin (2.5% sterile water suspension), 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 autoclaved PDA at about 50°C. The medium was poured in to petri dishes (18 mL per dish). One milliliter 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 soil (Marois *et al.*, 1984).

Investigation of the antagonistic effects of non-volatile extracts of *T. flavus* on *V. dahliae* in laboratory experiments: For preparation of non-volatile extracts, 20 different isolates of *T. flavus* were cultured on Czapek Dex Broth medium separately and placed on shaker in 50 rpm for ten days. In next step, the culture media were filtered through filter paper with pores in 0.45 µ diameter (Eziashi, 2006). The culture filtrates of fungal isolates were then mixed with PDA medium in 20% concentration separately. Culture filtrates mixed with PDA medium were transferred in to Petri dishes. *V. dahliae* which was previously isolated from cotton field soil (Naraghi *et al.*, 2007) was cultured on medium surface in every Petri dish when medium temperature attained to 50°C. After one week, diameter of *V. dahliae* colonies were recorded and growth inhibition percents were evaluated as follows:

$$\frac{\text{Colony diameter of } V. dahliae \text{ in culture medium mixed with filtrate} - \text{Colony diameter of } V. dahliae \text{ in culture medium without filtrate}}{\text{Colony diameter of } V. dahliae \text{ in culture medium mixed with filtrate}} \times 100$$

Preparation of *V. dahliae* inoculum for greenhouse experiment: In this experiment, one isolate was used (Naraghi *et al.*, 2007). Preparation of *V. dahliae* microsclerotia suspension of every strain was carried out separately according to the procedure described by Tjamos and Fravel (1995).

Pot soil inoculation was carried out as follows:

Two hundreds microsclerotia were used for each gram of pot soil. One milliliter of suspension containing microsclerotia was poured in to a 2 cm diameter petri plate and the number of microsclerotia was determined using stereo-dissecting microscope.

Preparation of seed treatment affected by *T. flavus* for pot soil: In this step, 10 isolate of *T. flavus* that showed more inhibitory effects on *V. dahliae* growth were selected for preparation of seed treatments. Cotton seed treatments were divided to two groups. First group was affected by ascospores suspension of *T. flavus* (2×10² ascospore mL⁻¹) and other group was affected by non-volatile extract of *T. flavus*. Preparation of non-volatile extracts was described in above section.

For preparation of ascospores suspension of *T. flavus*, this fungus was cultured on Potato Dextrose Agar (PDA) medium and incubated in 30°C for three weeks. In next step, 10 mL SDW was spread on medium surface containing ascospores and ascospores number of obtained suspension were determined in one milliliter (Marois *et al.*, 1984). This step was carried out for every *T. flavus* isolate separately.

Evaluation of the antagonistic effects of *T. flavus* as seed treatment on pathogenicity of *V. dahliae* in the greenhouse: A completely randomized design with 22 treatments and 3 replications was used. Each replication consisted of a 9 cm diameter and 20 cm height plastic pot containing 3 kg sterile soil of cotton field in Golestan province and five seeds of Varamin cotton cultivar. Treatments consisted of:

- Soil and seed without inoculum (negative control)
- Soil with *V. dahliae* inoculum and seed without inoculum (positive control)

From 3 to 12-soil with *V. dahliae* inoculum and seeds treated with non-volatile extracts of 10 *T. flavus* isolates.

From 13 to 22-soil with *V. dahliae* inoculum and seeds treated with ascospore suspension of 10 *T. flavus* isolates.

Leaf Infection Index (Iakutkin and Popov, 1979) was determined 8 weeks after sowing. Analysis of variance and mean comparison were performed using MSTATC statistical software.

RESULTS

Isolation of *Talaromyces flavus* from soil: As Table 1 shows twenty *T. flavus* isolates were obtained from cotton fields soil in different regions of Golestan province.

Investigation of the antagonistic effects of non-volatile extracts of *T. flavus* on *V. dahliae* in laboratory experiments: As Table 2 shows variable inhibitory percents on *V. dahliae* growth were induced by different *T. flavus* isolates. Maximum inhibitory percent was mediated by isolate TF-1 and minimum inhibitory percent belonged to isolate TF-16.

Selected isolates for greenhouse experiments included: TF-1, TF-17, TF-13, TF-2, TF-12, TF-3, TF-18, TF-20, TF-6 and TF-4.

Evaluation of the antagonistic effects of *T. flavus* as seed treatment on pathogenicity of *V. dahliae* in the greenhouse: As tables show seed treatments affected by ascospore suspension or non-volatile extracts of *T. flavus* isolates were placed in 9 statistical groups (Table 3, 4). Minimum infection index mean was 0 that belonged to treatments affected by non-volatile extracts of isolates

Table 1: Code of *T. flavus* isolates obtained from different regions of Golestan province

Code of <i>T. flavus</i> isolates	Collection regions
TF-1	Karkandeh
TF-2	Galikesh
TF-3	Bandar-e-Torkaman
TF-4	Karkandeh
TF-5	Emer
TF-6	Aliabad-e-Katool
TF-7	Jelin
TF-8	Emer
TF-9	Fenderesk
TF-10	Fenderesk
TF-11	Emer
TF-12	Emer
TF-13	Kaka
TF-14	Faselabad
TF-15	Emer
TF-16	Emer
TF-17	Emer
TF-18	Kaka
TF-19	Emer
TF-20	Faselabad

TF-1 and TF-17 (Table 3) and treatments affected by ascospore suspension of isolates TF-2, TF-12 and Tf-17 (Table 4). However, there was maximum infection index mean (26.9) in treatment affected by non-volatile extracts of isolate TF-4 (Table 3).

Table 2: Inhibitory percents on *V. dahliae* growth mediated by non-volatile extracts of *T. flavus* isolates

Code of isolates	Inhibitory percent
TF-1	95.00
TF-17	76.92
TF-13	63.00
TF-2	55.00
TF-12	51.00
TF-3	46.00
TF-18	42.30
TF-20	30.76
TF-6	29.00
TF-4	28.00
TF-19	23.07
TF-11	22.00
TF-14	22.00
TF-9	17.00
TF-5	15.00
TF-7	14.00
TF-10	11.00
TF-8	9.00
TF-15	3.00
TF-16	2.15

Table 3: The effect of seed treatment affected by non-volatile extracts of *T. flavus* isolates on Verticillium wilt infection index on cotton plants

Treatments	Infection index mean	Statistical grouping*
TF-4	26.90	a
TF-6	21.01	b
TF-20	15.97	c
Control (+)	15.38	c
TF-18	7.36	de
TF-3	5.96	def
TF-12	5.87	def
TF-2	2.42	efg
TF-13	0.55	fg
TF-17	0.00	fg
TF-1	0.00	fg
Control (-)	0.00	fg

*Treatments marked by the same letter(s) are not significantly different (p>0.01)

Table 4: The effect of seed treatment affected by ascospore suspension of *T. flavus* isolates on Verticillium wilt infection index on cotton plants

Treatments	Infection index mean	Statistical grouping*
Control (+)	15.38	c
TF-20	8.07	d
TF-3	4.75	defg
TF-4	4.37	defg
TF-1	4.33	defg
TF-6	3.00	defg
TF-18	2.60	efg
TF-13	1.45	fg
TF-17	0.00	fg
TF-12	0.00	fg
TF-2	0.00	fg
Control (-)	0.00	fg

*Treatments marked by the same letter(s) are not significantly different (p>0.01)

DISCUSSION

The overall results of this study show that it may be possible to manage cotton Verticillium wilt disease efficiently by seed treatment with non-volatile extracts or ascospore suspension of *T. flavus*. The inhibitory effect of metabolites of several bacterial and fungal microorganisms on growth and activity of important fungal pathogens have been shown in previous studies. For example, the efficiency of non-volatile metabolites of some bacteria and fungi such as *Aspergillus flavus*, *A. ochraceus*, *Penicillium aurantiogriseum*, *Bacillus subtilis* and *Trichoderma harzianum* for controlling bean antracnose disease have been reported (Adebanjo and Bankole, 2004).

In an other study, the effect of these metabolites on root rot disease of lettuce (*Lactuca sativa*) caused by *Sclerotinia minor* was shown in laboratory and greenhouse experiments. This study confirmed that the inhibitory effect of variable components of metabolites on pathogenic agents growth was different (EL- Tarabily *et al.*, 2000). According to conclusion of this study, among these components, chitinase enzyme produced by *T. harzianum* (Menendez and Godeas, 1998) and *T. flavus* (Madi *et al.*, 1997) were effective for controlling soybean stem white rot disease caused by *S. sclerotiorum* and bean stem rot caused by *S. rolfisii* respectively. Other examples, such as B-1,3 gluconase secreted by *Burkholderia cepacia* (Fridlender *et al.*, 1993) and glucose oxidase produced by *T. flavus* (Murray *et al.*, 1997) have been effective for decreasing seedling damping-off and Verticillium wilt diseases respectively. According to the above studies, for controlling different pathogens, used effective components are variable.

In other study, it has been shown that peroxide hydrogen prevented formation of microsclerotia of *V. dahliae* and *S. rolfisii* (Kim and Fravel, 1990). Since, glucose oxidase enzyme produced by *T. flavus* acts in glucose presence and this activity is resulted in peroxide hydrogen production therefore, glucose addition to non-volatile metabolite of *T. flavus* caused increase in inhibitory effect on microsclerotia formation (Murray *et al.*, 1997).

The differences between results of laboratory experiments of this study and the previous ones could be due to metabolite concentration. In this regard, it was reported that there was a minimum concentration for every effective component. For example, minimum concentration of effective component of non-volatile extracts of *Fusarium oxysporum* (Cyclosporin) used for growth inhibition of *S. sclerotiorum* was 0.1 µg mL⁻¹ (Rodriguez *et al.*, 2006).

In an other part of this study, using non-volatile extracts and ascospore suspension of *T. flavus* decreased Verticillium wilt infection index in greenhouse conditions. Results of present study in this part agree with those of some previous studies (Hashem and Ali, 2004; Chitarra *et al.*, 2004). The differences between the results of this study and those of the previous ones could be due to method of seed treatment with non-volatile extracts of *T. flavus* in intervals before sowing (Hashem and Ali, 2004).

Based on the overall results of this study, it is concluded that the use of antagonistic fungi including *T. flavus* and its metabolites and ascospore suspension could be an effective method for controlling Verticillium wilt disease of cotton and may be considered a major component of Integrated Pest Management (IPM) strategies for managing this disease which is a very important and destructive disease of cotton in all cotton growing area in the world.

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