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In vitro Antagonistic Mechanisms of *Trichoderma* spp. and *Talaromyces flavus* to Control *Gaeumannomyces graminis* var. *tritici* the Causal Agent of Wheat Take-all Disease

Leila Ghanbari, Seddighe Mohammadi*

Department of Plant Protection, Shiraz Islamic Azad University, Shiraz Branch, Shiraz, Iran

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* Corresponding Author:
E-mail: mohammadi.myco@gmail.com

A B S T R A C T

Wheat take-all disease caused by *Gaeumannomyces graminis* var. *tritici* has recently been detected in different regions of Iran. With respect to biocontrol effect of *Trichoderma* spp. on many pathogenic fungi, seven isolates of *Trichoderma* and four isolates of *Talaromyces* were *in vitro* evaluated in terms of their biological control against the disease causal agent. In dual culture test the five isolates showed efficient competition for colonization against pathogenic fungus and the highest percentages of inhibition belonging to *Talaromyces flavus* 60 and *Talaromyces flavus* 136 were 59.52 and 57.61%, respectively. Microscopic investigations showed that in regions where antagonistic isolates and *Gaeumannomyces graminis* var. *tritici* coincide, hyphal contact, penetration and fragmentation of *Gaeumannomyces graminis* var. *tritici* were observed. Investigating the effect of volatile and non-volatile compounds at 10 ml concentration showed that the highest inhibition percentage on mycelium growth of the pathogen caused by *T. harzianum* (44.76%) and *T. longibrachiatum* (52.38%) respectively.

Introduction

Wheat forms one-fourth of world cereal production and is the main calorie source of over 1.5 milliard people. On average, wheat provides one-fifth of the calorie required for the whole people on the earth (FAO, 2003). Wheat take-all is caused by *Gaeumannomyces graminis* var. *tritici*. This root disease has been reported from most of the wheat cultivations all over the world (Huber and McCay-Buis, 1993). Fahima and Henis (1997) listed the pathogenic fungus among the eight important pathogenic fungi of wheat in the world and introduced it as one of the most important wheat pathogens in Oceania. Take-all disease control in infected area was to use herbicide, fungicide, soil improvement, burning plan residues, nutrition management and crop rotation (Yarham, 1981). Use of fungicides for the control of soil borne diseases is costly and also they have undesirable effects on environment, humankind and beneficial microorganisms in soil (Dluzniewska, 2003). This has diverted the attention of plant pathologists towards alternative methods for the control of plant diseases. Biological control using antagonistic fungi and bacteria is one of the proper practices to control the disease (Maghsoodloo et al. 2009). For example, Duffy et al. (1996) observed that combination of fluorescent Pseudomonads and *Trichoderma koningii* is more effective to biologically control take-all disease than a combination of several bacterial isolates (Duffy et al. 1996). Also, Kucuk and Kivanc (2003) reported that ten *T. harzianum* isolates inhibited *in vitro* growth of the pathogenic fungus mycelium from 28 to 100% (Kucuk and Kivanc, 2003). In a study the isolates of *Talaromyces flavus* isolated from cotton farms in Gorgan were tested to control cotton Verticillium wilt *in vitro* and in greenhouse conditions (Naraghi et al., 2007). They showed that the tested isolates were effective for the disease control and increased the yield as well. In a greenhouse research by Mehrabi Koshki et al. (2008) the useful effect of five *T. virens* isolates and two *T. koningiopsis* and *T. viridescens* isolates and a *T. koningii* isolate as well as biological compounds including trichodermin and subtilin on the control of take-all disease was reported. Moreover, in a study it was found that antagonistic isolates including *T. harzianum* B-1 and *T. harzianum* G-4 as well as fungicides like rural TS, propiconazole, benomyl, and carboxin thiram had high effect against take-all disease (Foroutan, 2008). This study was carried out to investigate the *in vitro* antagonistic effects of *Trichoderma* spp. and *Talaromyces flavus* isolates against *Gaeumannomyces graminis* var. *tritici* (Ggt), the causal agent of wheat take-all disease in 2011-2012.

Materials and methods

Preparation of Biocontrol Agents

Trichoderma species used here were isolated by Iranian Pistachio Research Institute, Rafsanjan. *Trichoderma* species were grown on PDA media for further use.

Preparation of Pathogenic Fungus

Isolate T139 of *Gaeumannomyces graminis* var. *tritici* was provided by Zarghan Agriculture Research Center in Fars province, Iran.

Pathogen Preservation

To preserve the pathogen, PDA blocks were isolated from margins of 4-day old colonies of the pathogenic fungus and transferred into laboratory tubes containing PDA medium. These tubes were kept at 25ºC for one week. Then the cotton on top of the tubes was flamed and the tubes were sealed using parafilm and stored at 7ºC.

Determination of the in Vitro Antagonistic Activities of Biocontrol Agents

In order to investigate the ability of *Trichoderma* and *Talaromyces flavus* isolates for inhibition of mycelium growth, establishment and development of *Gaeumannomyces graminis* var. *tritici*, the causal agent of wheat take-all disease, and also to investigate if the antagonism is due to parasitism or antibiosis, the following experiments were conducted:

Dual culture test: This was carried out to compare the ability of different *Trichoderma* and *Talaromyces flavus* isolates for *in vitro* growth inhibition of Ggt and also their establishment and development on the pathogenic fungus. In dual culture, 5-mm rings from the margins of 4-day old colonies of the pathogenic fungus were placed on one side of 9-cm Petri dishes containing PDA and on the opposite side placed the 5-mm ring from the margins of 4-day old colonies belonging to *T. koningii, T. harzianum* S, *T. harzianum* M, *T. harzianum* A, *T. harzianum* D, *T. virens, T. koningii*, *Talaromyces flavus* 134, *Talaromyces flavus* 136, *Talaromyces flavus* 75, *Talaromyces flavus* 60 and *T. longibrachiatum* with 1 cm margins (Mohammadi et al. 2010). A 5-mm ring from the margins of pathogenic fungus was placed at the center of a plate as control. Petri dishes were kept at 25ºC and diametrical growth measurements were carried out for three times with 24, 72 and 120 hr intervals after incubation. The inhibitory percentage of mycelium growth was determined using the Abbott's formula (Abbott, 1925):

$$\mathrm{MGIP} = \frac{\mathrm{CDGC} - \mathrm{CDGT}}{\mathrm{CDGC}} \times 100$$

MGIP: mycelium growth inhibitory percentage
CDGC: colony diametrical growth of control
CDGT: colony diametrical growth of treatment

In this experiment in addition to mycelium growth inhibitory percentage of different *Trichoderma* and *Talaromyces flavus* isolates, against the pathogenic fungus after the growth was halted, development on take-all agent was investigated as well.

Direct contact:

i) By microscope slide: Sterile microscope slides were placed at the center of Petri dishes (10 cm) containing PDA media. A mycelial disc (5mm) from the margin of 4-day old colonies of *Gaeumannomyces graminis* var. *tritici* were placed each one side of each Petri dish and a mycelial disc (5mm) from margin of 4-day colonies of *Trichoderma* and *Talaromyces flavus* isolates were placed on the other side. For each isolate, three replicates were considered. Petri dishes incubated at 25°C for 7 days. Then microscope slides were removed from the media, their bottom side were cleaned and effect and interactions among fungi was investigated under the microscope with different magnifications (Mohammadi et al. 2010).

ii) By creation stria: Strip with one centimeter width was cut by a sterile scalpel at the center of the petri dishes (10 cm) containing the PDA media. A mycelial disc (5mm) from the margin of 4-day old colonies of *Gaeumannomyces graminis* var. *tritici* were placed one side of each Petri dish and a mycelial disc (5mm) from margin of 4-day old colonies of *Trichoderma* and *Talaromyces flavus* isolates were placed on the other side. After 5 days pathogenic and antagonistic isolates collided. Then hyphal contact and connection were investigated under the microscope (Mohammadi et al. 2010).

The effect of volatile compounds: Effect of volatile metabolites produced by *Trichoderma* and *Talaromyces flavus* isolates against test pathogen were evaluated by the method of Dennis and Webster (1971). The mycelia disc (5mm) from margins of 4-day colonies of *Trichoderma* and *Talaromyces flavus* isolates were placed on center of a 10-cm Petri dishes. A 5-mm ring from PDA was placed instead of 5-mm ring of antagonistic isolates the center of control plates. After 24 h, the top of each Petri dish was replaced with the bottom of *Gaeumannomyces graminis* var. *tritici* plates, so as test pathogen was directly exposed to the antagonistic environment created by antagonistic isolates. The pairs of each Petri dish were fixed and sealed together with paraffin tape and incubated at 25^0C. Radial growth of the pathogen was recorded after 24, 72 and 120 h of incubation and percentage inhibition of pathogenic mycelium growth was calculated by Abbott's formula (section 2.4.1).

The effect of non-volatile compounds : This test was conducted to investigate the effects of non-volatile compounds produced by *Trichoderma* and *Talaromyces flavus* isolates against the mycelial growth of the pathogenic fungus. Four 1-cm disks from the margins of *Trichoderma* colonies were transferred into 250-ml flasks containing PDB medium and four 1-cm disks from *Talaromyces flavus* isolates were also transferred into 250-ml flasks containing liquid Czapek Dox Broth medium. The flasks were shaken using a rotary shaker-oven at 25ºC with 120 shake/min for *Trichoderma* isolates, and 50 shake/min for *Talaromyces flavus* isolates. After 10 days the contents of the flasks were removed and extracted using autoclaved millipore and vacuum pump with 0.22-µm microfilters. The resulting solution was used to investigate the effects of liquid exudates from *Trichoderma* and *Talaromyces flavus* isolates on the mycelial growth of the pathogenic fungus.

In this experiment for each *Trichoderma* and *Talaromyces flavus* isolate two 5- and 10-ml treatments from the extract were added to 20 ml of PDA medium at room temperature and mixed well before transferring into the plates. When the plates were cooled enough, a 5-mm ring from the margins of 4-day colonies belonging to take-all pathogen was placed on the center of each plate and the plates were kept at 25ºC (Mohammadi et al. 2010).

The diametrical growth of pathogenic fungus was measured at 24, 72 and 120 hr after incubation. The percentage inhibition of mycelium growth was determined using the above formula.

Statistical Analysis

Dual culture test and investigation of volatile compound effects were performed in a completely randomized design consisting of 12 treatments with three replicates. The data from the experiments were statistically analyzed. The treatments were compared at $p \leq 0.01$ using Duncan multiple range test (Little and Hills, 1978).

Results

Dual Culture Test

All tested isolates were successful to inhibit the growth of pathogen and the isolates including *Talaromyces flavus* 60, *Talaromyces flavus* 134, *Talaromyces flavus* 136, *T. harzianum* A and *T. harzianum* S covered the pathogen colonies and became dominant. Thus *Trichoderma* and *Talaromyces flavus* isolates completely covered the pathogen colonies after 3-4 and 6 days, respectively.

Statistical analysis of the data showed that there is a significant difference between inhibitory effects of *Trichoderma* and *Talaromyces flavus* isolates ($P \leq 0.01$).

When the percentage of inhibition from *Trichoderma* and *Talaromyces flavus* isolates were compared it was determined that the highest levels of inhibition were achieved by using *Talaromyces flavus* 60 and *Talaromyces flavus* 136 after 120 hr (59.52 and 57.61%, respectively). No significant difference was observed between *Talaromyces flavus* 60 and *Talaromyces flavus* 136 (Table 1).

Direct Contact

The microscopic investigations showed that at earlier stages of contact, the hyphae of all tested antagonistic isolates contacted with the hyphae of the fungal pathogen in some regions and grew paralel to them. As the time passed the hyphal contact increased and direct penetration of antagonistic hyphae into the fungus hyphae was also observed. While using groove method, fragmentation was also observed in case of *Talaromyces flavus* 134. However, none of the antagonistic isolates formed hyphal coil.

The Effects of Volatile Compounds

There was a significant difference ($P \leq 0.01$) between *Trichoderma* and *Talaromyces flavus* isolates in terms of inhibiting the mycelium growth of *Gaeumannomyces graminis* var. *tritici.*

When the mean values of mycelium growth inhibitory effects on Ggt due to volatile compounds of *Trichoderma* and *Talaromyces flavus* isolates were compared, it was shown that the highest level of inhibition (44.76%) caused by *T. harzianum* after 120 hr. As time passed, the percentage of inhibition by *T. harzianum* increased. In case of other isolates this trend was decreasing from 72 to 120 hr (Table 2).

The Effect of Non-Volatile Compounds

The results from the statistical analysis showed that there was significant difference among *Trichoderma* and *Talaromyces flavus* isolates in terms of the effect of non-volatile compounds (25 and 50%) on the inhibition of the mycelial growth of Ggt.

When the means of inhibition rates of non-volatile compounds (25%) from *Trichoderma* and *Talaromyces flavus* isolates were compared, it was found that the highest levels of inhibition caused by *Talaromyces flavus* 136, *T. harzianum* and *T. harzianum* S as 51.41, 40.88 and 37.73%, respectively, after 72 hr. No significant difference was found between *Talaromyces flavus* 136, *T. harzianum* and *T. harzianum* S. Also, when the percentages of mycelium growth inhibition of Ggt by non-volatile compounds (50%) of *Trichoderma* and *Talaromyces flavus* isolates were compared, it was determined that *T. longibrachiatum* caused the highest inhibition rates of 52.83 and 52.38% at two recording intervals. In case of *T. harzianum* M and *T. koningi* the inhibition percentage increased as time passed while about other isolates this trend was first increasing from 24 to 72 hr and then decreasing from 72 to 120 hr (Table 3, 4).

Table 1 The mean percentage inhibition of the colony growth of wheat take-all agent *Gaeumannomyces graminis* var. *tritici* by different treatments, 24, 72 and 120 hours after incubation

Treatment	Mean (24 h)	Mean (72 h)	Mean (120 h)
Talaromyces flavus 60	52.22 a	52.28 a	59.52 a
Talaromyces flavus 136	51.11 a	52.28 a	57.61 a
Trichoderma harzianum S	51.11 a	50.32 ab	49.04 b
Trichoderma harzianum A	48.89 a	45.75 b	46.43 bc
Talaromyces flavus 134	45.55 a	39.21 c	44.28 c
Trichoderma harzianum	20.00 b	35.94 cd	43.81 c
Trichoderma harzianum M	16.66 bc	34.63 cde	35.23 d
Trichoderma longibrachiatum	14.44 bc	33.98 cde	31.90 de
Trichoderma koningi	13.33 bc	33.33 de	29.04 ef
Trichoderma virens	12.22 bc	30.06 e	26.19 f
Talaromyces flavus 75	8.89 c	20.26 f	21.43 g

Table 2 Mean rate of inhibition of the colony growth of wheat take-all agent *Gaeumannomyces graminis* var. *tritici* by the volatile metabolites of *Trichoderma* and *Talaromyces flavus* isolates, 24, 72 and 120 hours after incubation

Treatment	Mean (24 h)	Mean (72 h)	Mean (120 h)
Trichoderma harzianum S	53.57 a	57.74 a	40.95 b
Trichoderma koningi	51.19 ab	55.95 a	29.04 d
Trichoderma longibrachiatum	47.02 abc	45.24 b	34.76 c
Trichoderma harzianum	43.45 abcd	38.09 cd	44.76 a
Trichoderma virens	42.85 abcd	38.69 cd	21.43 e
Trichoderma harzianum M	40.47 bcd	38.69 cd	26.66 d
Talaromyces flavus 60	35.71 cde	48.81 b	20.95 e
Talaromyces flavus 75	34.52 cde	26.76 e	7.14 f
Talaromyces flavus 134	32.14 de	34.52 de	21.90 e
Talaromyces flavus 136	30.95 de	39.28 cd	36.90 c
Trichoderma harzianum A	25 e	43.45 bc	36.66 c

Table 3 Mean rates of inhibition of the colony growth of wheat take-all agent *Gaeumannomyces graminis* var. *tritici* by 25% concentration of the non-volatile exudates of *Trichoderma* and *Talaromyces flavus* isolates, 24, 72 and 120 hours after incubation

Treatment	Mean (24 h)	Mean (72 h)	Mean (120 h)
Talaromyces flavus 136	38.88 a	41.51 a	10.47 de
Talaromyces flavus 134	34.44 ab	27.67 c	7.61 e
Talaromyces flavus 75	31.11 abc	28.93 c	10.95 de
Talaromyces flavus 60	25.55 abc	32.70 bc	8.09 e
Trichoderma harzianum	24.44 abc	40.88 a	8.09 e
Trichoderma longibrachiatum	23.33 abc	30.19 bc	23.09 a
Trichoderma harzianum S	22.22 abc	37.73 ab	11.90 cd
Trichoderma harzianum A	20 bc	28.93 c	20.95 a
Trichoderma harzianum M	18.89 bc	32.07 bc	15.71 b
Trichoderma koningi	16.67 c	31.44 bc	20.47 a
Trichoderma virens	15.55 c	34.59 bc	14.76 bc

Table 4 Mean rates of inhibition of the colony growth of wheat take-all agent *Gaeumannomyces graminis* var. *tritici* by 50% concentration of the non-volatile exudates of *Trichoderma* and *Talaromyces flavus* isolates, 24, 72 and 120 hours after incubation

Treatment	Mean (24 h)	Mean (72 h)	Mean (120 h)
Trichoderma harzianum A	43.33 a	50.94 a	32.38 cd
Trichoderma longibrachiatum	41.11 ab	52.83 a	52.38 a
Talaromyces flavus 75	36.66 abc	42.77 b	15.23 fg
Talaromyces flavus 136	34.44 abcd	33.33 de	29.04 d
Trichoderma harzianum S	28.89 bcde	35.22 cde	23.81 e
Trichoderma virens	23.33 cdef	41.51 bc	30 d
Trichoderma koningi	22.22 def	34.59 cde	35.23 c
Trichoderma harzianum	22.22 def	40.88 bc	18.09 f
Trichoderma harzianum M	17.78 efg	40.25bcd	42.38 b
Talaromyces flavus 134	12.22 fg	30.81 e	14.28 fg
Talaromyces flavus 60	5.55 g	39.62 bcd	12.38 g

Discussion

Trichoderma is one of the biocontrol agents of take-all pathogenic fungus whose control mechanisms are competition, mycoparasitism, the function of non-volatile compounds and volatile compounds. These mechanisms were previously investigated and confirmed by other researchers such as Arianpour (2011) and Mehrabi Koushki et al. (2008). The important fact which was pointed out by many researchers is the presence of difference among species and special various isolates of a species in terms of control mechanisms and the intensity of them which is consistent with the present study. The fungus *Talaromyces flavus* is the biocontrol agent of several soil-borne pathogens whose isolates were not yet evaluated against take-all pathogenic fungus. The present research evaluated the antagonistic effects of seven *Trichoderma* isolates and four *Talaromyces flavus* isolates which were previously applied for biological control of other pathogenic agents, against *G. graminis*

var. *tiritici.*

Macroscopic investigations about the contact of *Trichoderma* and *Talaromyces flavus* isolates using dual culture showed that all tested isolates inhibited the growth of the pathogenic fungus as rapid growth of *Trichoderma* provided competitive conditions for place (medium) and food. The study by Kucuk and Kivanc (2003) and Mehrabi Koushki et al. (2008) confirmed these results. The isolates *T. harzianum* A, *T. harzianum* S, *Talaromyces flavus* 134, *Talaromyces flavus* 136 and *Talaromyces flavus* 60 were able to colonize the mycelium of the pathogenic fungus. The same results were reported by Mehrabi Koushki et al. (2008) and Naraghi et al. (2007). Hermosa et al. (2000) placed some pathogenic fungi against some isolates of *Trichoderma* and concluded that sporulation and growth of *Trichoderma* on colonies of the pathogenic fungi depend on the type of pathogen and combination of medium. The highest level of growth inhibition caused by *Talaromyces flavus* 60 and *Talaromyces flavus* 136 and it was found that these isolates have higher competitive potential against the take-all pathogenic fungus than other isolates. The results from Naraghi et al. (2010c) reflect high feeding ability of *Talaromyces flavus.*

Microscopic investigation on region where antagonistic *Trichoderma* and *Talaromyces flavus* isolates coincide with the take-all pathogenic fungus showed that hyphal penetration, contact and fragmentation occurred. This was consistent with the results of Naraghi et al. (2010a, 2010b and 2010c) and Heidari Faroughi et al. (2005). The hyphae of antagonistic isolates have positive tropism towards those of the pathogenic fungus. This tropism may be due to chemical compounds on the cell wall of the pathogenic fungus hyphae. The parasitism mechanism of *Trichoderma* is complicated and includes chemical tropism, identification of lactine presented on the cell wall of the pathogen (Inbar and Chet, 1995) and formation of appressorium, penetrating organs and pathogen-trapping rings (Elad and Chet 1983).

To investigate the antibiosis, two tests were carried out for evaluating the effects of volatile and non-volatile exudates from *Trichoderma* and *Talaromyces flavus* isolates against Ggt. These tests revealed that various *Trichoderma* and *Talaromyces flavus* isolates are different in terms of producing volatile and non-volatile compounds which inhibit the growth of host fungus. Due to this, some *Trichoderma* isolates were more effective to inhibit the growth of the pathogenic fungus. Our results were consistent with the results achieved by Iraqi et al. (2008). Generally, the results show that various *Trichoderma* species and even the different isolates of a species produce variety of volatile materials which have different effects on various fungi. These isolates may produce a series of volatile metabolites with different amounts or may produce completely different chemical compounds. The investigations have shown that *T. harzianum* produces a wide range of antibiotics and enzymes against various fungi. Volatile and non-volatile metabolites can disperse throughout soil pores so that there is no need for direct contact with the disease agent to affect. With respect to the results, it is shown that species and even isolates of a species act differently in terms of antagonistic mechanisms for biocontrol of the pathogenic agent (Lorito et al., 1994).

The results from investigating the effect of non-volatile exudates from *Trichoderma* and *Talaromyces flavus* isolates were consistent with the results reported by Iraqi et al. (2008). Moreover, Hashemi et al. (2012) reported the same results when he tested the effect of non-volatile exudates on prevented growth of *Fusarium oxysporum* f. sp. *sesame*.

Most *Trichoderma* strains produce toxic volatile and non-volatile anti-fungal compounds which halt the growth of pathogenic fungi (Vey et al., 2001). In another study, the role of *Trichoderma* spp. to produce antibiotics such as trichodermin, trichodermol, harzianum A and harizanulid was pointed out (Kucuk and Kivanc, 2004).

Experimental investigations are proper methods to elementary identification of antagonistic microorganisms. However, the usefulness of an antagonist cannot be validated only based on experimental data as the effect of antagonist directly against the pathogenic agent is mostly tested on rich medium while the effect of microorganisms in natural environment is highly affected by many factors such as temperature, pH, humidity, soil texture and the behavior of other microorganisms. For example, an isolate may be effective against a pathogen *in vitro* while it may fail to control the pathogen in natural environment due to the competition with other antagonists. Due to this, it is recommended that all *in vitro* tested species are investigated in greenhouse conditions as well. According to danger of fungicides these antagonistic isolates can be used as agents for the control of plant disease after *in vivo* trials.

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