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In Vitro Antifungal Activity of Strains of *Trichoderma harzianum*

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Abstract: Interactions between *Trichoderma harzianum* strains and some soilborne plant pathogens (*Gaeumannomyces graminis* var. *tritici*, *Fusarium culmorum* and *F. moniliforme*) were studied on PDA medium. All *T. harzianum* strains tested produced a metabolite that inhibited growth of plant pathogenic fungi on PDA medium. When grown in liquid cultures containing laminarin, chitin or fungal cell walls as sole carbon sources, 2 strains of *T. harzianum* produced 1,3-β-glucanase and chitinase in the medium. Higher levels of these enzymes were induced by *T. harzianum* T15.

Key Words: *Trichoderma harzianum*, plant pathogenic fungi, lytic enzymes

Trichoderma harzianum İzolatlarının In Vitro Antifungal Aktivitesi

Özet: PDA gelişme ortamında, bazı toprak kökenli bitki patojenleri (*Gaeumannomyces graminis* var. *tritici*, *Fusarium culmorum* ve *F. moniliforme*) ve *Trichoderma harzianum* streynleri arasındaki interaksiyonlar çalışılmıştır. Test edilen tüm *Trichoderma harzianum* streynleri, bitki patojeni fungusların gelişimini PDA ortamında inhibe eden uçucu metabolit üretmişlerdir. Karbon kaynağı olarak laminarin, kitin veya fungal hücre duvarı içeren sıvı ortamda geliştirildiklerinde, *Trichoderma harzianum*'un iki streyni ortamda 1,3-β-glukanaz ve kitinaz enzimleri üretmişlerdir. Bu enzimlerin en yüksek düzeyleri *Trichoderma harzianum* T15 tarafından üretilmiştir.

Anahtar Sözcükler: *Trichoderma harzianum*, bitki patojeni funguslar, litik enzimler

Introduction

Gaeumannomyces graminis var. *tritici* is the causal agent of take-all disease, the most important damaging root disease of wheat worldwide (1). *Fusarium culmorum* and *F. moniliforme* specifically attack many agricultural crops causing foot and root rot (2). Alabouvette et al. (3) showed that suppressiveness was reduced by natural saprophytic fungal communities of some soilborne plant pathogens. Many studies have proved the potential of *Trichoderma* spp. as biological agents antagonistic to several soilborne plant pathogens (4-6). Strains of *Trichoderma* can produce antifungal metabolites (6-8). They may also be competitors of fungal pathogens (7) which promotes plant growth (9). In addition, a number of *Trichoderma* strains are able to secrete lytic enzymes such as chitinases and β-(1,3)-glucanases when grown in liquid media supplemented with either polymers such as laminarin or chitin or with fungal cell walls (4,10, 11).

The purpose of this study was to evaluate the interaction between the pathogen and the fungal antagonist and the possibility of mycoparasitism under the biological control of *Gaeumannomyces graminis* var. *tritici, Fusarium culmorum* and *F. moniliforme* by 3 strains of *T. harzianum.*

Materials and Methods

Strain culture

Three *Trichoderma harzianum* strains were used: T8, T11 and T15. Cultures were maintained at -20 °C on potato dextrose agar (PDA) slants. *T. harzianum* strains were obtained from the culture collection of the Department of Microbiology, Anadolu University, Eskişehir.

Plant Pathogens

Gaeumannomyces graminis var. *tritici, Fusarium culmorum* and *F. moniliforme* were used as pathogens. Plant pathogens were kindly provided by the Anadolu Agricultural Research Institute in Eskişehir.

Dual Culture Tests

Interactions between antagonistic fungi and pathogenic fungi were determined by the method described by Küçük and Kıvanç (6). Mycelial disks (7 mm in diameter) of *Gaeumannomyces graminis* var. *tritici*, *Fusarium culmorum* and *F. moniliforme* were placed on one edge of a petri dish containing PDA, while mycelial disks of *T. harzianum* strain were placed on the opposite side of the plate. After the desired incubation time, at 28 °C, the overgrowth of colonies of the test fungi by the antagonist was determined.

Volatile Metabolites

The effect of volatile metabolites produced by the antagonistic microorganisms on *Gaeumannomyces graminis* var. *tritici*, *Fusarium culmorum* and *F. moniliforme* mycelial growth was determined by the method described by Dennis and Webster (12). Each antagonistic isolate was grown on a sterile cellophane disk lying on PDA in 9 cm petri dishes for 48 h. The cellophane with the mycelium was removed in the same position in which the microorganism was grown and a mycelial plug was inoculated pathogen with a 7 cm diameter. Diameter growth of the pathogen colonies was determined after 72 h and was compared with that of the plant pathogen grown on PDA without metabolites (control). The growth of the pathogens was measured daily.

Enzymatic Assay

G. graminis, *F. culmorum* and *F. moniliforme* were inoculated into 250 ml flasks with 50 ml of potato dextrose broth and incubated at 25 ± 2 °C for 7 days. The mycelia were then collected by filtration. The mycelia were thoroughly washed with distilled autoclaved water and homogenized on ice, with a homogenizer, for 5 min at the highest speed. The mycelial suspension was centrifuged at 30,000 xg for 20 min at 4 °C. The pellet was resuspended in distilled water and sonicated on ice 4 times for 5 min using a sonicator. The suspension was centrifuged at 800 xg for 10 min at 4 °C (4).

Trichoderma harzianum strains were cultured at 30 °C on a synthetic medium (SM) containing (grams per liter of distilled water); glucose, 15; $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; Fe^{2+}, 0.002; and Zn^{2+}, 0.002. Flasks containing 50 ml of liquid SM medium (4) were inoculated with 1 ml of a conidial suspension (1 x 10^7 conidia ml^{-1}) of *T. harzianum*. The glucose in the medium was substituted with one of the following carbon sources (each at 2 mg ml^{-1}): laminarin, colloidal chitin or plant pathogens' cell wall preparations. Cultures were incubated at 30 °C in a rotary shaker at 120 r.p.m. for 4 days, and then centrifuged at 15,000 xg at 4 °C for 10 min. The supernatant was dialyzed against distilled water. The dialyzate was lyophilized for enzymic activity (4).

β-Glucanase (EC 3.2.1.58) assay is based on the release of reducing glucose from laminarin as described by Elad et al. (4). Specific activity was expressed as mmol glucose h^{-1} (mg protein)$^{-1}$.

Chitinase activity (EC 3.2.1.14) was assayed by following the released of GlcNAc from colloidal chitin (4). Protein was determined by the method described by Bradford (13) using bovine serum albumin as the standard. Specific activity was defined as units of enzyme activity per milligram of protein.

Modification of Hyphal Cell Walls

A total of 40 mg of cell walls was suspended and shaken for 1 h at 50 r.p.m. in 60 ml of (a) 2 M NaOH at 25 °C, (b) chloroform/methanol (2:1, v/v) at 25 °C, (c) protease (300 mg ml^{-1} in 0.1 M phosphate buffer pH 7) at 37 °C, and (d) trypsin (100 mg ml^{-1} in 0.1 M phosphate buffer pH 7) at 37 °C. Modification of hyphal cell walls was determined using the method described by Sivan and Chet (14).

Results and Discussion

The filamentous fungus *T. harzianum* is one of the most potent agents for the biocontrol of plant pathogens (1). The antagonistic mode of action of the fungus has been proposed for the production of antibiotics (8) and fungal cell wall degrading enzymes such as chitinases, glucanases and proteases (4,6,10). All isolates of *T. harzianum* grew considerably faster on PDA than did the pathogens, in the same conditions of culture. Our isolates of *T. harzianum* T15 were more efficient than T8 and T11 in retarding growth and sporulation of *G. graminis.* Its rapid growth gives *Trichoderma* an important advantage in the competition for space and nutrients with plant pathogenic fungi, even before it deploys its arsenal of mycotoxins (7,8,12).

The metabolite of *T. harzianum* produced in agar culture inhibited the growth of all 3 soilborne plant

pathogens tested in vitro (Table 1). *Trichoderma* species are known to produce a number of antibiotics, such as trichodermin, trichodermol, harzianum A and harzianolide (7,8,12). Our study demonstrated the involvement of volatile metabolites in the inhibition of *G. graminis, F. culmorum* and *F. moniliforme*. Among the metabolites, hydrolytic enzymes such as chitinase and glucanase are thought to be closely related to mycoparasitism (10). In order to be able to degrade phytopathogen cell walls, the antagonistic *Trichoderma* induces the production of extracellular hydrolytic enzymes, responsible for the direct attack against the pathogen.

Table 1. Effect of volatile metabolites produced by *T. harzianum* strains on soilborne pathogens' mycelial growth (mm).

Plant Pathogens	*Trichoderma harzianum* strains		
	T8	T11	T15
F. culmorum	90 (± 0.30)	85 (± 0.10)	92 (± 0.20)
F. moniliforme	85 (± 0.10)	60 (± 0.10)	100 (± 0.20)
G. graminis	98 (± 0.35)	93 (± 0.10)	100 (± 0.10)

Values represent mean ± SD of 4 experiments each performed in duplicate.

The production of b-glucan-degrading enzymes is a characteristic attributed to a wide variety of fungi, with β-1,3-glucanases found in most strains examined. The majority of the fungal β-1,3-glucanases described are extracellular enzymes, secreted into the medium upon synthesis (1). *Trichoderma* β-1,3-glucanases are important for the enzymatic degradation of cell walls of phytopathogenic fungi during mycoparasitic attraction (4).

We compared the activity of lytic enzymes of *T. harzianum* strains. The chitinase and glucanase activies are summarized in Table 2. Extracellular glucanases and chitinase secretion were obtained during the growth of *T. harzianum* strains in liquid medium (Table 2). When T8, T11 and T15 were cultured in liquid medium containing laminarin as a sole carbon source, the highest level was obtained in T15. The maximal level of chitinase was obtained after 48 h in T15, but after 72 h in T11.

The specific activities of the 2 enzymes of the strains were tested the using cell walls of the pathogens as the substrate (Table 3). All tested strains showed chitinase and glucanase activity when grown in the *G. graminis* cell wall. The strains were cultured on cell walls of *G. graminis, F. culmorum* or *F. moniliforme* as the sole carbon source. The release of chitinase was higher in T15 than in T8 and T11. In addition, the enzymes were also produced in the presence of phytopathogen cell walls as the carbon source, suggesting that these substrates can also act as inducers of lytic enzyme synthesis. This result is similar to that by reported Sivan and Chet (14) in which *T. harzianum* produced high levels of chitinase and glucanase when grown on *Rhizoctonia solani* mycelia. Considerable variation has been reported with respect to biocontrol activity and host range among the *Trichoderma* species analyzed. The production of hydrolytic enzymes has been shown to be affected by culture conditions and by the host (8).

Some other studies demonstrated that *T. harzianum* revealed chitinase and gluconase activity when their cultures were supplemented with cell walls from *Sclerotium rolfsii* (4), *F. oxysporum, Rhizoctonia solani* (5) and *Botrytis cinerea* (8). We found that enzyme content degraded the hyphal wall of *G. graminis* but that *Fusarium* species cell walls were more resistant. Sivan

Table 2. Activity of enzymes produced by T. *harzianum* strains in liquid medium.

Strains	Chitinase Activity µmol GlcNAc h^{-1}(mg protein)$^{-1}$		Glucanase Activity µmol glucanase h^{-1}(mg protein)$^{-1}$	
	48 h	72 h	48 h	72 h
T8	2.60 ± 0.02	2.16 ± 0.04	67.0 ± 0.02	135.5 ± 0.10
T11	3.66 ± 0.03	3.57 ± 0.04	24.0 ± 0.03	127.1 ± 0.03
T15	4.75 ± 0.04	3.80 ± 0.02	85.0 ± 0.03	140.3 ± 0.02

Values represent mean ± SD of four experiments each performed in duplicate.

Table 3. Activity of enzymes produced by *T. harzianum* strains incubated with cell walls of pathogens.

Cell wall	Enzyme Activity*	Strains		
		T8	T11	T15
F. culmorum	Glucanase activity	10.0 ± 0.01	7.0 ± 0.02	13.0 ± 0.03
	Chitinase activity	0.27 ± 0.01	0.37 ± 0.01	0.88 ± 0.03
F. moniliforme	Glucanase activity	25.0 ± 0.02	14.0 ± 0.02	30.0 ± 0.01
	Chitinase activity	0.30 ± 0.04	0.34 ± 0.02	0.65 ±0.01
G. graminis	Glucanase activity	87.0 ± 0.001	84.0 ± 0.01	110 ± 0.01
	Chitinase activity	0.94 ± 0.01	1.05 ± 0.01	1.10 ± 0.01

* Glucanase activity of strains of *T. harzianum* incubated with cell walls of the 3 pathogens for 72 h. Chitinase activity of strains of *T. harzianum* incubated with cell walls of the 3 pathogens for 48 h. Values represent mean ± SD of 4 experiments, each performed in duplicate.

Table 4. Effect of NaOH and chloroform/methanol treatments on the release of monomers from cell wall pathogens by glucanase and chitinase of T15.

Cell wall	Release of monomers (μg ml^{-1}) after treatment					
	Control		2 M NaOH		Chloroform/methanol (2:1 v/v)	
	Glucose	GlcNAc	Glucose	GlcNAc	Glucose	GlcNAc
F. culmorum	9.6	35.5	138.0	177.9	3.0	31.0
F. moniliforme	7.0	28.5	177.9	193.5	6.1	40.6
G. graminis	57.5	70.5	31.0	80.3	67.6	50.3

and Chet (14) have argued that *Fusarium* species' cell walls contain more protein than do walls of other fungi. Our data seem to confirm this hypothesis; the lytic activity of chitinase and glucanase of T15 was higher than that of T8 and T11 when incubated with cell wall of *G. graminis* compared with *Fusarium* species. The possible interference of a *Fusarium* cell wall moiety in the mycoparasitic activity of *T. harzianum* strains was tested by treating cell walls with alkali or organic solvent, prior to incubation with the lytic enzymes (Table 4). β-1,3-glucanases incubated with NaOH treated cell walls of both *F. culmorum* and *F. moniliforme* released more glucose compared with incubation with untreated walls. In contrast, NaOH treatment had only a slight effect on the lytic enzymes (Table 4). This suggests that fusarial cell walls contain a proteinaceous interfering substance. Protease or trypsin treatment of walls of *Fusarium* species before incubation with lytic enzymes increased the release of monomers compared with untreated walls. However, proteolytic treatments had little effect on cell walls of *G. graminis* (Table 5). Sivan and Chet (5) similarly found that cell walls of *F. oxysporum* are more resistant to mycoparasitism.

Elad et al. (4) found the mycoparasites *Trichoderma harzianum* T35 and *Trichoderma harzianum* T203 were unable to degrade the cell wall of *F. oxysporum* and that these hydrolases of *Trichoderma* were actively involved in microbiological control (11). The 3 different strains of *T. harzianum* were grown in liquid medium containing *G. graminis* cell walls. Glucanase activities of strains T8, T11 and T15 were 87 ± 0.001, 84 ± 0.01, and 110 ± 0.01 mmol glucose/mg protein per hour, respectively. Chitinase activities of strains were 0.94 ± 0.01, 1.05 ± 0.01 and 1.10 ± 0.03 mmol *N*-acetylglucosamine/mg

Table 5. Effect of proteolytic enzyme treatments on the release of monomers from cell wals pathogens by glucanase and chitinase of T15.

Cell wall	Release of monomers (µg ml^{-1}) after treatment					
	Control		Protoease		Trypsin	
	Glucose	GlcNAc	Glucose	GlcNAc	Glucose	GlcNAc
F. culmorum	6.5	38	27.6	49	36	94
F. moniliforme	7.8	24	22.4	64.8	45	100
G. graminis	109	97	94.0	47	96	62

protein per hour, respectively. Enzymes of *T. harzianum* T15 degraded cell walls of *G. graminis*. This suggests that proteins in the cell walls of *Fusarium culmorum* and *F. moniliforme* may give these walls more resistance than in *G. graminis* to degradation by enzymes of *T. harzianum* strains. Alabouvette et al. (3) suggested that the very high protein content of clamydospores of *Fusarium* sp. (7%-28%) may be responsible for their ability to resist lysis in soil. Further experiments are being carried out to identify those enzymes important in *Trichoderma* biological control and to study their individual regulation.

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