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Mycoparasitism in the biological control of *Gibberella zeae* and *Aspergillus ustus* by *Trichoderma harzianum* Strains

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Trichoderma harzianum strains, several fungal cell wall degrading enzymes, amongst them chitinase and glucanase. These enzymes seem to play an important role in the antagonistic action of *Trichoderma* against a wide range of fungal plant pathogens. In this report we describe interaction between *T. harzianum*, *Gibberella zeae* and *Aspergillus ustus*. The strains produced a nonvolatile metabolite that inhibited growth of pathogenic fungi. When grown in liquid cultures containing laminarin, chitin or fungal cell walls as sole carbon source, strains of *T. harzianum* produced glucanase and chitinase in the medium. Higher levels of these enzymes were induced by *T. harzianum* Tm4.

Key words: *Trichoderma harzianum*, strains, fungal cell wall, degredation

Introduction

The soil fungus *Trichoderma harzianum* is active against a range of economically important aerial and soilborne plant pathogens and is successfully used as a biopesticide in greenhouse and field applications (Krause *et al.,* 2001; Tondje *et al.,* 2007). The antagonistic mechanism of *T. harzianum* is a complex process involving chemotropism (Harman, 2004), lectin mediated recognition (Woo *et al.,* 2006), and formation of trapping and penetration structures (Lorito *et al.,* 1994b). This process is further supported by the secretion of extracellular enzymes such as chitinases (Cherif and Benhamou, 1990; Kulling *et al.,* 2000), ß-glucanases (Schirmböck *et al*., 1994) and proteinases (Shakeri and Foster, 2007) as well as secondary metabolites (Ghisalberti and Sivasithamparam, 1991; Lorito *et al.,* 1994b).

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Phytopathogenic fungi species specifically attack many agricultural crops causing foot and root rot (Papavizas, 1985). Krause *et al.*, 2001) 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 plant pathogens (Kulling *et al.,* 2000; Tondje *et al.,* 2007; Woo *et al.,* 2006).
The purpose of this study was to evaluate the interaction between the pathogen and the fungal antagonist and possibility of mycoparasitism under the microbiological control of *Gibberella zeae* and *Aspergillus ustus* by four strains of *T. harzianum*.

Materials and methods

Microorganisms

Four *Trichoderma harzianum* strains were used: Tm4, Tm7, Tm9 and Tm10 were used, the cultures of pathogen, *Gibberella zeae* and *Aspergillus ustus* were obtained from the culture collections of the Department of Microbiology, Anadolu University, Eskişehir, Turkey.

Bioassays and properties of the possible antibiotics

T. harzianum strains were grown on cellophane membranes at the surface of 2% Malt Agar and incubated at 25 ^{o}C at 72 h. A disk (5 mm diameter) of a 6 day culture on Malt Agar of pathogen was then transferred to the same position occupied previously by the inoculum disk of the strain. Plates were incubated at 25 ^{o}C for 7 days and radial growth of pathogen was measured and compared with the control plates to determine the percent inhibition.

Heat stability of nonvolatile antibiotics was also determined using the technique of Dennis and Webster (1971) except for the sterilization at 121 ^{o}C for 15 min of the medium in petri plates after the removal of the cellophane membranes. Potato dextrose broth cultures (400 ml) of *T. harzianum* strains were shaked incubated at 25 ^{o}C for 5 days then filtrates were adjusted at pH 4 and extracted equal volumes of ethylacetate and evaporated. Contents were solubilizated in ehtylacetate and developed at TLC (chloroform-acetate-formic acid 70:28:2). Chromatograms were observed under UV (234 nm) for spots with Rf values.

Enzymatic activity

Gibberella zeae and *Aspergillus ustus* were transfered into 250 ml flasks with 50 ml of potato dextrose broth and incubated 25 ^{o}C for 5 days. The mycelia were collected by filtration and washed with distilled water and homogenized on ice, with a homogenizer for 5 min at the higher speed. The mycelial suspension was centrifuged at 30 000 x g for 20 min at 4 ^{o}C. The pellet was resuspended in distilled water and sonicated on ice 5 times for 5 min using a sonicator. The suspension was centrifuged at 800 x g for 10 min at 4 ^{o}C (Lorito *et al.,* 1994b). *T. harzianum* strains were grown in synthetic medium (SM) containing (grams per liter of distilled water); glucose, 15; $MgSO_4$. $7H_2O$, 0.2; KH_2PO_4, 0.9; KCI, 0.2; NH_4NO_3, 1.0; Fe^{2+}, 0.002; and Zn^{2+}, 0.002 (Elad nad Chet, 1989). Flasks containing 100 ml of liquid SM were inoculated with 1 ml of a conidial suspension (1 x 10^7 conidia ml^{-1}) of *T. harzianum* strains. The glucose in the medium was subsituted with one of the following carbon sources; laminarin, colloidal chitin, or plant pathogens cell walls (each at 2 mg ml^{-1}). Cultures were incubated at 30 ^{o}C in a rotary at 120 rpm for 5 days, and then centrifuged at 15.000 x g at 4 ^{o}C for 10 min. Content was lyophilized for enzymic activity. β-glucanases (E.C. 3.2.1.58) assay was based on the release of reducing glucose from laminarin as described by Elad and Chet (1989). Chitinase activity (EC 3.2.1.14) was assayed by following the released of GlcNAc from colloidal chitin (Elad and Chet, 1989; Lorito *et al.,* 1994b). Protein was determined by the method described by Bradford (1976) using Bovine Serum Albumin as the standard. Specific activity was defined as units of enzyme activity per miligram of protein.

Results and discussion

Non-volatile antibiotics capable of reducing *Gibberella zeae* and *Aspergillus ustus* growth were produced by the four *T. harzianum* strains. As for percent growth inhibition of *G. zeae* and *A. ustus*, there were no significant differences among the four strains even though the substance produced by *T. harzianum* Tm4 showed the strongest inhibitory activity (Table 1). In the test performed to detect the resistance of the antibiotics to steam sterilization, results presented on Table 1 showed that the substance produced by Tm7 was less affected by this treatment.

The non-volatile antibiotics could diffuse through the cellophone membrane and the solid culture medium. The results of the sterilization tests in solid medium showed that their inhibitory activity was considerably reduced after steam sterilization. Understanding the mechanisms involved in the

antagonistic effect of *T. harzianum* tested plant pathogens is important if the use of these strains as biocontrol agents is to be optimized. While the evidence of the role of mycoparasitism in biological control is convincing (Tondju *et al*., 2007) definite evidence establishing the importance of antibiosis has been more elusive antibiosis is the mechanism mediated by specific metabolites such as enzymes antibiotics and volatile or nonvolatile compounds (Schirmböck *et al*., 1994; Lorito *et al*., 1994b). In our study, on thin layer chromatography plates developed with chloroform-acetone-formic acid, spots were found at Rf 0.52 (Tm4), Rf 0.50 (Tm7), Rf 0.62 (Tm9) and Rf 0.49 (Tm10) respectively. These results indicate that strains were produced metabolites. It was suggested that the production of metabolites might be effectivity in biocontrol mechanism (Kulling *et al*., 2000). Also, metabolites could be competition or direct killing of the pathogen hyphae.

Several members to *Trichoderma* spp. were shown to be antagonistic to plant pathogens and producers of lytic enzymes (Elad and Chet, 1989; Witkowska and Maj, 2002). Witkowska and Maj (2002) showed that *T. harzianum* strains parasitized plant pathogens with the production of cell wall lytic enzymes. These investigators suggested that pathogen antagonist contact seemed essential in the production of these enzymes.

The chitinase and glucanase activities are summarized in Table 2. When Tm4, Tm7, Tm9 and Tm10 were cultured in liquid medium containing laminarin as a sole carbon source, the highest level was obtained in Tm4. The maximal level of chitinase was obtained after 72 h in Tm4, but after 96 h in Tm10.

The specific activities of the two enzymes of the *T. harzianum* strains were tested the using cell walls of the pathogens as the substrate (Table 3). The strains were cultured on cell walls of *G. zeae* or *A. ustus* as the sole carbon source. The release of chitinase was higher in Tm4 than Tm7, Tm9 and Tm10. ın addition the enzymes were also produced in the presence of pathogen 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 Elad and Chet, (1989); Tondje *et al*., (2007) and Woo *et al.,* (2006) in which *Trichoderma* strains produced high levels of chitinase and glucanase when grown on *Rhizoctonia solani* and *Phytophthora* species mycelia. The different strains of *T. harzainum* were grown in liquid medium containing *A. ustus* cell walls. Glucanase activities of strains Tm4, Tm7, Tm9 and Tm10 were 72.0 ± 0.01, 48.0 ± 0.01, 52.0 ± 0.002 and 57.0 ± 0.03 mmol glucose^{-1} mg protein per hour, respectively. Chitinase activities of mutant strains were 1.12 ± 0.03, 0.57 ± 0.02, 0.42 ± 0.01 and 0.55 ± 0.02 mmol N-acetylglucosamine^{-1} mg protein per hour, respectively. Enzymes of *T. harzianum* Tm4 degraded cell walls of *A.*

ustus. The lytic activity of chitinase and glucanase of Tm4 was higher than other strains when incubated with cell wall of *A. ustus* compared with *Gibberella zeae*. Woo *et al.* (2006) and Witkowska and Maj (2002) showed that one *T. harzianum* strain was not inhibitory to some plant pathogens. However, this fungus parazitized both pathogens with the production of cell wall lytic enzymes. These investigators suggested that pathogen-antagonist contact seemed essential in the production of these enzymes. Further experiments are being carried out to identify those enymes and metabolites important in *T. harzianum* biological control.

Table 1. Growth inhibition of pathogens induced by the nontreated and treated (sterilization 121 ^{o}C for 15 min) antibiotics produced by the *Trichoderma harzianum* strains.

strains	Radial growth of pathogen (cm)				% growth inhibition			
	Gibberella zeae		*A. ustus*		*Gibberella zeae*		*A. ustus*	
	Non sterilized	sterilized	Non sterilized	sterilized	Non sterilized	sterilized	Non sterilized	sterilized
Tm4	2.1	6.4	1.8	6.8	69.1	19.5	71.9	15.0
Tm7	3.2	6.2	2.4	5.6	52.9	4.3	62.5	30.0
Tm9	2.7	5.8	2.4	6.2	60.2	29.2	62.5	22.5
Tm10	3.4	7.0	2.7	6.8	50.0	14.6	57.8	15.0
control	6.8	8.2	6.4	8.0	0	0	0	0

Table 2. Activity of enzyme produced by mutant strains in liquid medium.

T.harzianum strains	Chitinase activity µmol GlcNAc h^{-1}(mg protein)$^{-1}$		Glucanase activity µmol glucanase h^{-1}(mg protein)$^{-1}$	
	72 h	96 h	72 h	96 h
Tm4	3.96 ± 0.01	3.80 ± 0.03	132.0 ± 0.01	110.0 ± 0.03
Tm7	2.12 ± 0.02	2.09 ± 0.03	118.0 ± 0.02	82.0 ± 0.04
Tm9	2.72 ± 0.01	2.60 ± 0.02	106.0 ± 0.02	58.0 ± 0.04
Tm10	3.10 ± 0.01	2.72 ± 0.01	122.0 ± 0.02	96.0 ± 0.02

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

Table 3. Activity of enzymes produced by *Trichoderma harzianum* strains incubated with cell walls of pathogen.

Cell wall	Enzyme activity*	*T. harzianum* mutant strains			
		Tm4	Tm7	Tm9	Tm10
Gibberella zeae	Chitinase	1.05 ± 0.03	0.27 ± 0.02	0.22 ± 0.01	0.20 ± 0.01
	Glucanase	13.0 ± 0.02	6.0 ± 0.02	9.0 ± 0.01	10.0 ± 0.01
A. ustus	Chitinase	1.12 ± 0.03	0.57 ± 0.02	0.42 ± 0.01	0.55 ± 0.02
	Glucanase	72.0 ± 0.01	48.0 ± 0.01	52.0 ± 0.02	57.0 ± 0.03

*Activity of strains of *T.harzianum* incubated with cell walls of the pathogens for 72 h.Values represent mean ± SD of four experiments each performed in duplicate.

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