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Effect of Carbon Source on Production of Lytic Enzymes by *Trichoderma Harzianum*

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Abstract

We investigated the effect of the different carbon sources and phytopathogenic fungal cell walls on production of enzymes by Turkish strain *Trichoderma harzianum* T15. *Trichoderma harzianum* T15 secretes β-1,3-glucanase and chitinase in the presence of different carbon sources. Maximal β-1,3-glucanases activity secreted was detected in media supplemented laminarin or *Dreschlera sorokiniana* and *Sclerotium rolfsii* purified cell walls. The highest chitinase activity was obtained in medium supplemented with chitin or *D. sorokiniana* and *S. rolfsii* purified cell walls. The properties of this enzymes suggest that the enzymes might play different roles in host cell wall lysis during mycoparasitism.

Key Words: Enzymes, Turkish isolate, *Trichoderma harzianum*, Carbon source

INTRODUCTION

There is a worlwide need to adopt the practice of sustainable agriculture using strategies that are environmental friendly, less dependent on agricultural chemicals and less damaging to soil and water resources. One of the key elements of such sustainable agriculture is the application of biocontrol agents for plant protection [1].

Many studies have proved the potential of *Trichoderma* spp. as biological agents antagonistic to several soilborne plant pathogens [2-5]. Strains of *Trichoderma* can produce antifungal metabolites [7,8-10]. They may also be competitors of fungal pathogens [1, 8, 9] which promotes plant growth [1].

In addition, a number of *Trichoderma* strains are able to secrete lytic enzymes such as chitinases and 1,3-β-glucanases when grown in liquid media supplemented with either polymers such as laminarin or chitin or fungal cell walls [2, 11-13]. These conditions have been described as simulated antagonism [14, 15]. These observations, together with the fact that chitin 1,3-glucan are the main skeletal polysaccharides of fungal cell walls. Elad et al. [16], De Marco et al. [17] and Vazquez-Garciduenaz et al. [18] suggest that chitinases and 1,3-β- glucanases act the key enzymes in the lysis of phytopathogenic fungal cell walls during the antagonistic action of *Trichoderma* spp.

The purpose of this study was to evaluate the effect of different carbon sources on the enzymatic activity of *Trichoderma harzianum* T15.

MATERIAL AND METHODS

Microorganisms

Trichoderma harzianum strain T15 and plant pathogens; *Fusarium solani, Fusarium culmorum, Dreschlera sorokiniana* and *Sclerotium rolfsii* used in the experiments were derived from the Microbiological collection of the University of Anadolu, Eskişehir, Turkey.

Cultural Conditions

All fungi 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 [12]. 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* T15. The glucose in the medium was subsituted with one of the following carbon sources; laminarin, colloidal chitin, cellulase, xylose or plant pathogens cell walls (each at 2 mg ml^{-1}). Cultures were incubated at 30 °C in a rotary at 120 rpm for 4 days, and then centrifuged at 15.000 x g at 4 °C for 10 min [19-21]. Content was lyophilized for enzymic activity.

Enzymic Assay

β-glucanases (E.C. 3.2.1.58) assay was based on the release of reducing glucose from laminarin as described by Elad et al. [16]. Chitinase activity was assayed by following the released of GlcNAc from colloidal chitin [16]. Protein was determined by the method described by Bradford [22] using Bovine Serum Albumin as the standard. An enzymatic unit (U) was defined as the amount of enzyme able to liberate 1µmol of product (as GlcNAc equivalent) per min, under the assay conditions.

RESULTS AND DISCUSSION

The filamentous fungus *Trichoderma harzianum* is one of the most potent agents for the biocontrol of plant pathogens [1,4,15]. The antagonistic mode of action of the fungus has been proposed for the production of enzymes [13,14,21]. The production of β-glucanases found in most strains examined. The majority of the fungal 1,3-β-glucans described are extracelluler

enzymes, secreted into the medium upon synthesis [21]. Most fungi attacked by *Trichoderma* spp. have cell walls that contain chitin as a structural backbone and laminarin (β-1,3-glucan) as a filling material. The other minor cell wall components are proteins and lipids [21]. Laminarin is a polymer of D-glucose in a β-1,3-configuration, arrenged as helical coils, from which minor polymer of β-1,6-D-glucose branch. Fungal cell walls contain more than 60 % laminarin. Laminarin is hydrolyzed mainly by 1,3-glucanases [16,21]. *Trichoderma* spp. β-1,3-glucanases are important for the enzymatic degradation of cell walls of phytopathogenic fungi during mycoparasitic attraction [15,21].

We have compared the activity of lytic enzymes of *Trichoderma harzianum* strain T15. The chitinase and glucanase activities of *T. harzianum* T15 grown in different carbon sources are summarized in Table 1. The level of production of lytic enzymes were varied depending on the carbon sources. The carbon sources for the enzymatic activity were found significant. On the other hand mean squares for carbon sources was larger than control vs, suggesting that carbon sources had larger effect on the enzymatic activity than control. Extracellular glucanases and chitinase secretion were obtained during the growth of *T. harzianum* T15 in liquid medium (Table 1-2). When all of the carbon sources tested were compared, the highest enzyme production was observed in laminarin. Glucanase activity of strain T15 in laminarin and glucose was 451 and 141 mU^{-1}mg $protein^{-1}$, respectively. Similar results in different strains have been observed for same enzymes in *Trichoderma* spp. [6,16,21].

A significant increase in activity to promote growth of mycelia in medium was observed when mycelium of *T. harzianum* T15 was incubated with chitin (Table 1). Chitinase activity of T15 in chitin was 7.54 mU^{-1}mg $protein^{-1}$.

Table 1. Mean squares of glucanase and chitinase activity production by *Trichoderma harzianum* T15 with different carbon sources

Treatment		Enzymatic Activity	
		Glucanase	Chitinase
		mU mg $protein^{-1}$	mU mg $protein^{-1}$
Control		12.5	0.18
Glucose		141	0.37
Laminarin		451	0.26
Chitin		8	7.54
Cellulose		16	0.41
Xylose		21	0.31
LSD		2.73	0.10
ANOVA			
	df		
Replication	1	1.34	0.03
Treatments	5	307956.7**	17.47**
Control vs.	1	21965.1**	4.24**
Carbon sources	4	71497.9**	21.83**
Error	5	1.13	0.002

** Significant at the 0.01 probability level

The specific activities of the two enzymes of the strain T15 was tested the using cell walls of the pathogens as the substrate (Table 2). The strain tested was showed high chitinase and glucanase activity when *D. sorokiniana* and *S. rolfsii* cell wall used as substrate (Table 2). Glucanase activity of strain T15 was 424 and 409 mU^{-1} mg $protein^{-1}$, respectively. Chitinase activity of strain in *D. sorokiniana* and *S. rolfsii* cell wall used as substrate was 1.23 and 1.16 mU^{-1} mg $protein^{-1}$, respectively. The level of activity secreted is depedent on the carbon sources present in the inducer. Strain of T15 was showed chitinase and glucanase activity when grown in *Fusarium solani, Fusarium culmorum, D. sorokiniana* and *S. rolfsii* as the sole carbon source. The release of chitinase and glucanase was higher in liquid medium containing *D. sorokiniana* and *S. rolfsii* (Table 2).

Table 2. Mean values and mean squares for activity of enzymes producted by *Trichoderma harzianum* T15 incubated with cell walls of plant pathogens

Treatment		Enzymatic Activity[a]	
		Glucanase	Chitinase
		mU mg $protein^{-1}$	mU mg $protein^{-1}$
Control		12.5	0.18
F. solani		47.5	0.25
D. sorokiniana		424	1.23
F. culmarum		42.5	0.27
S. rolfsii		409	1.16
LSD		3.2	0.12
ANOVA			
	df		
Replication	1	2.5	0.009
Treatments	4	88121.9**	0.561**
Control vs. pathogen walls	1	76212.9**	1.09**
Pathogen walls	3	92091.5**	0.584**
Error	4	0.75	0.002

[a]Glucanase activity of *T.harzianum* T15 incubated with cell walls of 4 pathogens for 72 h

Chitinase activity of *T.harzianum* T15 incubated with cell walls of 4 pathogens for 48 h

** Significant at the 0.01 probability level

The analysis of variance of the control and the pathogens cell walls showed that the pathogen walls were significantly different for enzymatic activity.

In addition to that enzyme content degraded the hyphal wall of *D. sorokiniana* and *S. rolfsii* but *Fusarium* species cell walls were more resistant. Sivan and Chet [15] 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 when incubated with wall of *D. sorokiniana* and *S. rolfsii* in compared with *Fusarium* species (Table 2). The results is similar to that reported by De Marco et al. [17], Innocenti et al. [4] and Vazquez-Garciduenaz et al. [18] in which *T. harzianum* produced high levels of chitinase and glucanase when *Mucor rouxii, Neurospora crassa, Saccharomyces cereviae, Rhizoctonia cerealis* mycelia. Considerable variation has been reported with respect to biocontrol activity and host range among the *Trichoderma* spp. analyzed. The production of lytic enzymes has been shown to be affected by culture conditions and the host [20].

The inhibitors cycloheximide and 8-hydroxyquindine were added to the cultures. The lower activity of the enzymes

(Table 3) observed in the presence of cycloheximide and 8-hydroxyquindine. It was not due to enzyme inactivition. The maximum enzymatic activity was recorded for cycloheximide treatments were significant for enzymatic activity. Our results were showed that the inhibitors cycloheximide and 8-hydroxyquindine, at concentrations used in filamentous fungi as reported by Ulhoa and Peberty [20] and Cruz et al. [19], impede the apperance of glucanase and chitinase (Table 3). Thus, this results suggest that enzyme activity lowered because of inhibition of enzyme synthesis rather than inhibition of enzyme secretion or enzyme inactivation.

Table 3. Effect of cycloheximide and 8-hydroxyquinoline on enzymatic activity of *Trichoderma harzianum* T15

Treatment		Enzymatic Activity[a]	
		Glucanase	Chitinase
		mU mg protein^{-1}	mU mg protein^{-1}
Control		12.5	0.18
Cycloheximide		56	2.4
8-hydroxyquinoline		17.5	2.0
LSD		1.77	0.10
ANOVA			
	df		
Replication	1	0.66	0.008
Treatments	2	1683.2**	2.805**
Control vs. inhibitors	1	3234.1**	5.5**
Inhibitors	1	132.25**	0.16**
Error	2	0.17	0.04

** Significant at the 0.01 probability level

Similar results have been described in *Penicillium* spp. with regards to β-glucanase production [9]. Lytic enzymes of *Trichoderma* spp. can take part in the digestion of the cell walls of plant fungal pathogens [10,15] are actively involved in pathogens control [9]. There were significant differences between various treatment in term of the enzymatic activities studied. Pathogen cell walls had the higher means as compared to other treatments for glucanase. Chitinase activity was affected the inhibitors and the carbon sources.

In this study, lytic activity was observed when using glucose, laminarin, chitin, xylose, cellulose, and cell walls of *Fusarium solani, Fusarium culmorum, D. sorokiniana, S. rolfsii* and inhibitors (Table 1-3). In compare with others, laminarin had the highest glucanase activity. Based on the secretion of these enzymes during mycoparasitism and considering that lytic enzymes including glucanase and chitinase secreted by *T. harzianum* have been shown to act synergistically [4]. At present, experiments are being performed to identify and characterize individual lytic enzymes production by *Trichoderma harzianum* strain T15.

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