Degradation of plant pathogenic fungi by *Trichoderma harzianum*

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Trichoderma harzianum excreted β-1,3-glucanase and chitinase into the medium when grown on laminarin and chitin, respectively, or on cell walls of the pathogen *Sclerotium rolfsii*, as sole carbon source. *Trichoderma harzianum* also showed high activity of both enzymes when grown on homogenized *S. rolfsii* sclerotia. Glucanase activity increased by 67% when the fungus was grown on a mixture of laminarin and glucose (3:1, v/v). Similarly high lytic activity was detected in wheat bran culture of the fungus and in soil inoculated with this culture. Protease and lipase activity were detected in the medium when the antagonist attacked mycelium of *S. rolfsii*.

Isolates of *T. harzianum* were found to differ in the levels of hydrolytic enzymes produced when mycelium of *S. rolfsii*, *Rhizoctonia solani*, and *Pythium aphanidermatum* in soil was attacked. This phenomenon was correlated with the ability of each of the *Trichoderma* isolates to control the respective soilborne pathogens.

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Trichoderma harzianum excrète de la β-1,3-glucanase et de la chitinase dans le milieu de croissance, si ce milieu contient respectivement de la laminarine et de la chitine, ou si le milieu contient de la paroi cellulaire du pathogène *Sclerotium rolfsii*, comme seule source de carbone. *Trichoderma harzianum* présente aussi une activité élevée des deux enzymes s'il est mis en croissance sur un homogénat sclérotial de *S. rolfsii*. L'activité glucanase augmente jusqu'à 67% lorsque le champignon croît en présence à la fois de laminarine et de glucose (3:1, v/v). De la même façon, une activité lytique élevée est décelée, si le champignon est mis à croître sur un milieu de son blé ou dans un sol inoculé de cette culture. Des activité lipolytique et protéolytique ont été décelées dans le milieu lorsque l'antagoniste attaquait le mycelium de *S. rolfsii*.

Les isolats de *T. harzianum* diffèrent dans le niveau de production d'enzymes hydrolytiques lorsque les mycéliums de *S. rolfsii*, *Rhizoctonia solani* et *Pythium aphanidermatum* sont attaqués dans le sol. Ce phénomène est corrélé avec l'aptitude de chacun des isolats de *Trichoderma* de contrôler les pathogènes respectifs du sol.

[Traduit par le journal]

Introduction

The cell walls of *Sclerotium rolfsii* and *Rhizoctoria solani* are composed of β-1,3-glucan and chitin (Chet *et al.* 1967; Bartnicki-Garcia 1973) whereas members of the Oomycetes, e.g., *Pythium* sp., also contain cellulose (Bartnicki-Garcia 1973). The fungus *Trichoderma harzianum* was able to grow on *R. solani* cell wall as a sole carbon source (Hadar *et al.* 1979).

The antagonistic ability of *T. harzianum* Rifai aggr. against several soilborne plant pathogens was reported by Dennis and Webster (1971). Application of the biocontrol agent to fields infested with *R. solani* Kühn and *S. rolfsii* Sacc. successfully reduced disease incidence (Elad *et al.* 1981*a*, 1981*b*; Elad *et al.* 1981).

This isolate produced extracellular β-1,3-glucanase and chitinase when grown on cell walls of *R. solani* (Chet *et al.* 1979; Hadar *et al.* 1979). It appears that the main mechanism involved in the antagonism of *T. harzianum* and pathogenic fungus is the release of lytic enzymes.

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The present work deals with the enzymatic basis of the interaction between *T. harzianum* and *S. rolfsii*, *R. solani*, and *Pythium aphanidermatum* (Edson) Fitzp. both in culture and in soil.

Methods

Growth medium and substances

Synthetic medium (SM) containing (grams per litre) $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; Fe^{2+}, 0.002; Mn^{2+}, 0.002; Zn^{2+}, 0.002; in distilled water, pH 6.3, prepared according to Okon *et al.* (1973), was used throughout this work.

Growth of T. harzianum *Rifai in liquid culture*

The fungus was grown in Erlenmeyer flasks (250 mL) containing 50 mL of SM on a rotary shaker (New Brunswick Scientific Co., New Brunswick, U.S.A.) at 180 rpm at 28°C. The medium was supplemented with either glucose, laminarin, chitin, *N*-acetylglucosamine (Sigma Chemicals Co., U.S.A.), peptone (Difco Laboratories, U.S.A.), fungal cell wall, casein hydrolyzate (Sigma), or triglycerides at the stated concentration. The flasks were seeded with 0.1 mL of a suspension containing 10^7 conidia/mL. The mycelium was then collected by centrifugation at 27 000 × *g* for 20 min at

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4°C. The supernatant was filtered through Whatman No. 1 filter paper and lyophilized.

Cell walls of the tested pathogenic fungi (*S. rolfsii* Sacc., *R. solani* Kühn, or *P. aphanidermatum* (Edson) Fitzp.) were prepared according to Chet *et al.* (1967), lyophilized, and ground to fine powder by milling in a coffee mill (Moulinex, France) for 1 min. The cell wall preparation served also as a sole carbon source for growing *T. harzianum* and determining its lytic enzymes.

Enzyme assay

β-1,3-Glucanase (EC 3.2.1.39) was assayed by following the release of free glucose from laminarin by lyophilized cultures using the glucose oxidase reagent (Sigma) according to the directions of the manufacturer. Specific activity (GU) was expressed as micromoles of glucose per milligram of protein per hour. The reaction mixture, which contained lyophilized culture in 0.2 mL of 0.1 *M* citrate buffer (pH 4.7) and 1.6 mg of soluble laminarin, was incubated at 40°C for 1 h and was stopped by boiling.

Cellulase (EC 3.2.1.4) was assayed by following the release of free glucose from carboxymethyl cellulose (British Drug Houses Ltd., England). Other assay procedures were as for β-1,3-glucanase.

Chitinase (EC 3.2.1.14) was assayed by following the release of *N*-acetylglucosamine according to Reissig *et al.* (1955). Specific activity (CU) was expressed as micromoles of *N*-acetylglucosamine per milligram of protein per hour. The lyophilized culture in 2.0 mL of 0.1 *M* phosphate buffer (pH 5.1) and 1.6 mg of colloidal chitin, was incubated at 37°C for 2 h and was stopped by boiling.

Cell-free extracts containing β-1,3-glucanase or chitinase were tested for enzyme stability during 1 h of incubation at a temperature range of 0–100°C and a pH range of 2.5–10 for 24 h at 20°C. Buffer (0.1 *M*) used was either citrate–phosphate (pH 2.5–5.5), phosphate (pH 6–8), or glycine–NaOH (pH 8.5–10.0).

Reaction mixture for determining protease (EC 3.4.24.4) activity contained 1 mL of 0.05 *M* phosphate buffer (pH 7.0), 0.1 *M* $MgCl_2$, 5 mg/mL Hide Azure (Calbiochem, Switzerland), and 1 mL of crude enzyme. This suspension was incubated in a rotary shaker (200 rpm) for 1 h at 37°C. The reaction was stopped by adding trichloroacetic acid to a final of 10% and incubating at 0°C for 30 min. Absorbance of the supernatant was measured at 595 nm. One enzyme unit (PU) was found to be the quantity which caused 0.1 absorbance change during 1 h.

Lipase (EC 3.1.1.3) activity was determined by incubating 10 mL of crude enzyme with 0.5 mL of soybean oil in a rotary shaker (200 rpm) at 37°C for 2 h. Then ethanol was added to the reaction mixture to a final concentration of 30%. Free fatty acids (FFA) were extracted by 25 mL of pure petroleum ether. The extractant was evaporated in Rotaevaporator (Büchi, Switzerland) and the FFA were dissolved in 15 mL of neutralized ethyl alcohol containing phenolphthalein at 60°C. Each sample was titrated with ethyl alcohol containing 0.005 *N* NaOH. The FFA were neutralized and one lipolytic unit (LU) was defined as micromoles of NaOH per milligram of protein per hour. Protein content of the enzyme solution was determined by the Folin phenol reagent, according to Lowry *et al.* (1951). Dry weight of mycelium was determined after incubating at 60°C for 1 week.

Laminarin concentration was determined after dialysis of the medium by Anthrone reagent (Johnson 1954). Glucose was determined by glucose oxidase reagent (Sigma).

Enzyme activity in soil

Loamy sand soil (Hadar *et al.* 1979) was washed twice in tap water for 1 h. Samples of 30 g each containing 15% moisture were autoclaved and incubated with mycelium discs of one of the tested pathogens. After 3–4 days of incubation the soil was infested with 1 mL of spore suspension of one of three *T. harzianum* isolates (3×10^6 spores/g soil): TH 203 (capable of parasitizing both *S. rolfsii* and *R. solani*), TH 290 (capable of parasitizing *R. solani*), and TH 294 (capable of parasitizing *P. aphanidermatum*) (Elad *et al.* 1981*b*). Three days later, 10 mL of 0.1 *N* phosphate buffer (pH 5) was added to the culture. The soil was shaken for 20 min (200 rpm) and centrifuged at 5000 × *g* for 10 min. The supernatant was examined for enzymatic activity.

Use of radioactive ^{14}C

D-[^{14}C]Glucose (0.1 μCi/mL, 1 Ci = 37 GBq) taken from a source of 29 mCi/mmol specific activity (Radiochemical Centre, Amersham, England) was added to 10 mL of medium of 1-day-old culture of *S. rolfsii* grown on SM (0.5 mg glucose/mL). Two days later the culture was harvested by centrifugation and washed twice in sterile water. The ^{14}C-labelled cell walls served as a carbon source for *T. harzianum* in SM liquid culture. $^{14}CO_2$ which was released by *T. harzianum* was captured on a 9-mm-diameter filter paper disc suspended from the stopper, containing 50 mL of Hyamine (methylbenzethonium hydroxide) (Sigma). determined in a Packard liquid scintillation spectrometer.

Parasitism in submerged culture

Growth and parasitism of *T. harzianum* on *S. rolfsii* were followed in submerged culture (Hadar *et al.* 1981). A spore suspension of the antagonist was inoculated into 3-day-old *S. rolfsii* submerged culture. One millilitre of medium containing propagules (mycelial pellets) of the fungi was dispersed on SM (supplemented with 5 g glucose/L) agar plates and incubated at 30°C for 72 h after which viable propagules of both fungi were counted.

All experiments were conducted in four replicates and were duplicated. In all cases the variation between experiments did not exceed 10%. When not otherwise mentioned, *T. harzianum* isolate TH 203 (Elad *et al.* 1981*b*) was used.

Greenhouse test

Control of soilborne plant pathogens under greenhouse conditions was examined according to Hadar *et al.* (1979) and Elad *et al.* (1980). Percentage of disease reduction (DR) was calculated according to the following formula: DR = [1 − (DC − DT)/(DC)] × 100, where DC and DT are of disease in control and treatment, respectively.

Results

When *T. harzianum* TH 203 was grown in liquid culture on laminarin as a sole carbon source, it produced extracellular β-1,3-glucanse. Enzyme concentration in the medium increased up to 3.3 GU after 72 h of

incubation, whereas only low activity (0.2 GU) of the enzyme was found in culture containing glucose as a sole carbon source (Fig. 1). Similarly, protein content in the supernatant was four to five times higher in the laminarin-supplemented medium than in the glucose-supplemented medium. Dry weight of mycelium in the glucose liquid medium was 1.4 times higher than in the laminarin-containing medium. Activity of β-1,3-glucanase was positively correlated with laminarin concentration in the medium up to 1 mg/mL, but decreased by three to five times at higher laminarin concentrations.

Mixtures of laminarin and glucose in the growth medium at ratios varying from 100% laminarin to 100% glucose (total carbohydrate concentration, 1.0 mg/mL) were tested for their effects on β-1,3-glucanase activity (Fig. 2). At a carbohydrate concentration of 1.0 mg/mL the highest enzyme activity (14 GU) was obtained at a laminarin to glucose ratio of 3:1 (w/w), while only 9 and 1.2 GU were detected when *T. harzianum* was grown on either laminarin or glucose, respectively.

Trichoderma harzianum grown on glucose (1.5 mg/mL) and laminarin (0.4 mg/mL) was added to the medium culture 24 h later. The glucose was utilized within 72 h whereas laminarin had already been exhausted within the first 24 h after its addition (Fig. 3).

Optimal temperature for enzyme excretion by *T. harzianum* was 30°C, while the optimal pH was 5.0. Maximal stability was found within a pH range of 7–9.

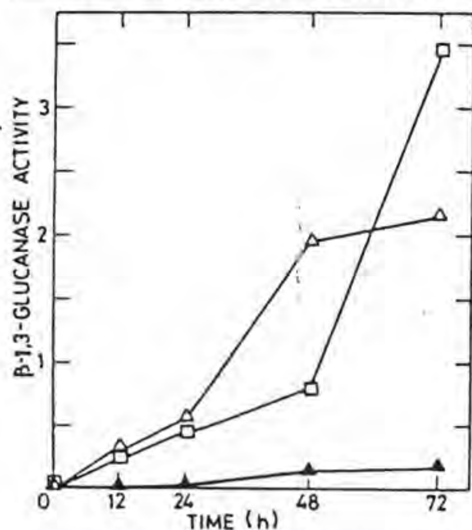

FIG. 1. β-1,3-Glucanase activity of *T. harzianum* (TH 203) excreted into liquid SM containing laminarin (□, 1 mg/mL; △, 2 mg/mL) or glucose (▲, 2 mg/mL) as a sole carbon source.

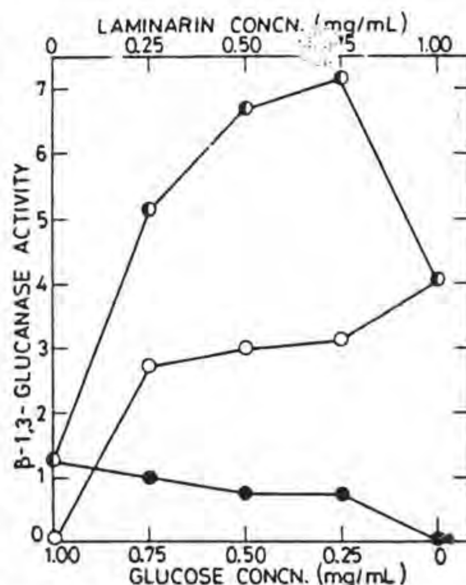

FIG. 2. β-1,3-Glucanase activity of *T. harzianum* (TH 203) after 44 h of incubation in liquid SM containing increasing amounts of glucose (●, bottom scale), laminarin (○, top scale), or both carbohydrates in a total concentration of 1 mg/mL at different ratios (◐, according to the combination of both scales at each point).

During incubation of the enzyme preparation at different temperatures partial loss of activity was found at 35°C, while a total loss was obtained at 90°C. Optimal activity of β-1,3-glucanase was obtained under reaction conditions of pH 3–5 and 40°C.

Chitinase excretion into the growth medium was enhanced by concentrations of chitin (0.5–10 mg/mL) up to 5 CU within 44 h of incubation. Only slight enzyme activity was detected when *N*-acetylglucosamine or glucose was the carbon source in the medium.

Optimal growth conditions of *T. harzianum* for chitinase excretion when grown on chitin were 28°C and pH 5. It was found that 45 and 64% of chitinase activity was lost after incubation at 40 and 60°C, respectively, and that activity was totally lost after incubation for 1 h at 70°C or higher. At pH 9 the enzyme activity was minimally damaged. Optimal activity of chitinase in cell-free extract was found at 35°C and pH 5–6 in the reaction mixture.

Trichoderma harzianum was tested for its ability to utilize protein as a sole carbon and nitrogen source. When grown on peptone or casein hydrolyzate (1 mg/mL) it produced 0.3 and 0.26 PU, respectively, after 36 h of incubation.

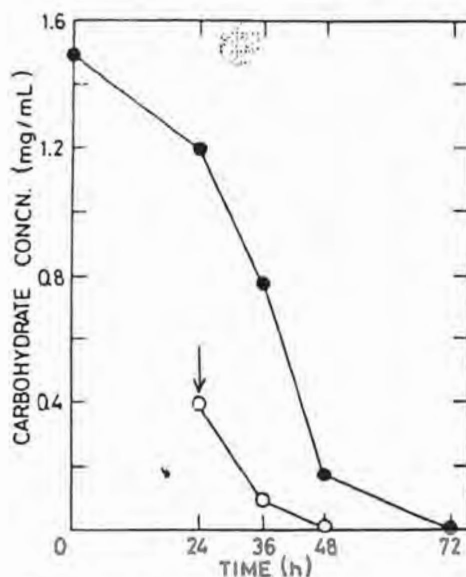

FIG. 3. Utilization of laminarin (○) in the presence of glucose (●) by *T. harzianum* (TH 203) in the growth liquid culture during 72 h of incubation. Arrow indicates time of laminarin application.

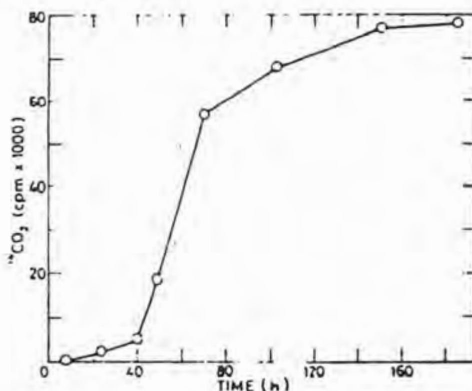

FIG. 4. Growth of *T. harzianum* (TH 203) on *S. rolfsii* ^{14}C-labelled cell wall. $^{14}CO_2$ that was released by *T. harzianum* during 72 h of incubation was captured on paper discs containing hyamine and counted in a liquid scintillation counter.

A cell-free extract of *T. harzianum* which was grown on triglyceride as a carbon source (0.2%, v/v) showed an activity of 2.4 LU. No lipase activity was detected on other media.

Spores of *T. harzianum* were inoculated into a 40-h-old submerged culture of *S. rolfsii*. Seventy-two hours later, the cell-free extract showed 0.68 PU and 5.0 LU compared with 0.09 PU and 0.7 LU of the noninoculated *S. rolfsii* control culture.

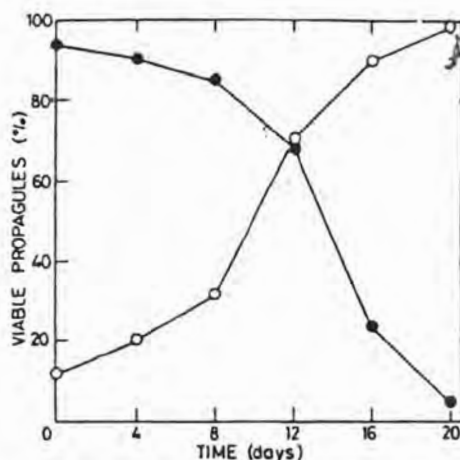

FIG. 5. Growth and parasitization of *T. harzianum* (TH 203) (○) in submerged culture of *S. rolfsii* (●). Viable propagules of both fungi were counted on SM agar plates during 20 days after inoculation of *T. harzianum* spores into the pathogen's culture.

Trichoderma harzianum was grown on *S. rolfsii* ^{14}C-labelled cell walls. Release rate of $^{14}CO_2$ (Fig. 4) was 1700 cpm per hour of growth 40–72 h after incubation.

Spores of the antagonist were inoculated into a 3-day-old *S. rolfsii* submerged culture. Viability of *S. rolfsii* propagules disappeared within 20 days. Conversely, during this period of time viable counts of *T. harzianum* increased up to 98% of the total 35/mL sampled propagules (Fig. 5).

The three different isolates of *T. harzianum* were grown in liquid medium containing 1 mg mycelial powder/g *S. rolfsii* sclerotia. β-1,3-Glucanase activities of isolates TH 203, TH 250, and TH 294 were 3.75, 1.5, and 1.75 GU, respectively. Chitinase activities of these isolates were 1.73, 0.6, and 0.9 CU, respectively. When grown on *S. rolfsii* cell walls, all three isolates showed 50% higher activities of glucanase and chitinase.

When grown on cell walls of *P. aphanidermatum*, isolate TH 294 showed 55% higher activities of both enzymes than did the other *Trichoderma* isolates.

The *T. harzianum* isolates were tested for their ability to secrete the hydrolytic enzymes mentioned above when grown on the pathogen's mycelium, which was previously grown in autoclaved soil. While growing on *S. rolfsii* or *R. solani* cultures in soil, the respective isolates (either TH 203 or TH 250) produced more

TABLE 1. Enzymatic activity (U) of *T. harzianum* isolates grown on mycelium of plant pathogenic fungi in soil*

Enzyme	*T. harzianum* isolate†	Plant pathogenic fungus		
		P. aphanidermatum	*R. solani*	*S. rolfsii*
β-1,3-Glucanase	TH 203	0.51*a*	1.03*b*	1.02*b*
	TH 250	0.53*a*	1.05*b*	0.24*a*
	TH 294	0.67*a*	0.31*a*	0.28*a*
Chitinase	TH 203	0.50*a*	0.80*b*	1.08*b*
	TH 250	0.62*a*	0.75*b*	0.08*a*
	TH 294	1.09*b*	0.39*a*	0.10*a*
Cellulase	TH 203	0.09*a*	0.01*a*	0.02*a*
	TH 250	0.52*b*	0*a*	0.03*a*
	TH 294	0.94*c*	0.03*a*	0.03*a*

*Data in each column of the three *Trichoderma* isolates which are followed by a common letter are not statistically different ($P = 0.05$).

†*Trichoderma harzianum* isolates were inoculated into 5-day-old cultures of the pathogens which had been grown in autoclaved soil.

TABLE 2. Control of soilborne diseases in bean seedlings, grown in a greenhouse, by *T. harzianum* wheat bran cultures*

T. harzianum isolate	Disease reduction of the following pathogens†		
	P. aphanidermatum	*R. solani*	*S. rolfsii*
TH 203	−14*a*	69*b*	96*b*
TH 250	−28*a*	76*b*	3*a*
TH 294	39*b*	20*a*	26*a*

*Three grams of preparation per 1 kg of soil.

†Percentage of disease reduction (DR) was calculated in 3-week-old seedlings. Data in each column which are followed by a common letter are not statistically different ($P = 0.05$).

glucanase and chitinase than isolate TH 294. This isolate, however, was superior in cellulase and chitinase activities when degrading the mycelium of *P. aphanidermatum* culture (Table 1).

Since *T. harzianum* (203) was successfully applied as a biocontrol agent when grown on wheat bran (Hadar *et al.* 1979; Elad *et al.* 1981*a*), the activity of lytic enzymes in this preparation was tested. The fungus excreted 4.1 GU within 48 h of incubation in wheat bran liquid culture (1 mg/mL). The cell-free extract of this culture was capable of releasing 101 mg glucose/mL from *S. rolfsii* cell wall, whereas cell-free extract of *T. harzianum* grown on glucose (1 mg/mL) was capable of releasing only 3 mg glucose/mL under the same conditions.

β-1,3-Glucanase activity was examined in sterile soil after applying a wheat bran preparation of *T. harzianum* at a rate of 10 g/kg soil. β-1,3-Glucanase activity in the amended soil was 180% higher than in soil inoculated with *T. harzianum* (TH 203) without the food base (1.8 GU).

When applied to infested soils as a wheat bran preparation, isolate TH 294 significantly reduced only *P. aphanidermatum*, whereas the other isolates even increased disease incidence. On the other hand, *S. rolfsii* disease was significantly reduced by isolate TH 203. Bean disease caused by *R. solani* was successfully controlled by both TH 250 and TH 203 isolates (Table 2).

Discussion

The enzyme β-1,3-glucanase is commonly produced by fungi mainly as a constitutive enzyme (Bull and Chester 1966) and is excreted into the growth medium in the presence of several carbon sources. Reese and Mandels (1959) found that *Trichoderma viride* excreted the enzyme when grown on starch, cellulose, maltose, laminarin, lactose, xylose, glucose, mannitol, and glycerol. In this work it was shown that *T. harzianum* excreted β-1,3-glucanase when grown on laminarin or starch (as a component of wheat bran) whereas glucose induced the enzyme only slightly.

Chester and Bull (1963) found that out of 21 different fungi, *T. viride* showed the highest glucanase activity. This enzyme differs from cellulase, which is inducible and is repressed by glucose (Bull 1967). When *T. harzianum* was grown on high concentrations of laminarin, the enzymic activity was repressed. Similar results were reported for *S. rolfsii* glucanase by Kritzman *et al.* (1976).

Chitinase was excreted by *T. harzianum* when chitin served as a sole carbon source. Chitinase and β-1,3-glucanase are the key enzymes in the lysis of fungal cell walls (Mitchell and Alexander 1963; Chet and Henis 1969; Henis and Chet 1975). The lytic extracellular enzymes are capable of degrading *S. rolfsii*, *R. solani*, and *P. aphanidermatum* cell walls. Both enzymes must

act simultaneously to decompose hyphal walls of *Schizophyllum commune* because of the linkage between the chitin and β-glucan (Sietsma and Wessels 1979). Sclerotia contain melanin in the rind, which apparently increases cell wall resistance to biodegradation (Chet and Henis 1969). *Trichoderma harzianum* enzymes even degrade sclerotia of *S. rolfsii*. Similarly, Jones *et al.* (1974) showed that *T. viride* solubilized the hyphae of *S. sclerotiorum* by β-1,3-glucanase.

Increased excretion of β-1,3-glucanase by *T. harzianum* was found when the fungus was grown on laminarin and glucose as compared with the same concentration of laminarin alone. Similarly, high lytic activity was found in the wheat bran culture of *T. harzianum*. This supports the idea of applying the antagonist into infested soil when carried on its food base.

The antagonistic fungus was shown to attack *S. rolfsii* mycelium in submerged culture and to utilize the hydrolysis products of ^{14}C-labelled cell walls.

Lipases and proteases were found in the growth medium when *T. harzianum* attacked mycelium of *S. rolfsii*. It can be assumed that *T. harzianum* attacks the pathogen's mycelium first by dissolving its cell wall in certain locations followed by hyphal penetration (Chet *et al.* 1981); it then uses other extracellular enzymes, e.g., lipase and protease.

Proteolytic activity of mixed soil culture infested with *S. rolfsii* was related by Rodriguez-Kabana *et al.* (1978) to *T. viride*. In this work we have demonstrated glucanase and chitinase in soil inoculated with *T. harzianum*.

The isolates of *T. harzianum* which were found to differ in their ability to attack *S. rolfsii*, *R. solani*, and *P. aphanidermatum* also differ in the levels of hydrolytic enzymes that they produce. This phenomenon was found to be correlated with the ability of each of these isolates to control soilborne diseases under greenhouse conditions. These differences may also explain the variability in the antagonistic activity of other *T. harzianum* isolates (Elad *et al.* 1981*b*). It might serve as a tool for an efficient screening of highly parasitic *Trichoderma* isolates from soil.

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