Degradation of Fungal Cell Walls by Lytic Enzymes of 
Trichoderma harzianum

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In in vitro tests, two strains of Trichoderma harzianum failed to parasitize colonies of Fusarium oxysporum f. sp. vasinfectum and F. oxysporum f. sp. melonis. However, these strains were strongly mycoparasitic on Rhizoctonia solani and Pythium aphanidermatum. When grown in liquid cultures containing laminarin, chitin or fungal cell walls as sole carbon sources, both strains of T. harzianum released 1,3-β-glucanase and chitinase into the medium. Higher levels of these enzymes were induced in strain T-203 than in T-35 by hyphal cell walls of F. oxysporum. When the lytic enzymes produced by T-35 were incubated with hyphal cell walls of the test fungi, more glucose and N-acetyl-D-glucosamine was released from cell walls of R. solani and Sclerotium rolfsii than from those of F. oxysporum. Treatment of F. oxysporum cell walls with 2 M-NaOH, protease or trypsin prior to their incubation with the lytic enzymes of T. harzianum significantly increased the release of glucose and N-acetyl-D-glucosamine. The effect of these treatments on R. solani and S. rolfsii cell walls was much lower. These results suggest that proteins in the cell walls of F. oxysporum may make these walls more resistant than those of R. solani or S. rolfsii to degradation by extracellular enzymes of T. harzianum.

INTRODUCTION

The direct mycoparasitic activity of Trichoderma spp. is one of the major mechanisms proposed to explain their antagonistic activity against soil-borne plant-pathogenic fungi (Dennis & Webster, 1971; Elad et al., 1982; Lynch, 1987; Ridout et al., 1986). The lytic activity of fungal, as well as of bacterial, antagonists is mainly due to the lytic enzymes 1,3-β-glucanase and chitinase (Mitchell & Alexander, 1963; Henis & Chet, 1975). 1,3-β-Glucanase is a semi-constitutive enzyme (Bull & Chesters, 1966) which may be induced by several inducers such as laminarin, starch, xylose, mannitol and glycerol (Reese & Mandels, 1959). However, in the presence of laminarin the excretion of this enzyme increases (Elad et al., 1982). Chitinase is an inducible enzyme excreted by many micro-organisms in cultures containing chitin or its oligomers as sole carbon source (Monreal & Reese, 1969).

Different strains of Trichoderma harzianum effective in controlling Rhizoctonia solani produced 1,3-β-glucanase and chitinase in cultures containing cell walls of this pathogen as a sole carbon source (Elad et al., 1982; Hadar et al., 1979; Ridout et al., 1986). Similarly, Tokimoto (1982) reported the production of these enzymes in dual cultures of T. harzianum and Lentinus edodes. It has been suggested that the lytic activity of several strains of T. harzianum on cell walls of Sclerotium rolfsii, Rhizoctonia solani and Pythium aphanidermatum can be correlated with the degree of biological control of those pathogens in vivo (Artigues & Davet, 1984; Elad et al., 1982).

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Abbreviations: SM, synthetic medium; YM, yeast-extract/glucose medium; PDA, potato dextrose agar; GlcNAc, N-acetyl-D-glucosamine.

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The objective of the present study was to evaluate the possible role of mycoparasitism in the biological control of *Fusarium oxysporum* obtained with a new strain of *T. harzianum*. This strain is one of the very few strains effective in controlling this pathogen (Sivan & Chet, 1986; Sivan *et al.*, 1987).

**METHODS**

Fungal strains and growth media. *Trichoderma harzianum* strains were cultured at 30 °C on a synthetic medium (SM; Okon *et al.*, 1973) containing (g per litre of distilled water): glucose, 15; MgSO₄, 7H₂O, 0.2; KH₂PO₄, 0.9; KCl, 0.2; NH₄NO₃, 1.0; FeCl₃·6H₂O, 0.002; ZnCl₂, 0.002; agar, 20 (in solid medium). The mycolytic activity of the biocontrol agent (*T. harzianum* strain T-35) against *Fusarium oxysporum* was compared with that of another *T. harzianum* strain (T-203) isolated by Elad *et al.* (1980) and reported as an effective mycoparasite of *Rhizoctonia solani* Kuhn and *Sclerotium rolfsii* Sacc. (Elad *et al.*, 1982). *Fusarium oxysporum* f. sp. *melonis* Syd. & Hans. (*F. o. melonis*) and *F. oxysporum* f. sp. *vasinfectum* (Atk.) Syd. & Hans. (*F. o. vasinfectum*) were isolated from infected melon and cotton plants, respectively (Sivan & Chet, 1986) and cultured at 27 °C on a yeast extract/glucose medium (YM) containing (g per litre of distilled water): yeast extract (Difco), 5; peptone (Difco), 5; glucose, 10; agar, 20 (in solid medium). *R. solani*, *S. rolfsii* and *Pythium aphanidermatum* were grown on SM at 30 °C.

Dual culture tests. Mycelial disks (5 mm in diameter) of *F. o. melonis*, *F. o. vasinfectum*, *R. solani* or *P. aphanidermatum* were placed on one edge of a Petri dish containing PDA, while mycelial disks of *T. harzianum* were placed on the opposite side of the plate. Because of the lower growth rate of *F. oxysporum*, in each test where this fungus was used, the inoculation with mycelial disks of *T. harzianum* was performed 72 h after that of *F. oxysporum*; otherwise the inoculation with *T. harzianum* was performed simultaneously with that of the test fungi. After the desired incubation time, at 27 °C, the overgrowth of colonies of the test fungi by the antagonist was determined.

Survival of mycelium of *F. oxysporum* and *S. rolfsii* was determined after exposure to conidia of *T. harzianum*. Mycelial disks (5 mm in diameter) were removed from 72-h-old cultures of the tested fungi on PDA, while mycelial disks of *T. harzianum* were placed on the opposite side of the plate. Because of the lower growth rate of *F. oxysporum*, in each test where this fungus was used, the inoculation with mycelial disks of *T. harzianum* was performed 72 h after that of *F. oxysporum*; otherwise the inoculation with *T. harzianum* was performed simultaneously with that of the test fungi. After the desired incubation time, at 27 °C, the overgrowth of colonies of the test fungi by the antagonist was determined.

Preparation of hyphal cell walls. Cell walls were obtained from *F. o. melonis*, *F. o. vasinfectum*, *R. solani* and *S. rolfsii* after culture at 27 °C for 5 d in 50 ml liquid YM. Flasks with cultures of *R. solani* or *S. rolfsii* were shaken in a rotary shaker at 120 r.p.m., while those of *F. oxysporum* were incubated without shaking, to reduce conidiation. After incubation, mycelia were thoroughly washed with distilled autoclaved water and homogenized on ice, with an Ultra Turax homogenizer (Ika-Werk, W. Germany) for 5 min each using a Heat Systems-Ultrasonics sonicator at full amplitude. The mycelial disks treated with autoclaved distilled water and incubated as described above served as controls. After the desired incubation time, mycelial disks of *F. oxysporum* were transferred to a Fusarium-selective medium (Nash & Snyder, 1962) containing 1 mg methyl 1-(butyrylcarbamoyl)-2-benzimidazolecarbamate (Benomyl) 1⁻¹, in order to inhibit growth of *T. harzianum* (Elad & Chet, 1983). Similarly, mycelial disks of *S. rolfsii* were transferred to SM plates containing the same concentration of Benomyl. The results were expressed as the percentage of the mycelial disks from where the test fungus grew.

Induction of extracellular lytic enzymes in *T. harzianum*. Erlenmeyer flasks (250 ml) each containing 50 ml liquid SM were inoculated with 1 ml of a conidial suspension (5 × 10⁷ 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 (Sigma), colloidal chitin (prepared according to Rodriguez-Kabana *et al.*, 1983), or one of the fungal cell wall preparations. Cultures were incubated at 28 °C in a rotary shaker at 120 r.p.m. for the desired time, then centrifuged at 15000 g at 4 °C for 10 min. The supernatant was dialysed against distilled water at 4 °C for 24 h to eliminate residual glucose or N-acetyl-D-glucosamine (GlcNAc). The dialysate was lyophilized or tested directly for enzyme activity. Filtrates from laminarin cultures also contained traces of 1,3-β-glucanase, but those from laminarin cultures contained no detectable chitinase.

Enzyme assays. The activity of extracellular lytic enzymes was tested according to Elad *et al.* (1982). 1,3-β-Glucanase (exo-1,3-β-D-glucosidase, EC 3.2.1.58) was assayed by following the release of free glucose from laminarin using the glucose oxidase reagent. Specific activity was expressed as µmol glucose h⁻¹ (mg protein)⁻¹. The reaction mixture, containing 1 ml crude 1,3-β-glucanase, 1 ml 0·1 M-citrate buffer (pH 5·1) and 1·6 mg soluble
Lytic activity of *Trichoderma harzianum* to laminarin, was incubated at 38 °C for 1 h. The reaction was stopped by placing the reaction mixture in boiling water.

Chitinase (1,4-β-poly-N-acetyl-β-glucosaminidase, EC 3.2.1.14) was assayed by following the release of GlcNAc from colloidal chitin (Reissig *et al.*, 1959). Specific activity was expressed as μmol GlcNAc h⁻¹ (mg protein)⁻¹. The reaction mixture, containing 1 ml crude chitinase, 1 ml 0.1 M-citrate buffer (pH 5.1) and 1.6 mg colloidal chitin, was incubated at 38 °C for 2 h and the reaction then stopped by boiling.

The release of monomers from fungal cell walls was also tested using lyophilized crude enzymes from cultures containing chitin or laminarin as sole carbon sources and designated chitinase or 1,3-β-glucanase, respectively. The reaction mixture (2 ml), containing 1.5 mg lyophilized enzyme and 1.6 mg cell walls ml⁻¹ was incubated at 38 °C for 24 h. Each test tube was amended with 10 μl methylbenzene (toluene) to prevent contamination.

The activity of these enzymes on a living mycelium was tested by using mycelial mats of *F. o. melonis* grown in liquid YM for 96 h. Each mycelial mat was washed with sterile citrate buffer (pH 5.1) and transferred to a 250 ml Erlenmeyer flask containing 25 ml of the same buffer and lyophilized enzyme (1 mg ml⁻¹) obtained from culture filtrates of *T. harzianum* grown on laminarin or chitin as sole carbon source.

Modification of hyphal cell walls. In some experiments, cell walls were treated prior to application of the lytic enzymes. In all these treatments, 40 mg of cell walls were 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 (Sigma type XXV) (300 μg ml⁻¹ in 0.1 M-phosphate buffer pH 7.0) at 37 °C or (d) trypsin (100 μg ml⁻¹ in 0.1 M-phosphate buffer pH 7.0) at 37 °C. The proteolytic activity was stopped by boiling for 10 min at 100 °C.

After each treatment the agent was removed and the hyphal walls were thoroughly washed by centrifugation (15000 g at 4 °C), suspended in flasks containing 20 ml 0.1 M-citrate buffer (pH 5.1) amended with lyophilized crude 1,3-β-glucanase or chitinase, and incubated for 24 h at 38 °C.

Reproducibility. In all experiments, three replicates of each treatment were used. All experiments were performed at least twice.

**RESULTS**

*In vitro tests*

Both strains of *T. harzianum* failed to parasitize colonies of *F. o. melonis* and *F. o. vasinfectum* even after 160 h incubation. However, when cultured on PDA plates with *R. solani*, *P. aphanidermatum* or *S. rolfsii*, *T. harzianum* strain T-35 overgrew the test fungal colonies. In dual culture plates of T-35 and *P. aphanidermatum*, after 120 h incubation the antagonist covered most of the test colony, while the rate of colonization of *R. solani* and especially of *S. rolfsii* was lower (Table 1). Similar results were obtained when *T. harzianum* T-203 – the mycoparasite of *R. solani* and *S. rolfsii* (Elad *et al.*, 1980) – served as the antagonist.

Dual culture tests performed with other media – SM, YM and Malt-extract agar (Sigma) – showed similar results.

After treatment of disks of *F. o. melonis* and *F. o. vasinfectum* with conidia of T-35 or T-203 and incubation for up to 16 d, hyphae survived in 100 and 75% of the disks, respectively. However, when *S. rolfsii* was subjected to the same treatment for 7 d, no hyphae survived (Fig. 1).

![Fig. 1. Survival of mycelial disks of *F. o. vasinfectum* (●), *F. o. melonis* (△) and *S. rolfsii* (■) after treatment with conidia of *T. harzianum* T-35 (10⁶ conidia ml⁻¹).](image-url)
Fig. 2. Specific activity of (a) 1,3-β-glucanase and (b) chitinase produced by *T. harzianum* T-35 (○) and T-203 (●) during growth in liquid SM containing cell walls of *F. o. melonis* (2 mg ml⁻¹) as sole carbon source.

### Table 1. In vitro mycoparasitism of test fungi by *T. harzianum* T-35

The data represent the average extent of growth of hyphae of T-35 over mycelium of the test fungus. *F. o. melonis* and *F. o. vasinfectum* were not overgrown by *T. harzianum*, even after 160 h incubation.

<table>
<thead>
<tr>
<th>Test fungus</th>
<th>Overgrowth of fungal colonies by <em>T. harzianum</em> (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation time (h): 72 96 120</td>
</tr>
<tr>
<td><em>S. rolfsii</em></td>
<td>0 2.1 4.5</td>
</tr>
<tr>
<td><em>R. solani</em></td>
<td>15.1 19.2 22.5</td>
</tr>
<tr>
<td><em>P. aphanidermatum</em></td>
<td>27.0 32.5 38.1</td>
</tr>
</tbody>
</table>

### Activity of cell-wall-degrading enzymes in *T. harzianum*

When *T. harzianum* T-35 and T-203 were cultured in liquid medium containing laminarin as sole carbon source, both strains produced high levels of 1,3-β-glucanase [165.5 and 210.3 μmol glucose h⁻¹ (mg protein)⁻¹ after 48 h incubation, respectively]. After growth on chitin as sole carbon source for 48 h, the level of chitinase released to the medium was similar in both strains [5.3 and 5.5 μmol GlcNAc h⁻¹ (mg protein)⁻¹ in T-35 and T-203, respectively].

When the *T. harzianum* strains were grown in media amended with hyphal cell walls of *F. o. melonis* as sole carbon source (Fig. 2a, b), the levels of 1,3-β-glucanase and chitinase secreted by T-203 were higher than those produced by T-35. In both strains, maximal induction of 1,3-β-glucanase was obtained after 48 h incubation (Fig. 2a). However, the maximal level of chitinase was obtained after 72 h in T-203, but after 48 h in T-35 (Fig. 2b).

The specific activities of the extracellular lytic enzymes of *T. harzianum* T-35 were also tested using cell walls of the four test fungi as the substrate (Fig. 3). The lytic activity of 1,3-β-glucanase was higher when incubated with cell walls of *S. rolfsii* or *R. solani* than with those of *F. o. vasinfectum* and *F. o. melonis*. Similarly, when incubated with chitinase, the release of GlcNAc from cell walls of *S. rolfsii* was the highest amongst the tested hyphal walls. On the other hand, there was little or no difference between the specific activity of chitinase after incubation with cell walls of *R. solani* and the fusaria (Fig. 3).

The release of monomers from a live mycelium of *F. o. melonis* after treatment with the crude lytic enzymes of T-35 was minimal (Fig. 4).
The possible interference of a fusarial cell wall moiety in the mycoparasitic activity of *T. harzianum* T-35 was tested by treating cell walls with alkali or organic solvent prior to incubation with the lytic enzymes (Table 2). 1,3-β-Glucanase incubated with NaOH-treated cell walls of both fusaria released more glucose (up to 13.5-fold) compared with incubation with untreated walls. Similarly, an improvement in chitinase activity was found with the NaOH-treated *Fusarium* walls. In contrast, NaOH treatment had only a slight effect on the susceptibility of walls of *R. solani* and *S. rolsii* to attack by the two lytic enzymes (Table 2). Treatment with chloroform/methanol did not increase the release of glucose or GlcNAc from walls of either *Fusarium* strain (Table 2).

The hypothesis that a protein or a protein-like constituent(s) is involved in the resistance of fusarial cell walls to lytic enzymes was tested using proteolytic enzymes (Table 3). Protease or trypsin treatment of walls of *F. o. melonis* or *F. o. vasinfectum* before incubation with 1,3-β-glucanase or chitinase increased the release of monomers, as compared with nontreated walls. However, proteolytic treatments had little effect on cell walls of *R. solani* and *S. rolsii* (Table 3).
Table 2. Effect of NaOH and chloroform/methanol treatments on the release of monomers from cell walls of F. oxysporum, R. solani and S. rolfsii by 1,3-β-glucanase and chitinase of T. harzianum T-35

<table>
<thead>
<tr>
<th>Cell walls of</th>
<th>None</th>
<th>2 M-NaOH</th>
<th>Chloroform/methanol (2:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>GlcNAc</td>
<td>Glucose</td>
</tr>
<tr>
<td>F. o. melonis</td>
<td>14.5</td>
<td>42.5</td>
<td>152.5</td>
</tr>
<tr>
<td>F. o. vasinfectum</td>
<td>12.0</td>
<td>32.3</td>
<td>162.5</td>
</tr>
<tr>
<td>R. solani</td>
<td>76.0</td>
<td>22.0</td>
<td>102.5</td>
</tr>
<tr>
<td>S. rolfsii</td>
<td>63.0</td>
<td>193.0</td>
<td>82.0</td>
</tr>
</tbody>
</table>

Table 3. Effect of proteolytic enzyme treatments on the release of monomers from cell walls of F. oxysporum, R. solani and S. rolfsii by 1,3-β-glucanase and chitinase of T. harzianum T-35

<table>
<thead>
<tr>
<th>Cell walls of</th>
<th>None</th>
<th>Protease</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>GlcNAc</td>
<td>Glucose</td>
</tr>
<tr>
<td>F. o. melonis</td>
<td>11.8</td>
<td>41.2</td>
<td>37.2</td>
</tr>
<tr>
<td>F. o. vasinfectum</td>
<td>7.4</td>
<td>42.0</td>
<td>44.7</td>
</tr>
<tr>
<td>R. solani</td>
<td>88.2</td>
<td>43.2</td>
<td>81.9</td>
</tr>
<tr>
<td>S. rolfsii</td>
<td>122.1</td>
<td>153.2</td>
<td>109.7</td>
</tr>
</tbody>
</table>

DISCUSSION

The mycoparasitic potential of Trichoderma spp. is well established (Dennis & Webster, 1971; Elad et al., 1982; Lynch, 1987). This trait has often been utilized as a means of in vitro screening for biocontrol candidates (Elad et al., 1980; Hadar et al., 1979). In the present study, using the same dual culture technique, neither of the tested T. harzianum strains showed a significant mycoparasitic interaction with Fusarium oxysporum. T-35, however, parasitized mycelium of Rhizoctonia solani, Pythium aphanidermatum and Sclerotium rolfsii. Moreover, the survival of mycelium of F. oxysporum treated with conidia of T-35 was markedly higher than that of S. rolfsii. Similar results were also obtained with T-203, a mycoparasite of R. solani and S. rolfsii (Elad et al., 1982). This was the first indication of the higher resistance of F. oxysporum to lysis. Lynch (1987) demonstrated the overgrowth of Fusarium spp. by two Trichoderma strains. Thus, it appears that the potential of Trichoderma spp. to parasitize Fusarium is strain dependent.

T. harzianum is, however, an effective biocontrol agent of F. oxysporum on several crops (Sivan & Chet, 1986, 1987). The lack of mycoparasitic interaction between T. harzianum and F. oxysporum indicates that this mechanism is unimportant in this specific system. Therefore, in vitro dual culture tests appear not to be a sufficient screen for effective biocontrol agents against F. oxysporum.

Two hypotheses for the ineffectiveness of mycoparasitism against F. oxysporum were evaluated: (1) lytic enzymes of T. harzianum (e.g. 1,3-β-glucanase and chitinase) are not excreted, or (2) lytic enzymes are produced and released but the cell walls of F. oxysporum are more resistant to lysis than other fungal cell walls.

To test the first hypothesis we compared the induction and activity of lytic enzymes of T. harzianum T-35 with those of the mycoparasite T-203. When grown in liquid medium containing laminarin or chitin as sole carbon source both strains secreted similar amounts of both enzymes. On the other hand, when the strains were cultured on cell walls of F. oxysporum as sole carbon source the release of chitinase (but not 1,3-β-glucanase) was 90% higher from T-203 than from T-35. Similarly, chitinase produced by T-203 released more GlcNAc from cell walls of F. o.
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melonis than did the chitinase of T-35. However, neither T-35 nor T-203 parasitizes fusaria; and only T-35 is an effective biocontrol agent of fusarial wilt diseases. Thus, the level of lytic enzyme production is unrelated to either mycoparasitism or biocontrol capability. Similar enzyme preparations did, however, degrade hyphal walls of S. rolfsii and R. solani, which suggests that fusarial cell walls are more resistant to lysis (hypothesis 2). These results were confirmed by the inability of T-35 to degrade cell walls of live mycelium of F. oxysporum. Similarly, the mycoparasite Pythium num was unable to degrade live mycelium of F. oxysporum f. sp. cucumerinum (Elad et al., 1985).

Thus our second hypothesis, that cell walls of F. oxysporum are more resistant to mycoparasitism, is probably correct. Treatment of hyphal walls of F. oxysporum with NaOH or proteolytic enzymes increased their susceptibility to lysis by chitinase and 1,3-β-glucanase of T. harzianum T-35. Pretreatment of the same hyphal walls with an organic solvent had almost no effect on their lysis. This suggests that fusarial cell walls contain a proteinaceous interfering substance. However, neither the alkali nor the proteolytic pretreatment gave such an effect with cell walls of R. solani or S. rolfsii. Elad et al. (1985) similarly found that lytic enzymes produced by Pythium num also acted more effectively on trypsin-treated walls of F. oxysporum f. sp. cucumerinum than on non-treated walls. They postulated the presence of a mucilaginous layer on hyphae of fusaria that protects cell walls against degradation.

We have recently found that Fusarium oxysporum cell walls contain more protein than walls of other fungi (unpublished results). Other fusaria also have high (7-28%) protein contents (Barran et al., 1975; Laborda et al., 1974; Schneider et al., 1977). Schneider et al. (1977) suggested that the very high protein content of chlamydospores of F. sulphureum (21%) may be responsible for their ability to resist lysis in soil.

Our present study suggests that the lack of mycoparasitic interaction between T. harzianum (T-35) and F. oxysporum may be a result of an outer layer of protein in the hyphal walls of the latter, thus increasing their resistance to lysis. The significant biological control of F. oxysporum obtained by this strain (Sivan & Chet, 1986; Sivan et al., 1987) may be due to other mechanisms such as competition (Sivan & Chet, 1989) or antibiosis.

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