Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii*—Scanning Electron Microscopy and Fluorescence Microscopy

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ABSTRACT

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Hyphal interactions between either *Trichoderma harzianum* or *T. hamatum*, and *Sclerotium rolfsii* or *Rhizoctonia solani* were observed by scanning electron microscopy. *Trichoderma* spp. attached to the host either by hyphal coils, hooks, or appressoria. Lysed sites and penetration holes were found in hyphae of the plant pathogenic fungi, following removal of parasitic hyphae. High β-(1,3) glucanase and chitinase activities were detected in dual agar cultures when *T. harzianum* parasitized *S. rolfsii*, compared with low levels found with either fungus alone. In the presence of cycloheximide, antagonism was prevented and enzymatic activity was diminished. Interaction sites were stained by fluorescein isothiocyanate-conjugated lectins or Calcofluor White M2R New. Appearance of fluorescence indicated the presence of localized cell wall lysis at points of interaction between the antagonist and its host.

Additional key words: biological control, soilborne plant pathogens.

Trichoderma spp. are active as hyperparasites (3). Successful biological control of *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kühn by infesting fields with cultures of *Trichoderma harzianum* Rifai has been described (1,9,11,12,24).

Hyphal interactions of *T. harzianum* with several fungi, including the above mentioned pathogens, has been reported (5,8). It was shown that *T. harzianum* excreted lytic extracellular β-(1,3) glucanase and chitinase into the growth medium and even into the soil (10). However, knowledge of the events occurring at the point where host and parasite hyphae interact is limited.

In this work, the interaction between *T. harzianum* and *S. rolfsii* or *R. solani* was observed by using SEM techniques. Application of fluorescein isothiocyanate conjugated lectins to the system provided evidence for localization of sites of lytic activity.

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MATERIALS AND METHODS

Strains and growth conditions. *T. harzianum* Rifai and *T. hamatum* (Bon.) Bain isolates capable of parasitizing *R. solani* Kühn and *S. rolfsii* Sacc. (5,10) were used. *S. rolfsii* type A, ATCC 26325 (6), *R. solani* and the antagonists were maintained on a synthetic medium (SM) (22) at 28 ± 1 C.

The following system was used to observe interaction sites. A cellophane membrane, well washed in boiling, distilled water, was placed on the surface of water agar (5). An agar disk (SM) covered with a mycelium of *T. harzianum* or *T. hamatum* was placed on one end of the cellophane membrane and a disk with one of the pathogenic fungi was placed on the other. The mycoparasite and its host grew towards each other (at 28 ± 1 C for 4 days) and the hyphae intermingled on the cellophane plates.

SEM procedures. Cellophane membranes from the interaction area were removed and the organisms were fixed in 3% glutaraldehyde (Sigma Chemicals Co., St. Louis, MO 63178) in 0.1 M phosphate buffer (pH 7.0). After 12 hr of refrigeration, the specimens were dehydrated in a graded acetone series. Critical-point dried specimens were coated with gold palladium in a Polaron E500 sputter coater (Polaron Equipment Ltd., Watsford WD1 8XG, England) and viewed in a scanning electron microscope (JEOL JSM 35C).

Lytic enzyme production by *Trichoderma harzianum*. *T. harzianum* was grown in 250-ml Erlenmeyer flasks, each containing 50 ml of liquid SM without glucose, in a rotary shaker (New Brunswick Scientific Co., New Brunswick, NJ 08903) at 180 rpm and 28 C for 48 hr. The medium was supplemented with *S. rolfsii* cell walls (1.5 mg/ml).

Cells walls of the tested pathogenic fungi (*S. rolfsii* and *R. solani*) were prepared according to Chet et al (7), lyophilized, and ground to fine powder by milling (Moulinex, Paris, France) for 1 min. This cell wall preparation served as a sole carbon source both for growing *T. harzianum* and for determining its extracellular lytic enzymes.

The flasks were seeded with 0.1 ml of a suspension containing 10^7 conidia per milliliter. The mycelium was collected by centrifugation at 27,000 *g* for 20 min at 4 C. The supernatant was filtered through Whatman No. 1 filter paper, dialyzed against 0.1 M citrate buffer (pH 5), and lyophilized. Enzymatic activity of *T. harzianum* in agar culture was detected after extracting crude enzyme from agar disks (1 cm in diameter) in 0.1 M citrate buffer, pH 5.1.

Assay procedures. β-1,3-glucanase (EC 3.2.2.39) was assayed by measuring the free glucose released from laminarin (U.S. Biochemical Corp., Cleveland, OH 44126) by using the glucose oxidase reagent (Sigma) according to the manufacturer's direction. Specific activity (GU) was expressed as micromoles of glucose per milligram of protein per hour. The reaction mixture, containing lyophilizate dissolved in 2.0 ml of 0.1 M citrate buffer (pH 5.1), and 1.6 mg of soluble laminarin was incubated at 40 C for 1 hr. The reaction was stopped by boiling.

Chitinase (EC 3.2.1.14) was assayed by following the release of *N*-acetyl-D-glucosamine according to the method of Reissig et al (23). Specific activity (CU) was expressed as micromoles of *N*-acetyl-D-glucosamine per milligram of protein per hour. The reaction mixture, containing lyophilizate dissolved in 2.0 ml of 0.1 M phosphate buffer (pH 5.1) and 1.6 mg colloidal chitin (Sigma), was incubated at 37 C for 2 hr. The reaction was stopped by boiling. Protein content of the enzyme solution was determined by the Folin phenol reagent, according to Lowry et al (19).

The antibiotic Actidione (=cycloheximide) (3-[2- (3,5-dimethyl-2-oxocyclohexyl) -2 hydroxyethyl]glutarimide) (Sigma) was added to liquid culture (50 μg/ml) or solid medium (100 μg/ml).

Fluorescence staining. Small hyphal sections were removed from the interaction region of the colonies by sticking them to Scotch tape (3M Co., St. Paul, MN 55101) (Y. Hadar, *unpublished*), and stained for 1 min in a 0.05% (w/v) solution of Calcofluor White M2R New (American Cyanamid Co., Bound Brook, NJ 08805). The mycelium was washed four times each for 1 min, in 0.1 M phosphate buffer, pH 7 (18).

Lectins. Binding of lectins to host hyphae was examined by using fluorescein isothiocyanate (FITC) conjugated derivatives. FITC-WGA (wheat germ agglutinin) and FITC-Con A (Concanavalin A) were purchased from Miles-Yeda (Rehovot 76326, Israel) and FITC-PNA (peanut agglutinin) was kindly supplied by D. Mirelman (Dept. of Biophysics, Weizmann Institute of Science, Rehovot, Israel). Culture labeling by FITC lectins (0.5 mg/ml) in buffered saline, pH 7.0, was carried out according to Mirelman et al (20). Parallel preparations were treated by FITC-conjugated lectins preincubated for 30 min at 25 C with their specific inhibitors—0.4 M methyl-α-D-glucoside for Con A and 2 mM (*N*-acetyl-D-glucosamine) for WGA. The preparations of both calcofluor and FITC-lectins were observed with a standard RA Zeiss (W. Germany) fluorescence microscope, using an excitation filter with 390–490 nm transmission and a barrier filter of 515 nm.

RESULTS

SEM observations. Mycelial samples from the interaction region of dual cultures of either *R. solani* or *S. rolfsii* and *T. harzianum* or *T. hamatum* were observed in a scanning electron microscope. The diameter of hyphae of *Trichoderma* spp. was 1.5–3 μm and the diameter of the plant pathogens was 5–7 μm, so they could easily be distinguished from each other (Figs. 1–4). Hyphae of *Trichoderma* often coiled around the host (Fig. 1), but while the coils of *T. hamatum* were very dense (Fig. 1), those of *T. harzianum* were rather loose. *T. harzianum* frequently grew parallel to the host and attached itself to host mycelium by forming hooks (Fig. 2). *T. hamatum* produced appressoria at the tips of short branches (Fig. 3). Following these interactions, the mycoparasite sometimes penetrated the host mycelium (Fig. 4) apparently by partially degrading its cell wall (Figs. 3 and 4). Detachment of a coiled hypha of *Trichoderma* from around the pathogen by gentle shaking, revealed a digested area and the penetration sites on the host mycelium (Fig. 5).

Glucanase and chitinase activity. Agar disks were sampled from the interaction zone where *T. harzianum* parasitized *S. rolfsii* in order to determine β-1,3-glucanase and chitinase activities. β-1,3-glucanase activity was 4.4 GU in the dual culture whereas its activities in single cultures of the same age of *S. rolfsii* and *T. harzianum* were 2.1 GU and 2.2 GU. Chitinase activity in the dual culture was 4.5 CU whereas its activities in separate pure cultures of *S. rolfsii* and *T. harzianum* were 2.6 CU and 2.08 CU, respectively.

When cycloheximide (100 μg/ml) was added to solid SM, both *S. rolfsii* and *T. harzianum* colonies grew towards each other normally, but at the meeting zone no antagonism of the *Trichoderma* on hyphae of *S. rolfsii* was observed.

T. harzianum was grown for 48 hr in a liquid salt medium containing *S. rolfsii* cell walls as a sole carbon source. Addition of cycloheximide (50 μg/ml) to the medium reduced β-1,3-glucanase activity from 4.85 to 0.77 GU and chitinase activity from 2.98 to 0.54 CU.

Host cell wall cytochemistry. Calcofluor binds to β-glucans and *N*-acetyl-D-glucosamine oligomers in regions of incomplete cell wall polymers (18). The interaction sites of both *Trichoderma* spp. and either *S. rolfsii* or *R. solani* were stained with this fluorescent dye and observed with ultraviolet light microscopy. Intense fluorescence was observed in both plant pathogens in regions of coiling and attachment of the hooklike bodies (Fig. 6).

FITC-lectins were applied to the dual culture. Their fluorescence showed binding of WGA and Con A to the coiling sites. PNA, however, did not show any evidence of binding at the interaction sites.

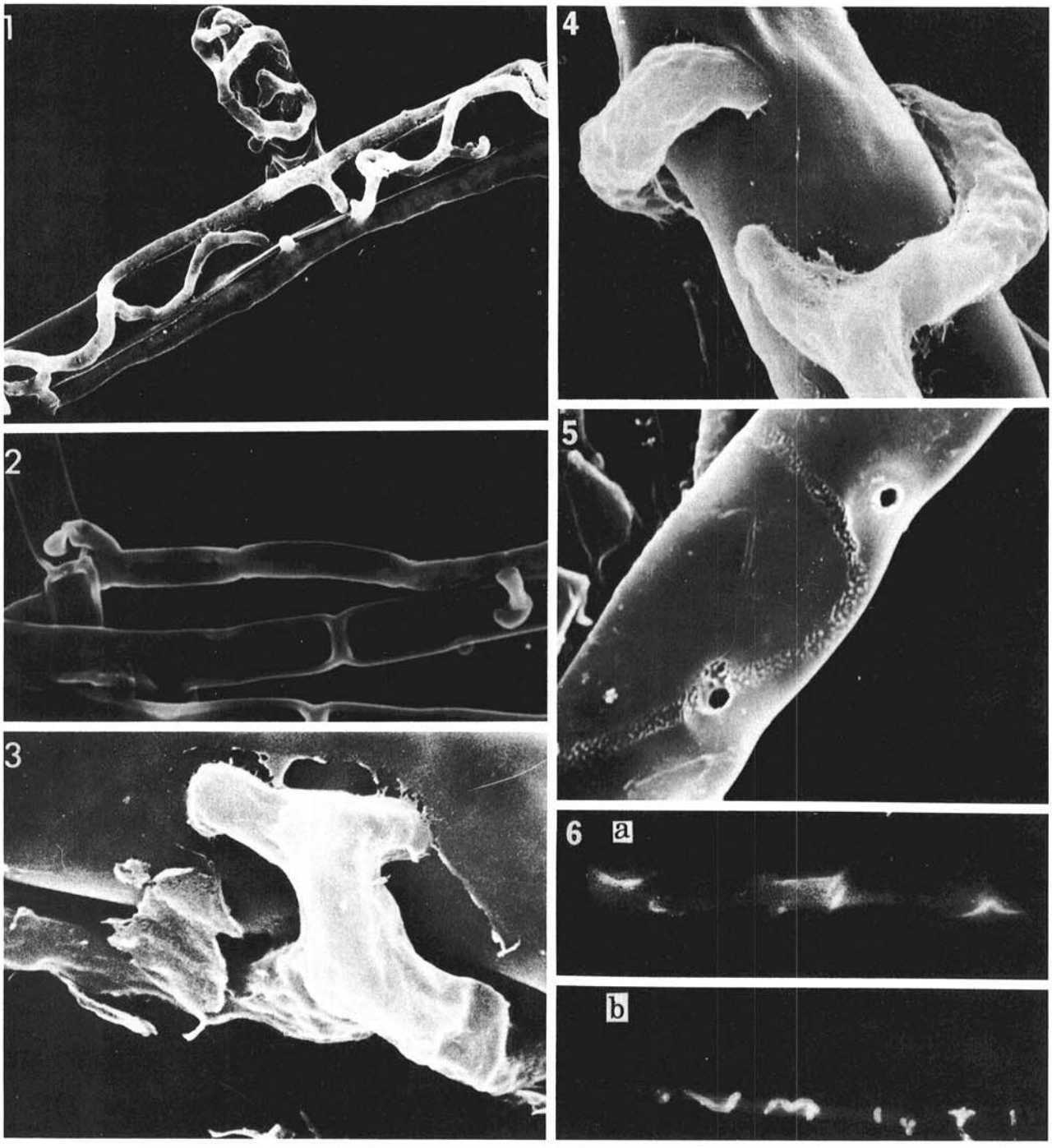
The sugars chitotriose $(GlcNAc)_3$ and methyl α-D-glucoside, which bind specifically to WGA and Con A, respectively, were incubated with the appropriate lectins. When the sugar-lectin mixtures were applied to the dual culture, no fluorescence was observed.

DISCUSSION

When either *T. harzianum* or *T. hamatum* grew toward *S. rolfsii* or *R. solani* contact was made and mycoparasitism occurred. Chet et al (5) showed that hyphae of *T. hamatum* grew directly towards

R. solani, which indicated that this is not a random phenomenon. Upon reaching host hyphae, the antagonistic fungus either coiled around the host or produced appressoria or hook-shaped contact branches. Sometimes *Trichoderma* was also observed to penetrate host hyphae (Fig. 4). Removal of the coiling hyphae of *T. harzianum* by gentle shaking revealed "footprints" of partial lysis on the host hyphae (Fig. 5). Moreover, holes that were observed in *S. rolfsii* hyphae apparently resulted from penetration by *T. harzianum* (Fig. 5). Scanning electron micrographs of host-parasite interactions have shown contact cells of *Stephanoma phaeospora* parasitizing *Fusarium* sp. and penetration of other fungi by *Pythium acanthicum* (15,16).

The cell walls of *S. rolfsii* and *R. solani* are composed of β-1,3-glucan (laminarin) and chitin (4,7). *T. harzianum* releases active lytic enzymes, that can digest these components (10,13). Microorganisms capable of lysing other organisms are widespread in natural ecosystems (21). Henis and Chet (14) have suggested that the extracellular enzymes may play a role in microbiological control. Jones et al (17) have shown that *T. viride* solubilized hyphae of *Sclerotinia sclerotiorum* by β-1,3-glucanase activity. In



Figs. 1–6. Scanning electron micrographs of *Trichoderma* spp. hyphae interacting with those of *Rhizoctonia solani* or *Sclerotium rolfsii*. **1,** Condensed coiling of *Trichoderma hamatum* around a hypha of *R. solani* (×1,700). **2,** Hooks of *T. hamatum* attached to hyphae of *R. solani* (×1,800). **3,** Appressoriumlike structure, formed by *T. hamatum*, attached to a hypha of *R. solani*. Note partial degradation of host cell wall (×8,300). **4,** Hypha of *T. hamatum* coiling around and penetrating one of *R. solani*. Partial degradation of host cell wall can be observed (×8,300). **5,** Hypha of *S. rolfsii*, from which a coiling hypha of *T. harzianum* was removed, showing digested zone with penetration sites caused by the antagonist (×5,500). **6,** Fluorescence light micrographs of interacting hyphae stained by Calcofluor White M2R New. **a,** Fluorescence in regions where *T. harzianum* coiled around a hypha of *S. rolfsii* (×800). **b,** Fluorescence in regions where hooks and appressoriumlike organs of *T. hamatum* attached to a hypha of *R. solani* (×350).

the present study, we found that adding cycloheximide to solid growth medium prevented hyphae of *T. harzianum* from invading colonies of *S. rolfsii* or *R. solani*. Enzymatic activity of *Trichoderma* was reduced in the presence of cycloheximide. These results are in agreement with the scanning electron micrographs (Fig. 5) showing lytic activity, which partially degraded the cell wall of the pathogen in the interaction regions.

Intense fluorescence was observed in coiled hyphae zones. Although calcofluor is not specific, it selectively binds to the edges of polysaccharide oligomers (18). Such binding sites appear to be present in places where enzymatic activity of *Trichoderma* has occurred (Fig. 6). The specific binding of the fluorescent FITC-WGA to the coiling zones indicate the presence of *N*-acetyl-D-glucosamine oligomers at these sites. This suggests that chitin fibrils are exposed in the cell walls of *S. rolfsii* and *R. solani* as a result of the extracellular β-1,3-glucanase excreted by *Trichoderma* at the contact sites.

Similarly, the binding of FITC-Con A revealed the presence of D-glucose and D-mannose in the lysed sites (2,20). The cell walls of the tested plant pathogenic fungi contained only low concentrations of galactose residues (2). Indeed, no binding of PNA was found after the attack by *Trichoderma*. Binding of FITC-lectins was shown to be sugar specific; no fluorescence was observed in the presence of both the lectins and their inhibitory sugars (2). Our results are in agreement with the studies of Mirelman et al (20) and Barkai-Golan et al (2), who found the binding of several lectins to hyphae to penicillia, aspergilli, and *T. viride*.

The use of both electron microscopy and specific binding proteins facilitated the study of the mode of antagonism and the localization of sites of interaction between hyphae of *Trichoderma* spp. and their hosts. This is in agreement with results of a recent study (10) that showed that nonparasitic isolates of *Trichoderma* have lower extracellular enzyme activity.

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