Saprotrophic and Mycoparasitic Components of Aggressiveness of *Trichoderma harzianum* Groups toward the Commercial Mushroom *Agaricus bisporus*

Josie Williams,¹ John M. Clarkson,^{1†} Peter R. Mills,² and Richard M. Cooper^{1*}

Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY,¹ and Department of Microbial Biotechnology, Horticultural Research International, Wellesbourne, Warwick CV35 9EF,² United Kingdom

Received 1 November 2002/Accepted 16 April 2003

We examined the mycoparasitic and saprotrophic behavior of isolates representing groups of *Trichoderma harzianum* to establish a mechanism for the aggressiveness towards *Agaricus bisporus* in infested commercial compost. Mycoparasitic structures were infrequently observed in interaction zones on various media, including compost, with cryoscanning electron microscopy. *T. harzianum* grows prolifically in compost in the absence or presence of *A. bisporus*, and the aggressive European (Th2) and North American (Th4) isolates produced significantly higher biomasses (6.8- and 7.5-fold, respectively) in compost than did nonaggressive, group 1 isolates. All groups secreted depolymerases that could attack the cell walls of *A. bisporus* and of wheat straw, and some were linked to aggressiveness. Growth on mushroom cell walls in vitro resulted in rapid production of chymotrypsin and trypsin-like proteases by only the Th2 and Th4 isolates. These isolates also produced a dominant protease isoform (pI 6.22) and additional chitinase isoforms. On wheat straw, Th4 produced distinct isoforms of cellulase and laminarinase, but there was no consistent association between levels or isoforms of depolymerases and aggressiveness. Th3's distinctive profiles confirmed its reclassification as *Trichoderma atroviride*. Proteases and glycanases were detected for the first time in sterilized compost colonized by *T. harzianum*. Xylanase dominated, and some isoforms were unique to compost, as were some laminarinases. We hypothesize that aggressiveness results from competition, antagonism, or parasitism but only as a component of, or following, extensive saprotrophic growth involving degradation of wheat straw cell walls.

Trichoderma species are ubiquitous in most soil types and are antagonistic towards many fungal species (14). *Trichoderma harzianum* is commonly acknowledged as a mycoparasite (5, 21) with the ability to secrete a wide complement of depolymerases (21, 25) and has been used as a biocontrol agent against several major plant pathogens. However, since 1985 *T. harzianum* green mold infestations of commercial mushroom compost in the Republic of Ireland, United Kingdom, United States, and France have been responsible for the considerable losses of yields of *Agaricus bisporus* (22, 31, 34, 36, 43, 44).

Muthumeenakshi et al. (35, 36) and Castle et al. (12) described the molecular classification of *T. harzianum* groups associated with mushroom compost. Groups Th2 and Th4 represent aggressive strains indigenous to Europe and North America, respectively, and nonaggressive European strains were designated Th1 and Th3. Aggressiveness is a term usually used to describe the degree of pathogenicity of plant pathogens, but here it refers to the reduction in *A. bisporus* yield resulting from unknown interactions with *T. harzianum*. For example, in controlled inoculations Th2 reduced quality and yield by up to 80% (46). The forms responsible for green mold have been recently separated from *T. harzianum* in growth rate

and in sequences of the ITS1 region of nuclear rRNA and a fragment of the protein-encoding translation elongation factor gene (EF-1 α) (42). On this basis, Samuels et al. (42) renamed Th4 and Th2 as *Trichoderma aggressivum* sp. nov. and *T. aggressivum* f. sp. *europaeum* f. sp. nov., respectively.

Mycoparasitism by *T. harzianum* can take the form of directed growth towards a potential host followed by attachment, coiling, formation of hooked branches, and sometimes appressorium-like structures and penetration (4, 15). Initial recognition of host fungi may involve a fucose-binding lectin (2). Penetration of the host cell wall could have both enzymatic and mechanical components (21). Chitinolytic degradation was localized to penetration sites under constricted hyphae in the invasion of *Sclerotium rolfii* sclerotia (5). Mycoparasitic structures have been induced to form on nylon fibers. Mycoparasitic signal transduction may involve G protein(s) and is mediated by cyclic AMP (37).

The role of depolymerases dominates reports of *T. harzianum* mycoparasitism to plant pathogens (11, 20, 25, 26, 27, 41). A serine protease, PRB1, was produced in the presence of cell walls or mycelium of several pathogenic fungi (32), and Flores et al. (23) obtained improved biocontrol of *Rhizoctonia solani* by *Trichoderma harzianum* transformants that overexpressed PRB1. An endochitinase (ECH42) was implicated in the mycoparasitism of *R. solani* based on improved disease control by multiple-copy transformants (11). Differential expression of *T. harzianum* chitinases during mycoparasitism of *R. solani* and *S. rolfii* was host dependent (26). Expression of *ech42* from *T. atroviride* occurred before mycoparasitic contact

* Corresponding author. Mailing address: Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, United Kingdom. Phone: 44 0 1225 383051. Fax: 44 0 1225 826779. E-mail: bssrmc@bath.ac.uk.

† Present address: Molecular Sensing plc, Melksham, Wiltshire SN12 8LH, United Kingdom.

following molecular exchange with the host *R. solani* (29), but in contrast, *chit 33* was expressed during, but not before, overgrowth of *R. solani* by *T. harzianum* (18). Also three to seven extracellular β -1,3-glucanases were produced by isolates of *T. harzianum* exposed to fungal cell walls, autoclaved mycelia, or laminarin, and some of these enzymes were linked to mycoparasitism (20, 40).

T. harzianum is a highly competitive fungus in the soil (52). The rapid growth rate of *Trichoderma* spp. and the abundant asexual production of numerous propagules could classify them as ruderals (38), or opportunists. However, their versatile complement of lytic enzymes suggests a more combative strategy (53). The ability to switch strategies based on environmental conditions and community structure might explain their ubiquity in soils. This hypothesis of versatility is consistent with investigations of *Trichoderma* spp. for intraspecific competition (54) and interspecific competition under field conditions (52). The outcomes of these interactions are dependent on many factors, such as resources, temperature (15, 28, 49), and tolerance to inhibitory bacteria from soil or compost (43). The mechanism(s) by which aggressive isolates of *T. harzianum* inhibit growth of *A. bisporus* is unknown.

Our objective was to identify traits linked with aggressiveness and, in particular, to test the hypotheses that saprotrophic growth of *T. harzianum* is a crucial factor and that extracellular depolymerases are required for aggressiveness. The role of depolymerases in the saprotrophic lifestyle of *T. harzianum* has not been widely studied. We used both aggressive and nonaggressive strains of *T. harzianum* to determine (i) the presence of mycoparasitic structures in interaction zones with *A. bisporus*, (ii) growth rates in compost in the presence and absence of *A. bisporus*, (iii) the presence of depolymerases induced by cell walls isolated from wheat straw (a major component of compost) and from *A. bisporus* (the putative host), and (iv) the presence of depolymerases produced in colonized compost.

MATERIALS AND METHODS

Fungal strains. *T. harzianum* isolates Th1(c), TD15, and T28JF (all group Th1); T7, Th2A, and KPNT (all group Th2); TD7, Th3c, and A006022 (all group Th3); and RM10c, BE, and RM10m (all group Th4) were supplied by Horticulture Research International (HRI), Wellesbourne, United Kingdom. *A. bisporus* isolate A12 spawn grain was also supplied by HRI, from which fungal growth was obtained on Oxoid (Basingstoke, United Kingdom) malt extract agar (MEA).

Fungal inoculation of compost. To simulate green mold disease, *T. harzianum* and *A. bisporus* were inoculated into tubes (3 by 5 cm, polypropylene) containing mushroom compost (formula 8; supplied by Mushroom Unit, HRI). *A. bisporus* spawn (infested rye grain) was rolled in profusely sporulating cultures of *T. harzianum* and coated with conidia (ca. 1×10^6 conidia per grain) to produce the *T. harzianum* inoculum. Three grains of this inoculum were placed at the bottom of each tube with three uninoculated spawn grains and covered with ca. 30 g of fresh mushroom compost, which was lightly compressed before the tube was sealed with a lid. There were no significant differences between *T. harzianum* strains in CFU load per grain (see below). To determine growth by *T. harzianum* in the absence of *A. bisporus*, conidia were coated onto rye grain as described above. Tubes were placed in a 25°C incubator with high relative humidity in the absence of light for 3 weeks, and lids were left loose to allow gaseous exchange.

Fungal growth was initially determined visually by using two scales. *A. bisporus* growth was measured on a scale of 0 (no visible growth) to 3 (full colonization of the tube). The growth of *T. harzianum* was determined by the distance (in millimeters) at which characteristic green sporulation was detected from the inoculum source. Subsequently, a *T. harzianum*-selective medium (THSM) was developed to quantify *T. harzianum* in compost (55).

Quantification of *T. harzianum* growth in mushroom compost. The growth of *T. harzianum* in compost was determined on THSM by counting CFU. Two or

three strains from each *T. harzianum* group were tested in three different batches of commercial compost. Compost was removed from the tube, and a top layer (1 cm thick) containing the inoculum source was discarded. A sample of 10 g was taken from the remaining compost and added to 100 ml of 100 mM sodium tetraphosphosphate (Sigma, Poole, United Kingdom) in distilled water (13). Samples were steeped for 60 min at room temperature followed by 2 min of continuous agitation in a Stomacher Lab-Blender 400 (Seward Laboratories, London, United Kingdom).

Colony counts were $\log_{10}$ transformed, treated as geometrical with a normal distribution, and subjected to one-way analysis of variance (ANOVA) with Minitab for Windows. The results were compared with analysis of the original counts, which had a Poisson distribution and were analyzed by using Genstat. The two methods gave comparable results, and therefore, ANOVA was chosen for convenience.

The grain inocula for all *T. harzianum* strains were tested for differences in numbers of conidia. A sample of 10 rye grains of each *T. harzianum* inoculum was subjected to spore retrieval in 100 mM sodium tetraphosphosphate (2-min agitation in the stomacher), and log dilutions were incubated on five replica plates of THSM. The resulting CFU per grain were analyzed by ANOVA, and no significant differences were found between *T. harzianum* strains and the numbers of conidia on the grain introduced to the compost. Therefore, direct comparison of the CFU retrieved from compost for each strain was made with no significant biased effects from different quantities of inoculum.

Dual cultures of *A. bisporus* and *T. harzianum*. Dual cultures were produced on agar media, in compost, and on spawn grain. Mycelial disks (5-mm diameter) of *A. bisporus* and *T. harzianum* were cut from the advancing edge of colonies and placed 3 cm apart on a cellophane disk on various agar media. Media included distilled water agar (DWA) (2% agar in distilled water), MEA (2% agar and 2% Oxoid malt extract), compost filtrate agar (CFA) (5% blended oven-dried compost was infused in 1 liter of distilled water for 3 h and filtered through four layers of muslin and 2% agar was added, adapted from reference 39), blended compost agar (1 g of blended, autoclaved compost per petri dish covered with 20 ml of DWA). In compost, *A. bisporus* and *T. harzianum* inocula were added at opposite ends of a cylinder containing hand-compressed compost. Interactions on spawn grains were followed after adding grains to profusely sporulating cultures of *T. harzianum* and incubating on 2% MEA. All combinations were incubated at 25°C in the absence of light.

SEM. Samples of cellophane were removed from the interaction zone, where the two fungal colonies made contact on agar media. Straw samples were taken from interaction zones in compost cylinders, and entire spawn grains were also examined. Samples were attached to a cryoscanning electron microscopy (SEM) stub and then flash frozen in liquid nitrogen. The sample stub was introduced to the initial chamber where ice was removed by sublimation. Subsequently, the sample was sputter coated with gold and viewed in a JEOL (Tokyo, Japan) JSM6310 SEM. Crystals on the *A. bisporus* mycelial surface were analyzed by energy dispersive X-ray microanalysis (Oxford Instruments AN10000 X-ray analyzer; Gatan, Osney Mead, United Kingdom). Three samples from the interaction zones of two replicates of aggressive and nonaggressive isolates were removed and examined for each treatment.

Induction of *T. harzianum* depolymerases in vitro. *T. harzianum* strains were cultured in 100 ml of basal medium (16) in 250-ml conical flasks and incubated at 25°C in the absence of light for 3 days. The basal medium was buffered to pH 6.0 with 50 mM morpholineethanesulfonic acid (MES) and supplemented with 1% (wt/vol) glucose to produce an extensive mycelium (ca. 500 mg). The resulting mycelium was washed in 50 ml of sterile, basal medium and transferred to an additional 100 ml of basal medium and starved overnight (ca. 16 h). Finally, the mycelium was transferred to 100 ml of basal medium supplemented either with blended (ca. 1-cm lengths) untreated wheat straw (1% wt/vol) or with extracted *A. bisporus* cell walls (1% wt/vol) (method adapted from reference 51) as the sole source of carbon. Samples of culture fluids were removed after 12, 24, 48, and 72 h and clarified by centrifugation at $3,000 \times g$.

Extraction of *T. harzianum* depolymerases from sterile, commercial mushroom compost. Commercial mushroom compost was autoclaved three times for 1 h at 121°C with intervals of 12 h at room temperature to allow germination of heat-activated thermophiles. Tubes containing compost were then inoculated with *T. harzianum* and incubated as described above. Samples were collected after 1, 2, and 3 weeks. Enzymes were extracted with a buffer designed to prevent denaturation of the enzymes and to allow maximum desorption from the compost substrate; the buffer comprised 5 mM dithiothreitol, 200 mM KCl, and 5% polyvinyl pyrrolidone in 50 mM sodium phosphate (pH 6.0) (Sigma) (16). Cold extraction buffer was added to the compost in a ratio of 5 ml:1 g (vol/wt [fresh weight]). Samples were agitated for 1 min in a Stomacher Lab-Blender 400, steeped on ice for 15 min, and agitated again for 1 min in the blender. Solid

matter was removed by filtration through two layers of muslin, and the filtrate was clarified by centrifugation at $4,000 \times g$ for 15 min then $23,000 \times g$ for 15 min. The supernatant was dialyzed exhaustively against 25 mM MES (pH 6) at 4°C overnight, and then extracts were concentrated ca. fivefold against 30,000- M_r polyethylene glycol (Sigma) at 4°C. Before isoelectric focusing on gels, samples were desalted with PD-10 columns (Amersham Biosciences, Rainham, United Kingdom) prepacked with G-25 Sephadex.

Enzyme assays. All enzyme activities were first characterized with respect to optimal pH, temperature, and time for assays (for linear kinetics). Chitinase, laminarinase, xylanase, and cellulase activities were assayed by following the release of reducing sugars from the appropriate polymeric substrates (16). The substrates, 1-mg/ml laminarin, 25-mg/ml colloidal chitin (crab shell chitin), birch wood xylan (all three from Sigma), and cellulose (comminuted, insoluble Whatman no. 1 filter paper) were prepared in 25 mM MES (pH 5.5). The temperatures and incubation times used were 37°C for laminarinase (20 min) and chitinase (60 min) and 50°C for cellulase (20 min) and xylanase (20 min). The general protease activity was determined with azocasein 3% (wt/vol) in 50 mM MES (pH 5.5). To 50- μ l test samples, 100 μ l of azocasein substrate (both preincubated at 37°C) was added, and the reaction mixtures were incubated for 180 min at 37°C (9). Chymoelastase-like and trypsin-like protease activities were measured against the nitroanilide substrates (Sigma) suc-(Ala)₂-Pro-Phe-pNA and benzoyl-Phe-Val-Arg-pNA, respectively (8, 47, 48). Reaction mixtures at 25°C contained 40 μ l of test sample, 160 μ l of Tris-HCl buffered to pH 8.0 (0.1 M), and 50 μ l of substrate (2 mM) in dimethyl sulfoxide. The release of nitroanilide was monitored for 10 min.

Protease inhibitors. We selected commercially available inhibitors (Sigma) to five classes of protease: metallo-, cysteine, trypsin-like, aspartic, and serine proteases (6). Samples of *T. harzianum* culture fluids were incubated with EDTA (50 mM), iodoacetic acid (50 μ M), leupeptin (50 μ M), pepstatin (1 μ M), and phenylmethylsulfonyl fluoride (1 mM) for 30 min before protease assays were conducted (47).

Isoelectric focusing of depolymerases. Samples containing 100 μ g of protein were flash frozen in liquid nitrogen, lyophilized, dissolved in 10 μ l of distilled water, focused on precast polyacrylamide gels, and calibrated with pI markers of pH 3 to 9.5 (Amersham Biosciences). Chitinase, laminarinase, cellulase, xylanase, and protease activities were detected in replica gels of 1% (wt/vol) agarose in 50 mM MES (pH 6.0) containing the corresponding dye-complexed substrates (final concentration, 3.2 mg/ml): carboxy-methyl-chitin-Remazol brilliant violet, Remazol brilliant blue (RBB)-curdlan, ostazin brilliant red-hydroxyethylcellulose, RBB-xylan, and RBB-gelatin (Loewe GmbH, Berlin, Germany). Other cellulase components, cellobiohydrolase and β -D-glucosidase were detected with 4-methylumbelliferyl β -D-cellobioside (Sigma); a replicate gel was soaked in 10 mM gluconolactone in 50 mM MES (pH 5.5) for 10 min, to inhibit β -glucosidase, before exposure to 4-methylumbelliferyl β -D-cellobioside (17). Agarose overlays were usually removed after 30 min of incubation at temperatures predetermined as optimal for enzyme assays (usually 37°C, but 50°C for xylanase) and destained (50 mM acetate buffer [pH 5.4]:ethanol [96%], 1:2 [vol/vol]) to reveal activity as achromatic bands on a colored background (7). Distances migrated by the isozymes were recorded with transmitted light to maximize the contrast between the background and the achromatic zones on destained gels.

RESULTS

Aggressiveness of *T. harzianum* strains. Aggressive *T. harzianum* groups (Th2 and Th4) were confirmed as such by the substantial inhibitory effects on the growth of *A. bisporus* in compost in polypropylene cylinders. No growth of *A. bisporus* was apparent in the presence of any of these isolates (T7, Th2, and RM10c). In contrast, nonaggressive *T. harzianum* strains (Th1 and Th3) (isolates A12, Th1c, and TD15) had no significant effect (ANOVA and Bonferroni's multiple comparison post test with 5 replicates) on *A. bisporus* growth, which by 3 weeks had almost fully colonized the compost cylinders (growth scale assessment, 2.7 to 2.8 [standard error, ± 0.1]). No significant difference was detected for Th2 or Th4 growth in the presence or absence of *A. bisporus* (Table 1). However, growth in compost of all three aggressive isolates was significantly greater (7- to 12-fold) than that of two nonaggressive isolates (Table 1).

TABLE 1. Growth of aggressive and nonaggressive *T. harzianum* isolates in commercial compost in the presence and absence of *A. bisporus* determined by qualitative and quantitative methods

<i>T. harzianum</i> strain	Genotype	Qualitative detection of <i>T. harzianum</i> (mean $\pm$ SEM) ^a		Quantitative detection of <i>T. harzianum</i> (mean no. of CFU) ^b in the absence of <i>A. bisporus</i>
		Absence of <i>A. bisporus</i>	Presence of <i>A. bisporus</i>	
Th1(c)	Th1	7 $\pm$ 1*	4 $\pm$ 1*	2.18 $\times$ 10 ⁴ *
TD15	Th1	6 $\pm$ 1*	7 $\pm$ 1*	1.00 $\times$ 10 ³ †
T28JF	Th1			1.41 $\times$ 10 ⁴ ‡
T7	Th2	50 $\pm$ 2†	50 $\pm$ 5†	
Th2A	Th2	50 $\pm$ 0†	50 $\pm$ 0†	1.00 $\times$ 10 ⁵ §,¶,***
KPNT	Th2			1.29 $\times$ 10 ⁵ §,
TD7	Th3			1.15 $\times$ 10 ⁵ §, ,¶
A006022	Th3			1.00 $\times$ 10 ⁵ ¶
RM10c	Th4	50 $\pm$ 5†	40 $\pm$ 5†	7.41 $\times$ 10 ⁴ ***
BE	Th4			1.12 $\times$ 10 ⁵ §¶

^a Growth (in millimeters) after 3 weeks was determined by visual assessment as described in Materials and Methods. Results are means of 5 replicates per treatment. * and †, values (within columns) were significantly different according to the Friedman test ($P < 0.05$) and Dunn's multiple comparison post test.

^b Results are given as CFU per gram (fresh weight) of compost. *, †, ‡, §, ||, ¶, and ***, values significantly different ($P \leq 0.05$) according to one-way ANOVA and Fisher's multiple comparison test. Colonies were detected on THSM (55). These data are representative of three separate experiments with three batches of commercial compost.

Mycoparasitism-associated structures. There was no evidence of mycelial interaction on nutrient-rich media such as MEA (Fig. 1a); a dense network of *T. harzianum* mycelium was addressed to the cellophane while the sparser *A. bisporus* hyphae were predominantly aerial. *T. harzianum* exhibited mycoparasitism-like behavior only on low nutrient agars (DWA and CFA); however, examples were sporadic. Isolate T7 occasionally produced hooked branches around and parallel growth along *A. bisporus* hyphae (Fig. 1b). Little fungal-fungal interaction was observed in spawn grains inoculated with *T. harzianum* conidia. *A. bisporus* mycelium grew from grains onto the agar medium, and *T. harzianum* spores germinated on the spawn grain. *T. harzianum* strain T7 rarely coiled around *A. bisporus* hyphae or produced parallel growth (Fig. 1c). *Trichoderma harzianum* and *A. bisporus* both colonized compost when inoculated at a distance; however, little *A. bisporus* growth was evident at the interface between the two fungi (Fig. 1d). Hyphal growth of *T. harzianum* isolate T7 was observed on the surface of the compost components, e.g., straw fragments, to which it adhered tightly, whereas *A. bisporus* produced more aerial growth (Fig. 1d). Swollen *T. harzianum* hyphae in contact with *A. bisporus* hyphae may indicate mycoparasitic behavior, but few such events were detected (Fig. 1e). We also observed calcium-rich crystals (previously identified on non-reproductive strands of *A. bisporus* in compost as calcium oxalate) (1, 32) on *A. bisporus* (Fig. 1b, c, and e) under all nutrient conditions studied.

Saprophytic growth of *T. harzianum* groups in commercial mushroom compost. *Trichoderma harzianum* strains varied in growth capabilities in nonsterile compost in the range of 1.00×10^3 to 1.29×10^5 CFU per g (fresh weight) of compost (Table 1). Group 1 strains (Th1c, TD15, and T28JF) had the lowest colonization. The difference in growth between group 1 and group 2 (typically 6.8-fold CFU per gram [fresh weight] of compost greater than group 1), group 3 (ca. 8.7-fold greater),

FIG. 1. SEMs of interactions between *A. bisporus* (A) and *T. harzianum* (T) isolate T7 on various media. (a) Growth on MEA; no interactions, as *T. harzianum* (closed arrow) colonized the surface of the medium and *A. bisporus* (open arrow) produced aerial hyphae. Bar, 10 μ m. (b) Occasional mycoparasitic-like behavior of *T. harzianum* towards *A. bisporus* on CFA exhibited as parallel growth and hooked branches (arrows). Bar, 10 μ m. (c) Coiling of *T. harzianum* around *A. bisporus* on spawn grain. Bar, 10 μ m. (d) Absence of interactions in inoculated, commercial compost. *T. harzianum* is tightly adpressed to the straw surface (arrows). Bar, 10 μ m. (e) Hyphal swelling of *T. harzianum* in contact with *A. bisporus* on spawn grain. Bar, 1 μ m. Note the extensive formation of crystals on *A. bisporus* hyphae, evident in all micrographs.

and group 4 (ca. 7.5-fold greater) was consistently significant for three different batches of commercial compost. This result is consistent with the increased saprophytic colonization by isolates of groups 2 and 4 based on growth rates (Table 1).

Depolymerases. (i) Total enzyme activities on *A. bisporus* cell walls. Isolates from all groups of *T. harzianum* secreted abundant extracellular chitinase, laminarinase, and protease when exposed to *A. bisporus* cell walls as the sole carbon source in liquid cultures. The concurrent appearances and maximum levels of protease (A_{405} of 0.37) and chitinase (2,033 nkat ml^{-1}) after 24 h of growth by isolate T7 were followed by that of laminarinase (1,123 nkat ml^{-1}) after 48 h. There was no significant difference between the groups in protease, chitinase, and laminarinase total activities.

The chymoelestatase-like and trypsin-like activities of two representative aggressive isolates were significantly higher after 12 h of induction than those from nonaggressive groups. Typically, chymoelestatase activity was 9.6- and 12.2-fold higher for Th2 and Th4, respectively, and trypsin-like activity was 4.9- and 6.3-fold higher for Th2 and Th4, respectively, than the activities of nonaggressive isolates. However, after 24 h, nonaggres-

sive isolates had produced similar maximal levels of protease activity (data not shown). For all isolates (three from each group), chymoelestatase-like activity was completely inhibited by phenylmethylsulfonyl fluoride, which is characteristic of serine proteases, and slightly inhibited by leupeptin (9.4%). The trypsin-like activity of all isolates (one per group) was significantly inhibited by leupeptin (89%), EDTA (58%), and pepstatin (37%).

(ii) Depolymerase isoforms on *A. bisporus* cell walls. The main isoforms identified on isoelectric focusing gels are summarized in Table 2. The European groups, Th1, Th2, and Th3, all produced (after 12 and 24 h) a dominant laminarinase isoform (pI 4.83) while Th4 (North American) secreted an isoform of pI 6.25 and several minor acidic bands (Fig. 2a).

All groups produced complex isoform profiles of protease and chitinase. Aggressive groups rapidly produced a dominant protease isoform (pI 6.22) after 12 and 24 h of exposure to *A. bisporus* cell walls (Fig. 2c). Th1 and Th3 differed with dominant isoforms of pI 5.53 and 7.17, respectively (Fig. 2c). Chitinase isozymes specific to aggressive isolates (pI 7.23 and 7.36) were detected after 12 h of induction. All isolates produced more-complex chitinase isoform profiles after 24 h, when the Th1 profile resembled those of Th2 and Th4, but Th3 isolates appeared distinct. An additional unique isoform of pI 7.83 was detected for Th2 and 4 (Fig. 2b).

(iii) Total enzyme activities on wheat straw. All groups of *T. harzianum* secreted cellulase, laminarinase, protease, and xylanase with wheat straw as the sole carbon source in liquid cultures. There were no significant differences between aggressive and nonaggressive strains with respect to the main activities, which were cellulase and xylanase (5 replicates per treatment). Cellulase levels were maximal at 48 h and ranged from 500 to 1,600 nkat/ml; xylanase was still increasing at 72 h when activities were 2,000 to 2,900 nkat/ml.

(iv) Depolymerase isoforms on wheat straw. The protease and laminarinase isoforms were very similar, after 24 h of growth, to those produced on *A. bisporus* cell walls, although the pIs differed slightly. The main protease isoform for Th1 and Th3 had a pI of 6.06 (although after 12 h there was a doublet that included a pI 6.25 isoform) while Th2 produced a dominant isoform with a pI of 7.08 (Fig. 2d). Laminarinase again had a simple profile with a major band with a pI of 4.98 for Th1, Th2, and Th3 and a main form with a pI of 6.19 for Th4 (Fig. 2e). There were numerous endo- β -1,4 glucanases (cellulases) for all groups after 24 h. The Th3 profile was distinct with ca. six acidic bands and one with a pI of 6.19. The Th4 profile also was clearly different except for a major alkaline band shared with Th1 and Th2. Th1 and Th2 isoforms were very similar with ≤ 4 main bands with pIs of 4.7 to 5.74 (Fig. 2f). Several β -glucosidase isoforms were detected after 12 h, and cellobiohydrolases were detected after 24 h of incubation. The profiles were not linked with aggressiveness, but Th3 again appeared distinct (data not shown). Xylanase profiles of all isolates were similar, with several predominantly acidic forms and a doublet at pIs 9.24 and 9.37 (Fig. 2g). However, Th3 was again distinct with two additional acidic forms.

(v) Total enzyme activities in compost. Significant activities of proteases and three main glycanases were readily detected from buffer-extracted compost colonized by *T. harzianum*. Total activities after 14 days of growth (data not shown) did not

TABLE 2. Depolymerase isoforms produced by aggressive and nonaggressive *T. harzianum* isolates in vitro on *A. bisporus* cell walls, on wheat straw, and in compost

Isoform	pI(s) of isoforms produced by <i>T. harzianum</i> genotypes on ^a :									
	<i>A. bisporus</i> cell walls					Wheat straw				
	Th1	Th2	Th3	Th4		Th1	Th2	Th3	Th4	
Protease	5.53	6.22	7.17	6.22		6.06	7.08	6.06	6.06	
Laminarinase	4.83	4.83	4.83	6.25		4.98	4.98	4.98	6.19	
Chitinase		7.23	4.25–5.00, 7.00–7.45	7.23						
	6.1, 6.8	7.36		7.36						
	4.23–5.20	7.83		7.83						
Cellulase						3.96–5.49, 7.97	3.96–5.49, 7.97	3.96–5.50, 6.19 ^b	4.5–5.87, 7.97	
Xylanase						4.66–5.74, 9.24	4.66–5.74, 9.24	4.66–6.00 ^b , 9.24	4.66–6.5, 9.24, 9.37	
						9.37	9.37	9.37	8.92	

^a The media from which *T. harzianum* enzymes were extracted were *A. bisporus* cell walls, wheat straw, and compost. The fungus was exposed as established mycelium for 12 or 24 h in vitro to mushroom cell walls or to straw, as inducers of enzyme synthesis, or inoculated in sterilized mushroom compost and grown for 3 weeks. Genotypes Th2 and 4 are aggressive to *A. bisporus*, and genotypes Th1 and 3 are nonaggressive. Numbers represent isoelectric points (pI) of depolymerase isoforms as determined from isoelectric focusing gels. Only major isoforms are shown, or in the event of many isoforms or insufficient resolution, a range of pIs is given. The absence of data indicates that an enzyme was not tested. ND indicates that the enzyme was not detected on isoelectric focusing gels but some were detected by enzyme assay (see text). Bold figures indicate isoforms that are apparently unique to both aggressive groups, Th2 and Th4.

^b Isoform pattern of Th3 is distinct from other isolates.

reveal consistent association with aggressiveness. Xylanase activity predominated at 600 to 1,000 nkat/ml and laminarinase activity ranged from 200 to 780 nkat/ml, whereas cellulase activity was only 40 to 150 nkat/ml. Group 3 was the only one that produced detectable chitinase activity (50 nkat/ml).

(vi) **Depolymerase isoforms in compost.** Isoenzyme profiles obtained from extracts after 14 days of growth, when activities were highest, were quite different from those secreted in culture, although some isoforms were common to both situations.

Group 3 (TD7) produced abundant (ca. 15) cellulase isoforms with a broad pI range of 4.43 to 8.99. The numerous alkaline forms were generally absent from liquid cultures. All groups produced an isoform with a pI of 4.69. Isolate Th1(c) secreted two additional isoforms with pIs of 6.97 and 7.30 (Fig. 2 h). As with cellulase, nonaggressive group 3 produced substantially more xylanase isoforms than the other three groups. The group 3 profile primarily consists of alkaline isoforms not present in vitro and not produced by any other isolates. Another band unique to growth in compost with a pI of 7.36 was from Th1 and Th4, and an isoform with a pI of 8.92 was from Th1, Th3, and Th4 (Fig. 2i). Laminarinase isoforms were similar from all groups and far more complex than the single dominant form (pI 4.98 or 6.19) observed in vitro. The latter isoform from liquid culture (pI 6.19) may be analogous to that of pI 6.25 in compost. From compost, >5 forms were present, but they were not well resolved (Fig. 2j).

DISCUSSION

Our data are the first quantitative data to assess saprophytic growth by *T. harzianum* groups in commercial mushroom compost in the absence of *A. bisporus*. We also observed substantial differences in colonization, which had previously been recorded only in spawned compost (for examples, see reference 52) and which may provide the basis for the aggressiveness of *T. harzianum* isolates. Our data contrast with those of Seaby (44, 45) but are consistent with microscopic assessments of mycelium in a spawned compost (31). Tolerance to inhibitory bacteria has been hypothesized to be the main component of compost colonization (43), but our finding of substantially greater growth in sterilized compost by aggressive isolates suggests that other factors are also important. Mycoparasitism by Th2, as judged by the formation of infection structures in vitro on spawn grain and in compost, was infrequent. Likewise, Fletcher (22) reported little evidence for mycoparasitism by aggressive biotypes, and Atkinson (1) found that hyphal interactions involving Th4 were rare.

Depolymerases of *T. harzianum* are probably essential for both saprotrophic and parasitic growth. We detected these enzymes in *T. harzianum*-infested compost and found that, in some cases, the speed of enzyme production or the presence of unique isoenzymes (Table 2) distinguished the aggressive and nonaggressive isolates. Similar isoforms were generally present whether wheat straw or mushroom cell walls were used as the inducing substrate. Chitinases, laminarinases, and proteases produced on *A. bisporus* walls could function in the degradation of the host's cell wall (3, 33). The difference in protease production between *T. harzianum* groups, apparent after only 12 h of induction and the much greater production of a protease isoform (pI 6.22) by aggressive biotypes, could be linked

FIG. 2. (a to c) Isozyme profiles produced by *T. harzianum* groups in vitro on *A. bisporus* cell walls after 24 h. Isoelectric focusing gels were overlaid with activity gels containing dye-linked substrates. (a) Laminarinase isoforms; (b) chitinase isoforms; (c) protease isoforms. Values denote pIs as determined from a calibration curve. (d to g) Isozyme profiles produced by *T. harzianum* in vitro on wheat straw after 24 h. (d) Protease isoforms; (e) laminarinase isoforms; (f) cellulase isoforms; (g) xylanase isoforms. (h to j) Isozymes detected in compost colonized by *T. harzianum* at 14 days postinoculation. (h) Cellulase isoforms; (i) xylanase isoforms; (j) laminarinase isoforms. Lane numbers 1 to 4 refer to the following *T. harzianum* isolates and groups: 1, isolate Th1(c) (group Th1); 2, isolate T7 (group Th2); 3, isolate TD7 (group Th3); 4, isolate RM10c (group Th4).

with mycoparasitism. Proteases have been implicated previously in the degradation of fungal host cell walls by mycoparasites (23, 25). Aggressive isolates of *T. harzianum* also had unique chitinase isoforms with pIs of 7.23 and 7.36 after 12 h of induction. The concurrent synthesis of chitinase and protease may result from the induction of some proteases by chitin-containing substrates (25). Some *T. harzianum* endochitinases appear particularly potent in terms of antifungal activity, which might justify further study of chitinase isoforms specific to aggressive isolates (30). Geographic origins of isolates could be distinguished by an acidic laminarinase isoform

(pI 4.83) in the European groups (Th1, Th2, and Th3) and a more alkaline isoform (pI 6.25) in the North American group (Th4). On straw in vitro and during axenic growth in compost, xylanase activity was higher than that of β -1,3 glucanase and cellulase. Cellulose is the key structural domain, and xylan is the major matrix component of cereal cell walls (10), with most fungal pathogens of cereals using arabinoxylanases as their main cell wall-degrading enzymes (9, 16). Noncellulosic, mixed-linkage β -D-glucans are a matrix component in wheat straw and may suffice to induce the glucanase (10).

This report is the first to characterize fungal depolymerase

isoenzymes from axenic growth of *T. harzianum* in compost. Glycosidases from *T. harzianum* isolates have been detected in compost (43), but total activities were insufficient to distinguish isolates differing in aggressiveness, as were the total activities of glycanases produced on straw and in compost measured in this study. Some of the isoforms in compost, e.g., most of the detected xylanases, had pIs similar to those produced on straw in vitro, which suggests that straw degradation is a key factor in compost colonization. However, some isoforms, especially that of laminarinase, were only produced by *T. harzianum* in compost. This pattern may reflect the induction of new isoforms or be an experimental artifact resulting from complexing with phenolic components of compost.

In all three forms of culture, Th3 (European nonaggressive) isolates had isozyme profiles that differed from those of the other groups. Chitinase activity was recovered only from compost colonized by Th3 isolates. This group has been reclassified as *T. atroviride* (based on ITS1 sequence homology) (35) and has a slower growth rate (42). Th3 generally grows poorly in spawned compost and does not affect yield (46), although there have been occasional reports of Th3 isolates extensively colonizing mushroom compost (22).

In conclusion, the only consistent feature linked with the aggressiveness of Th2 and Th4 isolates was their ability to colonize mushroom compost rapidly and extensively, even in the absence of *A. bisporus*. Prolific production of depolymerases could contribute to both mycoparasitic or saprotrophic growth. Thus, β -1,3 glucanases have substrates in *A. bisporus* and in wheat cell walls while chitinases and proteases may facilitate saprophytic growth on the rich fungal and bacterial compost microflora (some chitinases possess dual lysozyme activities) (50). Some depolymerases appeared to be associated with aggressiveness. Prima facie, these enzymes are candidates for analysis of their roles in aggressiveness by site-directed mutagenesis. However, this approach may be difficult because most fungi have multiple depolymerases and can compensate for the loss of any single enzyme with related enzyme(s) or by expressing a previously latent gene(s) (8). Our results strongly suggest that the antagonism of *T. harzianum* to *A. bisporus* is not primarily the result of mycoparasitism. Instead, the key factor seems to be best described as competitive saprophytic ability (24) and is consistent with the hypothesis of Deacon and Berry (19) that competition for nutrients is the most general mechanism in biocontrol. However, the ability of some Th3 isolates to colonize sterilized compost at levels similar to those of the aggressive groups and to produce equivalent levels and ranges of depolymerases indicates that these factors alone do not explain aggressiveness. Nonetheless, aggressiveness is dependent on extensive saprophytic colonization and, presumably, on associated competition, but competition and colonization could be a prerequisite to antagonism to *A. bisporus*, of which mycoparasitism could be but one of several components.

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