Biological Control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* Is Mediated by Different Mechanisms

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ABSTRACT

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Ten wild-type strains and two benomyl-resistant mutants of *Talaromyces flavus* were examined for their ability to secrete the cell wall-degrading enzymes chitinase, β-1,3-glucanase, and cellulase, to parasitize sclerotia of *Sclerotium rolfsii*, to reduce bean stem rot caused by *S. rolfsii*, and to secrete antifungal substance(s) active against *Verticillium dahliae*. The benomyl-resistant mutant Ben^{R}TF1-R6 overproduced extracellular enzymes and exhibited enhanced antagonistic activity against *S. rolfsii* and *V. dahliae* compared to the wild-type strains and other mutants. Correlation analyses between the extracellular enzymatic activities of different isolates of *T. flavus* and their ability to antagonize *S. rolfsii* indicated that mycoparasitism by *T. flavus* and biological control of *S. rolfsii* were related to the chitinase activity of *T. flavus*. On the other hand, production of antifungal compounds and glucose-oxidase activity may play a role in antagonism of *V. dahliae* by retardation of germination and hyphal growth and melanization of newly formed microsclerotia.

Additional keyword: antibiosis.

The soil-inhabiting ascomycete *Talaromyces flavus* (Klöcker) A.C. Stolk & R.A. Samson (anamorph *Penicillium dangeardii* J.I. Pitt; synonym *P. vermiculatum* Dang.) suppresses Verticillium wilt of tomato, eggplant, and potato (8,9,12,13,15,27) and parasitizes *Sclerotinia sclerotiorum* (28), *Rhizoctonia solani* (4), and *Sclerotium rolfsii* Sacc. (25). In general, mechanisms implicated in antagonism toward and biological control of phytopathogenic fungi include mycoparasitism, antibiosis, competition, and induced systemic resistance (11). Cell wall-degrading enzymes, such as β-1,3-glucanase, cellulase, and chitinase, are involved in the antagonistic activity of biocontrol agents against phytopathogenic fungi (6,7,10,17,20,31,35). In addition to secreting cell wall-degrading enzymes (25), *T. flavus* antagonizes *Verticillium dahliae* Kleb. by parasitism and antibiosis (14,26). Microsclerotia of *V. dahliae* also have been killed by culture filtrate of *T. flavus*, the toxicity of which has been attributed to the action of glucose oxidase (16,24). Although *T. flavus* produces several extracellular enzymatic activities, it is unknown which of the secreted enzymes, if any, are major determinants of its antagonistic capacity, or whether different enzymes are active against different pathogens.

In this study, 12 isolates of *T. flavus* were compared in terms of their extracellular cell wall-degrading enzymes and glucose oxidase activities, inhibition of sclerotial germination and hyphal growth of *S. rolfsii* and *V. dahliae* by their culture filtrates, and ability to parasitize sclerotia of *S. rolfsii* and control bean stem rot caused by this pathogen. The purpose of this study was to determine how the different extracellular enzymatic activities correlate with antagonism of *S. rolfsii* and *V. dahliae* by *T. flavus*. The process of parasitism of *S. rolfsii* by *T. flavus* strain Ben^{R}TF1-R6 also was studied by scanning and transmission electron microscopy.

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MATERIALS AND METHODS

Media. Potato dextrose agar (PDA; Difco Laboratories, Detroit) was used to maintain cultures of pathogenic and antagonistic isolates and produce sclerotia of *S. rolfsii*. The basal liquid medium used to grow *T. flavus* contained 0.1 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.8 g of KH_2PO_4, 0.4 g of KNO_3, and 0.5 g of yeast extract per liter of distilled water. It was supplemented with either glucose (autoclaved separately), colloidal chitin prepared according to Rodriguez-Kabana et al. (33), carboxymethylcellulose (CMC; Sigma Chemical Co., St. Louis), or laminarin (Sigma), each at a concentration of 2 mg ml^{-1}, and autoclaved. The medium used to enhance *T. flavus* antibiotic activity was described by Mizuno et al. (30) and Kim et al. (24). Molasses-corn steep agar (MCGA) (37) was used to produce conidia of *T. flavus*. Czapek-solution agar (2) was used to produce microsclerotia of *V. dahliae*.

Pathogens. *S. rolfsii* isolate SR-3 (19) and *V. dahliae* isolate VEP-1, obtained from eggplant with wilt (14), were used. Czapek-solution agar plates (85 mm diameter) were inoculated with mycelial plugs of *V. dahliae* and incubated at 25°C for 4 weeks in the dark. Microsclerotia (75 to 150 μm in diameter) were collected by wet sieving (25,26). Plates of PDA (85 mm diameter) were inoculated with mycelial plugs of *S. rolfsii* and incubated at 28°C for 3 to 4 weeks until mature sclerotia formed. The sclerotia were collected, dried in a desiccator (relative humidity < 20%), and stored at room temperature until needed.

Antagonists. Eight wild-type isolates of *T. flavus* (TF-1, TF-4, TF-17, TF-43, TF-46, TF-62, TF-64, and TF-72) and two benomyl-resistant mutants derived from TF-1 (Ben^{R}TF1-R3 and Ben^{R}TF1-R6) were supplied by G. C. Papavizas (Biocontrol of Plant Diseases Laboratory, Plant Protection Institute, USDA, Beltsville, MD). Additional wild-type isolates of *T. flavus*, TF-VI-L and TF-IX, were isolated from rhizosphere soils of a tomato plant in Sdeh Eliyahu and a potato plant in Gilat, Israel, respectively. Both wild-type isolates were sensitive to benomyl at a concentration of 5 μg ml^{-1} (22).

Preparation of *T. flavus* culture filtrates. *T. flavus* was grown on MCGA for 2 weeks at 30°C under continuous fluorescent light. Conidia were washed off the agar surface with sterile deionized water. Erlenmeyer flasks (250 ml) containing 50 ml of basal liquid medium supplemented with glucose (control treatment), chitin (inducer of chitinase), CMC (inducer of cellulase), or laminarin (inducer of β-1,3-glucanase) were inoculated with 10^6 conidia and incubated on a rotary shaker (150 rpm) at 30°C. After 5 days, cultures were filtered through Whatman (Maidstone, England) No. 1 filter paper, and the filtrates were used as crude preparations for chitinase, cellulase, and glucanase assays. Erlenmeyer flasks (250 ml) containing 50 ml of antibiotic activity-enhancing medium (24,30) were inoculated and incubated as above for 50 h. The cultures were filtered through Whatman No.1 filter paper, and the filtrates were sterilized by filtration through a Millipore (Bedford, MA) filter (0.45 μm). These filtrates were used as crude preparations for determining glucose oxidase and antibiotic activities (24). Protein concentration in all crude preparations was determined as described by Sedmak and Grossberg (34).

Enzymatic activity assays. Cell wall-degrading enzymes in the culture filtrates were assayed as described by Madi et al. (25). Glucanase (exo-1,3-β-D-glucosidase, EC 3.2.1.58) was assayed by monitoring the release of free glucose from laminarin with glucose oxidase reagent (Sigma) according to the manufacturer's directions. Specific activity was expressed as micromoles glucose per hour per milligram of protein. Cellulase (exo-1,4-β-D-glucosidase, EC 3.2.1.4) was assayed by monitoring the release of free glucose from CMC by the same procedure as that used for glucanase. Chitinase (β-*N*-acetyl-D-glucosaminidase, EC 3.2.1.14) was assayed by monitoring the release of *N*-acetyl-D-glucosamine (NAGA), as described by Reissig et al. (32). Specific activity was expressed as micromoles NAGA per hour per milligram of protein. Glucose oxidase (β-D-glucose: oxygen oxidoreductase, EC 1.1.3.4) was assayed spectrophotometrically at 25°C, using a coupled peroxide-*o*-dianisidine system, according to Worthington Biochemicals Corp. (Freehold, NJ) (1). Specific activity was expressed as micromoles H_2O_2 per minute per milligram of protein. Each assay was replicated four times (four different culture filtrates of each strain served as four replicates). The experiment was repeated three times, and the data were pooled.

Mycoparasitism of *S. rolfsii* by *T. flavus*. Strain Ben^{R}TF1-R6 was used to compare the abilities of different types of *T. flavus* propagules (ascospores, conidia, and mycelium) to parasitize sclerotia of *S. rolfsii*. To prepare a mycelial suspension, strain Ben^{R}TF1-R6 was grown in basal liquid medium containing glucose for 5 days, as described above. Mycelia were collected by filtration on Whatman No. 1 filter paper, washed three times with distilled water, macerated in a Waring blender for 30 s, and the concentration was adjusted to 1.4 mg of mycelium per ml. Ascospores were prepared as described by Katan (21). Dried sclerotia of *S. rolfsii* were immersed for 30 min in either a conidial suspension (10^7 ml^{-1}), an ascospore suspension (10^7 ml^{-1}), or a mycelial suspension (1.4 mg ml^{-1}). Natural sandy soil (rhodoxeralf) from Rehovot, Israel (80 g, adjusted to 70% moisture-holding capacity, which corresponded to a matric potential of 0.3 bars), was placed in petri dishes, and its surface was smoothed by gentle pressure with the back of a smaller petri dish. The treated *S. rolfsii* sclerotia were placed on the soil surface and gently pushed into the soil. Twenty sclerotia were added to each plate of soil, and each treatment was replicated four times, with each plate serving as a replicate. Treatments were arranged in a completely randomized design. The plates were incubated at 30°C for 3 to 4 days. Mycoparasitism was considered positive based on the development of yellow colonies of *T. flavus* on *S. rolfsii* sclerotia, which is associated with loss of sclerotial germinability. To compare the mycoparasitic ability of the different *T. flavus* strains, the dried sclerotia were immersed in conidial suspensions (10^6 ml^{-1}) and incubated in soil, as described above. The percentages of colonized sclerotia were calculated, and the data were arcsine-transformed before statistical analysis. The experiment was repeated three times, and the data were pooled.

Microscopic examination of parasitism of *S. rolfsii* sclerotia by *T. flavus*. To prepare for scanning electron microscopy (SEM), sclerotia of *S. rolfsii* treated with *T. flavus* were incubated on 2% water agar at 30°C for 3 to 4 days, until yellow colonies of *T. flavus* developed on the sclerotia. Sclerotia were placed on brass stubs and fixed for 3 days in a sealed chamber containing an opened vial with 5% osmium tetroxide (OsO_4) in 0.1 M sodium phosphate buffer (pH 7.4) and a second opened vial with 25% glutaraldehyde. After fixation, the samples were air-dried for 24 h and coated with gold in a Polaron E5000 SEM coating apparatus (Bio-Rad, Polaron Division, Watford, England). The samples were observed under a Jeol (Tokyo) JSM 35 microscope. To prepare for transmission electron microscopy (TEM), the parasitized sclerotia were fixed in 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.1) for 3 h and washed twice with 20 mM Tris buffer (pH 7.1). The sclerotia were immersed in 0.05% dimethylsulfoxide for 10 min, washed again three times with Tris buffer, and postfixed in 2% osmium tetroxide in water for 2 h at 4°C. The samples were dehydrated with graded ethanol solutions and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome (LKB 8800; LKB Products, Bromma, Sweden) and observed in a Jeol JSM 100CX transmission electron microscope at 80 kV.

Suppression of bean stem rot caused by *S. rolfsii*. Biological control was assayed in the greenhouse, as described previously (25). Briefly, plastic pots (10 cm in diameter) were filled with 350 g of Rehovot sandy soil, and five bean seeds were placed on the soil surface in each pot. Dried sclerotia of *S. rolfsii* were immersed in a conidial suspension (10^6 ml^{-1}) of *T. flavus*, and one sclerotium was placed 0.5 cm from each bean seed. Seeds and sclerotia were covered with 150 g of soil, and the pots were incubated in a greenhouse at temperatures ranging from 25 to 30°C. Symptoms of stem rot were recorded every day for 3 weeks after planting. Disease reduction by the *T. flavus* treatment was assessed by comparison with plants inoculated with nontreated sclerotia. About 95% of the noninoculated control seeds developed into healthy plants. Calculation of percent disease reduction was based on considering this number of healthy control plants as a 100% reduction. The calculated data were arcsine-transformed before statistical analysis. The experiment was conducted three times, with 6 to 10 replicates per treatment, in which each pot served as a replicate, and the data were pooled. Treatments were arranged in a completely randomized design.

Antibiotic activity. Antibiotic activity was assayed as described by Madi et al. (25). Twofold dilutions of culture filtrates of *T. flavus* (0.5 ml) were mixed with 0.5 ml of molten Czapek-solution agar to give a total volume of 1 ml per well of a 24-well tissue-culture plate (Nunclon, Delta, Netherlands). After the agar solidified, 25 μl of the *V. dahliae* microsclerotial suspension was seeded on the agar surface at a rate of 5 to 10 microsclerotia per well. Similarly, one sclerotium of *S. rolfsii* was placed in each well. Plates were incubated at 28°C for 3 weeks. The most dilute solution of culture filtrate that completely inhibited sclerotial germination was considered the dilution end point. The effect of H_2O_2, at concentrations from 0.35 to 147 μM, on germination and melanization of *S. rolfsii* and *V. dahliae* was determined, as described above. The experiment was conducted three times. Treatments were replicated four times (four different culture filtrates of each strain) and arranged in a completely randomized design. The data shown are from a single representative experiment. The results were identical when the experiment was repeated.

Statistical analyses. The data were analyzed by one-way analysis of variance (after arcsine-transformation for proportions). Means were separated by Duncan's multiple range test ($P = 0.05$). The significance of Pearson correlation coefficients from 0 was tested by the Fisher method (38).

RESULTS

Enzymatic activities in culture filtrates of *T. flavus*. The specific activities of cell wall-degrading enzymes and glucose oxidase were determined in culture filtrates of 12 *T. flavus* isolates. The enzymatic activities varied considerably (Table 1), with the greatest difference among the isolates found for chitinase (25-fold), followed by glucanase (16-fold), cellulase (11-fold), and glucose oxidase (7-fold). In Table 1, the isolates are listed in descending order of chitinase activity. Statistical analyses with Pearson correlation coefficients, revealed no significant correlation between any two of the enzymatic activities. Although strain Ben^{R}TF1-R6 exhibited the highest activities for all enzymes and TF-46 showed very low activities for all of them, this apparent trend did not hold true when additional isolates were examined. For example, TF-17 and TF-62 showed similar glucanase activities but differed significantly in chitinase and cellulase activities. Similarly, TF-64 and TF-46 resembled each other in cellulase activity but differed significantly from each other in chitinase, glucanase, and glucose oxidase activities.

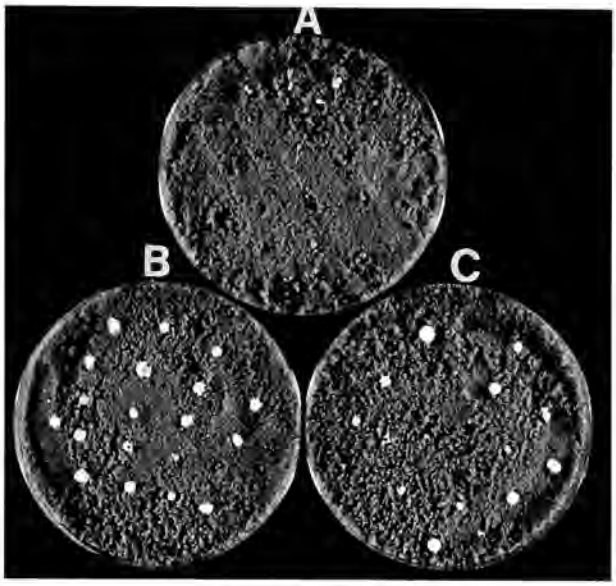

Fig. 1. Mycoparasitism of *Sclerotium rolfsii* sclerotia by different propagules of *Talaromyces flavus*. Sclerotia of *S. rolfsii* were immersed in suspensions of **A,** heat-activated ascospores; **B,** mycelial fragments; or **C,** conidia of *T. flavus*. The sclerotia were incubated in soil at 30°C for 3 to 4 days until bright-yellow colonies of *T. flavus* became evident.

Mycoparasitism of *S. rolfsii* sclerotia. Parasitism of sclerotia is considered an important mechanism of suppression of *S. rolfsii* by *T. flavus*. Of the 12 isolates compared (Table 1), the conidia of Ben^{R}TF1-R6 exhibited the highest mycoparasitic ability, with 63% of sclerotia colonized, whereas TF-62 and TF-46 colonized no sclerotia. The other isolates examined exhibited various levels of mycoparasitic ability to colonize sclerotia, ranging from 4 to 56%. Mycelial and conidial suspensions of Ben^{R}TF1-R6 colonized sclerotia of *S. rolfsii* more efficiently than did the ascospores (100, 95, and 5%, respectively) (Fig. 1).

SEM examination of the cellular interaction between *T. flavus* strain Ben^{R}TF1-R6 and *S. rolfsii* showed that *T. flavus* heavily colonized the surface of *S. rolfsii* sclerotia. Incubation of the inoculated sclerotia in the dark induced the teleomorphic state, leading to the development of *T. flavus* cleistothecia (Fig. 2A). Incubation in the light induced conidiation of *T. flavus* on the surface of *S. rolfsii* sclerotia (Fig. 2B). When the inoculated sclerotia were only partially colonized by *T. flavus* and succeeded in producing hyphal strands, some of the *S. rolfsii* hyphae also were parasitized by *T. flavus* hyphae, which grew along, coiled around (Fig. 2C), and penetrated the hyphae (Fig. 2D). Along the *T. flavus* hyphae, primarily at the contact sites with *S. rolfsii* hyphae, swollen segments occurred. The penetration pegs also appeared as swollen, appressorium-like structures (Fig. 2D and E). TEM revealed that, after *T. flavus* hyphal penetration of the thick cell walls of *S. rolfsii* sclerotia, the host-cell organelles disintegrated and the cytoplasmic content disappeared (Fig. 2F and G).

Biological control of bean stem rot caused by *S. rolfsii*. The ability of *T. flavus* isolates to control bean stem rot caused by *S. rolfsii* was examined in the greenhouse. The greatest reduction in disease (64%) was obtained by treating sclerotia with conidia of Ben^{R}TF1-R6, whereas TF-62 and TF-46 did not reduce disease (Table 1). The remaining isolates exhibited low to moderate abilities to control the disease, ranging from 5 to 52%.

Inhibitory activity of the culture filtrates. The antibiotic activity of *T. flavus* culture filtrates was examined by comparing their effect on the germination of *V. dahliae* microsclerotia. The

TABLE 1. Enzymatic activities and antagonism of strains of *Talaromyces flavus* toward *Sclerotium rolfsii* and *Verticillium dahliae*

	Specific activity[u]				*S. rolfsii*		*V. dahliae*
Strain[t]	Chitinase	Glucanase	Cellulase	Glucose oxidase	Parasitism[w] (%)	Bean stem rot reduction (%)	antibiotic activity[v]
Ben^{R}TF1-R6[x]	15.1 a[y]	1,724 a	55.1 a	529 a	63 a	64 a	512
TF-64	10.0 b	813 g	8.1 e	280 b	56 b	52 b	128
TF-17	8.9 c	970 e	25.3 bc	117 d–g	44 c	43 c	64
TF-1	8.9 c	1,010 d	27.6 bc	129 d–f	18 e	32 cd	64
TF-IX	8.9 c	849 f	54.3 a	187 c	33 d	37 cd	64
TF-43	8.2 c	1,225 b	28.7 b	108 e–g	ND[z]	ND	64
TF-VI-L	7.9 c	551 i	26.3 bc	158 c–e	30 d	38 cd	128
TF-4	6.9 d	1,128 c	22.6 cd	74 g	10 f	16 d	64
Ben^{R}TF1-R3[x]	4.2 e	710 h	18.2 d	165 cd	4 g	5 e	128
TF-62	0.9 f	952 e	5.0 e	131 d–f	0 h	0 f	64
TF-72	0.9 f	541 i	18.8 d	71 g	ND	ND	64
TF-46	0.6 f	110 j	6.4 e	96 fg	0 h	0 f	8

[t] Strains are listed in descending order of chitinase activity.
[u] Chitinase: micromoles *N*-acetylglucosamine per hour per milligram of protein; cellulase and glucanase: micromoles glucose per hour per milligram of protein; glucose oxidase: micromoles H_2O_2 per minute per milligram of protein.
[v] The maximal dilution of a culture filtrate that completely inhibited germination of *V. dahliae* microsclerotia.
[w] Sclerotia of *S. rolfsii* parasitized by *T. flavus* in soil.
[x] Benomyl-resistant mutants derived from TF-1.
[y] In each column, values not followed by the same letters are significantly different at $P = 0.05$, according to Duncan's multiple range test. Except for the antibiotic study, all other experiments were conducted three times, and the data were pooled. The data shown for the antibiotic study represent a single experiment, because the results were identical for all experiments.
[z] Not determined.

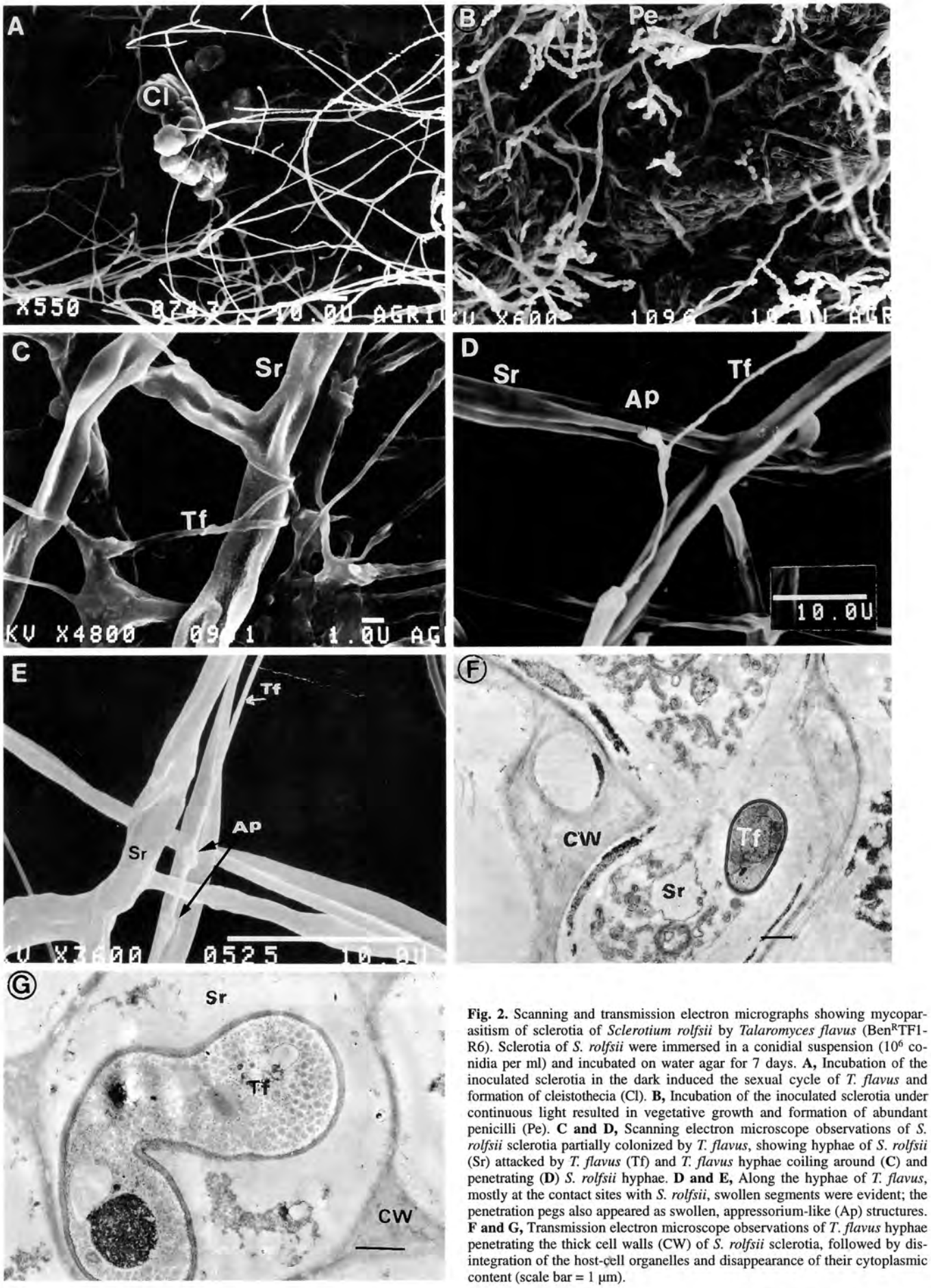

Fig. 2. Scanning and transmission electron micrographs showing mycoparasitism of sclerotia of *Sclerotium rolfsii* by *Talaromyces flavus* (Ben^RTF1-R6). Sclerotia of *S. rolfsii* were immersed in a conidial suspension (10^6 conidia per ml) and incubated on water agar for 7 days. **A,** Incubation of the inoculated sclerotia in the dark induced the sexual cycle of *T. flavus* and formation of cleistothecia (Cl). **B,** Incubation of the inoculated sclerotia under continuous light resulted in vegetative growth and formation of abundant penicilli (Pe). **C and D,** Scanning electron microscope observations of *S. rolfsii* sclerotia partially colonized by *T. flavus*, showing hyphae of *S. rolfsii* (Sr) attacked by *T. flavus* (Tf) and *T. flavus* hyphae coiling around (**C**) and penetrating (**D**) *S. rolfsii* hyphae. **D and E,** Along the hyphae of *T. flavus*, mostly at the contact sites with *S. rolfsii*, swollen segments were evident; the penetration pegs also appeared as swollen, appressorium-like (Ap) structures. **F and G,** Transmission electron microscope observations of *T. flavus* hyphae penetrating the thick cell walls (CW) of *S. rolfsii* sclerotia, followed by disintegration of the host-cell organelles and disappearance of their cytoplasmic content (scale bar = 1 μm).

isolates differed considerably in ability to inhibit germination, with Ben^{R}TF1-R6 exhibiting the highest antibiotic activity (Table 1). The culture filtrate of isolate TF-43 was used to study the effect of filtrates on mycelial growth and melanization of sclerotia of *V. dahliae* and *S. rolfsii*. Whereas germination and hyphal growth of *V. dahliae* were both inhibited by a 1:64 dilution of the culture filtrate, melanization of newly formed microsclerotia was more sensitive and was inhibited by a 1:128 dilution (Fig. 3A). Because the antibiotic effect has been attributed to toxic peroxide generated by glucose oxidase in the culture filtrate (16,24), we examined the relationship between antibiotic and glucose oxidase activities (Table 1) and found a significant correlation ($r = 0.940$, $P = 0.05$) between the two. We also tested the effect of H_2O_2 added directly to the agar medium. Microsclerotial germination and hyphal growth of *V. dahliae* were inhibited by 1.4 μM H_2O_2, whereas melanization of newly formed microsclerotia was inhibited by as little as 0.7 μM H_2O_2 (Fig. 3B).

In contrast to *V. dahliae*, sclerotial germination, hyphal growth, and melanization of new *S. rolfsii* sclerotia were not inhibited in the presence of the culture filtrate of *T. flavus* (data not shown). Germination of *S. rolfsii* sclerotia was affected only at 147 μM H_2O_2, mycelial growth was strongly inhibited at concentrations higher than 14.7 μM H_2O_2, and melanization was inhibited at 2.8 μM H_2O_2.

Correlation analyses between extracellular enzymes, mycoparasitism, and disease control. To determine which of the secreted enzyme(s) are involved in mycoparasitism, the enzymatic activities of the isolates tested and their ability to parasitize *S. rolfsii* sclerotia in the soil were correlated. A significant positive correlation ($r = 0.895$, $P = 0.05$) occurred between mycoparasitism and chitinase activity; no correlation occurred with glucanase, cellulase, or glucose oxidase (Table 2). A significant positive correlation ($r = 0.916$, $P = 0.05$) occurred between the mycoparasitic ability of the isolates and their ability to reduce bean stem rot caused by *S. rolfsii* (Fig. 4). Similarly, bean stem rot suppression correlated positively with chitinase activity ($r = 0.952$, $P = 0.05$) but not with glucanase, cellulase, glucose oxidase, or antibiotic activities (Table 2).

TABLE 2. Correlations between enzymatic activities in culture filtrates of *Talaromyces flavus* strains and their ability to parasitize sclerotia of *Sclerotium rolfsii* or to reduce bean stem rot in greenhouse experiments

	Correlation coefficient[y] (r)	
Enzymatic activity	Mycoparasitism	Disease reduction
Chitinase	0.895[z]	0.952[z]
Glucanase	0.283	0.323
Cellulase	0.546	0.387
Glucose oxidase	0.544	0.426

[y] Based on data presented in Table 1.
[z] Significant from 0 at $P = 0.05$.

DISCUSSION

Isolates of *T. flavus* vary greatly in their ability to suppress Verticillium wilt (16) and bean stem rot (Table 1). This study shows that isolates also vary in production of cell wall-degrading enzymes and antibiotic compounds and in mycoparasitic ability to antagonize *S. rolfsii*. Comparing these traits among isolates enabled us to assess whether biocontrol activity toward the target pathogens, *V. dahliae* and *S. rolfsii*, is correlated with any of the in vitro activities.

Results obtained with 12 strains of *T. flavus* suggest that different mechanisms are involved in the biological control of *S. rolfsii* and *V. dahliae*. Mycoparasitism seems to be the main mechanism controlling *S. rolfsii*, as indicated by the colonization of its sclerotia and hyphae by *T. flavus*, as well as the high positive correlation found between mycoparasitism and disease suppression (Fig. 4). Of the *T. flavus* extracellular enzymes tested, only chitinase activity correlated positively with both mycoparasitism and bean stem rot reduction. These findings concur with reports that chitinase plays a role in the degradation of hyphal tips of *S. rolfsii* by *Serratia marcescens* and the ability of *Escherichia coli* trans-

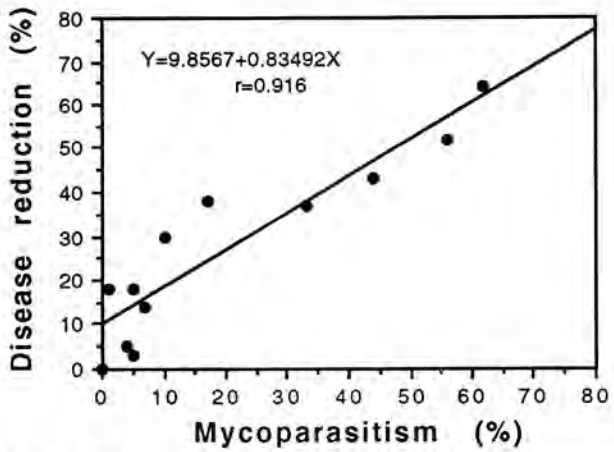

Fig. 4. The relationship between the ability to parasitize sclerotia of *Sclerotium rolfsii* and bean stem rot reduction by different strains of *Talaromyces flavus*. Pearson correlation coefficient (r) based on data presented in Table 1.

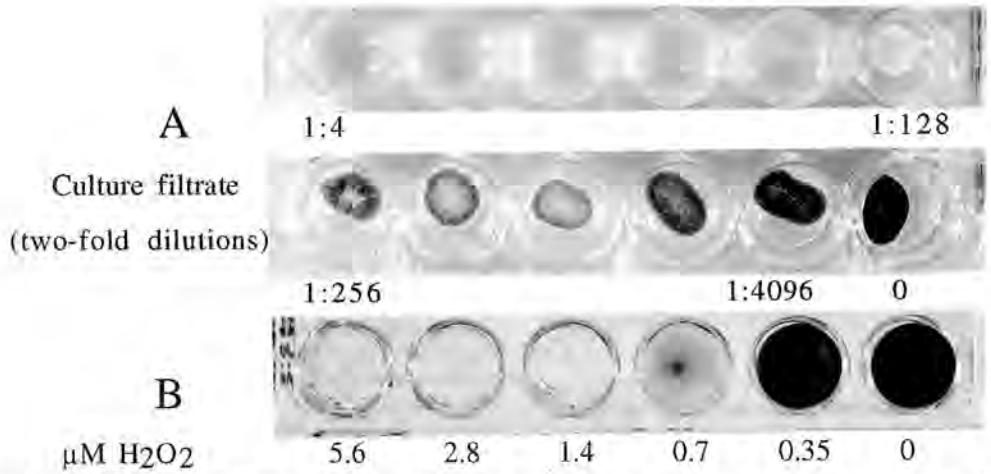

Fig. 3. Effect of *Talaromyces flavus* culture filtrate and H_2O_2 on *Verticillium dahliae* growth and sclerotial melanization. Series of twofold dilutions of the culture filtrate of *T. flavus* (TF-43) and H_2O_2 were used. **A,** Upper and middle rows, effect of culture filtrate at various dilutions. **B,** Effect of H_2O_2 at concentrations of 0.35 to 5.6 μM.

formed with the *chi A* gene from *Serratia marcescens* to reduce disease caused by *S. rolfsii* in beans (31,35). Similarly, transgenic tobacco plants expressing a bean chitinase gene showed enhanced resistance to *R. solani* (5). Further proof of the involvement of chitinase in mycoparasitism will require the generation of a *T. flavus* mutant lacking chitinase activity and comparing its activity with the parent strain. Antibiotic metabolite(s) secreted by *T. flavus* did not affect sclerotial germination, hyphal growth, or melanization of *S. rolfsii* in vitro.

In contrast to *S. rolfsii*, mycelium and microsclerotia of *V. dahliae* were very sensitive to the antibiotic activity present in the culture filtrate of *T. flavus*. The antibiotic metabolite(s) secreted by *T. flavus* inhibited melanization of newly formed *V. dahliae* microsclerotia, whereas melanization of newly formed *S. rolfsii* sclerotia was not affected. Although melanization in both fungi was inhibited by H_2O_2, that of *V. dahliae* was fourfold more sensitive to inhibition by H_2O_2 than that of *S. rolfsii*.

The results regarding the inhibitory effect of glucose oxidase activity on germination and melanin formation agree with the finding of Kim et al. (24) that glucose oxidase, secreted by *T. flavus*, retards hyphal growth and kills microsclerotia of *V. dahliae* in vitro, probably by generating toxic peroxide. McLaren et al. (29) reported partial disintegration of melanin near hyphae of *T. flavus* that colonized sclerotia of *Sclerotinia sclerotiorum*. Therefore, it seems that *T. flavus* is capable of degrading already produced melanins and inhibiting their production by maturing sclerotia. Previous results (25), as well as the high positive correlation between antibiotic and glucose oxidase activities shown here, support this hypothesis. Prevention of microsclerotial melanization could affect their survival in soil, rendering the sclerotia more sensitive to ultraviolet radiation (18) and attack by antagonistic microorganisms (3,18). Tjamos and Fravel (36) reported inhibition of germination and melanin formation in sublethally heated microsclerotia of *V. dahliae* and additive suppression by sublethal heating and *T. flavus* treatment. The possibility that, in soil, antibiotic activity plays a role in enhancing parasitic activity via a weakening effect, therefore, should be considered (14,23,24,36). Our results suggest that biological control of *S. rolfsii* and *V. dahliae* by *T. flavus* is mediated by different mechanisms. Although mycoparasitism is the main mechanism of suppression of *S. rolfsii*, antifungal compounds and glucose oxidase activity probably play a role in biological control of *V. dahliae*, as indicated by our in vitro studies.

The ability of *T. flavus* to colonize plant roots (12,13) may contribute further to disease suppression by reducing the proliferation of the pathogens on the roots by direct mycoparasitism or competition for inoculation sites or plant cell exudates. The Ben^RTF1-R6 mutant exhibited generally high activities of extracellular enzymes, including chitinase, as well as mycoparasitism and biological control of *S. rolfsii*. Microscopic examination of the parasitic process revealed the presence of swollen segments and appressorium-like structures, which have not been observed in wild-type strains of *T. flavus* in previous studies (4,14,28,29). Ben^RTF1-R6 also exhibited the highest glucose oxidase and antibiotic activities against *V. dahliae*. The antagonistic properties of this mutant demonstrate its potential as a highly effective biocontrol agent of both *S. rolfsii* and *V. dahliae*. This potential now needs to be examined further under field conditions.

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