Viability of Microsclerotia of *Verticillium dahliae* Reduced by a Metabolite Produced by *Talaromyces flavus*

D. R. Fravel, K. K. Kim, and G. C. Papavizas

Soilborne Diseases Laboratory, Plant Protection Institute, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705.

We sincerely thank Carolyn Sanford for her dedicated assistance and useful suggestions.
Accepted for publication 6 November 1986 (submitted for electronic processing).

ABSTRACT

Fravel, D. R., Kim, K. K., and Papavizas, G. C. 1987. Viability of microsclerotia of *Verticillium dahliae* reduced by a metabolite produced by *Talaromyces flavus*. Phytopathology 77:616-619.

Talaromyces flavus produced a metabolite that retarded radial growth and killed microsclerotia of *Verticillium dahliae* in vitro. Microsclerotia of two isolates of *V. dahliae* were killed (LD_{95}) in less than 3 hr in vitro. Maximum titer of the metabolite was produced by *T. flavus* after 5 days in a high-carbon liquid medium that was shaken at 120 rpm and incubated at 27 C. The metabolite was also able to kill microsclerotia in each of three sterile soils and in sand, indicating that the physical components of the soils did not inactivate the metabolite. The production of this metabolite may be involved in the biological control of *V. dahliae* by *T. flavus*.

Additional key words: *Penicillium dangeardii, Penicillium vermiculatum.*

Talaromyces flavus (Klöcker) Stolk & Samson (anamorph *Penicillium dangeardii* Pitt, usually reported as *P. vermiculatum* Dangeard) has been demonstrated to suppress Verticillium wilt of potato and eggplant (1,4,13) and to increase yield of eggplant under commercial production conditions (13). Loss of viability of microsclerotia of *Verticillium dahliae* Kleb. in the presence of *T. flavus* may account for, or contribute to, the observed disease control (12). Mechanisms postulated for the loss of viability of microsclerotia include antibiosis, mycoparasitism, and competition.

T. flavus is known to produce four antibiotics (5–7,15). One of these, talaron, has activity against fungi including *Hormodendrum pedrosoi* Brumpt (=*Cladosporium pedrosoi*) and *Pyricularia oryzae* Cav. (15). The present experiments were performed to determine whether antibiosis contributes to the loss of viability of microsclerotia of *V. dahliae*.

MATERIALS AND METHODS

Preliminary experiments indicated that culture filtrates of *T. flavus* were toxic to *V. dahliae* (3). To determine the time necessary to produce the highest titer of the inhibitory metabolite, two in vitro experiments were performed: effect of the metabolite on radial growth of *V. dahliae* and effect on microsclerotial germination.

Radial growth. Cultures of *T. flavus* isolate Tf-1 (also reported as 282-3 [13]) were maintained on potato-dextrose agar (Difco Laboratories, Detroit, MI) in the dark at 30 C. Cleistothecia and ascospores were formed by 3 wk. For production of the inhibitory metabolite, two 1.2-cm-diameter agar disks from 3-wk-old cultures of *T. flavus* were transferred to 100 ml of a liquid culture medium containing 8% glucose (15). Four replicate flasks of these liquid cultures were maintained at 27 ± 0.2 C on a rotary shaker at 120 rpm. Daily, for a total of 8 days, samples were filtered through a 0.45-μm filter to remove hyphae and spores of *T. flavus*. The resulting cell-free culture filtrates, autoclaved culture filtrates, uninoculated medium (broth), dilutions of each of these, or sterile distilled water were incorporated into double-strength Czapek solution agar (Difco Laboratories, Detroit, MI). These liquids were incorporated at the rate of 1:1 (v:v) so that the final concentration of the Czapek solution agar was at the recommended strength and the final dilution rates of the culture filtrate, autoclaved culture filtrate, or broth were 1:2, 1:10, 1:50, 1:100, and 1:200. There was one dilution series for each replicate flask. *V. dahliae* (isolate 1) was grown for 2 wk on Czapek solution agar at 22 C. Mycelial disks 0.5 cm in diameter were transferred to the amended agar plates. Radial growth of the colonies developing from the mycelial disks was recorded every other day for 2 wk after seeding plates with agar disks. The experiment was repeated twice.

Microsclerotial germination. *T. flavus* was grown in liquid shake culture as in the previous experiment, and the cell-free culture filtrate, autoclaved culture filtrate, broth, dilutions of these, or sterile distilled water was incorporated into Czapek solution agar medium as in the radial growth experiment. Microsclerotial suspensions were prepared from cultures of *Verticillium* grown as in the previous experiment by comminuting 4-wk-old agar cultures in sterile distilled water in a Sorvall mixer and filtering through 200-μm pore-size nylon mesh to remove large particles of fungal material and agar. Amended 9-cm-diameter Czapek solution agar plates were seeded with 1 ml of a suspension containing 50 microsclerotia per milliliter. The number of developing colonies was counted after 7 days. Treatments were replicated four times, and the experiment was done twice.

Time required to kill microsclerotia. Two isolates of *V. dahliae* were used in this experiment. Isolate 1 was originally obtained from eggplant and isolate 6 from potato, although isolate 6 is more pathogenic on eggplant than isolate 1. Both isolates were grown and microsclerotial suspensions prepared as described previously. Nylon mesh (42-μm pore size) was cut into 0.5-cm^2 squares, and microsclerotia were embedded in the squares by vacuum filtration (11). Each square contained about 15 microsclerotia. Culture filtrates from 5-day-old cultures, autoclaved culture filtrates, broth, or sterile distilled water were incorporated into Czapek solution agar at the same concentrations used in the previous experiment. Ten nylon squares with *V. dahliae* were placed on each of four replicate plates. After 1, 2, 4, 8, 32, and 64 hr, one nylon square per replicate per treatment was removed, rinsed vigorously for 20 sec in sterile distilled water, and placed on unamended Czapek solution agar. After 7 days, the number of squares developing colonies of *Verticillium* was recorded. Although each nylon square contained about 15 microsclerotia, each single square was recorded as plus or minus for growth. To determine whether

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the nylon squares were adsorbing the inhibitory metabolite, sterile mesh squares without *Verticillium* were placed on the medium containing the 1:2 dilution of culture filtrate. These squares were removed after 24 or 72 hr. After removal, microsclerotia of *Verticillium* were embedded in these squares and placed on Czapek solution agar. The experiment was repeated twice.

Soil columns. An experiment was performed to determine whether the metabolite produced by *T. flavus* was capable of reducing viability of microsclerotia of *V. dahliae* in sterile soil. Microsclerotia of *V. dahliae* (isolate 1) were embedded in 0.5-cm^2 pieces of nylon mesh as in the two previous experiments. Mesh squares were buried in cylindrical Lucite columns containing one of three sterile soils or fine white sand washed with distilled water. Soils used were a Galestown gravelly loamy sand (pH 5.8, 77.8% sand, 9.6% clay, 12.6% silt, 0.6% organic matter), a Hatboro loamy sand (pH 4.5, 59.4% sand, 11.8% clay, 28.8% silt, 3.2% organic matter), or a Rumford loamy sand (pH 5.4, 74.3% sand, 8.6% clay, 17.1% silt, 0.3% organic matter). The Hatboro loamy sand was selected for its relatively greater clay and organic matter content since these components could potentially bind the metabolite. Soils were collected 2–3 days before use, passed through a 3-mm pore-size screen to remove large particles, and autoclaved for 1 hr each day on two consecutive days. The 3-cm-diameter Lucite columns were cut into 2-cm-high sections that could be taped together to form taller columns. Nylon mesh (60-μm pore size) was attached to the bottom of the column with a rubber band. The bottom section (2 cm tall) was then filled with soil, and five mesh squares were placed on the soil surface of each column. An additional two 2-cm sections were then taped to the top and the resulting column was filled with soil.

The columns were placed on a wire basket over a sink. Twenty-five milliliters of culture filtrate, broth, or sterile distilled water was poured into the top of each column. Excess liquid was permitted to escape through the bottom of the column. After 24 hr of incubation at room temperature, the meshes were removed, rinsed in sterile distilled water, and plated onto a medium semiselective for *Verticillium*. The medium contained 25 ml of soil extract, 975 ml of water, 4 g of K_2HPO_4, 1.5 g of KH_2PO_4, 15 g of agar, 2 g of polygalacturonic acid, 0.05 g of streptomycin, 0.05 g of aureomycin, and 0.05 g of chloramphenicol. After 10 days, the number of nylon squares producing colonies of *V. dahliae* was counted. Columns were considered replicates and mesh squares as subsamples. Treatments were replicated five times, and the experiment was repeated once.

RESULTS

Radial growth. Radial growth of *V. dahliae* was similar on Czapek solution agar and on the medium amended with autoclaved culture filtrate or broth for all days sampled and for the 2-, 3-, and 4-day samplings on the medium amended with culture filtrate of the antagonist (Fig. 1). For clarity, only the growth curve for the 8-day culture filtrate is presented in Figure 1. The growth rate (slope) was similar ($P > 0.05$) on agar amended with 5-, 6-, 7-, or 8-day culture filtrate, and these were significantly slower than all other treatments ($P > 0.05$). In addition, cultures on the 5-, 6-, 7-, or 8-day culture-filtrate-amended agar initially started growing 4 days later (initial slope = 0) than cultures on other treatments. A white translucent halo, approximately 1 cm in width, was evident surrounding many of the cultures that did not begin growing immediately.

Germination of microsclerotia. Microsclerotia of *V. dahliae* within the population were equally sensitive to the metabolite. Either all microsclerotia germinated and formed colonies on a plate, or none germinated at the filtrate concentrations tested. There were no cases where only some of the microsclerotia germinated; however, occasionally all the microsclerotia germinated weakly, forming colonies containing only a few hyphae. Microsclerotia were not killed by 1- or 2-day culture filtrate at any dilution rate (Fig. 2). Maximum activity was detected in 5-day culture filtrate that killed all microsclerotia, even at dilutions up to 1:120. Although data are not shown, neither the autoclaved culture filtrate nor the broth significantly affected germination of microsclerotia.

Time required to kill microsclerotia. At the higher concentrations of culture filtrate, microsclerotia were killed relatively quickly (Table 1). For example, LD_{50} values for the 1:2 dilution were 1.5 and 1.7 hr for isolates 1 and 6, respectively. At the

TABLE 1. Effect of culture filtrate of *Talaromyces flavus*[a] on survival of microsclerotia of *Verticillium dahliae* in vitro

	Hours required to kill microsclerotia[b]				
	1:2[c]	1:10	1:50	1:100	1:200
LD_{50} (hr)					
Isolate 1	1.5	3.9	5.3	9.8	43.5
Isolate 6	1.7	2.6	7.0	8.6	19.1
LD_{95} (hr)					
Isolate 1	2.2	6.9	10.2	12.6	47.0
Isolate 6	2.7	3.8	9.4	12.2	43.5

[a] *T. flavus* was grown in high-carbon liquid medium incubated at 27 C with shaking. After 5 days, culture was filtered through 0.45-μm filter.
[b] Microsclerotia were embedded in 0.5-cm^2 pieces of nylon mesh and placed on amended Czapek solution agar. They were removed after various lengths of time, rinsed, plated onto Czapek solution agar, and assessed for germination.
[c] Dilution of culture filtrate in Czapek solution agar.

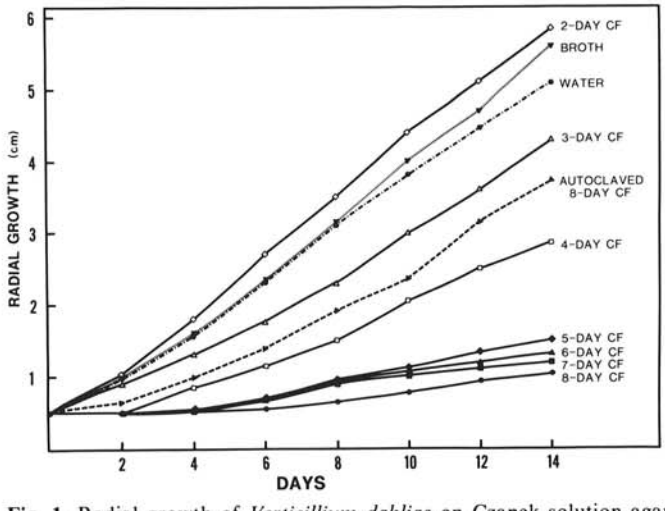

Fig. 1. Radial growth of *Verticillium dahliae* on Czapek solution agar amended with 1:2 dilution of culture filtrate (CF) of *Talaromyces flavus*. Antagonist was grown for 2–8 days to produce culture filtrate.

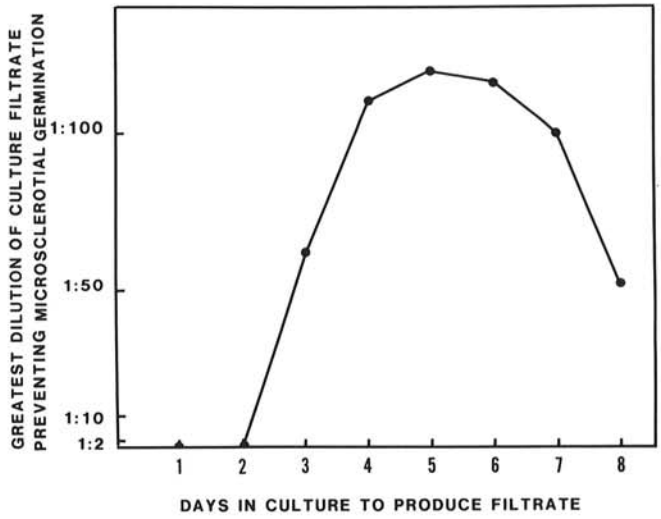

Fig. 2. Number of days of culture of *Talaromyces flavus* in liquid medium necessary to produce highest titer of compound preventing germination of microsclerotia of *Verticillium dahliae*.

1:200 dilution, the two isolates of *V. dahliae* differed in their responses to the metabolite, and LD_{50} values were 43.5 and 19.1 hr, respectively, for isolates 1 and 6. Microsclerotia were not killed on agar amended with either autoclaved culture filtrate or broth. Colonies were produced from all nylon mesh squares to which microsclerotia of *V. dahliae* were added after exposure to the Czapek solution agar amended with the metabolite. This indicated that the nylon squares did not adsorb the toxic metabolite.

Soil columns. Survival of microsclerotia was affected significantly ($P > 0.01$) by both medium (culture filtrate, medium, or water) and soil type (Table 2). In addition, there was a significant medium × soil interaction ($P > 0.01$). The main source of variance in the medium component resulted from reduced survival in the presence of the culture filtrate. With culture filtrate, 0.05 squares out of five tested (treatment mean from five squares from each of five replicates from each of four soils) showed evidence of growth compared with 4.05 for broth and 4.50 for the water (Table 2; Fig. 3). In addition. the broth reduced survival compared with the water (Table 2; Fig. 3). The Hatboro loamy sand reduced survival compared with the other two soils and sand.

DISCUSSION

It is evident that *T. flavus* produces a metabolite that kills microsclerotia and suppresses radial growth of *V. dahliae* in vitro. Microsclerotia were considered dead because they did not germinate after a rinse in sterile distilled water and incubation on fresh Czapek solution agar for 10 days. Maximum titer of the metabolite was obtained after incubation for 5 days with the culture conditions described here.

A preliminary report (3) indicated that this metabolite was able to kill microsclerotia in two freshly collected, nonsterile field soils. The high nutrient content of the culture filtrate and broth stimulated growth of many soil microorganisms, making it difficult to observe the relatively slow-growing *Verticillium* in field soils. Because of these difficulties, sterile soil was used for the current study. The metabolite may have the potential to function in soil since it was not inactivated by the physical components of any of the three soils tested.

Solvent extraction procedures, thin-layer chromatography, and molecular sieving through an open column or on high-performance liquid chromatography indicated that the crude culture filtrate contained only one compound exhibiting activity against *V. dahliae* (10). The metabolite produced by *T. flavus* isolate Tf-1 is thought to be different from any of the four antibiotics previously reported to be produced by *T. flavus*. The molecular weight of the metabolite, which is a polar but neutral compound, is approximately 400 daltons, which is smaller than other known antibiotics produced by *T. flavus* (10).

T. flavus was previously demonstrated to reduce populations of viable microsclerotia of *V. dahliae* in field soils (12). The production of a metabolite by *T. flavus* that kills microsclerotia may explain in part this reduction. Antibiosis has been demonstrated to be involved in other biological control systems, including the suppression of *Pythium ultimum*-induced damping-off by *Pseudomonas fluorescens* (8) and the control of *Agrobacterium tumefaciens* (E. F. Sm. & Towns.) Conn by *A. tumefaciens radiobacter* (9). Recently, direct evidence was provided for the production of the antibiotic geldanamycin in sterile soil by *Streptomyces hygroscopicus* var. *geldanus* (16). Mycoparasitism is also involved in the control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *T. flavus* (14) but apparently not in the control of *V. dahliae* by *T. flavus* (J. Beagle-Ristaino, *unpublished data*). Evidence presented here indicates that antibiosis may be involved in the mechanism of control of *V. dahliae* by *T. flavus*. A collection of isolates from 17 countries and 33 states in the United States (2) is currently being screened for metabolite production and biological control ability. Conventional or molecular genetic techniques may be used to improve the ability of *T. flavus* to produce this metabolite and to control *V. dahliae*. The metabolite alone, or an analog of it, may be an effective fungicide that could be used in agronomic production conditions.

TABLE 2. Analysis of variance table for the effect of culture filtrate of *Talaromyces flavus* and soil on survival of microsclerotia of *Verticillium dahliae*

ANOVA source (Contrast source)	ANOVA df (contract df)	ANOVA SS (contrast source)
Medium	2	240.33**[a]
(Filtrate vs. others)	(1)	(238.01)**
(Broth vs. water)	(1)	(2.02)*
Soil	3	29.73 **
(Hatboro vs. others)	(1)	(28.80)**
(Sand vs. Galestown and Rumford)	(1)	(0.10)
(Galestown vs. Rumford)	(1)	(0.83)
Medium × soil	6	15.57 **

[a]Asterisks ** and * denote $P > F$ at $\alpha = 0.05$ and 0.01, respectively.

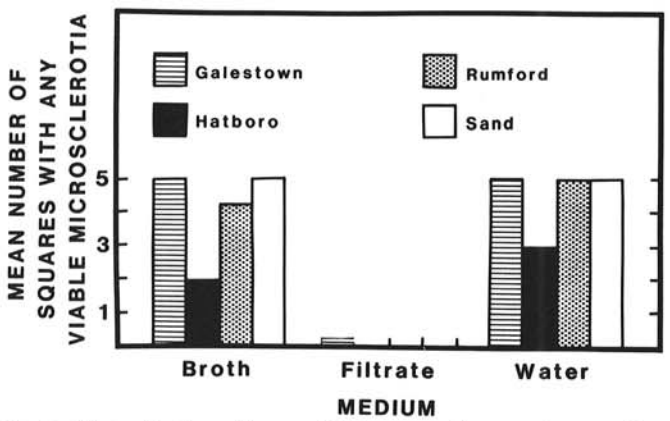

Fig. 3. Effect of culture filtrate of *Talaromyces flavus*, culture medium (broth), or water on survival of microsclerotia of *Verticillium dahliae* in three soils and in sand. Each replicate contained five 0.5-cm^2 pieces of nylon mesh, each embedded with about 15 microsclerotia. Meshes were buried in soils, treated with media, and recovered 24 hr later.

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