Identification of a Metabolite Produced by *Talaromyces flavus* as Glucose Oxidase and its Role in the Biocontrol of *Verticillium dahliae*

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

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

We thank Chester E. Holmlund and Malcolm J. Thompson for critical discussions and suggestions and Carolyn Sanford for her excellent technical assistance.

The research was supported in part by Grant US-815-84 from BARD—The United States-Israel Binational Agricultural Research and Development Fund.

Trade names and company names are included for the benefit of the reader and do not imply endorsement or preferential treatment of the product.

Accepted for publication 21 October 1987 (submitted for electronic processing).

ABSTRACT

Kim, K. K., Fravel, D. R., and Papavizas, G. C. 1988. Identification of a metabolite produced by *Talaromyces flavus* as glucose oxidase and its role in the biocontrol of *Verticillium dahliae*. Phytopathology 78:488-492.

A metabolite produced in liquid culture by *Talaromyces flavus* that mediated inhibition of radial growth and germination of microsclerotia of *Verticillium dahliae* was identified as glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4). A semipurified preparation of glucose oxidase per se from the culture filtrate or of a commercial preparation of glucose oxidase per se was not active against microsclerotia. However, both exhibited antibiotic activity against microsclerotia when glucose was added to the preparation. Thus, antibiotic activity present in crude culture filtrates may be due to the action of hydrogen peroxide released by the reaction catalyzed by glucose oxidase. The minimum in vitro concentration of hydrogen peroxide necessary to inhibit germination of microsclerotia was approximately 12 $\mu g\ ml^{-1}$. The molecular weight of the glucose oxidase in filtrates was estimated to be 173,000. The enzyme displayed a very high specificity for D-glucose as a substrate with apparent K_m of 100 mM of α and β anomeric mixture of glucose when the activity was monitored by a bioassay against microsclerotia of *V. dahliae*. The optimum activity of the enzyme occurred in a solid agar matrix of pH 5.0.

Additional key words: antibiosis, enzymes, *Penicillium dangeardii*, *P. vermiculatum*.

Talaromyces flavus (Klöcker) Stolk & Samson (anamorph: *Penicillium dangeardii* Pitt, usually reported as *P. vermiculatum* Dangeard) is a potential biocontrol agent of plant diseases caused by *Verticillium* and *Sclerotinia* spp. (3,6,19,20). Control of *Rhizoctonia solani* Kühn, *Verticillium albo-atrum* Reinke & Berth., and *Sclerotinia sclerotiorum* (Lib.) de Bary by *T. flavus* has been attributed to mycoparasitism, although parasitism of *Verticillium dahliae* Kleb. by *T. flavus* has not been observed in our laboratory (J. Beagle-Ristaino, *unpublished*). *T. flavus* is known to produce an antibiotic-like compound that kills microsclerotia of *V. dahliae* in vitro and in soil (7).

T. flavus is reported to produce four antibiotics: vermiculine (11), vermicillin (10), vermistatin (9), and talaron (21). Only talaron has been reported to be antifungal (21). The metabolite responsible for the inhibition of microsclerotia of *V. dahliae* is different from the previously reported antibiotics (15). In this paper we report characteristics and identity of the metabolite produced by *T. flavus* that mediates inhibition of radial growth and germination of microsclerotia of *V. dahliae*. A preliminay report has been published (16).

MATERIALS AND METHODS

Preparation and fractionation of culture filtrate. *T. flavus* isolate Tf-1 was maintained on potato-dextrose agar (PDA) at 28 C and cultured in a liquid medium containing 8% glucose (Dextrose, Sigma Chemical Co., St. Louis, MO) for 5 days (21). Culture filtrates were prepared as described previously (7) and fractionated according to the scheme presented in Figure 1. Cold acetone was added slowly with stirring to the filtrates in an ice bath at a ratio of 1.5 to 1 (v/v). The mixture was stirred for an additional 30 min and stored without stirring overnight at 4 C. All subsequent operations were done at 4 C unless otherwise indicated. The mixture was centrifuged at 4,000 *g* for 15 min, and the acetone in fraction A1 was removed by flash evaporation at 35 C. Ten-milliliter aliquots of the supernatant were loaded onto cartridge columns bearing C18 ligands (C18 Sep-Pak, Waters Chromatography Division, Millipore Corp., Milford, MA) at room temperature.

The acetone precipitate was extracted twice with water in a total volume equal to 1/10th of the original culture filtrate volume with stirring followed by centrifugation at 4,000 *g* for 10 min. The water extracts were pooled (fraction B1), and an aliquot was flash evaporated to remove the residual acetone for bioassay. The remaining pooled water extract was fractionated again with the addition of 1.5 volumes of cold acetone followed by centrifugation

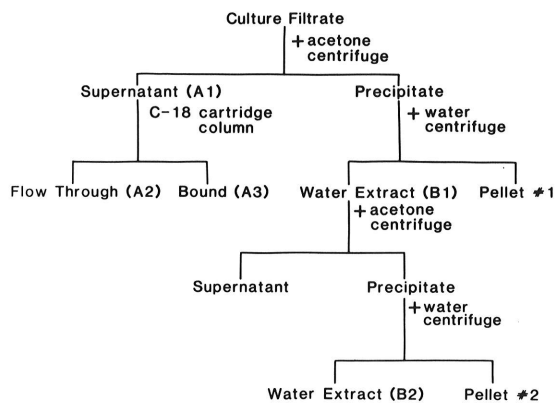

Fig. 1. Detailed scheme of fractionation of culture filtrate from *Talaromyces flavus*. The fungus was grown for 5 days in a liquid medium containing 8% glucose, and the culture filtrates were fractionated as shown in the figure.

as described above. An aliquot of the water extracts from the second acetone fractionation (fraction B2) was dialyzed in 3,500 MW cut-off Spectra Por #6 semipermeable membrane against 500 volumes of sterile water with stirring for 20 hr with one change of water at midpoint in the dialysis. The dialyzed acetone-precipitable fraction was further fractionated based on net charge of the molecule using cartridge columns bearing quaternary methylamine ligands (Accell QMA anion exchange Sep-Pak, Millipore Corp.). The dialyzed fraction was diluted 1:10 with 25 mM Tris-HCl buffer, pH 8.0, and loaded onto the prepared cartridges at a rate of 2 ml per cartridge at room temperature. The cartridges were washed four times with 3-ml aliquots of the buffer, and sequentially eluted with approximately 2 ml of the buffer containing 0.1, 0.2, 0.4, 0.5, and 1.0 M NaCl. All of the above fractions and controls were sterilized by filtration using 0.45 μm alpha Metricel membrane filters (Gelman Sciences, Inc., Ann Arbor, MI) and bioassayed.

Reconstitution of culture filtrate. Acetone-soluble fractions A1, A2, or A3 (Fig. 1) were combined with acetone-precipitable fractions B1 or B2 in ratios approximating the concentrations present in the original culture filtrate. Similarly, the culture medium was combined with the acetone-soluble fraction or the acetone-precipitable fraction. These combined fractions were then bioassayed against *V. dahliae*.

Effect of culture medium components on activity. Aqueous solutions of each components such as 8% glucose, 1% cornsteep liquor, 0.65% $NaNO_3$, 0.5% $CaCl_2$, and 0.01% $MnSO_4$ of the culture medium used for the production of the inhibitory metabolite from *T. flavus* were prepared individually and autoclaved. The individual components were combined with the acetone-precipitable fraction B2 at a ratio of 20:1 (v/v) and the combined fractions were bioassayed against microsclerotia of *V. dahliae*.

Effect of carbohydrates and their derivatives on activity. Glucose, mannose, levulose, fucose, ribose, glucosamine, gluconic acid (hemicalcium salt), α-methyl-D-glucopyranoside, glyceraldehyde, maltose, melibiose, cellobiose, sucrose, raffinose, starch, cellulose, and polygalacturonic acid were combined individually with the acetone-precipitable fraction B2 to determine their effect on bioactivity of the fraction B2 against microsclerotia of *V. dahliae*. Ten percent (w/v) solutions or suspensions of the carbohydrates and their derivatives were prepared in water and autoclaved, the cooled preparations were mixed with fraction B2 at 8% (w/v), and the mixtures were bioassayed within 2 hr of preparation. The solutions or suspensions without the B2 fraction and the B2 fraction alone were used as controls. Partially soluble or insoluble compounds such as hemicalcium gluconate, cellulose, and starch were added to the acetone precipitable fraction in the ratio of 80% (v/v) of saturated solutions to the fraction and bioassayed.

Interaction of glucose with the unknown metabolite. An experiment was performed to determine the time sequence of the interaction of glucose with the unknown metabolite in culture filtrates and fractions resulting in the inhibition of germination of microsclerotia of *V. dahliae*. Microsclerotia were embedded by vacuum in pieces of nylon mesh (Tetko, Elmsford, NY) squares, 0.5 cm on each side, with a pore size of 25 μm at a rate of 15–20 microsclerotia per 0.25 cm^2 (17). Water, crude culture filtrate, glucose, acetone-precipitable fraction B2, and sterile glucose solution plus fraction B2 were mixed individually in petri dishes (100 × 15 mm) with molten water agar (70–80 C). The final concentrations of the inhibitor ranged from 125 to 250 units with or without 4% glucose in 1.5% agar. Twenty nylon mesh squares with embedded microsclerotia were placed on each of four replicate plates containing water agar with treatments. At 4 and 24 hr after exposure, five of the nylon meshes were rinsed three times in sterile distilled water and transferred to Czapek solution agar to determine the viability of the microsclerotia. In addition, after 4 and 24 hr of exposure, five of the mesh squares from each of glucose or fraction B2 treatments were transferred to Czapek solution agar plates containing the fraction B2 or glucose.

Effect of metal compounds on activity. Acetone-precipitable fraction B2 was dialyzed against water as described earlier. Stock solutions of $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $FeSO_4$, $CoCl_2$, $AgNO_3$, and $CuSO_4$ were each prepared at concentrations of 100 mM in water and filter-sterilized. A stock solution of EDTA was prepared at a concentration of 100 mM in water, and the pH was adjusted to 6.0 with 2 N NaOH. The metal compound solutions were added to the dialyzed fraction B2 to give a final concentration of 1 mM. The EDTA solution was added at a concentration of 5 mM. All of the samples were bioassayed in the presence or absence of 8% glucose. The fractions without glucose or fraction B2 alone served as negative controls. The dialyzed fraction B2, bioassayed with glucose, served as a positive control.

High performance liquid chromatography. Aliquots of 20–50 μl of acetone-precipitable fraction B2 containing approximately 10 μg of protein were subjected to high performance gel permeation chromatography on Spherogel TSK 2000 SW or TSK 3000 SW columns (7.5 × 300 mm, Toyo Soda, Tokyo) at room temperature. Fifty millimolar potassium phosphate buffer (pH 7.0) or 50 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl was used as the mobile phase at a flow rate of 0.3 ml/min. The absorbance of the effluent was monitored at 280 nm. The elution position of the inhibitory metabolite was determined by the bioassay, and the elution position of glucose oxidase (β-D-glucose : oxygen oxidoreductase, EC 1.1.3.4) was determined by enzyme assay of the individual fractions from the gel-permeation column. The relative molar mass of the metabolite or the enzyme was determined from a standard calibration curve of $\log_{10}$ molecular weight versus mobility on TSK 3000 SW column of the following standard proteins: thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1,350) (Bio-Rad Laboratories, Richmond, CA).

Effect of commercial glucose oxidase on microsclerotial germination. A stock solution of glucose oxidase from *Aspergillus niger* (Sigma) was prepared at a concentration of 1 mg/ml in 50 mM sodium acetate buffer (pH 5.0). The enzyme solution was filter-sterilized and bioassayed with or without 8% glucose against microsclerotia of *V. dahliae*.

Effects of hydrogen peroxide and its scavangers on activity. Hydrogen peroxide (H_2O_2) was diluted with sterile water to 6% and immediately bioassayed against microsclerotia of *V. dahliae*. Stock solutions of horseradish peroxidase (Donor : H_2O_2 oxidoreductase, EC 1.11.1.7) and bovine liver catalase (H_2O_2: H_2O_2 oxidorreductase, EC 1.11.1.6) (Sigma) were prepared at a concentration of 1 mg/ml in water and in 50 mM potassium phosphate buffer (pH 7.0), respectively. The enzyme solutions were appropriately diluted in the water or the buffer and filter-sterilized. Bioassays of peroxidase and catalase were carried out by mixing the enzyme solutions in 24-well tissue culture plates with molten Czapek solution agar (70–80 C) in the presence or absence of 250 units of the acetone-precipitable fraction B2 with 8% glucose.

Bioassay. The cultures of *V. dahliae* were maintained on Czapek solution agar at 22 C. Aqueous suspension of microsclerotia of *V. dahliae* were prepared as described previously (7).

Culture filtrates of *T. flavus* and fractions were bioassayed against microsclerotia of *V. dahliae* in 24-well tissue culture plates. A series of twofold dilutions of filtrates and fractions were mixed with molten Czapek solution agar in a total assay volume of 0.5 ml per well. After the agar solidified, an aqueous suspension of microsclerotia of *V. dahliae* was seeded onto the surface of the agar at a rate of 5–10 microsclerotia per square centimeter of the agar surface. The highest dilution of the sample that completely inhibited the growth of *V. dahliae* after 6 or 7 days was considered the dilution end point, and activity was computed from this dilution. One unit of antifungal activity is defined as the amount of metabolite in the dilution end point that completely inhibits the germination of microsclerotia of *V. dahliae*.

Glucose oxidase assay of filtrates and fractions. Glucose oxidase activity was determined by a spectrophotometric assay using a coupled peroxide-*o*-dianisidine system (27). One unit of glucose oxidase activity is defined as the amount of enzyme needed to liberate one micromole of H_2O_2 per minute at 25 C.

Protein determination. Protein concentration of the filtrate and

fractions was estimated by the method of Bradford (4) with bovine serum albumin as a standard.

RESULTS

Acetone fractionation and reconstitution of culture filtrates. When culture filtrate from *T. flavus* was fractionated with acetone as in Figure 1, less than 3% of the total bioactivity in the culture filtrate was obtained by acetone precipitation (Table 1). Essentially all of the activity was restored by combining the acetone-soluble fraction (supernatant A1) with the water soluble component of the acetone-precipitable fraction (fraction B1). Combining the acetone-precipitable fraction B1 with the culture medium also resulted in restoration of greater than 90% of the original activity.

Identification of the active component of the acetone-soluble fraction as glucose. The component of the acetone-soluble fraction responsible for restoration of the inhibitory activity was polar, since it did not bind to the C-18 Sep-Pak (Table 1, Fig. 1). This polar component was also present in the culture medium, since addition of the culture medium to the acetone-precipitable fraction B1 resulted in complete restoration of the original activity. When all of the components of the culture medium, for example, glucose, cornsteep liquor, and salts were combined individually with the acetone-precipitable fraction B2 and bioassayed, only glucose restored the inhibitory activity. Among the carbohydrates tested, only a few monosaccharides such as mannose and fucose and a few of disaccharides including sucrose restored small percentages (0.4–3%) of activity to the acetone-precipitable fraction B2.

Characteristics of acetone-precipitable fraction and its interaction with glucose. The bioactivity was restored only when glucose and the acetone precipitable fraction were present simultaneously. Addition of glucose 4 or 24 hr after treatment of microsclerotia of *V. dahliae* with acetone-precipitable fraction did not result in restoration of the inhibitory activity. Therefore, bioassays of inhibitory activity against microsclerotia of *V. dahliae* in various fractions such as the acetone-precipitable fraction and column effluents were performed with concentrations of the inhibitor that gave a linear inhibitory response with respect to 4–10% glucose. When a fixed amount of the acetone-precipitable fraction B2 (125 or 250 units) was bioassayed in the presence of varying concentrations of a mixture of α and β anomers of D-glucose (0.1–9.5%), a Michaelis-Menton type of saturation curve was observed. The apparent K_m for glucose was approximately 100 mM of the mixture of α and β anomers as calculated from Lineweaver-Burk plots of reciprocal values of bioactivity versus reciprocal values of the varying glucose concentrations.

TABLE 1. Effects of acetone fractionation of the culture filtrate of *Talaromyces flavus* and subsequent reconstitution on activity against microsclerotia of *Verticillium dahliae*

Fraction[a] and combinations	Total activity against microsclerotia (units)[b]	Recovery of activity (%)
Culture medium	0	
Culture filtrate	4.0×10^4	100
Water extract (B1)	1.0×10^2	0.3
Water extract (B2)	0	0
Supernatant (A1)	8.0×10^2	2
C-18 Sep-Pak flowthrough (A2)	8.0×10^2	2
C-18 Sep-Pak bound (A3)	0	0
A1 + B1 (7:1)	3.9×10^4	96
A1 + B2 (20:1)	2.6×10^4	65
A2 + B1 (7:1)	3.9×10^4	96
Culture medium + A1 (1:1)	1.6×10^3	4
Culture medium + B2 (7:1)	3.9×10^4	96

[a] Fractions were obtained from acetone precipitation of culture filtrate as outlined in Figure 1. The fractions were combined in ratios to approximate their concentrations in the original culture filtrate.

[b] One unit of activity is the amount of inhibitor capable of complete inhibition of germination of microsclerotia under the conditions of bioassay described in Materials and Methods.

Restoration of activity to the acetone-precipitable fraction by glucose was optimum at pH 5.0 (Fig. 2).

The interaction of the acetone-precipitable fraction and glucose was not significantly affected by addition of $CaCl_2$, $MgCl_2$, $ZnCl_2$, or $CoCl_2$, but was affected by the addition of $FeSO_4$ or $CuSO_4$. The addition of $FeSO_4$ increased the inhibition activity twofold, while $CuSO_4$ suppressed the inhibition to half of that in the controls. The effects of $AgNO_3$ and EDTA on the activity of acetone-precipitable fractions could not be assessed, since they were inhibitory to *V. dahliae* in the absence of the fraction.

The inhibitory metabolite in the acetone-precipitable fraction has a net negative charge since all of the activity recovered from the Accell QMA Sep-Pak was bound to the anion exchanger. The bulk of the recovered activity was eluted with 0.2 M NaCl in 25 mM Tris buffer at pH 8.0.

Molecular weight of the inhibitory metabolite. The molecular weight of the metabolite in the acetone-precipitable fraction B2 was estimated to be approximately 173,000. Under the conditions of elution, thyroglobulin (670,000) with other larger contaminating proteins eluted in the void and vitamin B-12 (1,350) eluted with the salts.

Elution profile of activity in the acetone-precipitable fraction. The elution of the metabolite activity and glucose oxidase activity were compared on HPLC. The elution profile of the activity of the metabolite in the acetone-precipitable fraction was coincident with the elution profile of the glucose oxidase activity (Fig. 3).

Effect of commercial glucose oxidase, hydrogen peroxide, and gluconic acid on germination of microsclerotia. Because glucose was specifically and simultaneously required for bioactivity of the acetone-precipitable fraction, the metabolite in the fraction suggests an enzyme whose substrate is glucose. Glucose oxidase is one such enzyme. When a preparation of commercial glucose oxidase was combined with glucose in the bioassay system, activity was observed even with 0.0014 units per milliliter of glucose

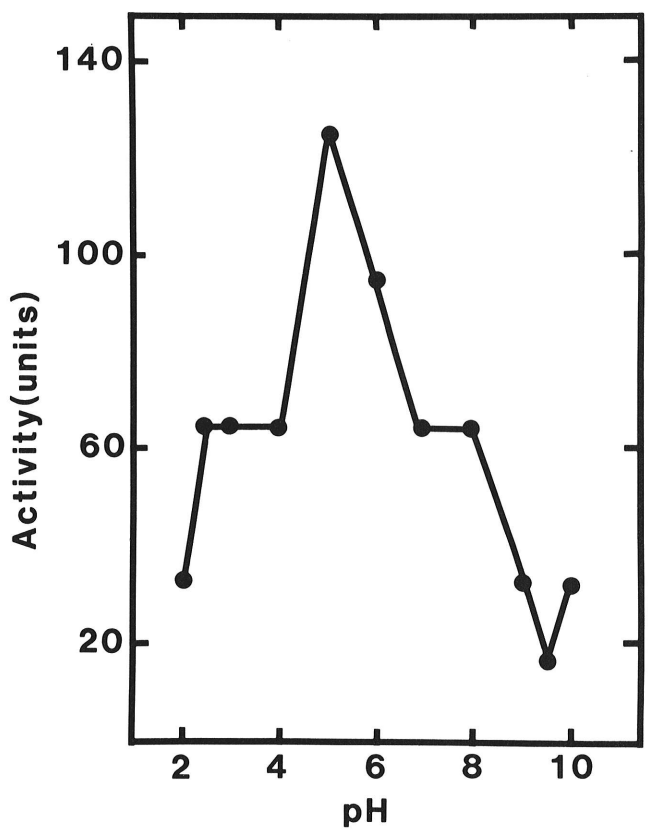

Fig. 2. Effect of pH of acetone-precipitable fraction B2 from culture filtrate of *Talaromyces flavus* on the antibiotic activity against germination of microsclerotia of *Verticillium dahliae*. Aliquots of the fraction B2 were adjusted to various pH values with 2 N HCl or 2 N NaOH and bioassayed against microsclerotia.

oxidase (Table 2). The activity increased with an increase in the amount of enzyme from 0.0014 to 14 units per milliliter in the bioassay media. No appreciable inhibitory activity was observed in the absence of glucose.

Because the acetone-precipitable fraction or the commercial preparation of glucose oxidase was not inhibitory to microsclerotia in the absence of glucose, the products of the reaction catalyzed by glucose oxidase such as gluconic acid and H_2O_2 were assayed for activity against microsclerotia. Gluconic acid did not affect germination of microsclerotia. Hydrogen peroxide was highly inhibitory (Table 3). In fact, a 6% H_2O_2 solution had approximately 5,000 units of activity against microsclerotia. Under these conditions, the minimum inhibitory concentration of H_2O_2 was 12 μg ml^{-1}. In addition, bioactivity of the metabolite in the acetone-precipitable fraction decreased in the presence of catalase or peroxidase, which are known peroxide scavangers (Table 3) (18). For instance, inclusion of 0.0003 and 0.00031 units of peroxidase and catalase caused reduction of the bioactivity from 250 units/ml to 32 and 64 units/ml, respectively.

DISCUSSION

Our data strongly indicate that the metabolite produced by *T. flavus* in liquid culture, which mediates inhibition of radial growth and germination of microsclerotia of *V. dahliae*, is glucose oxidase. Extracellular or intracellular glucose oxidases are known to be produced by a number of fungi. However, Nakamatsu et al (22) have reported that the production of extracellular glucose oxidase may be specific for *Penicillium* spp.

In the present study, the semipurified glucose oxidase from the culture filtrate of *T. flavus* was not inhibitory in the absence of glucose. However, the inclusion of glucose resulted in inhibition of germination of microsclerotia of *V. dahliae* equivalent to that obtained with crude culture filtrates. Culture filtrates apparently contain sufficient residual glucose needed for the activity of the enzyme. Similarly, a commercial preparation of glucose oxidase was highly inhibitory only in the presence of glucose. The slight inhibitory effect of the commercial preparation in the absence of glucose may be explained partly by the presence of invertase in the preparation (Sigma), which may have hydrolyzed sucrose present in Czapek solution agar to glucose and fructose.

Our results also indicate that the observed antibiotic activity of glucose oxidase was caused by the action of H_2O_2, which is one of the two products of the reaction catalyzed by the enzyme. The second product, gluconic acid, did not affect germination of microsclerotia of *V. dahliae*. Additionally, the peroxide scavangers peroxidase and catalase included in the bioassay resulted in a scavenger-concentration-dependent reduction in inhibitory activity. Similarly, White et al (26) attributed the antibacterial action of inhibine from honey to H_2O_2 which originated from the honey glucose oxidase system. Hydrogen peroxide produced by inhibine at levels of 0.0017% and higher were inhibitory to *Staphylococcus aureus*. The minimum concentrations of H_2O_2 necessary for inhibition of microsclerotia of *V. dahliae* was approximately 12 μg ml^{-1} (0.0012%), which was comparable to those indicated by White et al (26).

Glucose oxidases from various fungal sources have high specificities for β-D-glucose, and molecular weights range from 150,000 (23) to 192,000 (24). Additionally, these glucose oxidases may have residual activity with other monosaccharides including 2-deoxy-D-glucose (5). Consistent with the other fungal glucose oxidases, the enzyme from *T. flavus* has a specific substrate requirement for D-glucose. However, the specific anomer of D-glucose required is not known at the present time, since the results were obtained with a mixture of α and β anomers of D-glucose. The apparent Michaelis-Menton constant, K_m, for D-glucose for the *T. flavus* enzyme was 100 mM of the mixture of α and β anomers. This value of K_m is within the high range of the values for K_m

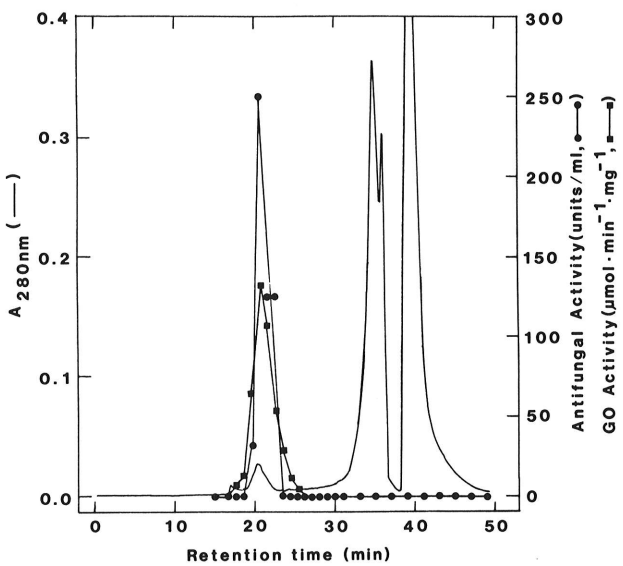

Fig. 3. Elution profiles of antifungal and glucose oxidase activities from *Talaromyces flavus* culture filtrates on a Spherogel TSK 2000-SW column (7.5 × 300 mm). An aliquot of 50 μl of the acetone-precipitable fraction B2 was injected. A mobile phase consisted of 50 mM potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl. The flow rate was 0.3 ml/min and 1-min fractions were collected. Selected fractions were analyzed for protein, antifungal, and glucose oxidase activities.

TABLE 2. Effect of a commercial preparation of glucose oxidase on the inhibition of germination of microsclerotia of *Verticillium dahliae*[a]

Glucose oxidase (units/ml)[b]	Activity against microsclerotia (units/ml)[c]	
	without glucose	with glucose
0.00014	0	0
0.0014	0	2
0.014	2	4
0.14	2	16
1.4	8	125
14.0	≥32	250

[a] Bioassay was performed by incorporation of the glucose oxidase in Czapek solution agar with or without 8% glucose.
[b] One unit of glucose oxidase activity is that amount of the enzyme capable of liberating 1 micromole of H_2O_2 per minute at 25 C.
[c] One unit of activity is the amount of inhibitor capable of complete inhibition of germination of microsclerotia under the conditions described in Materials and Methods.

TABLE 3. Effect of gluconic acid, peroxide, peroxidase, and catalase on the germination of microsclerotia of *Verticillium dahliae* with or without the acetone-precipitable fraction B2

Treatment	Amount added	Activity against microsclerotia (units/ml)[a]	
		without fraction B2	with fraction B2
None		0	250
Gluconic acid (hemicalcium salt)	80% saturation	0	0
Hydrogen peroxide	6%	5,000	ND[b]
Peroxidase	0.0003–150 units[c]	0	32
Catalase	0.00031–3.1 units	0	≥64
	31 units	0	32
	310 units	0	16

[a] Bioassays of all the treated samples contained 8% glucose in the Czapek solution agar with exceptions for gluconic acid and hydrogen peroxide treatments. One unit of activity is the amount of inhibitor capable of complete inhibition of germination of microsclerotia under the conditions of bioassay.
[b] Not determined.
[c] One unit is the amount capable of decomposing 1 micromole of H_2O_2 per minute at 25 C.

determined with other fungal sources, which ranged from 9.6 mM for *P. notatum* (25) to 110 mM for *A. niger* (12). The molecular weight of glucose oxidase from *T. flavus* was estimated to be approximately 173,000, and the activity of the enzyme was optimum at pH 5.0.

The use of glucose oxidase has been limited to preservation of color and flavor or stabilization of the microbial flora of food-related products because of the antioxidant and glucose depleting nature of the enzyme (2,8,24) and to the use of the enzyme in sugar analysis. This is the first time that glucose oxidase has been implicated as an intermediary in the biocontrol of a plant pathogen by reducing growth and propagule germination.

Even though much new information has been provided by the research described in this paper about glucose oxidase produced by *T. flavus*, no definite statements can be made about production of the enzyme by the antagonist in nature. If glucose oxidase is involved in the killing of microsclerotia of *V. dahliae* in soil, a source of glucose must be available to the biocontrol agent. Carbohydrates such as glucose are known to be exuded by plant roots (13). Polysaccharides such as glucan are also known to be present in sclerotia of species of *Sclerotinia* (1). In vitro culture of *V. dahliae* and *T. flavus* showed no evidence of mycoparasitism of the pathogen by the antagonist (J. Beagle-Ristaino, *unpublished*). In contrast, Fahima and Henis (T. Fahima and Y. Henis, *unpublished*) observed invasion of microsclerotia by the hyphae of *T. flavus* only in the presence of dead eggplant roots or other food sources. In light of these observations, it is perhaps reasonable to assume that production of glucose oxidase by *T. flavus*, with subsequent production of H_2O_2 in the presence of nutrients may precede the invasion of microsclerotia by the *T. flavus* hyphae. Attenuation of microsclerotia by such a process probably predisposes them to invasion by the antagonist. In a parallel situation, Henis and Papavizas (14) found that a brief exposure of sclerotia of *Sclerotium rolfsii* Sacc. to small amounts of a fumigant made them very susceptible to invasion by *Trichoderma harzianum* Rifai. The advent of new microtechniques in soil and rhizosphere microecology will provide more information on the interactions between glucose, *T. flavus*, and the pathogen at the rhizosphere and microsclerotia level.

LITERATURE CITED

1. Adams, P. B., and Ayers, W. A. 1983. Histological and physical aspects of infection of sclerotia of two *Sclerotinia* species by two mycoparasites. Phytopathology 73:1072-1076.
2. Barton, R. R., Rennert, S. S., and Underkofler, L. A. 1957. Glucose oxidase in the protection of foods and beverages. Food Technol. 11:683-686.
3. Boosalis, M. G. 1956. Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology 46:473-478.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
5. Dixon, M., and Webb, E. C. 1979. Enzymes. 3rd ed. Academic Press, New York. 243 pp.
6. Dutta, B. K. 1981. Studies on some fungi isolated from the rhizosphere of tomato plants and the consequent prospect for the control of Verticillium wilt. Plant Soil 63:209-216.
7. 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.
8. Frels, J. M., Samuelson, K. J., Froning, G. W., and Rupnow, J. H. 1984. Evaluation of glucose oxidase-catalase treatment to improve the microbiological quality of poultry meat. Poult. Sci. 63:841-843.
9. Fuska, J., Fuskova, A., and Nemec, P. 1979. Vermistatin, an antibiotic with cytotoxic effects produced from *Penicillium vermiculatum*. Biologia (Bratislava) 34:735-740.
10. Fuska, J., Nemec, P., and Fuskova, A. 1979. Vermicillin, a new metabolite from *Penicillium vermiculatum* inhibiting tumor cells in vitro. J. Antibiot. 32:667-669.
11. Fuska, J., Nemec, P., and Kuhr, I. 1972. Vermiculine, a new antiprotozoal antibiotic from *Penicillium vermiculatum*. J. Antibiot. 25:208-211.
12. Gibson, Q. H., Swoboda, B. E. P., and Massey, V. 1964. Kinetics and mechanism of action of glucose oxidase. J. Biol. Chem. 239:3927-3934.
13. Hale, M. G., Moore, L. D., and Griffin, G. J. 1978. Root exudates and exudation. Pages 163-203 in: Interactions between Non-Pathogenic Soil Microorganisms and Plants. Y. R. Dommergues and S. V. Krupa, eds. Elsevier Scientific Publishing Co., New York.
14. Henis, Y., and Papavizas, G. C. 1983. Factors affecting germinability and susceptibility to attack of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* in field soil. Phytopathology 73:1469-1474.
15. Kim, K. K., Fravel, D. R., and Papavizas, G. C. 1986. A novel metabolite from the culture filtrate of *Talaromyces flavus*. (Abstr.) Fed. Proc. 45:1796.
16. Kim, K. K., Fravel, D. R., and Papavizas, G. C. 1987. Identification and characterization of a metabolite produced by *Talaromyces flavus* which mediates biocontrol of *Verticillium dahliae*. (Abstr.) Phytopathology 77:172.
17. Lumsden, R. D. 1981. A nylon fabric technique for studying the ecology of *Pythium aphanidermatum* and other fungi in soil. Phytopathology 71:282-285.
18. Maehly, A. C., and Chance, B. 1954. The assay of catalases and peroxidases. Pages 357-424 in: Methods of Biochemical Analysis Vol. 1. E. Glick, ed. Interscience Publishers, New York.
19. Marois, J. J., Johnston, S. A., Dunn, M. T., and Papavizas, G. C. 1982. Biological control of Verticillium wilt of eggplant in the field. Plant Dis. 66:1166-1168.
20. McLaren, D. L., Huang, H. C., and Rimmer, S. R. 1986. Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus*. Can. J. Plant Pathol. 8:43-48.
21. Mizuno, K., Yagi, A., Takada, M., Matsuura, K., Yamaguchi, K., and Asano, K. 1974. A new antibiotic, Talaron. J. Antibiot. 27:560-563.
22. Nakamatsu, T., Akamatsu, T., Miyajima, R., and Shiio, I. 1975. Microbial production of glucose oxidase. Agric. Biol. Chem. 39:1803-1811.
23. Pazur, J. H., Kleppe, K., and Cepure, A. 1965. Glycoprotein structure for glucose oxidase from *Aspergillus niger*. Arch. Biochem. Biophys. 111:351-357.
24. Richter, G. 1983. Glucose oxidase. Pages 428-436 in: Industrial Enzymology. T. Godfrey and J. Reichelt, eds. Nature Press, New York.
25. Swoboda, B. E. P., and Massey, V. 1965. Purification and properties of the glucose oxidase from *Aspergillus niger*. J. Biol. Chem. 240:2209-2215.
26. White, J. W., Jr., Subers, M. H., and Schepartz, A. I. 1963. The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. Biochim. Biophys. Acta 73:57-70.
27. Worthington Biochemicals Corp. 1977. Worthington Enzyme Manual. Freehold, NJ. 346 pp.