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FINAL REPORT

PROJECT NO. US-815-84

Genetic Improvement of *Talaromyces Flavus*
and its Mechanism of Action on *Verticillium*

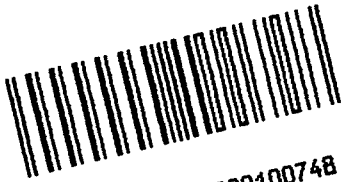
G. C. Papavizas, T. Katan, Y. Henis

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Genetic Improvement of Talaromyces flavus and its
Mechanism of Action on Verticillium

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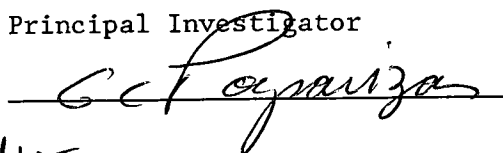
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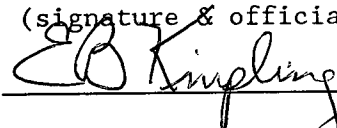
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Principal Investigator



Institution's Authorized Official
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c. Abstract

Talaromyces flavus (anamorph: Penicillium dangeardii), the biocontrol fungus that suppresses the wilt pathogen Verticillium dahliae, produced a metabolite that retarded radial growth and killed microsclerotia of the pathogen (LD₉₅) in less than 3 hr in vitro. The metabolite also killed microsclerotia in sterile soils and in sand, indicating that the physical components of the soil did not inactivate the metabolite. The metabolite was identified as glucose oxidase (β -D-glucose: oxygen oxidoreductase, E.C. 1.1.3.4). A semipurified preparation of glucose oxidase per se from the culture filtrates of T. flavus or of a commercial preparation of glucose oxidase per se was not active against microsclerotia. Both preparations exhibited antibiotic activity against microsclerotia when glucose was added to the preparation. The 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 $\mu\text{g ml}^{-1}$. 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. The enzyme was purified by a combination of acetone precipitation and HPLC. The relative molecular weight of the native enzyme was 164,000 and the enzyme appears to be a dimer of identical Mr subunits. The optimum pH for the enzyme reaction was 5.0, but the enzyme was stable in buffer from pH 3 to 7. The purified enzyme is composed of relatively high amounts of Ala, Asp, Glu, Gly, and Leu, with relatively low amounts of Cys. The antibiotic taloron produced by T. flavus, isolated by Japanese scientists but not identified biochemically, was shown to be glucose oxidase.

Transmission electron micrographs of V. dahliae microsclerotia parasitized by T. flavus revealed a unique cell-to cell invasion by the hyphae of T. flavus. Lysis of host cells took place only at the contact sites between the host cells and T. flavus hyphal tips. Microsclerotia of the pathogen on the surface of eggplant roots were colonized by T. flavus which developed the anamorphic stage on the microsclerotia but on root tissue.

In the genetic improvement program, several matings between strains of T. flavus yielded infertile cleistothecia. From 11 fertile crosses, 112 plates were found to contain ascospores of both parental types. About half of these plates were analyzed further. All the cleistothecia examined resulted from selfing as it became evident by the uniform phenotypes of their ascospores.

d. Objectives of the Original Research Proposal

The objectives of the original research proposal were as follows:

- a. Investigate the mechanism by which Talaromyces flavus reduces the ability of Verticillium dahliae to cause disease. Research included the pathogen-antagonist relationship between T. flavus and Verticillium at the physiological and morphological level of organization.
- b. Produce by mutagenesis and somatic hybridization and recombination, using conventional genetic approaches and intraspecific protoplast fusions, new biotypes of T. flavus tolerant to selected fungicides, possessing enhanced biocontrol capabilities.

No deviations were made from the original objectives.

e. Body of the Report

INTRODUCTION

Talaromyces flavus (klöcker) Stolk & Samson (anamorph: Pennicillium dangeardii Pitt, usually reported as P. vermiculatum Dangeard) is an ascomycete widely distributed in soils of temperate and subtropical areas (Fravel and Adams, 1986) that is antagonistic against several soilborne plant pathogens (Boosalis, 1956; Dutta, 1981; McLaren et al., 1982). The fungus suppressed Verticillium wilt and increased yield of eggplant in agricultural production systems (Marois et al., 1982). When applied either as a dust to potato seed pieces or as alginate-Pyrax pellets in furrow, T. flavus suppressed potato wilt (V. dahliae kleb.) in field tests (Davis et al., 1986; Fravel et al., 1986). Also, T. flavus suppressed damping-off of cotton induced by Pythium ultimum (G. C. Papavizas, unpublished). The antagonist survived well in soil and proliferated when added to soil as alginate-bran pellets but not when added as alginate-Pyrax pellets (Papavizas et al., 1987).

If T. flavus is to be used for biocontrol, its mechanism of action against Verticillium must be elucidated and its strains be improved by genetic manipulation. The objectives of this research were to (a) investigate the mechanism by which T. flavus reduces the ability of V. dahliae to cause disease and (b) produce by mutagenesis and somatic hybridization and recombination, using conventional genetic approaches and intraspecific protoplast fusions, new biotypes of T. flavus tolerant to selected fungicides, possessing enhanced biocontrol capabilities.

MATERIALS AND METHODS

Identification of a metabolite produced by *T. flavus* - *T. flavus* isolate Tf-1 was maintained on potato-dextrose agar at 28 C and cultured in a liquid medium containing 8% glucose for 5 days described by Mizuno et al.(1974). Culture filtrates were prepared by a method described by Fravel et al.(1987) and fractionated according to the scheme in Figure 1 (Kim et al.,1988). Cold acetone was added to the filtrates in an ice bath at a ratio of 1.5 to 1 (v/v). The mixture was stirred and centrifuged at 4,000 g for 15 min., and the acetone in fraction A1 was removed by flash evaporation at 35 C. 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. 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.

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 bioassayed against *V. dahliae*.

Bioassay - Culture filtrates of *T. flavus* were bioassayed against microscolorotia of *T. flavus* in 24-well tissue culture plates (Kim et al.,1988). 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. 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 defined as the amount of metabolite in the dilution end point that completely inhibited the germination of microsclerotia.

The effect of metabolites from T. flavus on viability of microsclerotia of V. albo-atrum was also assayed in sterile soil (Fravel et al., 1987). Microsclerotia were embedded in 0.5-cm² pieces of nylon mesh and these were buried in cylindrical Lucite columns containing one of three sterile soils or fine white sand washed with distilled water. The 3-cm-diam Lucite columns were cut into 2-cm-high sections that could be taped together to form taller columns. Nylon mesh (60- μ m 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 2-cm sections were then taped to the top and the resulting column was filled with soil. Twenty-five ml of culture filtrate, broth or sterile distilled water was poured into the top of each column. After 24 hr of incubation, the meshes were removed, rinsed in sterile distilled water, and placed onto a medium semiselective for Verticillium. After 10 days, the number of nylon squares producing colonies of V. dahliae was counted. Columns were considered replicates and mesh squares as subsamples.

Biochemical determinations - Several biochemical determinations, including interaction of glucose with the unknown metabolite, effect of carbohydrates and their derivatives on activity, effect of hydrogen peroxide and its scavengers on activity, protein determination, and high performance liquid chromatography (HPLC) were performed according to established procedures (Kim et al., 1988, 1989). For HPLC, aliquots of 20-50 μ l of acetone-precipitable fraction B2

containing about 10 μ g of protein were subjected to high performance gel permeation chromatography. Fifty millimolar potassium phosphate buffer (pH 7.0) 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 relative molar mass of the metabolite was determined from a standard calibration curve of \log_{10} molecular weight vs. mobility on TSK 3000 SW column of several standard proteins.

Purification and properties of glucose oxidase - Water extracts of the acetone precipitates of *T. flavus* metabolite were adjusted to 50 mM sodium acetate (pH 5.0) and chromatographed on a preparative HPLC gel filtration column (Kim et al., 1989). Protein was eluted from the column with sodium acetate containing 0.2 M NaCl at a flow rate of 4 ml/min. The fractions containing glucose oxidase activity were pooled, dialyzed, and concentrated by ultra-filtration. The retentate was adjusted to 20 mM Tris (pH 8.0) and chromatographed on an HPLC anion exchange column. The enzyme was eluted from the column with a gradient of 0.050 to 0.8 M NaCl at a flow rate of 0.8 ml/min. The native enzyme was analyzed by polyacrylamide gel electrophoresis (PAGE) in 7.5% gels as described by Davis (1964). Amino acid composition of the purified glucose oxidase produced by *T. flavus* was determined by methods developed by Schaeffer and Sharpe (1981) with a Beckman 118 BL amino acid analyzer.

Electron microscopy of *V. dahliae* sclerotia parasitized by *T. flavus* - Microsclerotia of *V. dahliae* embedded in nylon mesh were incubated for 2 weeks in soil amended with bran preparations of *T. flavus*. The microsclerotia were recovered, washed, and prepared for electron microscopy work. Specimens were mounted on copper stubs, coated with gold-palladium, and examined and photographed with a scanning electron microscope. Also, microsclerotia colonized by *T. flavus* in vitro were fixed, sectioned, and examined with a transmission electron microscope.

Microscopic observations of roots invaded by *T. flavus* - The interactions among eggplant roots, *V. dahliae* and *T. flavus* was studied in sterile vermiculite in 30-mm diam test tubes incubated at 25 C under light (3000 lux). Seeds of eggplant were placed on the vermiculite and inoculated with an ascospore suspension of *T. flavus*. After 1 week, the tubes were inoculated with *V. dahliae* microsclerotia. After 2 weeks, the roots were washed thoroughly, stained with trypan blue, and observed under a light microscope.

Genetic studies - In an attempt to find strain combinations or conditions that would result in hybrid cleistothecia, a series of experiments were performed with 19 pairs of strains of *T. flavus*. Conidia of each strain were harvested from apple-juice agar cultures, suspended in water and diluted to 10^4 , 10^5 , and 10^6 /ml. The conidial dilutions of two isolates to be mated were mixed in all nine possible combinations of spore density and ratios, and each mixture was spread over four PDA plates (36 plates per cross). The plates were incubated in the dark at 25 C or 28 C for several weeks until mature cleistothecia developed. Each pair included one wild-type (Ben-S) isolate and one benomyl-resistant (Ben-R) mutant.

From each plate 15-25 cleistothecia were harvested, pooled and crushed to release the ascospores. After heat activation (30 min at 60 C), aliquots of the ascospore suspension were plated on PDA to determine germinability, and on PDA plus benomyl to determine benomyl sensitivity. A plate in which all the ascospores were either Ben-S or Ben-R was considered to contain the products of selfing only, and was not processed further. On the other hand, the presence of both Ben-S or Ben-R ascospores indicated that both parental strains were represented, and the plates were passed to the next step. Cleistothecia were picked individually, using a sterile needle and microscopic observation, and treated as above but on an individual basis. This technique enabled to

distinguish between cleistothecia that resulted from selfing and therefore contained ascospores of a uniform phenotype, and hybrid cleistothecia with segregating ascospore progeny. Only hybrid cleistothecia were useful in genetic analyses and breeding.

RESULTS

Identification of a metabolite involved in biocontrol - When culture filtrate from *T. flavus* was fractionated with acetone (Fig. 1), less than 3% of the total bioactivity in the culture filtrate was obtained by acetone precipitation (Table 1). 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). 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 (Fig.1, Table1). This polar component was also present in the culture medium, since addition of the culture medium to the acetone-precipitable fraction B1 restored the original activity. When all of the components of the culture medium were combined individually with the acetone-precipitable fraction B2 and bioassayed, only glucose restored the inhibitory activity. The bioactivity was restored only when glucose and the acetone precipitable fraction were present simultaneously. When a fixed amount of the acetone-precipitable fraction B2 was bioassayed in the presence of various concentrations of a mixture of α and β anomers of glucose (0.1-9.5%), a Michaelis-Mentor 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 vs. reciprocal values of varying glucose concentrations.

Restoration of activity to the acetone-precipitable fraction by glucose was optimum at pH 5.0 (Fig. 2). Because glucose was specifically and simultaneously required for bioactivity of the acetone-precipitable fraction, the metabolite in the fraction suggested an enzyme whose substrate is glucose. Since it was assumed that the metabolite was glucose oxidase, the elution of the metabolite

activity and an authentic sample of 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). When a preparation of commercial glucose oxidase was combined with glucose in the bioassay system, activity was observed even with 0.0014 units per ml of glucose oxidase (Table 2). The activity increased with an increase in the amount of enzyme from 0.0014 to 14 units per ml 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 $12 \mu g \text{ ml}^{-1}$. In addition, bioactivity of the metabolite in the acetone-precipitable fraction decreased in the presence of catalase or peroxidase, which are known peroxide scavengers (Table 3). 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.

Purification and properties of glucose oxidase from *T. flavus* - Comparison of the levels of the glucose oxidase activity in mycelial extracts and culture filtrates indicated that greater than 90% of the total glucose oxidase produced by different strains of *T. flavus* was present in the respective culture filtrates. Therefore, the enzyme appears to be excreted into the fermentation broth.

The purification of glucose oxidase from the culture filtrates is summarized in Table 4. Usually 15 to 25 mg of the purified glucose oxidase was obtained from 2 liters of culture. During the final column purification using DEAE-5PW, the enzyme was eluted completely between 0.20 and 0.35 M NaCl. The fraction collected at 29 min contained 60% of the total loaded enzyme and the fraction was homogeneous based on SDS-PAGE (Fig. 4). The homogeneous enzyme showed specific activity of 400 to 500 $\mu\text{mol/min per g}$ of protein.

The apparent molecular weight of the native enzyme was determined by gel filtration on an analytical HPLC column in 50 mM sodium acetate containing 0.2M NaCl. The gel filtration standards used were thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B-12. The purified enzyme migrated as a single protein band with molecular weight of 71,000 on a SDS-PAGE gel. This indicated that the native enzyme is a dimer of identical molecular weight (M_r) subunits. The native enzyme eluted as a protein of apparent molecular weight of $164,000 \pm 4000$ from an analytical TSK-3000 SW gel filtration column (Fig. 5). Electrophoresis studies under SDS-PAGE conditions combined with staining for glycoprotein by the dansyl hydrazine method indicated that the purified enzyme from T. flavus was identical to a commercial glucose oxidase preparation from Aspergillus niger. Also, the amino acid composition of glucose oxidase from T. flavus was similar to that from A. niger and Penicillium amagasakiense.

Comparison of glucose oxidase produced by T. flavus with an authentic sample of the antibiotic talaron - An authentic sample of the antibiotic talaron produced by T. flavus and obtained from Japan displayed varying degrees of antimicrobial properties towards bacteria and fungi only in the presence of glucose in the bioassay media. When the talaron sample was analyzed by HPLC, the elution profile of the antimicrobial peak coeluted with that of glucose

oxidase. Approximately 30-40% of the solid talaron sample was glucose oxidase.

Fluorescence emission and excitation wavelength maxima for the talaron sample were similar to that of glucose oxidase from T. flavus. Molecular weight of the glucose oxidase from the talaron was 152,000 with the subunit molecular weight being 80,000. The pI of the enzyme was 4.2. Therefore, glucose oxidase is the active ingredient of the previously reported antifungal-antibiotic talaron produced by T. flavus.

Effect of the metabolite on viability of microsclerotia of V. dahliae - Microsclerotia of two isolates of V. dahliae were killed (LD₉₅) in less than 3 hr in vitro by the metabolite produced by T. flavus (Table 5). Maximum titer of the metabolite was produced by the biocontrol fungus after 5 days in a high-carbon liquid medium that was shaken at 120 rpm and incubated at 27 C. Microsclerotia were not killed on agar amended with either autoclaved culture filtrate or both. The metabolite was also able to kill microsclerotia of V. dahliae in sterile soils and in sand (Fig. 6), indicating that the physical components of the soils did not inactivate the metabolite.

Electron microscopy - Microsclerotia of V. dahliae recovered from soil amended with T. flavus were extensively colonized by T. flavus hyphae which were seen growing toward the microsclerotia (Fig. 7). At a later stage, an extensive sporulation of T. flavus took place on the surface of colonized microsclerotia (Fig. 8). No sporulation was observed on the surface of unloaded, empty nylon meshes buried in T. flavus-amended soil, or on microsclerotia incubated in soil amended with T. flavus, but without an added food base (bran). The morphology of conidiophores and conidia observed on colonized microsclerotia was identical to that of T. flavus observed in pure cultures. Bright yellow cleistothecia of T. flavus were also observed on colonized microsclerotia.

Transmission electron microscopy showed hyphae of the biocontrol agent within the microsclerotia medulla and within individual cells. However, even parasitized sclerotia contained some intact cells with dense cytoplasm and lipid droplets. Penetration of the microsclerotia by the mycoparasite was followed by shrinking of the host cell protoplast, disintegration of the cell organelles, and disappearance of the lipid droplets. The invading hyphae use the contents of the degrading cells as a food base to produce more penetration pegs which invade neighboring host cells by penetrating their walls at specific sites rather than causing total lysis of the walls (Fig. 9, A-B.). Following penetration, the invading thin pegs undergo reexpansion and regain the normal dimensions of the mother cells. Another way of penetration of T. flavus into microsclerotial cells is by using natural passages such as septal pore connections.

Microscopic observations of roots - Great masses of hyphae of V. dahliae were observed on the surface of roots of eggplants grown in the vermiculite mixture without T. flavus. No mycelium of V. dahliae could be seen on roots when T. flavus had also been added to the vermiculite mixture. Microsclerotia seen on the root surfaces were parasitized by the biocontrol agent. Typical structures of the anamorph of T. flavus (Penicillium dangeardii) were produced on the parasitized microsclerotia (Fig. 10), but not on the root surfaces.

Genetic Studies - Nine wild type and seven Ben-R strains were used in 19 pairwise matings. The combined results were as follows: 343 plates contained ascospores of one type only (231 plates contained only Ben-S ascospores and 112 plates contained only Ben-R ascospores). Matings involving strains 26015 and TF1-5 were infertile as were these strains themselves. Matings involving strains 32908 and 10-1C yielded only few cleistothecia, most of which were empty or contained nongerminating ascospores. From 11 crosses, 112 plates were found to

contain ascospores of both parental types at varying ratios. About half of these plates, which contained at least 30% ascospores of the minor component, were analyzed further by examining individual cleistothecia. All the cleistothecia examined so far had resulted from selfing as evident by the uniform phenotypes of their ascospores, and are not useful for this research. More cleistothecia are being examined after the completion of the project.

d. Description of Cooperation

Cooperation consisted of information exchanges by telephone, frequent correspondence, and exchange of cultures and manuscripts. Dr. Henis, one of the two Israeli cooperating scientists who spent his sabbatical leave at Cornell University, visited Beltsville in October, 1986 and spent a week discussing research results and ideas. He presented a seminar to the scientists of the Biocontrol of Plant Diseases Laboratory and showed that T. flavus penetrates and colonizes microsclerotia of V. dahliae, an important discovery that assisted us to further elucidate the mechanism of action. Dr. Talma Katan, the second Israel scientist, also visited Beltsville in July, 1987 and exchanged ideas with the scientists involved in this project. At Beltsville, Dr. Fravel developed isolates of T. flavus which are deficient in glucose oxidase production. These isolates were sent to Dr. Katan for use in genetic crosses to determine the inheritance of the glucose oxidase gene. Soils from Israel were sent to Beltsville to isolate new strains of Talaromyces on selective media.

g. Evaluation of the Research Achievements

The main thrust of objective #1, "Understand the mechanism by which Talaromyces flavus reduces the ability of Verticillium dahliae to cause disease", was the responsibility of scientists at Beltsville. The research performed during the 3-year period accomplished the objective by identifying hydrogen peroxide as the killing agent of microsclerotia of the wilt pathogen. Hydrogen peroxide is released by a reaction catalyzed by a metabolite produced by T. flavus, identified as glucose oxidase. This metabolite was purified and its molecular weight, amino acid composition, and other characteristics were determined. Research at Beltsville also showed that the active ingredient of the antibiotic talaron produced by T. flavus, and first described in Japan, is

glucose oxidase.

Additional research on objective #1, performed at the Hebrew University of Jerusalem in Rehovot, showed that the biocontrol agent parasitizes and destroys the microsclerotia of V. dahliae in soil and at the rhizosphere level and that the antagonist uses root exudates rather than root tissue as a food base for parasitizing and destroying the microsclerotia.

The findings of the research on objective #1 will be of great value to agriculture because a biocontrol marker, glucose oxidase, was identified which can be used in a further genetic improvement program of the biocontrol agent. If biocontrol operates by a metabolite that destroys cells, then the gene(s) coding for such metabolite can be determined and the information used to improve strains of T. flavus by recombinant DNA technology. The findings will also be useful for registration purposes. The Environmental Protection Agency of the United States requires that the mechanism of action of microbial pest control agents be identified in most cases. Also the marker can be used to develop a rapid and meaningful screen for potential biocontrol strains of T. flavus and avoid the laborious and time-consuming practice of testing strains in soils.

h. List of Publications

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* Publications indicated by an asterisk resulted from this Bard Project.

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 x 10 ⁴	100
Water extract (B1)	1.0 x 10 ²	0.3
Water extract (B2)	0	0
Supernatant (A1)	8.0 x 10 ²	2
C-18 Sep-Pak flowthrough (A2)	8.0 x 10 ²	2
C-18 Sep-Pak bound (A3)	0	0
A1 + B1 (7:1)	3.9 x 10 ⁴	96
A1 + B2 (20:1)	2.6 x 10 ⁴	65
A2 + B1 (7:1)	3.9 x 10 ⁴	96
Culture medium + A1 (1:1)	1.6 x 10 ³	4
Culture medium + B2 (7:1)	3.9 x 10 ⁴	96

^aFractions 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.

^bOne 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.

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

^aBioassay was performed by incorporation of the glucose oxidase in Czapek solution agar with or without 8% glucose.

^bOne unit of glucose oxidase activity is that amount of the enzyme capable of liberating 1 micromole of H₂O₂ per minute at 25 C.

^cOne 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

^aBioassays 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.

^bNot determined.

^cOne unit is the amount capable of decomposing 1 micromole of H₂O₂ per minute at 25 C.

TABLE 4. Purification of glucose oxidase from culture filtrates of Talaromyces flavus^a

Steps	Total protein (mg)	Total Units ($\mu\text{mol}/\text{min}$)	Specific Activity ($\mu\text{mol}/\text{min per mg}$)	Yield (%)
Cultures filtrates	123.7	2.39×10^4	193	100
Acetone precipitation	37.4	1.39×10^4	370	58.4
TSK-3000SW	32.9	1.42×10^4	430	59.5
DEAE-5PW	15.8	7.71×10^3	487	32.3

^aThe results were obtained from 2 liter cultures grown for 4 days.

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TABLE 5. 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	19.9
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

^aT. flavus was grown in high-carbon liquid medium incubated at 27 C with shaking. After 5 days, culture was filtered through 0.45- μ m filter.

^bMicrosclerotia were embedded in 0.5-cm² 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.

^cDilution of culture filtrate in Czapek solution agar.

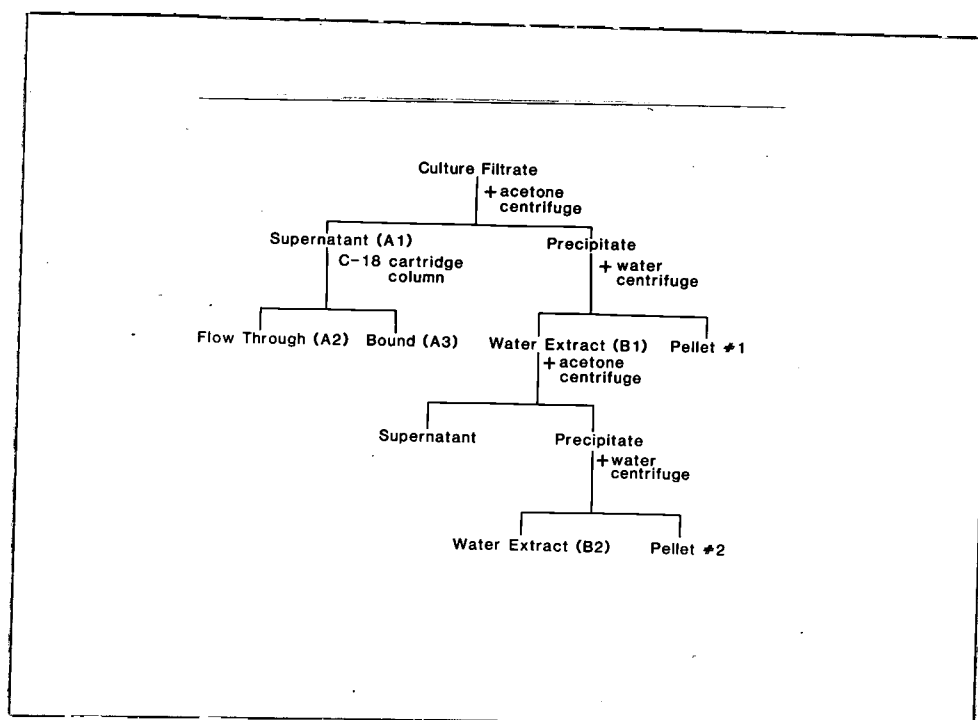


Fig. 1. Detailed scheme of fractionation of culture filtrate from Talaromyces flavus. The fungus was grown for 5 days in a liquid medium containing 8% glucose, and the culture filtrates were fractionated as shown in the figure.

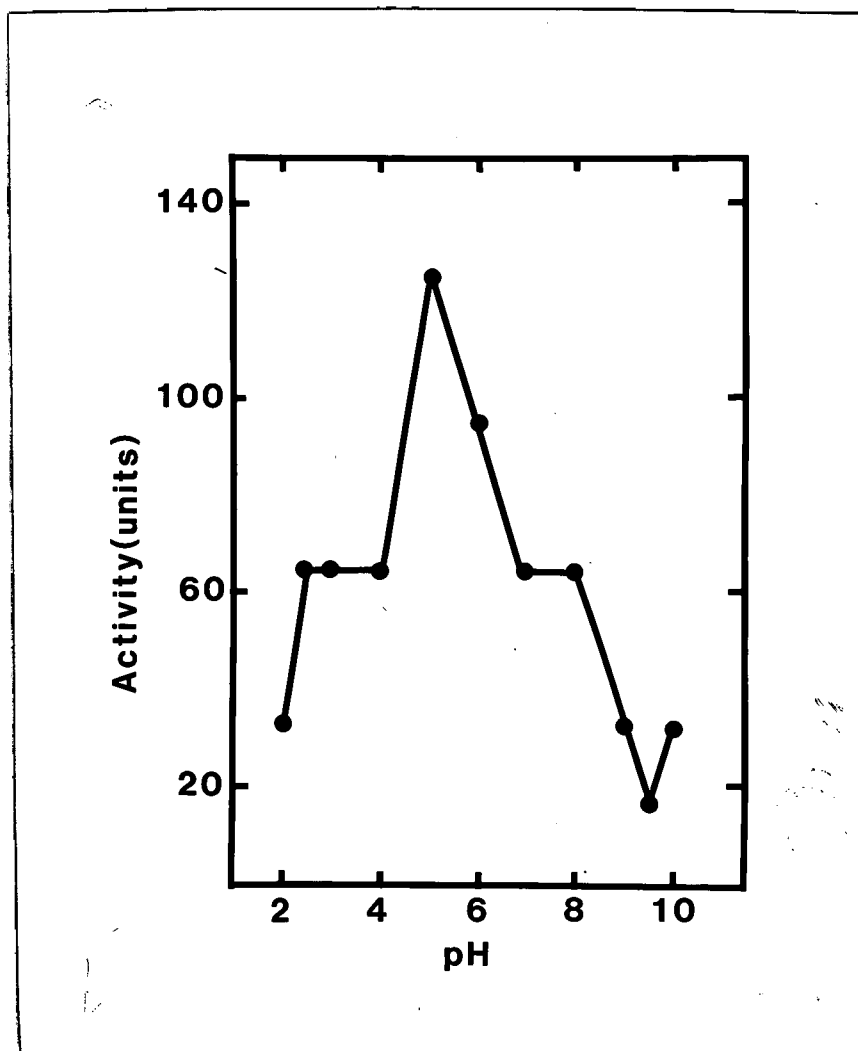


Fig. 2. Effect of pH of acetone-precipitable fraction B2 from culture filtrate of *Talaromyces flavus* on the antibiotic activity against germination of microsclerotia of *Verticillium dahliae*. Aliquots of the fraction B2 were adjusted to various pH values with 2 N HCl or 2 N NaOH and bioassayed against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

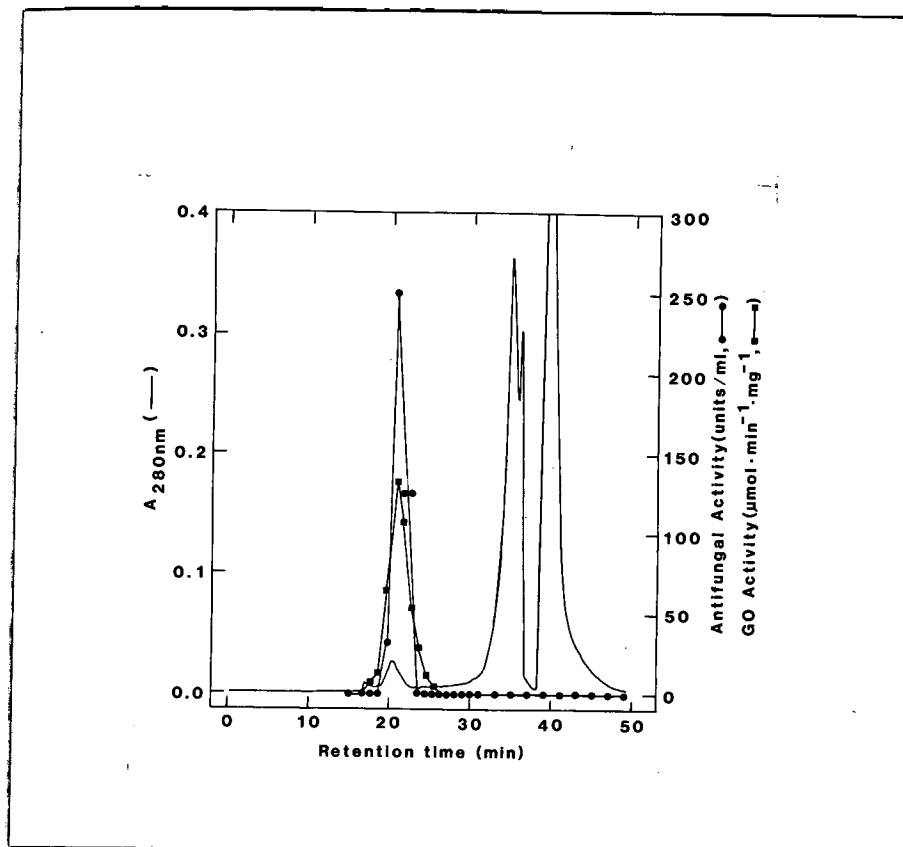


Fig. 3. Elution profiles of antifungal and glucose oxidase activities from *Talaromyces flavus* culture filtrates on a Sperogel TSK 2000-SW column (7.5 x 300 mm). An aliquot of 50 μ l of the acetone-precipitable fraction B2 was injected. A mobile phase consisted of 50 mM potassium phosphates buffer, 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.

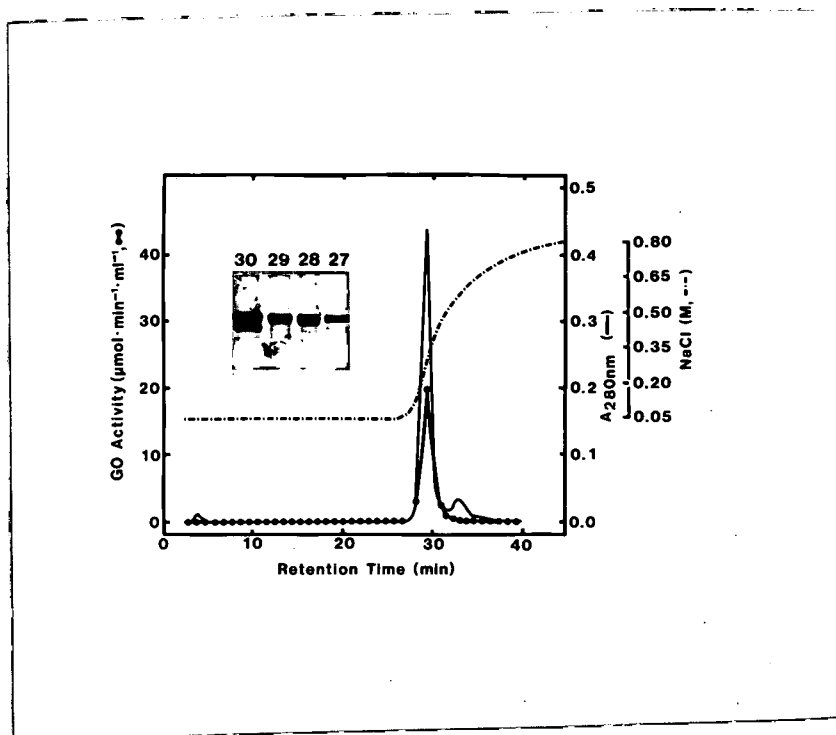


Fig. 4. Elution profile of glucose oxidase activity on a DEAE-5PW column. Aliquots of 100 μl (approximately 50-100 μg of protein) were injected, and 1 min fractions were collected and assayed for protein and enzyme activity. Inset represents the SDS-PAGE patterns of the active fractions 27 through 30 min.

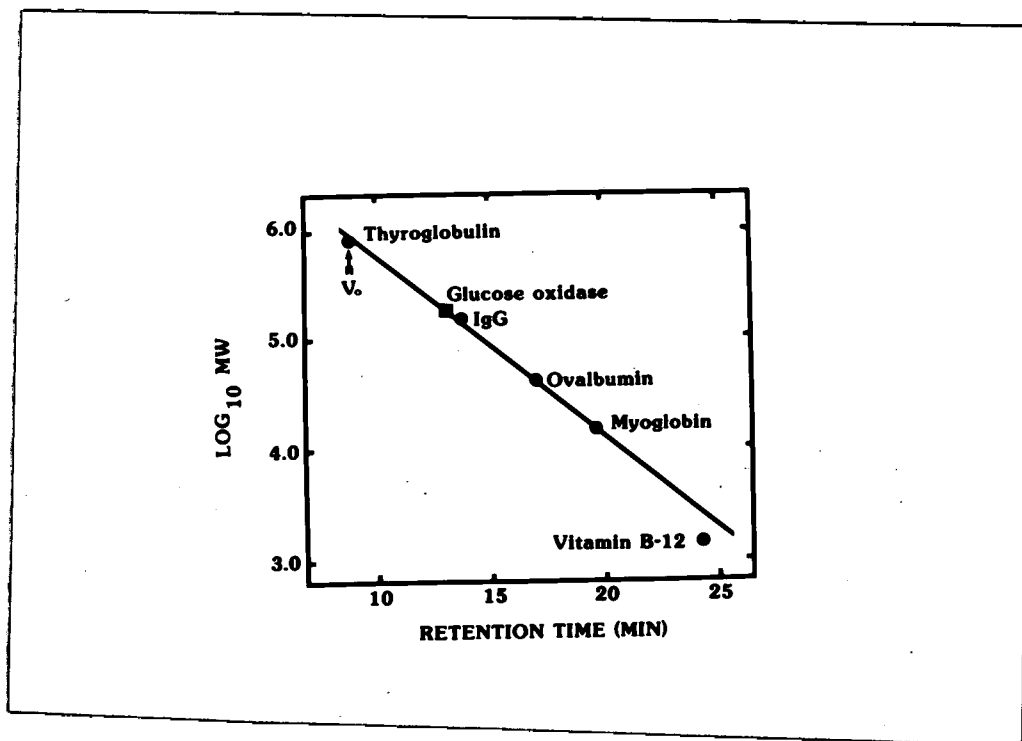


Fig. 5. Molecular weight determination of glucose oxidase on an analytical gel filtration column, TSK-3000SW (7.5 X 300 mm). An aliquot of 20 μ l (approximately 20 μ g of protein) of the pure enzyme was injected. On min fractions were collected. The protein standards were injected under similar conditions.

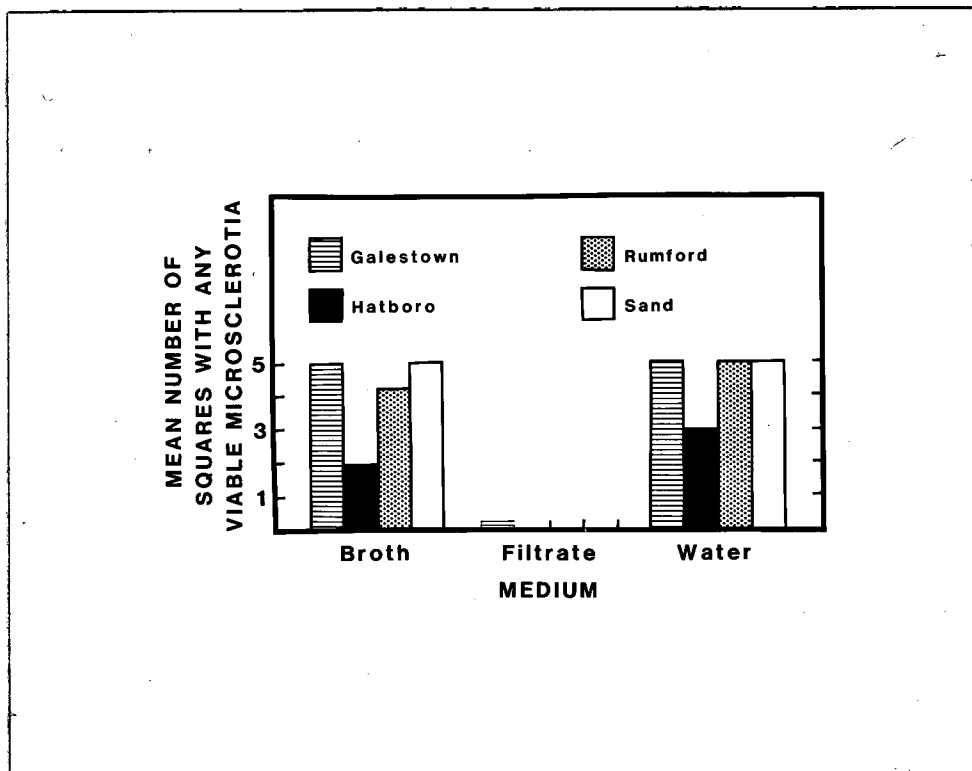


Fig. 6. Effect of culture filtrate of Talaromyces flavus, culture medium (broth), or water on survival of microsclerotia of Verticillium dahliae in three soils and in sand. Each replicate contained five 0.5-CM² pieces of nylon mesh, embedded with about 15 microsclerotia. Meshes were buried in soils, treated with media, and recovered 24 hr later.

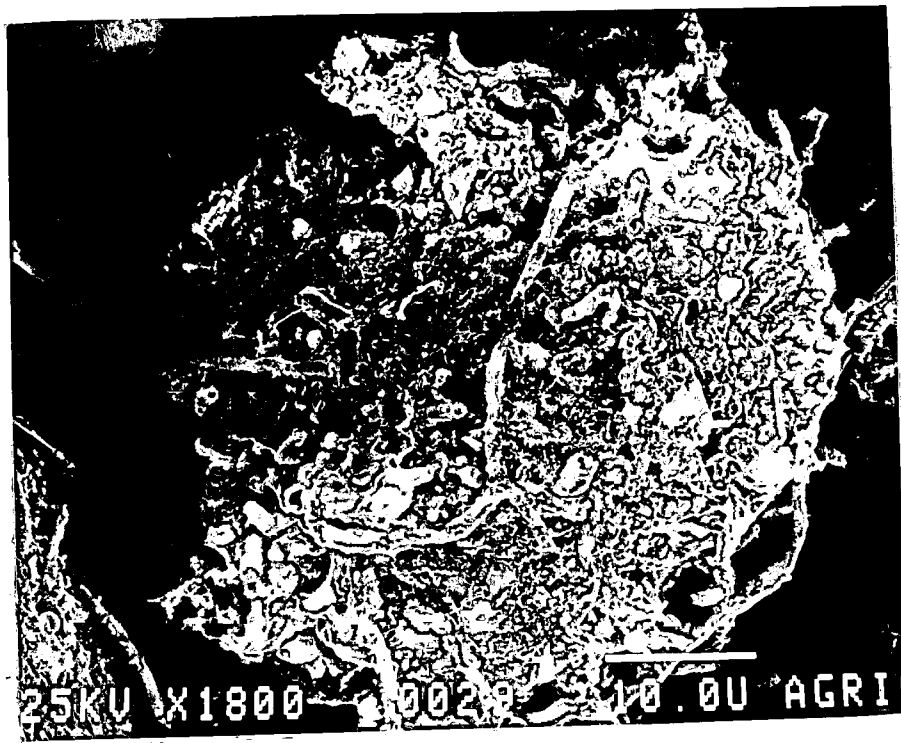


Fig. 7. Hyphae of *Talaromyces flavus* growing towards, around, and on a microsclerotium of *Verticillium dahliae*.



Fig. 8. Conidia of Penicillium dangeardii, the anamorphic stage of Talaromyces flavus on a colonized microsclerotium of Verticillium dahliae.

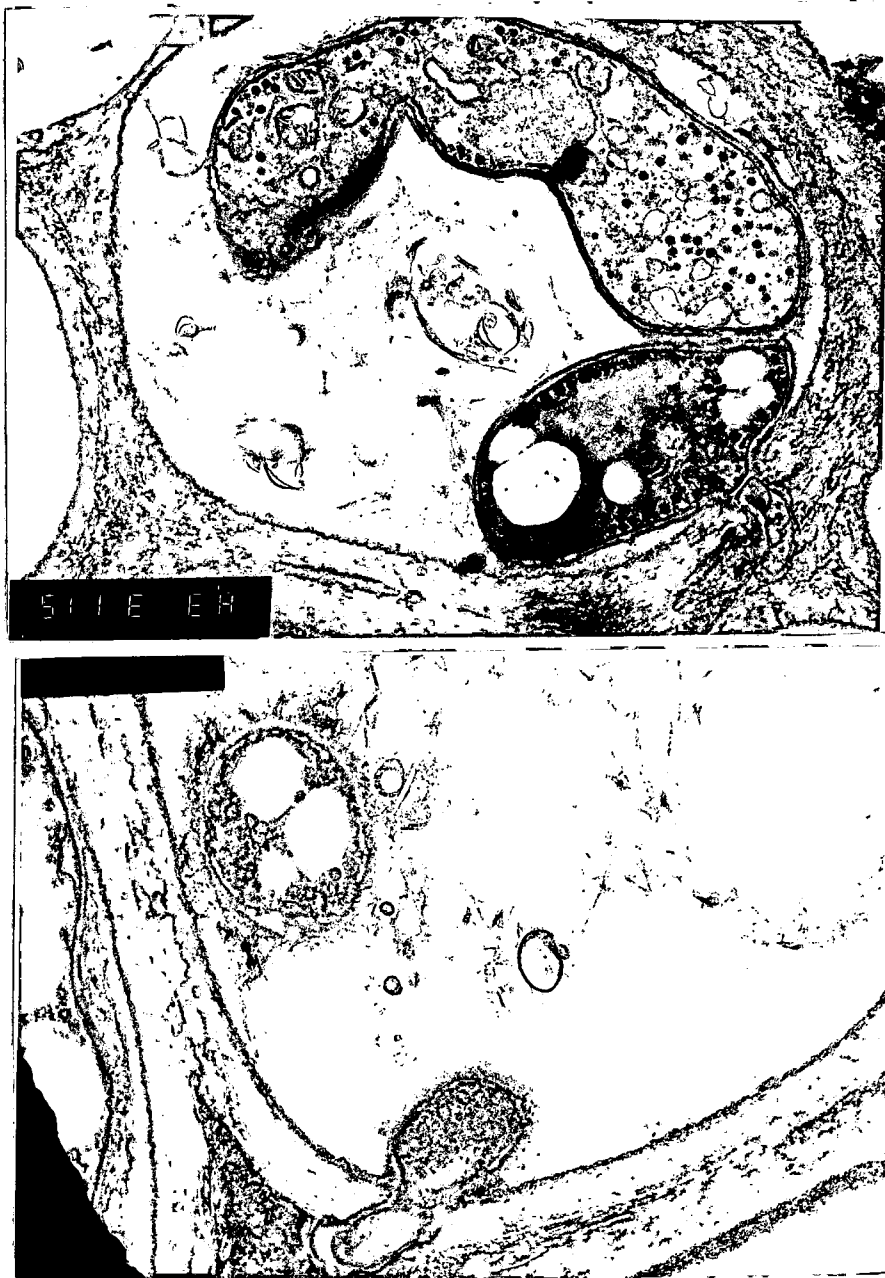


Fig. 9, A-B. A) Transmission electron microscope photograph showing a hypha of Talaromyces flavus within a cell of a microsclerotium of Verticillium dahliae invading a neighboring host cell. B) Reexpansion of an invading hypha of T. flavus.



Fig. 10. Conidiophores of Penicillium dangeardii, the anamorph of Talaromyces flavus produced on parasitized microsclerotia of Verticillium dahliae on the root surface of an eggplant.