BIOLOGICAL CONTROL OF SOYBEAN ROOT ROT CAUSED BY THIELAVIOPSIS BASICOLA (BERK. & BR.) FERR.

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
MICHAEL ABDI ZAKARIA
1975

ABSTRACT

BIOLOGICAL CONTROL OF SOYBEAN ROOT ROT CAUSED BY THIELAVIOPSIS BASICOLA (BERK. & BR.) FERR.

By

Michael Abdi Zakaria

The effects of temperatures ranging from 16°C-28°C and inoculum concentrations ranging from 0.31 g-5 g of inoculum/kg soil were tested on the development of soybean root rot caused by Thielaviopsis basicola. Disease development was enhanced both by lower temperatures and higher inoculum concentrations, but temperature showed a much greater effect than inoculum concentration.

A bilayer method was used for the collection of antagonists. In this method, soybean roots with closely adhering soil were mixed with selective agar media for bacteria, actinomycetes, and fungi as the bottom layer. PDA seeded with T. basicola endoconidia was used as the top layer. Rhizospheres of soybean grown in soil amended with either chitin, ground dried alfalfa, or non-amended were used as sources of antagonists. Several bacterial, actinomycete, and fungal antagonists were also collected by random selection directly from soybean rhizosphere soil and from lysing Fusarium hyphae.

In paired cultures, antagonism involving overgrowth of T. basicola was shown by fungal isolates; and production of inhibition zones by bacterial, actinomycete, and several fungal isolates. In many

instances, fungal overgrowth of T. basicola resulted in loss of viability of the pathogen. The inhibition zones were associated with either antibiotic production, inhibition of endoconidial germination, bursting of endoconidia, lysis of hyphae and endoconidia, or combinations of the four characters. Inhibition zones from antibiotic production were smaller as the nutrient content of the agar medium decreased. Larger inhibition zones were produced on PDA than on seed exudate agar, and some isolates producing inhibition zones on PDA failed to do so on seed exudate agar.

Promising results were obtained from biological control experiments by applying antagonists to seeds sown in steamed soil or natural Conover loam. Three bacteria, five actinomycetes, and four fungi showed significant reduction in disease severity in steamed soil mix; and three bacteria, no actinomycetes, and one fungus, in Conover loam. Of the three bacteria, one reduced disease significantly in both soils. However, antagonists isolated directly from the rhizosphere through random selection gave greater success in biological control than those isolated by the bilayer method or from the lysed Fusarium hyphae. Six of twelve antagonists selected randomly from the rhizosphere significantly reduced disease severity in steamed soil, and four did so in Conover loam; success in biological control obtained by antagonists isolated otherwise was only 14%. Greater success in biological control was also shown by the antagonists which did not produce diffusible antibiotics (34%), as compared to those that did (14%). Isolates which produced distinct inhibition zones in paired cultures on seed exudate agar (29%) showed greater success than those which produced none or indistinct inhibition

zones (17%) in steamed soil mix. However, success in Conover loam was about equal for the two groups, i.e., 6% and 8%, respectively.

Other characteristics of antagonism, either individually or in combinations, were not consistently associated with success in biological control.

An approach to evaluation of antagonists using water agar seeded with T. basicola endoconidia as the growth medium for the coated soybean seeds was tested. Reduction in disease severity obtained using this method showed some correlation with the success in biological control in soil. This method has a short test period and it provides a transparent medium, making possible observations of disease development and possibly of the interactions of the antagonists with the pathogen in the rhizosphere.

BIOLOGICAL CONTROL OF SOYBEAN ROOT ROT CAUSED BY *THIELAVIOPSIS BASICOLA* (BERK. & BR.) FERR.

By

Michael Abdi Zakaria

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1975

To my wife and parents

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my major professor, Dr. J. L. Lockwood, for his guidance and constant encouragement, and especially for his patience and assistance in the preparation of this manuscript.

Thanks are also conveyed to Dr. W. G. Fields and Dr. A. W. Saettler for advice and for critical evaluation of the manuscript.

I would also thank Dr. B. Sneh for his useful suggestions and comments, and Mr. D. M. Weller for his assistance in the identification of several bacteria.

To all my laboratory-mates, for their cooperation, I express my gratitude.

TABLE OF CONTENTS

LIST OF TABLES

Table | Page

LIST OF FIGURES

Figure Page

INTRODUCTION

Thielaviopsis basicola (Berk. & Br.) Ferr. is a common pathogen of many crops, e.g., tobacco, cotton, citrus, but it was first reported causing root rot of soybeans in Michigan in 1970 (49). This disease occurs widespread throughout the eastern portion of the lower half of the lower peninsula. Since then, work has been done at Michigan State University on the epidemiology of the disease. Lower temperatures have been found to favor disease development, while temperatures above 24°C markedly decreased disease severity (Chen and Lockwood, unpublished results). Soybean plants of ages ranging from 0-21 days have been shown to be susceptible to severe infection provided low temperature requirements for disease development are met (Zakaria and Lockwood, unpublished results). Under low temperatures, disease can be produced on plants grown in soil infested with an inoculum concentration as low as 10^2 propagules per gram of soil (Maduewesi and Lockwood, unpublished results). Although different soybean varieties showed different degrees of response to the pathogen, none proved to be immune.

Work has been done on the influence of antagonistic fungi on T. basicola attacking tobacco (73). Although the fungi maintained their antagonistic properties in laboratory experiments as well as in sterilized soil, they were not effective in unsterilized soil.

With increasing knowledge of the manipulation of soil environments and their microorganisms, this study was undertaken to

investigate the possibilities of performing biological control of T. basicola root rot of soybeans.

LITERATURE REVIEW

The pathogen, its taxonomic status and epidemiology

According to the Saccardo system of classification, the genus Thielaviopsis belongs to the family Dematiaceae, of the order Moniliales (8,30). No perfect stage of this fungus has so far been found. The conidia are rod-shaped, one-celled, hyaline, and borne endogenously from sporogenous cells, the phialides. Besides the hyaline conidia, this fungus also produces dark chlamydospores (aleuriospores), each consisting of several cells arising from transverse septation. According to the Hughes-Tubaki-Barron system of classification, this fungus is grouped in the series Phialosporae (8,9).

Thielaviopsis basicola is the only species of the genus recorded from soil (9). The fungus is known as an aggressive pathogen causing root rot on a large host range, although variations in virulence exist among isolates from different hosts (45,46). This fungus has also been found associated with root necrosis of Phytophthora-resistant soybeans (68).

T. basicola survives in soil mainly as chlamydospores which remain viable over a long period of time at low moisture and low temperatures (61,76). Under similar soil conditions a considerable number of endoconidia were found to survive after eight months, but a great reduction in number occurred in moist soil incubated at higher

temperatures. In moist soil the chlamydospores break up into segments each of which is capable of causing infection (44,61,62). In the absence of host plants, germinability of chlamydospores declined rapidly after 20-45 days at 40-45% of moisture-holding capacity (MHC). No decline in germinability was observed at 15% of MHC with a temperature maintained at 20°C. In air-dry soil germinability declined to 90% at 10°C and 18°C and to 30% at 34°C after three months (61). Clough and Patrick (20) found that loss in chlamydospore viability in soil was associated with naturally occurring perforations in the spore walls. Bacteria were often seen in association with the perforations, but a causal relationship has not yet been established.

From laboratory studies, it was found that chlamydospore germination is stimulated by glucose, sucrose, mannose, and especially by root exudates (52). Some chlamydospores, however, were capable of germinating in the absence of exogenous nutrient (44). In nature, germination mainly occurs in the immediate vicinity of the host (45). During infection, the disease progresses as the hyphae grow within the host tissue and upon the root surface, giving rise to new infection loci (45). On soybeans, black to brown necrotic symptoms are produced on the cortex of the below-ground portion of the hypocotyl and of the tap and fibrous roots (49). Secondary inocula are produced after the development of root rot symptoms, resulting in a marked increase in the number of propagules in the rhizosphere (10,62).

In general, disease development for high temperature plants like tobacco, cotton, and Navy beans is favored by a temperature range of 18°C-23°C; temperatures above 26°C markedly lower the disease

severity (52,73). However, disease development in peas, a cool temperature plant, is favored by higher temperatures (46). Most severe disease in peas has been obtained at a temperature of 28°C. The relationship between disease severity and temperature, therefore, appears to depend on the optimum temperature for the host plants. Disease development is also favored by pH values higher than 5.6, although on cotton severe disease can be obtained at a pH value as low as 4.0 (52,73).

Antagonism

Antagonism among microorganisms may be attributed to antibiosis, competition, or exploitation (5,31). On agar plates antagonism may be exhibited by the formation of inhibition zones. Lytic zones have been observed on agar plates both by antibiotic-producing and nutrient-depriving actinomycetes (33).

Antagonism through antibiosis requires that the organisms producing the toxic substances have adequate nutrients (13). Production of antibiotics and other staling products may enhance the competitive ability of the organisms producing them (16,24).

In the case of competition, energy-yielding nutrient seems most likely to be of limited supply (19). That a sizable proportion of the soil microorganisms should be dormant at any given time reflects the lack of suitable and available energy-yielding nutrient. The importance of exogenous and endogenous nutrients on the germination of nutrient-dependent and nutrient-independent spores in soil has been discussed in detail by Lockwood and his co-workers (34,39,47,48,75,78).

Failure of germination in soil can be attributed to lack of essential exogenous nutrients or rapid loss of essential endogenous nutrients by competing microorganisms. Rapid germination followed by rapid growth provides advantages in the process of competition (23,75). Spores with long germination times may not produce germ tubes if the nutrients in their surroundings are rapidly exhausted due to the competitive activities of other microorganisms. Other attributes suggested for a successful competitor include the capability of producing toxic substances, tolerance to toxic substances produced by other microorganisms, and possession of appropriate enzyme systems for substrate decomposition (24,28).

Antagonism in the form of exploitation involves predation and hyperparasitism (5). There are two means suggested by which hyperparasitism occurs (7,12). In necrotrophic parasitism, toxic substances may be produced while the parasite is making contact with its host. The parasite will thus kill the host and utilize the released nutrients. In biotrophic parasitism the parasite obtains nutrients from living host cells without previously killing them. The latter form of parasitism is most often found in nature. Phenomena of haustorial formation, internal growth of parasitic hyphae, and coiling of parasitic hyphae around the host have been observed in vitro (7), but their occurrence in soils is still questionable.

Biological control

Biological control of soil-borne plant pathogens could be achieved if antagonism in favor of the non-pathogens could be maintained

in the soil (5). One important mechanism of such antagonism involves soil fungistasis which is of widespread occurrence in natural soil. It is characterized by a non-specific inhibition affecting almost all species tested, coexistence with microbial activities , and can be nullified by nutrients (47). Germination of spores of fungi in soil is generally restricted to the nutrient-rich environments of plant rhizospheres and plant residues, or follows artificial amendment of soil with energy-yielding nutrients (78).

Ko (38) successfully utilized the soil fungistatic phenomena in the biological control of seedling root rot of papaya caused by Phytophthora palmivora. He used pathogen-free soil to replace a core of pathogen-containing soil in the field as a growth medium for the papaya during the susceptible stage. The pathogen was excluded due to the fungistatic effect of the pathogen-free soil.

Papavizas and his co-workers (1,59,60) used organic amendments such as alfalfa hay, corn stover, and oat straw, to stimulate germination of T. basicola chlamydospores in soil. However, germination was followed by lysis due to antagonistic activities of soil microorganisms which were also stimulated by the amendments. They also showed that the fungistatic effect of the soil was increased after the amendments.

The importance of plant exudates in relation to diseases has widely been studied. Plant exudates directly affect the pathogens by inducing their germination and by contributing to their nutritional status prior to infection (11,26,41,69,71). For example, Rai and Strobel (63) observed that attraction of zoospores of Aphanomyces

cochlioides to roots of sugar beets, and germination was stimulated by different components of root exudates. Kraft (42) related resistance of peas to Fusarium and Pythium root rots to qualitative differences in the seedling exudates. On the contrary, Keeling (35) found no qualitative differences in the exudates of soybean seeds of resistant and susceptible cultivars to Pythium ultimum and P. debaryanum, but more than twice the quantity of soluble carbohydrates was exuded from seeds of the susceptible cultivar.

Plant pathogens are affected indirectly through competition and antibiosis by the epiphytic microorganisms whose activities also are mediated by plant exudates (21,25,29,43,70,71). Germlings of Fusarium solani f. phaseoli lysed due to microbial activity in the nutrient-rich environment around exuding bean seeds, but lysis did not occur and hyphae continued to develop around the hypocotyl where exudation was less (21). PCNB-tolerant microbes have been shown to increase more rapidly in nutrient-amended soil containing PCNB than in soil without PCNB (25). This reduced competition by PCNB-sensitive microorganisms was suspected to be a mechanism for PCNB-induced disease accentuation by tolerant fungi.

Plant pathogens can establish themselves in the rhizospheres, particularly when favored by a host as a selective substrate (5). However, if a high number of microorganisms were for some reason already growing actively when an exuding plant part first appeared, the nutrient liberated might quickly be absorbed and a steep gradient consequently maintained from the outset, resulting in fewer germinated pathogenic propagules. Successful performance in biological control using resident

antagonists has been achieved by several workers. Fokkema and Lorbeer (27) used saprophytic mycoflora of onion leaves to reduce infection in Alternaria porri. A Fusarium species isolated from colonized tissues below a pruning wound was found capable of controlling die back of apricot caused by Eutypa armeniacae (17). Macroconidial suspensions of the Fusarium applied to wounds immediately after pruning reduced infection by the pathogen. Rishbeth (65,66) used Peniophora gigantea, a natural stump colonizer to prevent invasion of freshly cut pine stumps by Fomes annosus. The antagonist was also capable of replacing, to some extent, the pathogen in already colonized stumps. In another approach to biological control, planting grass leys prior to wheat stimulated the development of Phialophora radicicola, a non-pathogenic ectotrophic root parasite of grasses (22). This fungus has a growth habit similar to that of the pathogen, Gaeumannomyces graminis, and was found capable of reducing infection by the pathogen.

Marx and Davey (51) found that fully formed mycorrhizal roots of pine seedlings were resistant to infection by zoospores of Phytophthora cinnamomi. Although the zoospores were attracted to excised root tips, infection did not occur on mycorrhizal roots even with artificially exposed cortical tissue. Marx (50) suggested several possible mechanisms for resistance, viz., nutrient deprivation, physical barrier, stimulation of rhizosphere microbial growth, and production of inhibitors by the plant and antibiotics by the mycorrhizal fungus. Similar mechanisms were suggested by Stack and Sinclair (74) from their work on protection of Douglas-fir seedlings against Fusarium root rot by mycorrhizal fungi.

Antagonism through the production of antibiotics requires that sources of nutrient such as plant residues, laimosphere, and rhizosphere be available (6,77). Although antibiotics can rapidly be inactivated by soil particles or microbial degradation, they may be important for limited periods while the environment favors their production (15). For example, production of bulbiformin by Bacillus subtilis has been observed on seed coats, spermatospheres, and rhizospheres of bacterized pigeon-pea seeds in sterile and unsterile soil (72). Production was enhanced when molasses and groundnut cake were applied to the seeds with the bacteria, and this resulted in reduction of wilt by Fusarium udum.

Biological control using rhizosphere antagonists applied through seed-coating and root-dipping has been carried out with some success (2,36,55,56,58). Increase in yield derived from applications of antagonistic microorganisms has also been claimed (40,53,54). Atkinson et al. (3,4), however, found that although antagonists were more prevalent in the rhizosphere and in the laimosphere of resistant varieties of wheat, seed bacterization with the antagonists did not increase resistance of the susceptible varieties to Cochliobolus sativus. Moreover, one breeding line, although susceptible, was also shown to harbor antagonists in a comparable number to those of the resistant line. Thus, antagonists and resistance were non-related in their system.

Antagonism in agar media does not necessarily predict antagonistic activity in soil (6,36). Since a very low percentage of antagonists isolated from soil proved effective under commercial

conditions, Broadbent et al. (14) suggested that large scale screening methods are essential. However, screening for antagonists may be made more efficient using better approaches in the evaluation technique (32, 57), accompanied by better specifications in environmental requirements such as substrate (15,17,36) and temperature (17,18) suitability.

MATERIALS AND METHODS

Preparation of inocula

Thielaviopsis basicola isolate 157 (from Monroe Co., Mich.) was used throughout the experiments. The fungus was maintained as a stock culture on potato dextrose agar (PDA) slants at 4°C. For production of inocula, the fungus was grown either on soybean-sand medium, in carrot-extract broth, or on PDA slants.

The soybean-sand medium was prepared by mixing 200 g ground soybean seeds (prepared by grinding 200 g dried soybean seeds in a Waring Blendor for about five minutes), 1000 g white silica sand, and 250 ml distilled water; followed by sixty-minute steaming. The steamed mixture was broken up with a fork before putting it into Erlenmeyer flasks for sterilization. *T. basicola* was grown in the medium for 4-6 weeks at 24°C before use.

Carrot-extract broth was prepared by autoclaving 200 g carrot root slices in 1000 ml distilled water for fifteen minutes. The mixture was then passed through four layers of cheese cloth and the cheese cloth containing the carrot was squeezed until most of the liquid was extracted. The extract was resterilized in 250 ml Erlenmeyer flasks which, after cooling, were inoculated with *T. basicola*. The flasks were incubated at 24°C for 2-3 weeks. The liquid cultures were then homogenized in a Servall Omnimixer for ten minutes and the homogenates were washed by repeated centrifugation. The washed homogenates were resuspended in

distilled water and the propagule (mixture of endoconidia and chlamydospores) concentration was measured using a hemacytometer. An intact chlamydospore chain, a single segment, or a short chain of several segments, each was counted as a single propagule.

Slant cultures were prepared by inoculating T. basicola on PDA slants in test tubes, followed by one week's incubation at 24°C. Endoconidial suspension was prepared by adding 5 ml distilled water into each test tube, followed by shaking vigorously using a Vortex Jr. mixer for one minute. Inoculum concentration was measured using a hemacytometer. This endoconidial suspension was used for work on antagonism on agar plates. Unless otherwise stated, the final concentration was adjusted to 10^6 endoconidia/ml and 0.05 ml was applied to each plate using a De Vilbiss atomizer.

Effect of soil temperature and inoculum concentration on disease severity

An experiment was carried out in the greenhouse to test the effect of different temperatures and inoculum concentrations on disease severity.

Controlled temperature water baths at 16°C, 20°C, 24°C, and 28°C were used. The inoculum concentrations used were 5 g, 2.5 g, 1.25 g, 0.63 g, and 0.31 g of soybean-sand culture per kg of steamed soil mix (compost, peat, coarse sand; 1:1:1 v/v/v). Uninfested steamed soil mix was used as control. Four to five 'Harosoy-63' soybean seeds were planted in each of four plastic pots for each treatment. The pots were incubated for two days on a greenhouse bench and then transferred

to the water baths for three week's additional incubation before disease severity was assessed. Watering was done daily by maintaining constant weight in each treatment: 1160 g/pot for 16°C, 1180 g/pot for 20°C, 1190 g/pot for 24°C, and 1200 g/pot for 28°C. Disease in each plant was rated on a scale of 0-5; 0 being disease-free, 1 slightly diseased, 2 slightly to moderately diseased, 3 moderately diseased, 4 moderately to severly diseased, and 5 severely diseased.

Sources of antagonists

Conover loam (46) amended either with 1% (w/w) chitin or 1% (w/w) ground dried alfalfa hay, or non-amended was used as sources of antagonists. Four soybean seeds were planted in single plastic pots containing the amended or non-amended soil and incubated in a water-tank at 19±1°C in a greenhouse for fifteen days. The seedlings were then uprooted, the roots washed with running tap water to remove adhering soil and ground in a mortar. The ground roots were made into 50 ml suspensions which were further diluted with distilled water from 10^{-2} to 10^{-3} for plating purposes.

Serial dilutions were also prepared from rhizospheres of soybeans grown in Conover loam in the greenhouse and from mycelial mats of Fusarium solani placed on small pieces of nylon net and buried in soil for two days. These were also then used as sources of antagonists.

Evaluation of antagonists

Evaluation of antagonists from the root suspensions was carried out using a bilayer technique according to the method of Henis

et al. (32). Three different selective media were used for the basal layer. For actinomycetes the medium contained 4 g colloidal chitin, 20 g Difco agar, and 1000 ml distilled water (pH 7), and will be referred to as chitin agar. For bacteria the medium contained 100 ml soil extract, 1 g glucose, 0.5 g K_2HPO_4, 20 g Difco agar, 900 ml distilled water, and 0.05 g pentachloronitrobenzene (PCNB); the pH was adjusted to 6.8-7.0--this medium will be referred to as soil-extract agar. For fungi, PDA with 0.5 ml TMN detergent (Union Carbide Co.) and 0.25 g chloramphenicol (added after autoclaving) was prepared--this medium will be referred to as PDA-chloramphenicol. The serial dilutions from the root suspensions were plated using these selective media. The plates were incubated at 24°C for 2-4 days before applying the top layer.

PDA seeded with T. basicola endoconidia was prepared in Petri plates and incubated for 1-2 days. This agar layer was transferred to a sterilized cellophane sheet and then was aseptically transferred onto the bottom layer. These bilayer cultures were incubated for two or more additional days and colonies showing production of inhibition zones and/or overgrowth of T. basicola were isolated. Antagonistic bacteria and fungi were transferred to PDA and actinomycetes to chitin agar.

Similar selective media were used for the isolation of bacteria, actinomycetes, and fungi from soybean rhizospheres and the Fusarium mycelial mats. Several of these isolates were chosen for evaluation of antagonism, using the paired culture technique according

to the method of Hsu and Lockwood (33). Antagonists were isolated and transferred to the same media.

Preservation of isolates

Isolates were preserved either on agar slants or in glycerol solution. Fungal isolates were grown on PDA slants for a few days in screw-capped test tubes at 24°C before being kept at 4° C. Actinomycete isolates were preserved in a similar manner, except that yeast-extract agar (1000 ml distilled water: 2 g yeast extract and 20 g Difco agar) slants were used. Bacterial isolates were grown in 5 ml nutrient broth for several days before 2 ml of 80% glycerol was added. These cultures were maintained in glass vials and kept at -2°C.

Antagonism on agar plates

The various isolates were tested for antagonism to T. basicola on PDA and on seed exudate agar. The latter medium was prepared by soaking 200 g surface-sterilized (fifteen minutes in 1000 ppm NaOCl) soybean seeds in 600 ml distilled water for twenty-four hours, then passing the exudate solution successively through Whatman No. 5 and No. 50 filter papers. Distilled water was added to the filtrate to make a final volume of 500 ml, into which was then added 10 g Difco agar before autoclaving.

1. Production and assays of inhibition zones. Isolates were inoculated individually at the centers of PDA and seed exudate agar plates, and incubated for two days before application of an endoconidial suspension of T. basicola using a De Vilbiss atomizer. After another two

days' incubation the plates were examined for zones of inhibition in the otherwise uniform growth of T. basicola. Inhibition zones were measured from the margins of the antagonistic colonies to the margins of the inhibition zones.

Microscopic observation of the inhibition zones was carried out one, two and three days after spraying by directly observing the plates under the microscope. Occurrence of poor germination of T. basicola endoconidia, poor growth, bursting of endoconidia, lysis of hyphae, and parasitism was noted.

Detection of antibiotic production and nutrient deprivation in the agar was carried out by transferring agar discs (7 mm in diameter), cut with a cork borer, from the inhibition zones to the surface of PDA plates. For those isolates producing smaller inhibition zones, agar squares (5 mm x 5 mm) were cut with a spatula and transferred. After application of an endoconidial suspension of T. basicola, the plates were incubated at 24°C for two days and the presence of new inhibition zones was then observed to see if diffusible inhibitory substances were produced. Inhibition zones were measured from the margins of the agar discs or squares to the margins of the inhibition zones.

For those isolates producing very small inhibition zones in paired cultures and those showing overgrowth of T. basicola, another method for detection of antibiotic production was used. The isolates were grown in seed exudate broth for one week when culture filtrates were tested for the presence of antibiotics. Two ml of each culture filtrate was passed through a Nuclepore filter (0.22 um; 10 mm diameter)

in a Swinnex adapter attached to a hydrodermic syringe. Three drops of each culture filtrate were applied to sterilized filter paper discs (13 mm diameter) and the discs were placed on PDA plates. The plates were sprayed with an endoconidial suspension of T. basicola and formation of inhibition zones was observed after two days. Inhibition zones were measured from the margins of the filter paper discs to the margins of the inhibition zones.

2. Viability tests. Viability of T. basicola from the inhibition zones produced by the transferred agar discs and by the filter paper discs impregnated with the culture filtrates was analyzed. Agar discs (7 mm diameter) or agar squares (5 mm x 5 mm, for those isolates producing smaller inhibition zones) were taken from the inhibition zones, inverted, and placed on fresh PDA plates. The plates were incubated at 24°C for one week and growth of T. basicola from the transferred agar discs or squares was observed.

Viability of T. basicola in the areas overgrown by fungal isolates was also determined. Seed exudate agar in Petri plates was seeded with T. basicola endoconidia and incubated for two days at 24°C. Individual fungal isolates were then inoculated at the centers of the plates. The plates were incubated for fifteen days before discs (7 mm diameter) taken from the overgrown areas next to the inoculation points were ground in 5 ml distilled water for one minute using a glass tissue homogenizer. Each homogenate was made into a final volume of 10 ml by adding distilled water, and 0.5 ml was then plated with 5 ml of a selective medium for T. basicola (Maduewesi et al., unpublished results).

Colonies of T. basicola arising from the plates were counted after 6-7 days. Control plates were prepared from similar suspensions, but agar discs from pure T. basicola plates were used instead.

Antibiotic production and nutrient deprivation on diluted PDA

PDA either undiluted or diluted to 1/2, 1/4, 1/8, 1/16, 1/64, and 1/128 normal strength was used in the assay. Isolates known to produce inhibition zones through antibiosis and nutrient deprivation were individually streaked at the centers of PDA plates.

Agar discs (7 mm diameter) were taken from the margins of antibiotic-producing colonies incubated at 24°C for 3-4 days and were transferred onto fresh undiluted PDA plates. The plates were then sprayed with an endoconidial suspension of T. basicola. Inhibition zones produced were measured two days after spraying. Isolates producing inhibition zones through nutrient deprivation were individually streaked on PDA plates prepared at various dilution from 1/1 to 1/128. After 3-4 days the plates were sprayed with a suspension of T. basicola endoconidia. Inhibition zones were measured three days after spraying. Measurement of inhibition zones was done as previously described.

Assays of biological control of T. basicola of soybean

1. Preparation of cell and spore suspension. Bacterial and fungal isolates were grown on PDA slants in test tubes for about ten days before 3 ml of 0.1% water agar was added to each tube. The tubes were vigorously shaken using a Vortex Jr. mixer for one minute, and the

resulting suspensions were collected in glass vials. Spore suspensions of actinomycetes were prepared in a similar manner except that yeast-dextrose agar slants and 0.1% chitin agar were used. Yeast-dextrose agar contained, per liter: yeast extract 10 g, dextrose 10 g, $CaCO_3$ 2.5 g, and Difco agar 20 g. Isolates which did not produce abundant spores were scraped from the slants and homogenized in a glass tissue homogenizer for two minutes with the same amount of 0.1% agar solution.

2. Seed coating. Homogeneous soybean seeds (cultivar 'Hark') were selected. Sixteen seeds were soaked in the cell or spore suspensions for three minutes before placing them individually in soil in polystyrene test tubes 10 cm long x 1.6 cm diameter (Maduewesi and Lockwood, unpublished results). Control seeds were similarly soaked in 0.1% water agar or 0.1% chitin agar.

3. Soil preparation and planting. Steamed soil mix was passed through a 2 mm sieve before infesting it with T. basicola spore (mixture of endoconidia and chlamydospores) suspension. Infestation was done by spraying the spore suspension onto the soil in a drum rotating on an electric-powered rotary mixer. Unless otherwise stated, the final inoculum concentration was 10^3-10^4 propagules per g soil. Natural Conover loam was similarly prepared, except that 20% (w/w) of white silica sand was mixed with the soil before infestation and the final inoculum concentration was adjusted to 10^4-10^5 propagules per g soil. Uninfested soils were used for comparison.

The soil was wetted to about 50% MHC before transferring about 15 g to each polystyrene test tube. To compact the soil, each tube was gently tapped three times on a hard surface. Coated seeds

were placed on the top of the soil columns and enough soil to cover the seeds was added. The tubes were incubated at 25°C under continuous fluorescent light (8,600 lux) for three days, during which time the seeds germinated. The seedlings were further incubated in a growth chamber maintained at 20°C, with a photoperiod of fifteen hours per day and a light intensity of 10,200 lux. Disease assessment was carried out after fifteen days.

4. Disease assessment. Fifteen days after planting, the seedlings were removed from the polystyrene tubes. The roots were then washed with running tap water. Disease was assessed on a scale of 0-6. 0 indicates a disease-free state of the root system; 1, lesions present on up to 25% of the root system but no girdling of tap root; 2, lesions present on up to 50% of the root system but no girdling of the tap root; 3, lesions on more than 50% of the root system or a girdled lesion of up to 1 cm in length; 4, girdling as in 3 but with additional lesions on up to 25% of the root system, or a girdled lesion of 1.1-1.5 cm in length; 5, a girdled lesion of 1.1-1.5 cm in length with additional lesions on up to 25% of the root system, or a girdled lesion of 1.6-2.0 cm in length; and 6, a girdled lesion of 1.6-2.0 cm in length with additional lesions covering up to 25% of the root system, or a girdled lesion of more than 2 cm in length (Figure 1).

An alternative method for evaluating antagonists for biological control

Soybean seeds (cultivar 'Hark') were surface-sterilized by soaking them in 1000 ppm NaOCl for fifteen minutes. After rinsing three

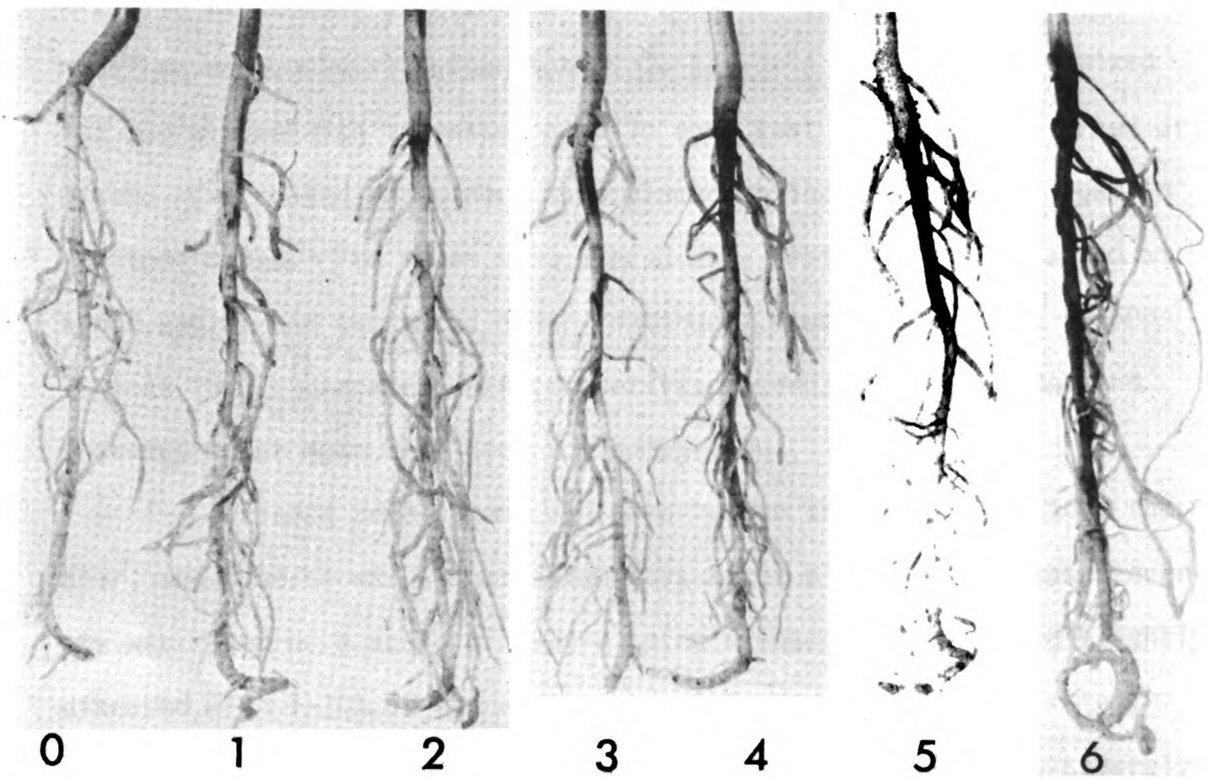

Figure 1. Disease index scale used for estimating severity of Thielaviopsis basicola root rot in soybean plants grown individually in test tubes. Disease was rated on a scale of 0-6, with 0 indicating no disease and 6, maximum disease severity.

times with sterile distilled water, the seeds were then coated with aqueous suspensions of the antagonists.

Glass test tubes (15 cm x 2.3 cm) were one-third filled (20 ml) with 0.8% water agar and maintained at 45°C in a water bath. One ml of an endoconidial suspension of T. basicola was vigorously mixed for one minute with the water agar in each test tube using a Vortex Jr. mixer. The coated seeds were then placed on the seeded water agar in the test tubes. The tubes were placed on a test tube rack slanted at a 60° angle, and incubated under continuous fluorescent light (8,600 lux) at 25°C. Uncoated surface-sterilized seeds sown on seeded and unseeded water agar were used for comparison.

Disease development was observed as the roots grew into the agar, and disease was assessed ten days after planting. Disease severity was rated on a scale of 0-3; 0 being disease free, 1 being slightly diseased with 1-10% of the root system infected, 2 being moderately diseased with 11-25% of the root system infected, and 3 being severely diseased with more than 25% of the root system infected.

RESULTS

Effect of soil temperature and inoculum concentration on disease severity

Soybeans (cultivar 'Harosoy'63') were grown in soil infested with different inoculum concentrations from 0.31 g to 5 g of soybean-sand culture/kg soil and incubated in 16°C, 20°C, 24°C, and 28°C water tanks. After three weeks disease severity was rated on a scale from 0-5, 0 being disease-free and 5 being most severely diseased.

Disease severity decreased markedly as the temperature increased, but less markedly with decrease in inoculum concentrations (Figure 2). At 16°C each inoculum concentration used produced at least four times as severe a disease index as did corresponding inoculum concentrations at 28°C. At all temperatures tested, except at 28°C, disease severity at 0.31 g inoculum/kg soil was at least half that at 5 g inoculum/kg soil. At 28°C, 5 g inoculum/kg soil was required to produce as severe a disease index as 0.31 g inoculum/kg soil at 16°C. Results obtained by Chen and Lockwood (unpublished results) on the effect of soil temperatures on disease severity of soybeans caused by *T. basicola* were thus confirmed. These temperature effects provided important clues for the biological control of the disease: a) if antagonists were to be used, they should be able to grow well and perform antagonism at lower temperatures where conditions for disease development are suitable; b) protection against the pathogen may be needed only during the early period of the growing season when soil temperatures are in the range favorable for disease development.

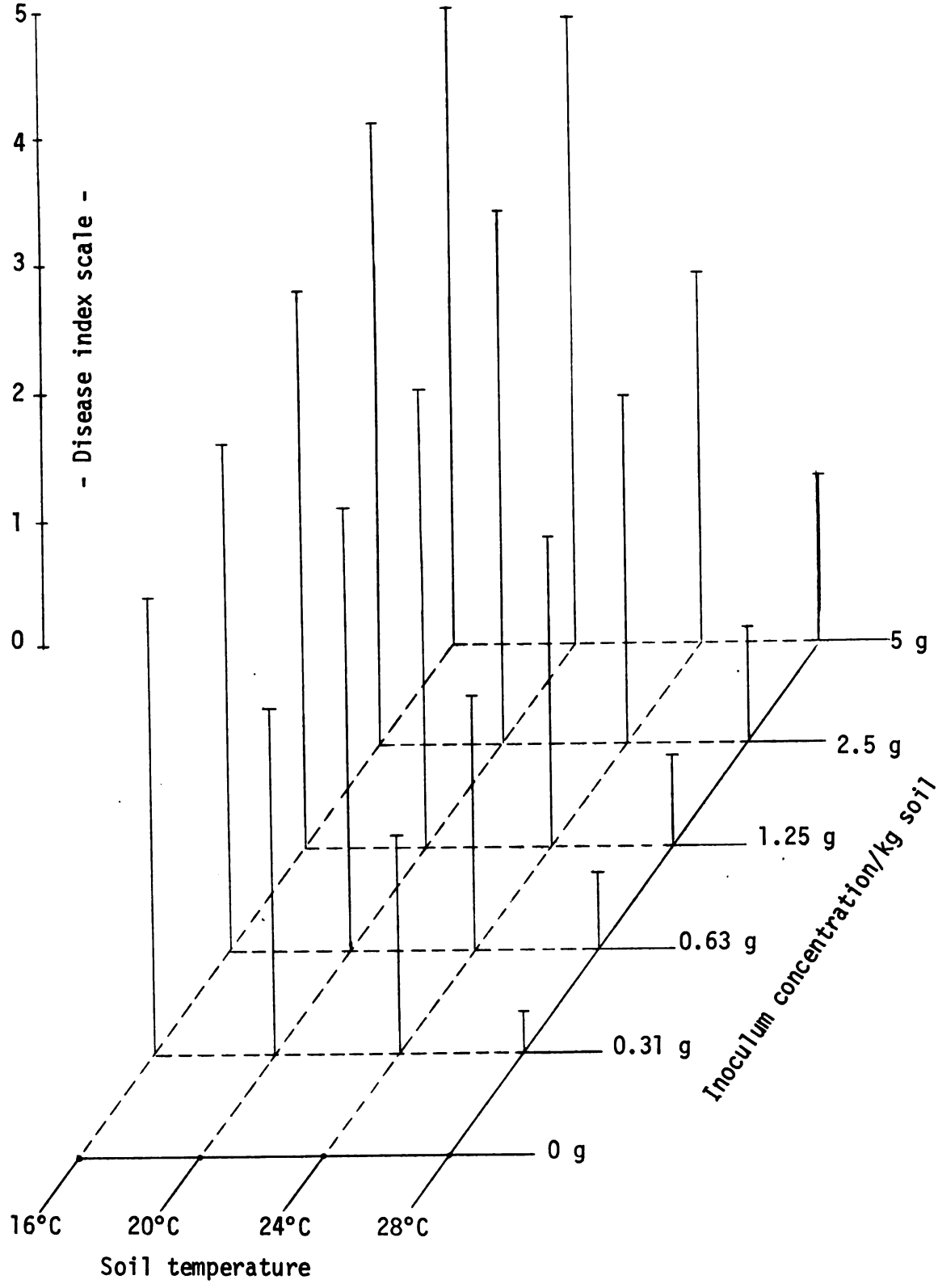

Figure 2. Effect of different soil temperatures and inoculum concentrations on root disease severity of soybean cultivar 'Harosoy-63' caused by Thielaviopsis basicola. Each bar represents the mean of four replicates with 4-5 measurements in each replicate.

Collection of antagonists

Seventeen bacteria, forty-six fungi and five actinomycetes which produced inhibition zones against T. basicola or overgrew the pathogen were isolated by the bilayer method from rhizospheres of soybeans grown in soils with different amendments. Of the forty-six fungal isolates, twenty-two belonged to the genus Trichoderma (8), which were then grouped into species aggregates (64): T. harzianum (thirteen isolates), T. pelluliferum (six isolates), and T. hamatum (three isolates). Based on the Saccardo system of classification (8), except for two asporogenous deuteromycetes, the other twenty-two isolates were grouped into the following genera: Penicillium, Aspergillus, Gliocladium, Fusarium, Alternaria, Myrothecium, Rhizopus, and Actinomucor. The actinomycetes were all of the genus Streptomyces. Bacterial isolates were not identified.

Using the paired culture technique, four bacteria, two fungi (both of the genus Fusarium), and five actinomycetes (Streptomyces spp.) from the rhizospheres of soybeans grown in the greenhouse, and eight actinomycetes (Streptomyces spp.) from the Fusarium mycelial mats showed antagonism to T. basicola either through the production of inhibition zones or overgrowth.

Isolates obtained by the bilayer method are designated by the prefixes 'b' (bacteria), 'T' (Trichoderma spp.), 'f' (fungi other than Trichoderma), and 'a' (actinomycetes). Those obtained directly from the rhizosphere are designated by the prefixes 'sb' (bacteria), 'bf' (fungi), and 'sa' (actinomycetes); and those obtained from the Fusarium

mycelial mats are designated by 'L' (actinomycetes). After purification through successive transferring, the antagonists were preserved.

Analysis of antagonism on agar plates

1. Bacterial isolates. Spraying a suspension of T. basicola endoconidia onto individual bacterial colonies grown on PDA or seed exudate agar in most cases resulted in the production of inhibition zones. All twenty-one isolates produced inhibition zones on PDA although three of them were indistinct. Seventeen isolates also produced inhibition zones on seed exudate agar but two of them were indistinct. Two isolates with distinct inhibition zones and two of the three with indistinct ones on PDA produced none on seed exudate agar. Six other isolates produced weaker inhibition zones on seed exudate agar than on PDA.

Microscopic observations were made around the inhibition zones on PDA one, two and three days after application of T. basicola endoconidia. Production of (distinct or indistinct) inhibition zones by nineteen isolates was associated with poor germination, twelve produced inhibition zones associated with bursting of spores or spores in chains, and all twenty-one isolates lysed hyphae and endoconidia. Nine isolates showed a spreading growth habit both on seed exudate agar and on PDA (Table 1).

Agar discs or squares transferred from the inhibition zones produced on seed exudate agar to fresh PDA indicated that eight of twenty-one isolates produced diffusible antibiotics. Production of larger inhibition zones on PDA or seed exudate agar seemed correlated

Table 1. Production of inhibition zones, antibiotic production, characteristics of the inhibition as determined microscopically, and growth habit of bacterial isolates three days after spraying agar plates containing individual isolates with a suspension of Thielaviopsis basicola endoconidia.

Isolate[a]	Inhibition zones[b]		Antibiotic production[b]	Microscopic observation			Growth habit
	PDA	SEA[c]		Germination inhibition	Bursting of spores	Lysis[d]	
b1	±	-		+	+	+	Spreading
b2	+++	+++	++	+	+	+	Spreading
b3	++	-	-	+	+	+	Spreading
b4	+++	+++	++	+	-	+	Spreading
b5	++	+	-	+	-	+	Spreading
b6	++	+	-	+	-	+	Spreading
b7	+	+	-	+	-	+	Spreading
b8	+++	++	++	+	-	+	Spreading
b9	+++	+++	++	+	+	+	Not spreading
b10	++	±		+	+	+	Not spreading
b11	++	+	-	+	+	+	Not spreading
b12	+++	+++	+++	+	+	+	Not spreading
b13	+++	+++	+++	+	+	+	Not spreading
b14	+	-	-	+	+	+	Not spreading
b15	+++	+++	++	+	+	+	Not spreading
b16	++	+	-	+	+	+	Not spreading
b17	+++	+++	++	+	+	+	Not spreading
sb1	±	±		-	-	+	Not spreading
sb2	+	+	-	+	-	+	Not spreading
sb3	±	-		-	-	+	Not spreading
sb4	+	+	-	+	-	+	Spreading

[a]Isolates with 'b' prefixes were obtained by the bilayer method; those with 'sb' prefixes, directly from the rhizosphere.
[b]-= no inhibition zone; ±= inhibition zone not distinct; += inhibition zone 0.1-0.5 cm wide; ++= inhibition zone 0.6-1.0 cm wide; +++= inhibition zone > 1 cm wide.
[c]SEA= seed exudate agar.
[d]Lysis of hyphae and endoconidia.

with antibiotic production (Tables 1 and 2). T. basicola endoconidia on agar discs or squares from the newly formed inhibition zones showed low viability when transferred to fresh PDA. Only two of the eight agar pieces transferred showed growth of T. basicola during a one-week's incubation period (Table 2). Bursting of spores appeared to correlate positively with loss of viability (Tables 1 and 2).

Seeds coated with bacterial antagonists were placed on carrot-extract agar plates (prepared by adding 1.5% Difco agar to the carrot-extract broth) seeded with 10^6 T. basicola endoconidia/ml, to determine the effect of inoculum from coated seeds on growth of the antagonist colonies and on inhibition zone formation. Coating was done by soaking surface-sterilized (fifteen minutes in 1000 ppm NaOCl) soybean seeds in individual cell suspensions for one minute. Comparable plates were prepared similarly, but the seeds were removed from the plates after one minute. Plates treated with seeds soaked in sterile distilled water were also prepared as the control. The plates were incubated at 25°C under continuous fluorescent light (8,600 lux) for five days before diameters of inhibition zones and of bacterial growth were measured. Each treatment consisted of two to three replications.

Growth of the bacterial colonies and production of inhibition zones were more prominent on agar plates with the coated seeds after five days of incubation; the diameters of growth plus inhibition zones were wider by 7-53 mm than those where the seeds were removed (Table 3). The diameters of growth alone (excluding the inhibition zones) were wider by 5-32.7 mm. One isolate (sb3) even failed to grow on the seeded agar in the absence of the seeds.

Table 2. Reproduction of inhibition zones by seed exudate agar taken from inhibition zones of the bacterial isolates obtained by the bilayer method, and viability of *Thielaviopsis basicola* endoconidia in the reproduced inhibition zones.

Isolate	Inhibition zone[a]	Viability[b]
b2	++	-
b4	++	+
b8	++	+
b9	++	-
b12	+++	-
b13	+++	-
b15	++	-
b17	++	-

[a] ++ = Inhibition zone 0.6-1.0 cm wide; +++ = inhibition zone > 1 cm wide.

[b] + = Growth of *T. basicola* was observed from the agar pieces transferred from the reproduced inhibition zones to fresh PDA; - = no growth of *T. basicola*.

Table 3. Inhibition zone production and growth of bacterial antagonists applied with seeds to carrot-extract agar seeded with Thielaviopsis basicola endoconidia. Seeds were either left on the agar for five days, or were removed after one minute.

Bacterial antagonist[a]	Diameter (mm)[b]			
	Seed removed		Seed not removed	
	Growth of bacteria	Growth + inhibition zone	Growth of bacteria	Growth + inhibition zone
b9	41.5	66.0	46.5	73.0
b12	22.7	45.7	46.7	70.0
sb2	16.0	16.0	29.2	29.2
sb3	0	0	32.7	53.2
Control[c]	0	0	0	0

[a]Isolates with 'b' prefixes were obtained by the bilayer method; and those with 'sb' prefixes, directly from the rhizosphere.

[b]Means of two to three replications.

[c]Seeds soaked in sterile distilled water instead of bacterial cell suspensions.

The results showed that the presence of the seeds enhanced growth of antagonists and resulted in formation of wider inhibition zones, although variation among treatments occurred. This phenomenon was no doubt due to seed exudate and it suggested that an important consideration in the selection of antagonists might be their ability to make use of the substances exuded from the seeds and possibly roots.

2. Actinomycetes. Inhibition zones produced by this group of microorganisms were also larger on PDA than on seed exudate agar. The inhibition zones were also associated with poor germination, bursting of spores, and lysis of hyphae or endoconidia. Of the eighteen inhibitory isolates, poor germination was associated with thirteen, lysis with nine, and bursting of spores with four (Table 4).

Tests for antibiotic production in seed exudate broth showed that only three of the eighteen isolates produced inhibition zones when filter paper discs impregnated with culture filtrates placed on PDA were sprayed with a suspension of T. basicola endoconidia (Table 4). Culture filtrates of two additional isolates inhibited growth of T. basicola on the filter paper discs, but no zones were produced.

Agar squares containing T. basicola endoconidia transferred from the inhibition zones of the three isolates to fresh PDA showed loss of viability associated with two isolates (Table 5). Loss of viability in this assay did not involve bursting of endoconidia.

3. Fungi. All fungal isolates collected overgrew T. basicola in paired cultures. Six slower growing fungi (Penicillium sp., Myrothecium sp., and Gliocladium sp.) produced expanding inhibition zones into which the antagonists continued to grow. Two of these isolates (both of the

Table 4. Production of inhibition zones by actinomycetes, antibiotic production, and characteristics of the inhibition as determined microscopically three days after spraying agar plates containing individual isolates with a suspension of Thielaviopsis basicola endoconidia.

Isolate[a]	Inhibition zones[b]		Antibiotic production[b]	Microscopic observation		
	PDA	SEA[c]		Germination inhibition	Bursting of spores	Lysis[d]
a1	+	±	-	+	-	+
a2	+	±	±	+	-	+
a3	+	+	-	+	+	+
a4	+	±	-	+	+	+
a5	++	++	++	+	-	-
L1	+++	++	++	+	-	+
L2	±	±	-	-	-	-
L3	±	±	-	+	-	-
L4	-	±	-	-	-	-
L5	+	±	-	+	+	-
L6	±	±	-	-	-	-
L7	++	+	-	+	-	-
L8	++	++	++	+	-	+
sa1	++	+	±	+	+	+
sa2	±	±	-	-	-	-
sa3	±	±	-	-	-	-
sa4	±	±	-	+	-	+
sa5	++	-	-	+	-	+

[a]Isolates with 'a' prefixes were obtained by the bilayer method; those with 'L' prefixes, from the Fusarium mycelial mats; and those with 'sa' prefixes, directly from the rhizosphere.

[b]- = No inhibition zone; ± = inhibition zone not distinct; + = inhibition zone 0.1-0.5 cm wide; ++ = inhibition zone 0.6-1.0 cm wide; +++ = inhibition zone > 1 cm wide.

[c]SEA: seed exudate agar.

[d]Lysis of hyphae and endoconidia.

Table 5. Production of inhibition zones by filter paper discs impregnated with culture filtrates of actinomycete isolates, and viability of Thielaviopsis basicola endoconidia in the inhibition zones surrounding the filter paper discs.

Isolate[a]	Inhibition zone[b]	Viability[c]
a2	±	
a5	++	-
L1	++	-
L8	++	+
sa1	±	

[a]Isolates with 'a' prefixes were obtained by the bilayer method; those with 'L' prefixes, from the Fusarium mycelial mats; and that with the 'sa' prefixes, directly from the rhizosphere.

[b]± = No T. basicola growth on discs, but no inhibition zone on agar; ++ = inhibition zone 0.6-1.0 cm wide.

[c]+ = Growth of T. basicola was observed from agar pieces transferred from the inhibition zones to fresh PDA; -: no growth of T. basicola; viability of T. basicola associated with isolates a2 and sa1 was not tested.

genus Gliocladium) were observed to parasitize T. basicola. Again, the inhibition zones were more prominent on PDA than on seed exudate agar, and all were associated with lysis of hyphae and endoconidia of T. basicola. Bursting of endoconidia was associated with inhibition zones produced by four, and poor endoconidial germination was associated with inhibition zones produced by five of these six isolates (Table 6).

Diffusible antibiotics were produced by eleven of twenty-seven isolates growing in seed exudate broth (Table 7). Trichoderma pelluliferum ($T2_1$) and T. hamatum ($T3_1$) produced strong inhibition zones when filter paper discs were impregnated with their culture filtrates, while T. harzianum ($T1_1$) produced none.

Paired cultures of T. basicola and individual fungal isolates were prepared to test the viability of T. basicola in the overgrown areas. Homogenates of agar discs taken from the overgrown areas showed variation in viability, as indicated by the numbers of T. basicola colonies arising on the selective medium (Figure 3, Table 7). Homogenates of agar discs taken from T. basicola monocultures (control plates) yielded an average of seven colonies of the fungus. Of twenty-seven fungal isolates, sixteen caused complete loss of viability of the overgrown T. basicola. Of these sixteen isolates, five produced indistinct inhibition zones while two failed to produce inhibition zones. One isolate which produced a strong inhibition zone yielded sixteen T. basicola colonies, a number much higher than that of the control. Production of antibiotics in culture, or size of inhibition zones, therefore, could not be related to loss of viability.

Table 6. Production of inhibition zones by fungi isolated by the bilayer method, antibiotic production, and characteristics of the inhibition as determined microscopically three days after spraying agar plates containing individual isolates with a suspension of Thielaviopsis basicola endoconidia.

Isolate	Inhibition zones[a]		Antibiotic production[a]	Microscopic observation			
	PDA	SEA[b]		Germination inhibition	Bursting of spores	Lysis[c]	Parasitism
f1 (Myrothecium sp.)	++	+	++	+	+	+	-
f2 (Myrothecium sp.)	++	+	++	+	+	+	-
f4 (Penicillium sp.)	±	-	+	-	-	+	-
f8 (Gliocladium sp.)	+	±	±	+	+	+	+
f9 (Penicillium sp.)	+	±	++	+	-	+	-
f15 (Gliocladium sp.)	+	±	±	+	+	+	+

[a] -: No inhibition zone; ± = inhibition zone not distinct; + = inhibition zone 0.1-0.5 cm wide; ++ = inhibition zone 0.6-1.0 cm wide.

[b] SEA = seed exudate agar.

[c] Lysis of hyphae and endoconidia.

Table 7. Production of inhibition zones by filter paper discs impregnated with culture filtrates of fungal isolates, and viability of *Thielaviopsis basicola* in fifteen-day-old overgrown cultures.

Isolates[a]	Inhibition zones[b]	*T. basicola* colonies[c]
Control	-	7
f1 (*Myrothecium* sp.)	+	0
f2 (*Myrothecium* sp.)	+	0
f3 (Asporogenous deuteromycete)	±	10
f4 (*Penicillium* sp.)	+	0
f5 (*Fusarium* sp.)	+	1
f6 (*Penicillium* sp.)	±	0
f7 (*Fusarium* sp.)	-	2
f8 (*Gliocladium* sp.)	±	0
f9 (*Penicillium* sp.)	++	0
f10 (*Fusarium* sp.)	+	0
f11 (*Fusarium* sp.)	±	3
f12 (*Aspergillus* sp.)	±	0
f13 (*Alternaria* sp.)	++	16
f14 (Asporogenous deuteromycete)	-	13
f15 (*Gliocladium* sp.)	±	0
f16 (*Fusarium* sp.)	+	5
f17 (*Fusarium* sp.)	±	0
f18 (*Actinomucor* sp.)	-	1
f19 (*Rhizopus* sp.)	-	13
f20 (*Actinomucor* sp.)	-	0
f21 (*Penicillium* sp.)	-	1
f22 (*Aspergillus* sp.)	-	0
bf1 (*Fusarium* sp.)	-	3
bf2 (*Fusarium* sp.)	++	0
$T1_1$ (*Trichoderma harzianum*)	-	0
$T2_1$ (*Trichoderma pelluliferum*)	++	0
$T3_1$ (*Trichoderma hamatum*)	++	0

[a]Isolates with prefixes 'f' and 'T' were obtained by the bilayer method and those with 'bf' prefixes, directly from the rhizosphere.

[b]- = No inhibition zone; ± = no growth of *T. basicola* on discs, but no inhibition zone produced; + = inhibition zone 0.1-1.0 cm wide; ++ = inhibition zone > 1 cm wide.

[c]Mean number of colonies of two plates; homogenates of agar discs taken from the overgrown areas were plated on a medium selective for *T. basicola*.

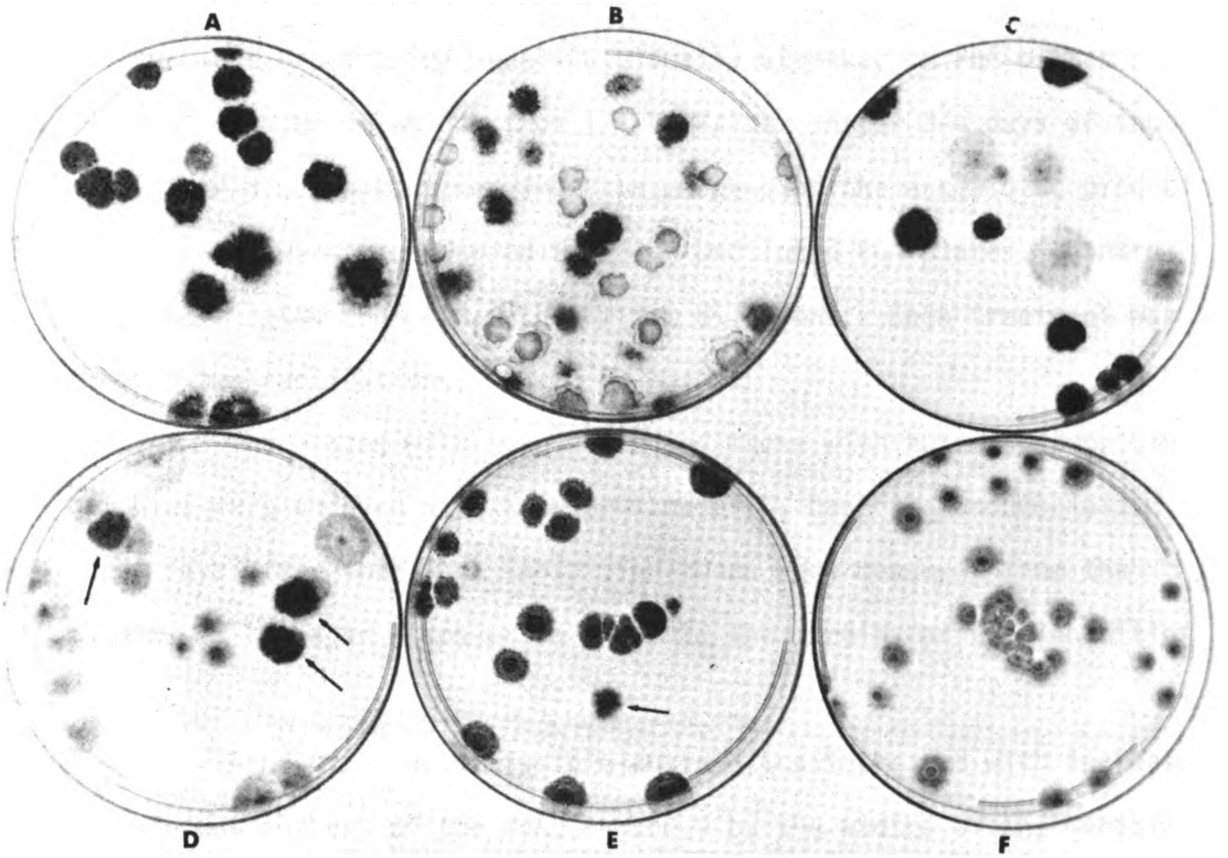

Figure 3. Viability of overgrown Thielaviopsis basicola in paired cultures with six different fungal antagonists as shown by the number of colonies arising on a selective medium. A) Rhizopus sp., B) an asporogenous deuteromycete, C & D) Fusarium spp., E & F) Penicillium spp. Darkest colonies are T. basicola, some of which are indicated by arrows.

Antibiotic production and nutrient deprivation on diluted PDA

Isolates known to produce inhibition zones through antibiosis and nutrient deprivation were individually streaked on PDA plates prepared at various dilutions from 1/1 to 1/128. After 3-4 days of incubation, agar discs were taken from the margins of the antibiotic producing colonies and were transferred to fresh undiluted PDA plates. Control discs were taken from PDA with similar dilutions. Each treatment was done in two replications.

The plates with agar discs and those with nutrient-depriving colonies were sprayed with a suspension of T. basicola endoconidia. Three days after spraying, inhibition zones were measured from the margins of the agar discs or colonies to the margins of the inhibition zones.

Production of diffusible antibiotics increased with increase in nutrient content of the PDA, as shown by the widths of the inhibition zones produced (Figure 4). In some cases increase in dilution resulted to some extent in a slight increase in widths of inhibition zones produced by the nutrient-depriving colonies (Table 8), but this phenomenon was not reproducible in repeated experiments. In other cases the more diluted the medium the narrower were the inhibition zones produced.

Biological control experiments with soybean root rot caused by T. basicola

Sieved, steamed soil mix was infested with a suspension of T. basicola endoconidia and chlamydospores and the final inoculum

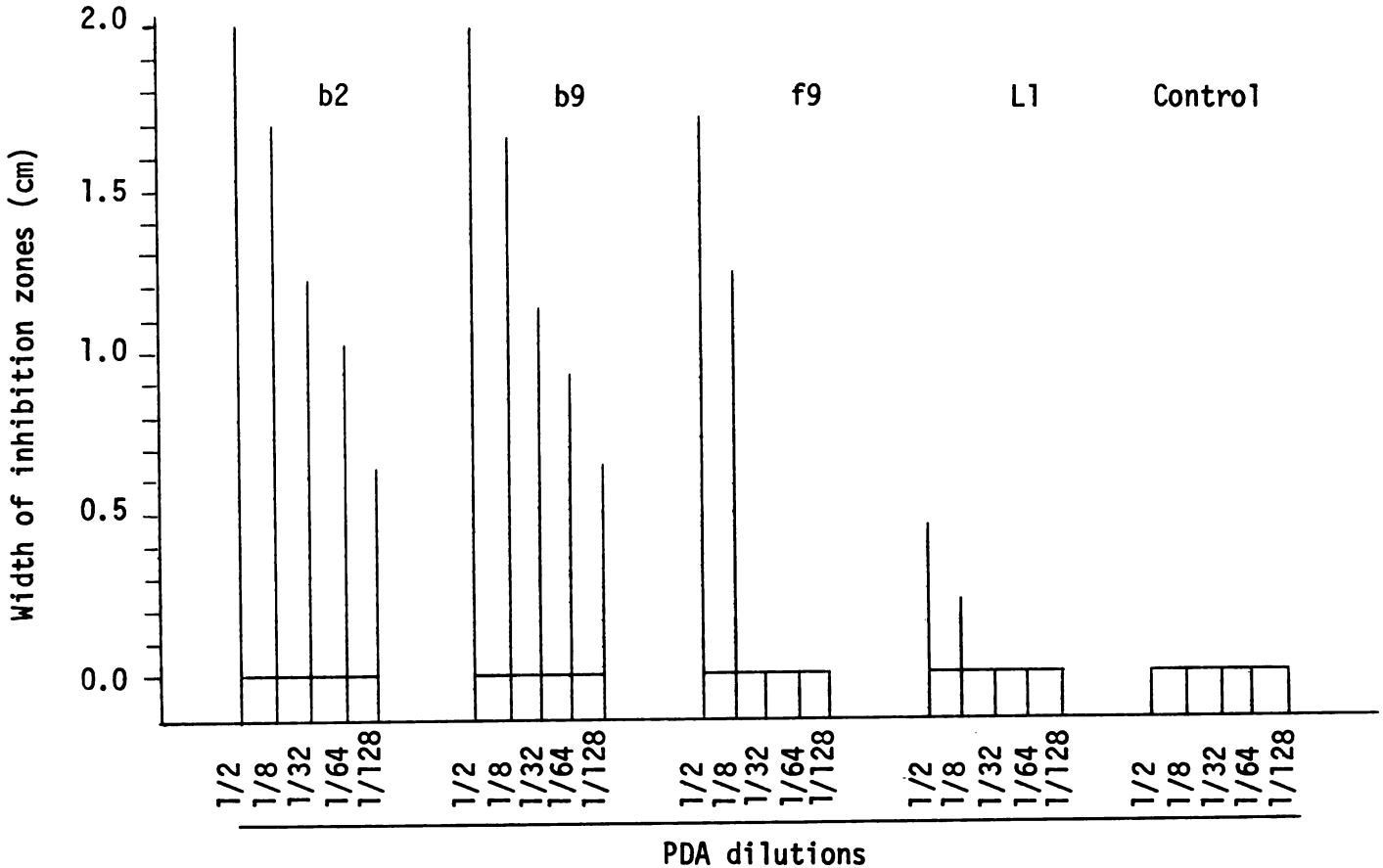

Figure 4. Effect of various dilutions of PDA on production of diffusible antibiotics, as expressed by the width of the inhibition zones produced. b2 and b9 were bacteria, f9 was a fungus (Penicillium), and L1 was a Streptomyces.

Table 8. Effect of various dilutions of PDA on formation of inhibition zones against *Thielaviopsis basicola* by nutrient-depriving colonies of actinomycetes.

Isolates[a]	Width of inhibition zones (mm)[b] on diluted PDA					
	1/1	1/4	1/16	1/32	1/64	1/128
a2	4.5	4.0	3.0	3.0	2.5	3.0
a3	0.5	0.5	1.0	2.0	2.0	2.0
L7	5.5	5.5	6.0	7.5	5.5	5.5

[a]Isolates with 'a' prefixes were obtained by the bilayer method and that with the 'L' prefix, from the *Fusarium* mycelial mats.

[b]Means of two measurements.

concentration was adjusted to 10^3-10^4 propagules per g of soil. This soil was used in the first four experiments.

In the first experiment ten bacterial and eighteen fungal isolates were tested, each with 9-12 single plant replications. One bacterial isolate (sb2) showed an average disease index of 1.8, as compared to 2.9 for the control ($\underline{P}$=0.05) (Figure 5, Table 9).

In the second experiment, nine bacterial and thirteen fungal isolates were tested, each with 7-10 single plant replications. One bacterial isolate (b9) showed an average disease index of 2.3, as compared to 4.0 for the control ($\underline{P}$=0.01); another bacterial isolate (sb4) significantly reduced disease index to 2.5 ($\underline{P}$=0.05) (Figure 6, Table 10).

In the third and fourth experiments, nineteen actinomycete and four fungal isolates were tested, each with 6-10 single plant replications. Four actinomycete isolates (sa4, sa5, L8, and a3) showed average disease indices of 1.9, 2.1, 2.1, and 2.1, respectively, as compared to 3.9 for the control ($\underline{P}$=0.01) (Table 11). One other actinomycete isolate (sa2) significantly reduced disease index to 2.4 ($\underline{P}$=0.05). Three fungal isolates (f20, f21, and f22) gave average disease indices of 1.9, 2.0, and 2.4, respectively, as compared to 4.5 for the control ($\underline{P}$=0.01) (Table 11). One fungal isolate (bf2) significantly reduced disease index to 3.2 ($\underline{P}$=0.05).

Sieved Conover loam-white silica sand mix was infested as previously described, but the final concentration was adjusted to 10^4-10^5 propagules per g of soil. Three bacterial, two actinomycete, and three fungal isolates which had reduced disease severity significantly in the previous experiments were tested using this soil. In addition,

Figure 5. Reduction in severity of soybean root rot caused by Thielaviopsis basicola, by antagonists applied to the seeds. The seeds were sown in steamed soil mix infested with the pathogen. Left, control; center, seed-coating with bacterial isolate sb2; right, seed-coating with fungal isolate Tl_3 (Trichoderma harzianum).

Figure 6. Reduction in severity of soybean root rot caused by Thielaviopsis basicola, by antagonists applied to the seeds. The seeds were sown in steamed soil mix infested with the pathogen. Left, control; center, seed-coating with bacterial isolate b9; right, seed-coating with bacterial isolate sb4.

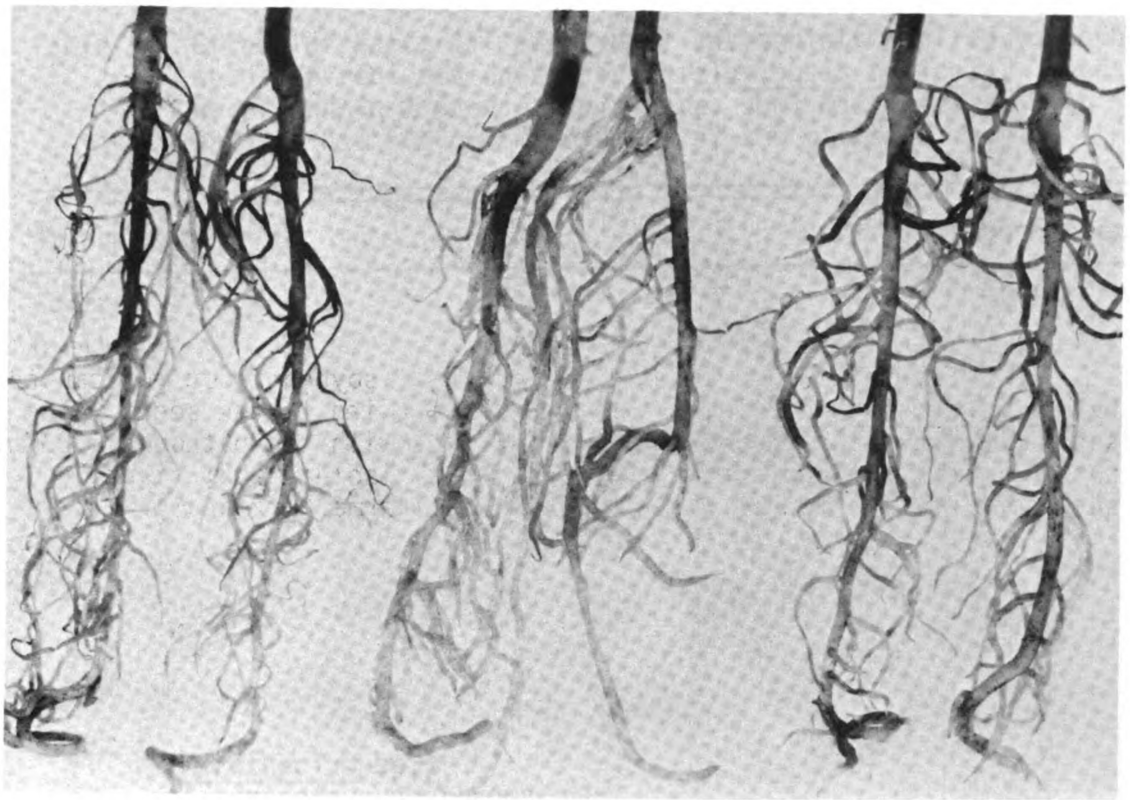

Table 9. Effect of bacterial and fungal antagonists applied to soybean seeds on root rot caused by *Thielaviopsis basicola*. Seeds were sown in a previously steamed soil mix infested with endoconidia and chlamydospores of the pathogen.

Antagonists[a]		Disease Index[b]
Control		2.9
Bacteria		
b1		3.3
b2		2.7
b3		2.4
b4		3.0
b5		2.9
b7		3.8
b8		2.5
sb1		2.1
sb2		1.8*
sb3		2.1
Fungi		
f1	(*Myrothecium* sp.)	3.7
f3	(Asporogenous deuteromycete)	2.8
f4	(*Penicillium* sp.)	2.9
f5	(*Fusarium* sp.)	2.7
f6	(*Penicillium* sp.)	2.6
f7	(*Fusarium* sp.)	2.4
f8	(*Gliocladium* sp.)	2.8
f9	(*Penicillium* sp.)	3.1
f10	(*Fusarium* sp.)	2.9
bf1	(*Fusarium* sp.)	2.3
$T1_1$	(*Trichoderma harzianum*)	3.0
$t1_2$	(*Trichoderma harzianum*)	2.4
$T1_3$	(*Trichoderma harzianum*)	2.4
$T1_4$	(*Trichoderma harzianum*)	3.2
$T1_5$	(*Trichoderma harzianum*)	2.8
$T1_6$	(*Trichoderma harzianum*)	3.4
$T2_1$	(*Trichoderma pelluliferum*)	2.1
$T3_3$	(*Trichoderma hamatum*)	2.8
Shortest Significant Range (SSR)[c], P=0.05		

[a]Each treatment had 9-12 single plant replications; isolates with prefixes 'b', 'f', and 'T' were obtained by the bilayer method; and those with prefixes 'sb' and 'bf', directly from the rhizosphere.

[b]Disease was estimated on a scale of 0-6, with 0 indicating no disease and 6, severe disease.

[c]Duncan's Multiple Range test; the disease index for each isolate was compared with that of the control only.

Table 10. Effect of bacterial and fungal antagonists applied to soybean seeds on root rot caused by Thielaviopsis basicola. Seeds were sown in a previously steamed soil mix infested with endoconidia and chlamydospores of the pathogen.

Antagonists[a]		Disease Index[b]
Control		4.0
Bacteria		
sb4		2.5*
b9		2.3**
b10		3.9
b11		2.8
b12		2.8
b13		3.7
b15		3.9
b16		4.3
b17		3.1
Fungi		
f11	(Fusarium sp.)	3.4
f12	(Aspergillus sp.)	3.2
f13	(Alternaria sp.)	3.7
f14	(Asporogenous deuteromycete)	3.9
f15	(Gliocladium sp.)	3.7
f16	(Fusarium sp.)	3.1
f17	(Fusarium sp.)	2.9
f18	(Actinomucor sp.)	3.6
f19	(Rhizopus sp.)	4.0
$T1_5$	(Trichoderma harzianum)	3.0
$T1_7$	(Trichoderma harzianum)	3.1
$T2_2$	(Trichoderma pelluliferum)	3.3
$T3_4$	(Trichoderma hamatum)	3.6
Shortest Significant Range (SSR)[c], P=0.05		1.3
P=0.01		1.6

[a]Each treatment had 7-10 single plant replications; isolates with prefixes 'b', 'f', and 'T' were obtained by the bilayer method; and that with the 'sb' prefix, directly from the rhizosphere.

[b]Disease was estimated on a scale of 0-6, with 0 indicating no disease and 6, severe disease.

[c]Duncan's Multiple Range test; the disease index for each isolate was compared with that of the control only.

Table 11. Effect of actinomycete and fungal antagonists applied to soybean seeds on root rot caused by Thielaviopsis basicola. Seeds were sown in a previously steamed soil mix infested with endoconidia and chlamydospores of the pathogen.

Antagonists[a]	Disease index[b]	SSR[c] P=0.05	SSR[c] P=0.01
Control	3.9		
Actinomycetes			
L1	3.2		
L2	2.7		
L3	3.1		
L4	4.4		
L5	4.2		
L7	3.3		
L8	2.1**		1.7
a1	3.1		
a2	3.4		
a3	2.1**		1.7
a4	4.0		
a5	3.2		
sa1	2.9		
sa2	2.4*	1.3	
$sa3_1$	3.6		
$sa3_2$	3.8		
$sa4^2$	2.1**		1.7
sa5	1.9**		1.9
Control	4.6		
Fungi			
f20 (Actinomucor sp.)	2.4**		1.5
f21 (Penicillium sp.)	1.9**		1.9
f22 (Aspergillus sp.)	2.0**		1.5
bf2 (Fusarium sp.)	3.2*	1.2	

[a]Each treatment had 6-10 single plant replications; isolates with prefixes 'a' and 'f' were obtained by the bilayer method; those with prefixes 'sa' and 'bf', directly from the rhizosphere; and those with the 'L' prefixes, from the Fusarium mycelial mats.

[b]Disease was estimated on a scale of 0-6, with 0 indicating no disease and 6, severe disease.

[c]Shortest Significant Range from Duncan's Multiple Range test; the disease index for each isolate was compared with that of the control only.

five bacterial and four fungal isolates which had produced lower disease indices (but not significantly), and one avirulent T. basicola isolate 173 (Maduewesi and Lockwood, unpublished results) were included. Each treatment consisted of 8-16 single plant replications. Of the eighteen isolates tested, plants from seeds coated with three bacterial isolates (sb1, sb2, and sb3) and one fungal isolate (Fusarium sp.) had disease indices of 1.5, 1.4, 1.6, and 1.6, respectively, as compared to 2.6 for the control (P=0.05) (Table 12). Although only four isolates showed statistically significant differences from the control, all except one (L8) produced disease indices lower than that of the control.

The fungal isolate which gave significant biological control in Conover loam mix was identified as Fusarium solani (29). The two bacterial isolates sb2 and sb3 were identified as Bacillus spp. and the bacterial isolate sb1 was identified as Achromobacter sp. Several characteristics of those bacterial isolates are listed in Table 13.

Effect of coated soybean seeds on root rot development in water agar

Water agar in glass test tubes was seeded with T. basicola endoconidia and soybean seeds coated with individual antagonists were placed on the top of the agar columns. Uncoated seeds and unseeded water agar were used for comparison. After ten days' incubation under continuous fluorescent light (8,600 lux) at 25°C, root rot development was observed. Root rot severity was rated on a scale of 0-3, with 0 indicating no disease and 3, severe disease.

Table 12. Effect of bacterial, actinomycete, and fungal antagonists applied to soybean seeds on root rot caused by Thielaviopsis basicola. Seeds were sown in a Conover loam-white silica sand mix infested with endoconidia and chlamydospores of the pathogen.

Antagonists[a]	Disease index[b]	SSR[c] P=0.05
Control	2.6	
Bacteria[d]		
sb1	1.5*	0.9
sb2	1.4*	0.9
sb3	1.6*	0.9
sb4	2.5	
b8	2.0	
b9	2.5	
b11	2.2	
b12	1.9	
Fungi[d]		
Thielaviopsis basicola isolate 173	2.3	
f17 (Fusarium sp.)	1.9	
f20 (Actinomucor sp.)	2.4	
f21 (Penicillium sp.)	2.4	
f22 (Aspergillus sp.)	1.9	
bf1 (Fusarium sp.)	1.6*	0.9
$T1_2$ (Trichoderma harzianum)	1.8	
$T2_1$ (Trichoderma pelluliferum)	2.0	
Control	2.4	
Actinomycetes[e]		
a3	2.2	
L8	2.6	

[a]Isolates with prefixes 'b', 'f', 'T', and 'a' were obtained by the bilayer method; those with prefixes 'sb' and 'bf', directly from the rhizosphere; and that with the 'L' prefix, from the Fusarium mycelial mats.

[b]Disease was estimated on a scale of 0-6, with 0 indicating no disease and 6, severe disease.

[c]Shortest Significant Range from Duncan's Multiple Range test; the disease index of each isolate was compared with that of the control only.

[d]Each treatment had 9-16 single plant replications.

[e]Each treatment had 8-12 single plant replications.

Table 13. Several morphological and physiological characteristics of three bacterial antagonists giving significant biological control of soybean root rot caused by Thielaviopsis basicola in Conover loam.

Characteristics	Isolates		
	sb1	sb2	sb3
Gram stain	Negative	Positive	Positive
Shape	Small rods	Rods	Rods in chains
Size (microns)	0.5 x 0.7	2.7 x 0.6	8.0 x 1.5
Motility	Motile	Motile	Motile
Endospore production	Negative	Positive	Positive
Litmus milk reaction	Non-acidic No clearing	Non-acidic No clearing	Non-acidic Clearing
Oxygen requirement	Obligate aerobe	Obligate aerobe	Obligate aerobe
H2S test	Negative	Negative	Negative
Reaction in phenol-red dextrose agar	Slightly basic	Basic	Basic
Fluorescence in King's B medium	Negative	Negative	Negative
Catalase test	Weakly positive	Positive	Positive

One bacterial antagonist (b9) significantly reduced root rot severity with an average disease index of 1.7, as compared to 2.7 for the control ($\underline{P}$=0.05) (Figure 7, Table 13). Two fungal antagonists (bf1 and Tl_3) also significantly reduced root rot severity with disease indices of 1.7 and 0.2 ($\underline{P}$=0.05 and $\underline{P}$=0.01, respectively) (Figure 7, Table 14). The avirulent $\underline{T}$. $\underline{basicola}$ isolate 173 again failed to reduce root rot severity significantly. One bacterial (sb2) and one fungal (f9) antagonists gave disease indices higher than that of the control.

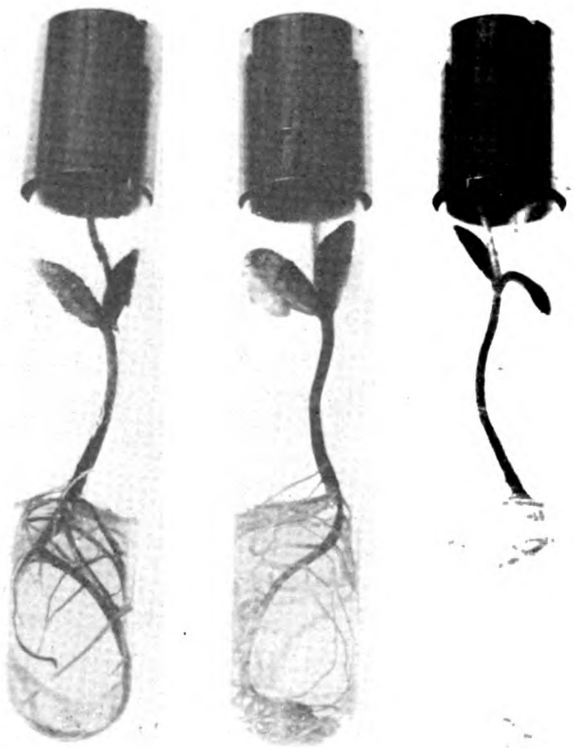

Figure 7. Reduction in severity of soybean root rot caused by Thielaviopsis basicola, by antagonists applied to the seeds. The seeds were sown in water agar seeded with the pathogen. Left, control; center, seed-coating with bacterial isolate b9; right, seed-coating with fungal isolate $T1_3$ (Trichoderma harzianum).

Table 14. Effect of soybean seeds coated with antagonists on root rot development in water agar seeded with endoconidia of *Thielaviopsis basicola*.

Antagonists[a]	Disease index[b]	SSR[c] P=0.05	SSR[c] P=0.01
Control	2.7		
Bacteria			
b9	1.7*	0.9	
sb1	2.2		
sb2	3.0		
Fungi			
T. basicola isolate 173	2.3		
f9 (*Penicillium* sp.)	3.0		
bf1 (*Fusarium* sp.)	1.7*	0.8	
Tl_3 (*Trichoderma harzianum*)	0.2**		1.2
Actinomycete			
L8 (*Streptomyces* sp.)	2.0		

[a]Each treatment had 2-4 single plant replications; isolates with prefixes 'b', 'f', and 'T' were obtained by the bilayer method; those with prefixes 'sb' and 'bf', directly from the rhizosphere; and that with the 'L' prefix, from the *Fusarium* mycelial mats.

[b]Disease was estimated on a scale of 0-3, with 0 indicating no disease and 3, severe disease.

[c]Shortest Significant Range from Duncan's Multiple Range test; the disease index for each isolate was compared with that of the control only.

DISCUSSION

Broadbent et al. (14) found that of more than 3,500 bacterial and actinomycete isolates tested, about 40% inhibited one or more of nine root pathogens on agar and about 4% were effective in steamed soil; even fewer would be expected to be effective under commercial conditions. They chose non-rhizosphere soils as the sources of antagonists. Due to the very low probability of obtaining antagonists effective in field conditions, they suggested that large-scale screening methods are essential for success in biological control. Even if an introduced organism became established in a soil at a level of population higher than in the uninoculated soil, there is still no guarantee that it would be antagonistic to a pathogen on or near the root surface, because this might not be a suitable substrate for the introduced organism (36).

One hundred percent of the seventeen bacteria and five actinomycetes isolated by the bilayer method were antagonistic to T. basicola in paired cultures. Antagonism was either associated with inhibition of endoconidial germination, bursting of endoconidia, lysing hyphae and endoconidia, or with a combination of the three mechanisms (Tables 1 and 4). The success in biological control in steamed soil was also greater than that found by Broadbent et al. (14). Of these twenty-two isolates, one bacterium and one actinomycete (9.1%) significantly reduced root rot in steamed soil mix.

The bilayer method also was highly efficient in obtaining fungal antagonists. Of the forty-six fungi isolated, all overgrew T. basicola in paired cultures; sixteen of twenty-five isolates tested caused a complete loss of viability of the pathogen in overgrown areas, while an additional four caused a marked reduction in viability (Table 7). Of thirty-five isolates tested for biological control (Tables 9-12), three (about 8%) significantly reduced root rot in steamed soil.

On the other hand, antagonists obtained directly from the rhizosphere by random selection showed a higher proportion of success in the biological control experiments. Of the four bacteria so obtained, two (50%) gave significant biological control in steamed soil and three (75%) in Conover loam; one of these reduced disease significantly in both soils (Table 15). Of the six actinomycetes thus isolated, three (50%) gave significant biological control in steamed soil and none in Conover loam (Table 16). Of the two fungi thus isolated, one (50%) gave significant biological control in steamed soil and one (50%) in Conover loam. Overall, six of twelve isolates reduced disease in steamed soil, and four of twelve did so in Conover loam.

Actinomycetes isolated from the lysed Fusarium hyphae resulted in 14.3% success in steamed soil (Table 16).

Success in biological control was not closely related to antibiotic production on agar plates or in liquid medium. Of the twelve antagonists significantly reducing disease severity in steamed soil (Tables 15-17), only three produced antibiotics and these three antagonists failed to reduce disease severity significantly in Conover loam. Only one of eight bacteria, one of three actinomycetes, and one of ten

Table 15. Results of laboratory observation of bacterial antagonists as related to their performance in the biological control experiments with soybean root rot caused by Thielaviopsis basicola.

Isolates[a]	Inhibition zone[b]	Antibiotic production[b]	Viability[c]	Germination inhibition[d]	Bursting of endoconidia[d]	Lysis of hyphae or endoconidia[d]	Disease index[e]			
							Greenhouse mix	Control	Conover loam mix	Control
b1	-	-		+	+	+	3.3	2.9		
b2	+++	+++	-	+	+	+	2.7			
b3	-	-		+	+	+	2.4			
b4	+++	++	+	+	-	+	3.0			
b5	+	-		+	-	+	2.9			
b6	+	-		+	-	+				
b7	+	-		+	-	+	3.8			
b8	++	++	+	+	-	+	2.5		2.0	2.6
sb1	±	-		-	-	+	2.1		1.5*	
sb2	+	-		+	-	+	1.8*		1.4*	
sb3	-	-		-	-	+	2.1		1.6*	
sb4	+	-		-	-	+	2.5*	4.0	2.5	
b9	+++	++	-	+	+	+	2.3**		2.5	
b10	±	-		+	+	+	3.9			
b11	+	-		+	+	+	2.8		2.2	
b12	+++	+++	-	+	+	+	2.8			
b13	+++	+++	-	+	+	+	3.7			
b14	-	-		+	+	+				
b15	+++	++	-	+	+	+	3.9			
b16	+	-		+	+	+	4.3			
b17	+++	++	-	+	+	+	3.1			

[a]Isolates with 'b' prefixes were obtained by the bilayer method and those with 'sb' prefixes, directly from the rhizosphere.

[b]Based on seed exudate agar; - = no inhibition zone, ± = inhibition zone not distinct; + = inhibition zone 0.1-0.5 cm wide; ++ = inhibition zone 0.6-1.0 cm wide; +++ = inhibition zone > 1 cm wide.

[c]- = Loss in viability; + = T. basicola remained viable.

[d]Microscopic observation on PDA plates; + = respective mechanism present; - = respective mechanism absent.

[e]Scale from 0 to 6; 0 being disease free and 6 being most severely diseased. *Indicates statistical significance at P=0.05; **indicates statistical significance at P=0.01.

Table 16. Results of laboratory observation of actinomycete antagonists as related to their performance in the biological control experiments with soybean root rot caused by Thielaviopsis basicola.

Isolates[a]	Inhibition zone[b]	Antibiotic production[b]	Viability[c]	Germination inhibition[d]	Bursting of endoconidia[d]	Lysis of hyphae or endoconidia[d]	Disease index[e]			
							Greenhouse mix	Control	Conover loam mix	Control
L1	++	++	-	+	-	+	3.2	3.9		
L2	±	-		-	-	-	2.7			
L3	±	-		+	-	-	3.1			
L4	±	-		-	-	-	4.4			
L5	±	-		+	+	-	4.2			
L7	+	-		+	-	-	3.3			
L8	++	++	+	+	-	+	2.1**		2.6	2.4
a1	±	-		+	-	+	3.1			
a2	±	±		+	-	+	3.4			
a3	+	-		+	+	+	2.1**		2.2	
a4	±	-		+	+	+	4.0			
a5	++	++	-	+	-	-	3.2			
sa1	+	±		+	+	+	2.9			
sa2	±	-		-	-	-	2.4*			
$sa3_1$	±	-		-	-	-	3.6			
$Sa3_2$	±	-		-	-	-	3.8			
$sa4^2$	±	-		+	-	-	2.1**			
sa5	-	-		+	-	+	1.9**			

[a]Isolates with 'a' prefixes were obtained by the bilayer method; those with 'L' prefixes, from the lysed Fusarium hyphae; and those with 'sa' prefixes, directly from the rhizosphere.

[b]Based on seed exudate agar or culture filtrate with seed exudate broth as growth medium; - = no inhibition zone; ± = inhibition zone not distinct; + = inhibition zone 0.1-0.5 cm wide; ++ = inhibition zone 0.6-1.0 cm wide.

[c]- = Loss in viability; + = T. basicola remained viable.

[d]Microscopic observation on PDA plates; + = respective mechanism present; - = respective mechanism absent.

[e]Scale from 0 to 6; 0 being disease free and 6 being most severely diseased. *Indicates statistical significance at P=0.05; **indicates statistical significance at P=0.01.

Table 17. Results of laboratory observation of fungal antagonists as related to their performance in the biological control experiments with soybean root rot caused by Thielaviopsis basicola.

Isolates[a]	Inhibition zone[b]	Antibiotic production[b]	Viability[c]	Parasitism[d]	Germination inhibition[d]	Bursting of endoconidia[d]	Lysis of hyphae or endoconidia[d]	Disease index[e]			
								Greenhouse mix	Control	Conover loam mix	Control
f1	++	++	0		+	+	+	3.7	2.9		
f3		±	10					2.8			
f4	±	+	0		-	-	+	2.9			
f5		+	1					2.7			
f6		±	0					2.6			
f7		-	2					2.4			
f8	+	±	0	+	+	+	+	2.8			
f9	+	++	0		+	-	+	3.1			
f10		+	0					2.9			
bf1		-	3					2.3		1.6*	2.6
$T1_1$		-	0					3.0			
$T2_1$		+++	0					2.1		2.0	
$T3_1$		+++	0								

f12		±	0					3.2			
f13		+++	16					3.7			
f14		-	13					3.9			
f15	+	±	0	+	+	+	+	3.7			
f16		+	5					3.1			
f17		±	0					2.9		1.9	
f18		-	1					3.6			
f19		-	13					4.0			
f20		-	0					2.4*	4.6	2.4	
f21		-	1					1.9**		2.4	
f22		-	0					2.0**		1.9	
bf2		+++	0					3.2*			

[a]Isolate numbers, for reference to generic names and prefixes, consult Table 6.

[b]Based on seed exudate agar or culture filtrate with seed exudate broth as growth medium; - = no inhibition zone; ± = inhibition zone not distinct; + = inhibition zone 0.1-0.5 cm wide; ++ = inhibition zone 0.6-1.0 cm wide; +++ = inhibition zone > 1 cm wide.

[c]Number of colonies of T. basicola arising from the overgrown areas, as compared to seven colonies in the control.

[d]Microscopic observation on PDA plates; + = respective mechanism present; - = respective mechanism absent.

[e]Scale from 0-6; with 0 indicating disease free and 6, severely diseased. *Indicates statistical significance at P=0.05; **indicates statistical significance at P=0.01.

fungi which clearly produced diffusible antibiotics on agar plates or liquid medium reduced disease significantly in steamed soil; while four of thirteen bacteria, four of thirteen actinomycetes, and four of nine fungi which did not produce antibiotics reduced disease significantly either in steamed soil or in Conover loam. Therefore, antagonism by virtue of nutrient competition may be more important than antibiotics in biological control of T. basicola. Other characteristics of antagonism, either individually or in combinations, were also not consistently associated with success in biological control (Tables 15-17).

Success in biological control in steamed soil appeared to be somewhat greater with isolates producing distinct inhibition zones in paired cultures on seed exudate agar (29%) than with those producing indistinct or no zones (17%) (Tables 1, 4, 6, 15-17). However, success in natural Conover loam was about equal for the two groups, i.e., 6% and 8%, respectively.

Production of inhibition zones on a too-rich medium may not reflect antagonistic ability in a medium similar to root exudate. Isolates that produced inhibition zones on PDA often produced none or smaller zones on seed exudate agar (Tables 1 and 4). However, of the twenty-nine isolates producing distinct inhibition zones on PDA, 21% reduced disease significantly in either steamed soil or Conover loam, whereas of those producing distinct inhibition zones on seed exudate agar, 24% did so (Tables 1, 4, 15, 16). Therefore, the expected advantage of using seed exudate agar in the evaluation of antagonists was not clearly shown.

In the absence of a set of antagonistic characters clearly predicting success in biological control, selection of antagonists from soybean rhizospheres at present appears to be the best course. Rhizosphere organisms may have a better capability than non-rhizosphere ones to colonize roots, and may more quickly and effectively establish a fungistatic environment in the rhizosphere, where germination of spores of root-infecting fungi occurs (78).

Failure of biological control by many antibiotic- and the non-antibiotic-producing antagonists in natural soil may be due to their sensitivity to inhibitory substances produced by indigenous microorganisms, or to inability to compete successfully for substrate (28). Failure of the antibiotic-producing antagonists may also be due to microbial degradation of their antibiotics. Indigenous antagonists of T. basicola may also have been eliminated due to their sensitivity to the antibiotics produced by the introduced antagonists, resulting in a weaker overall antagonism to the pathogen (13). Exhaustion of nutrient in the rhizosphere may also occur quickly, resulting in lack of substrate for the production of antibiotics. Moreover, isolation of antagonists from the rhizosphere does not necessarily assure that they are rhizosphere residents, unless the frequencies of their occurrence there are high.

The results of the experiments using soil mixes and those using water agar seeded with T. basicola endoconidia showed some correlation. For example, of the eight isolates tested in agar, three produced disease indices significantly lower than the control (Table 14); and of these, bacterial isolate b9 significantly reduced disease

in steamed soil and reduced disease, but not significantly, in Conover loam. The reverse was true for fungal isolate bf1. Trichoderma isolate $T1_3$, that significantly reduced disease severity in water agar, also reduced disease in steamed soil (Tables 9 and 14). However, two bacterial isolates (sb2 and sb3) which gave significant biological control in Conover loam (Table 12) failed to do so in water agar; one even gave a disease index higher than that of the control (Table 14). These two bacteria did not produce diffusible antibiotics (Table 1), and might not have been able to grow along the soybean roots in the water agar to maintain antagonism in the rhizosphere.

The technique using seeded water agar should be further developed and evaluated as an alternative approach for screening antagonists for biological control. Its advantages are that the test period is short (ten days), and it provides a transparent medium making possible continued observations of disease development and possibly of the interactions of the antagonists with the pathogen in the rhizosphere.

Promising results have been obtained in the biological control experiments, and further work toward field testing should be carried out. To increase the probability of obtaining antagonists applicable for biological control under commercial scale, further screening from soybean rhizospheres with the aim of selecting those with high populations and those showing ability to colonize the root surfaces should be done. Suitable combinations of antagonists and suitable food bases applied together with the antagonists may provide higher degree of antagonism in the rhizospheres.

Since pathogenic propagules of roots must germinate in the rhizosphere in order to cause infection, continued effort to find antagonists capable of rapidly colonizing the rhizosphere and depleting its nutrients, and possibly at the same time producing inhibitory substances, may lead to further success in biological control.

LITERATURE CITED

LITERATURE CITED

1. Adams, P. B. and G. C. Papavizas. 1968. Survival of root-infecting fungi in soil. X. Sensitivity of propagules of Thielaviopsis basicola to soil fungistasis in natural and alfalfa-amended soil. Phytopathology 59: 135-138.

2. Aldrich, J. and R. Baker. 1970. Biological control of Fusarium roseum f. sp. dianthi by Bacillus subtilis. Plant Dis. Rep. 54: 446-448.

3. Atkinson, T. G., J. L. Neal, Jr. and R. I. Larson. 1974. Root rot reaction in wheat: resistance not mediated by rhizosphere or laimosphere antagonists. Phytopathology 64: 97-101.

4. Atkinson, T. G., J. L. Neal, Jr. and R. I. Larson. 1975. Genetic control of the rhizosphere microflora of wheat, pp. 116-122. In Biology and control of soil-borne plant pathogens. (G. W. Bruehl, ed.) Am. Phytopathol. Soc., St. Paul, Minn.

5. Baker, K. F. and R. J. Cook. 1974. Biological control of plant pathogens. W. H. Freeman and Co., San Francisco, 433 p.

6. Baker, R. R. 1968. Mechanisms of biological control of soil-borne pathogens. Annu. Rev. Phytopathol. 6: 263-294.

7. Barnett, H. L. and F. L. Binder. 1973. The fungal host-parasite relationship. Annu. Rev. Phytopathol. 11: 273-292.

8. Barnett, H. L. and B. B. Hunter. 1972. Illustrated genera of imperfect fungi. Burgess Publishing Co., Minn., 241 p.

9. Barron, G. L. 1968. The genera of Hyphomycetes from soil. Williams and Wilkins Co., Baltimore, 364 p.

10. Bateman, D. F. 1963. Influence of host and non-host plants upon populations of Thielaviopsis basicola in soil. Phytopathology 53: 1174-1177.

11. Beute, M. K. and J. L. Lockwood. 1968. Mechanism of increased root rot in virus-infected peas. Phytopathology 58: 1643-1651.

12. Boosalis, M. G. 1964. Hyperparasitism. Annu. Rev. Phytopathol. 2: 363-375.

13. Brian, P. W. 1957. The ecological significance of antibiotic production. pp. 168-188. In Microbial ecology. (R. E. O. Williams and C. C. Spicer, eds.) Cambridge Univ. Press, London.

14. Broadbent, P., K. F. Baker and Y. Waterworth. 1971. Bacteria and actinomycetes antagonistic to fungal root pathogens in Australian soils. Aust. J. Biol. Sci. 24: 925-944.

15. Brown, M. E. 1974. Seed and root bacterization. Annu. Rev. Phytopathol. 12: 181-197.

16. Bruehl, G. W. 1975. Systems and mechanisms of residue possession by pioneer fungal colonists. pp. 77-83. In Biology and control of soil-borne plant pathogens. (G. W. Bruehl, ed.) Am. Phytopathol. Soc., St. Paul, Minn.

17. Carter, M. V. 1971. Biological control of Eutypa armeniacae. Aust. J. Exp. Agr. and An. Husb. 11: 687-692.

18. Chang, I. and T. Kommedahl. 1968. Biological control of seedling blight of corn by coating kernels with antagonistic microorganisms. Phytopathology 58: 1395-1401.

19. Clark, F. E. 1965. The concept of competition in microbial ecology. pp. 339-347. In Ecology of soil-borne plant pathogens. (K. F. Baker and W. C. Snyder, eds.) Univ. Calif. Press, Berkeley, Calif.

20. Clough, K. S. and Z. A. Patrick. 1972. Naturally occurring perforations in chlamydospores of Thielaviopsis basicola in soil. Can. J. Bot. 50: 2251-2253.

21. Cook, R. J. and W. C. Snyder. 1965. Influence of host exudates on growth and survival of germlings of Fusarium solani f. phaseoli in soil. Phytopathology 55: 1021-1025.

22. Deacon, J. W. 1973. Control of the take-all fungus by grass leys in intensive cereal cropping. Plant Pathol. 22: 88-94.

23. Dix, N. J. 1972. Effect of soil fungistasis on spore germination and germ-tube growth in Penicillium species. Trans. Brit. Mycol. Soc. 58: 59-66.

24. Dwivedi, R. S. and S. D. Garrett. 1968. Fungal competition in agar plate colonization from soil inocula. Trans. Brit. Mycol. Soc. 51: 95-101.

25. Farley, J. D. and J. L. Lockwood. 1969. Reduced nutrient competition by soil microorganisms as a possible mechanism for pentachloronitrobenzene-induced disease accentuation. Phytopathology 59: 718-724.

26. Flentje, N. T. and H. K. Saksena. 1964. Pre-emergence rotting of peas in South Australia. III. Host-pathogen interaction. Aust. J. Biol. Sci. 17: 665-675.

27. Fokkema, N. J. and J. W. Lorbeer. 1974. Interactions between Alternaria porri and the saprophytic mycoflora of onion leaves. Phytopathology 64: 1128-1133.

28. Garrett, S. D. 1970. Pathogenic root-infecting fungi. Cambridge Univ. Press, Cambridge, 294 p.

29. Garrett, S. D. 1975. Seed- and soil-borne pathogens. pp. 2-4. In Biology and control of soil-borne plant pathogens. (G. W. Bruehl, ed.) Am. Phytopathol. Soc., St. Paul, Minn.

30. Gilman, J. C. 1956. A manual of soil fungi. The Iowa State Univ. Press, Iowa, 450 p.

31. Griffin, D. M. 1972. Ecology of soil fungi. Syracuse Univ. Press, pp. 44-53.

32. Henis, Y., B. Sneh and J. Katan. 1967. Effect of organic amendment on Rhizoctonia and accompanying microflora in soil. Can. J. Microbiol. 13: 643-650.

33. Hsu, S. C. and J. L. Lockwood. 1969. Mechanisms of inhibition of fungi in agar by Streptomyces. J. Gen. Microbiol. 57: 149-158.

34. Hsu, S. C. and J. L. Lockwood. 1973. Soil fungistasis: behavior of nutrient-independent spores and sclerotia in a model system. Phytopathology 63: 334-337.

35. Keeling, B. L. 1974. Soybean seed rot and the relation of seed exudate to host susceptibility. Phytopathology 64: 1445-1447.

36. Kerr, A. 1961. A study of tomato root surface organisms antagonistic to Verticillium albo-atrum. Trans. Brit. Mycol. Soc. 44: 365-371.

37. Kerr, A. 1972. Biological control of crown gall: seed inoculation. J. Appl. Bacteriol. 35: 493-497.

38. Ko, W. H. 1971. Biological control of seedling root rot of papaya caused by Phytophthora palmivora. Phytopathology 61: 780-782.

39. Ko, W. H. and J. L. Lockwood. 1967. Soil fungistasis: relation to fungal spore nutrition. Phytopathology 57: 894-901.

40. Kommedahl, T. and I. C. Mew. 1975. Biocontrol of corn root infection in the field by seed treatment with antagonists. Phytopathology 65: 296-300.

41. Kosuge, T. and W. B. Hewitt. 1964. Exudates of grape berries and their effect on germination of conidia of Botrytis cinerea. Phytopathology 54: 167-172.

42. Kraft, J. M. 1974. The influence of seedling exudates on the resistance of peas to Fusarium and Pythium root rot. Phytopathology 64: 190-193.

43. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 3: 209-230.

44. Linderman, R. G. and T. A. Toussoun. 1967. Behavior of chlamydospores and endoconidia of Thielaviopsis basicola in nonsterilized soil. Phytopathology 57: 729-731.

45. Linderman, R. G. and T. A. Toussoun. 1968b. Pathogenesis of Thielaviopsis basicola in nonsterile soil. Phytopathology 58: 1578-1583.

46. Lloyd, A. B. and J. L. Lockwood. 1963. Effect of soil temperature, host variety and fungus strain on Thielaviopsis root rot of peas. Phytopathology 53: 329-331.

47. Lockwood, J. L. 1964. Soil fungistasis. Annu. Rev. Phytopathol. 2: 341-362.

48. Lockwood, J. L. 1975. Quantitative evaluation of a leaching model system for soil fungistasis. Phytopathology 65: 460-464.

49. Lockwood, J. L., D. L. Yoder and N. A. Smith. 1970. Thielaviopsis basicola root rot of soybeans in Michigan. Plant Dis. Rep. 54: 849-850.

50. Marx, D. H. 1972. Ectomycorrhizae as biological deterrents to pathogenic root infections. Annu. Rev. Phytopathol. 10: 429-454.

51. Marx, D. H. and C. B. Davey. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. IV. Resistance of naturally occurring mycorrhizae to infections by Phytophthora cinamomi. Phytopathology 59: 559-569.

52. Mathre, D. E. and A. V. Ravenscroft. 1966. Physiology of germination of chlamydospores and endoconidia of Thielaviopsis basicola. Phytopathology 56: 337-342.

53. Merriman, P. R., R. D. Price and K. F. Baker. 1974. The effect of inoculation of seed with antagonists of Rhizoctonia solani on the growth of wheat. Aust. J. Agr. Res. 25: 213-218.

54. Merriman, P. R., R. D. Price, J. F. Kollmorgen, T. Piggott and E. H. Ridge. 1974. Effect of seed inoculation with Bacillus subtilis and Streptomyces griseus on the growth of cereals and carrots. Aust. J. Agr. Res. 25: 219-226.

55. Michael, A. H. and P. E. Nelson. 1972. Antagonistic effect of soil bacteria on Fusarium roseum 'Culmorum' from carnation. Phytopathology 62: 1052-1056.

56. Mitchell, R. and E. Hurwitz. 1965. Suppression of Pythium debaryanum by lytic rhizosphere bacteria. Phytopathology 55: 156-158.

57. Neal, J. L., Jr. 1971. A simple method for enumeration of antibiotic producing microorganisms in the rhizosphere. Can. J. Microbiol. 17: 1143-1145.

58. New, P. B. and A. Kerr. 1972. Biological control of crown gall: field measurements and glass house experiments. J. Appl. Bacteriol. 35: 279-287.

59. Papavizas, G. C. 1968. Survival of root-infecting fungi in soil. VI. Effect of amendments on bean root rot caused by Thielaviopsis basicola and on inoculum density of the causal organism. Phytopathology 58: 421-428.

60. Papavizas, G. C. and P. B. Adams. 1969. Survival of root infecting-fungi in soil. XII. Germination and survival of endoconidia and chlamydospores of Thielaviopsis basicola in fallow soil and in soil adjacent to germinating bean seed. Phytopathology 59: 371-378.

61. Papavizas, G. C. and J. C. Lewis. 1971. Survival of endoconidia and chlamydospores of Thielaviopsis basicola as affected by soil environmental factors. Phytopathology 61: 108-113.

62. Patrick, Z. A., T. A. Toussoun and H. J. Thorpe. 1965. Germination of chlamydospores of Thielaviopsis basicola. Phytopathology 55: 466-467.

63. Rai, P. V. and G. A. Strobel. 1966. Chemotaxis of zoospores of Aphanomyces cochlioides to sugar beet seedlings. Phytopathology 56: 1365-1369.

64. Rifai, M. A. 1969. A revision of the genus Trichoderma. Mycological papers, No. 116, Commonwealth Mycological Institute, pp. 1-56.

65. Rishbeth, J. 1963. Stump protection against Fomes annosus. III. Inoculation with Peniophora gigantea. Ann. Appl. Biol. 52: 63-77.

66. Rishbeth, J. 1975. Stump inoculation: a biological control of Fomes annosus. pp. 158-162. In Biology and control of soil-borne plant pathogens. (G. W. Bruehl, ed.) Am. Phytopathol. Soc., St. Paul, Minn.

67. Rovira, A. D. 1972. Studies on the interactions between plant roots and microorganisms. J. Aust. Inst. Agr. Sci. 38: 91-94.

68. Schmitthenner, A. F. 1964. Fungi associated with root necrosis of Phytophthora-resistant soybeans. Phytopathology 54: 96.

69. Schreiber, L. R. and R. J. Green, Jr. 1963. Effect of root exudates on germination of conidia and micro-sclerotia of Verticillium albo-atrum inhibited by soil fungistatic principle. Phytopathology 53: 260-264.

70. Schroth, M. N. and D. C. Hildebrand. 1964. Influence of plant exudates on root-infecting fungi. Annu. Rev. Phytopathol. 2: 101-123.

71. Short, G. E. and M. L. Lacy. 1974. Germination of Fusarium solani f. sp. pisi chlamydospores in the spermosphere of pea. Phytopathology 64: 558-562.

72. Singh, P., R. S. Vasudeva and B. S. Bajaj. 1965. Seed bacterization and biological activity of bulbiformin. Ann. Appl. Biol. 55: 89-97.

73. Smith, J. G. 1960. The influence of antagonistic fungi on Thielaviopsis basicola. Acta Botanica Neerlandica 9: 59-118.

74. Stack, R. W. and W. A. Sinclair. 1975. Protection of Douglas-fir seedlings against Fusarium root rot by a mycorrhizal fungus in the absence of mycorrhiza formation. Phytopathology 65: 468-472.

75. Steiner, G. W. and J. L. Lockwood. 1969. Soil fungistasis: sensitivity of spores in relation to germination time and size. Phytopathology 59: 1084-1092.

76. Tsao, P. H. and J. L. Bricker. 1966. Chlamydospores of Thielaviopsis basicola as surviving propagules in natural soils. Phytopathology 56: 1012-1014.

77. Wright, J. M. 1956. The production of antibiotics in soil. IV. Production of antibiotics in coats of seeds sown in soil. Ann. Appl. Biol. 44: 561-566.

78. Yoder, D. L. and J. L. Lockwood. 1973. Fungal spore germination on natural and sterile soil. J. Gen. Microbiol. 74: 107-117.

Typed and Printed in the U.S.A.
Professional Thesis Preparation
Cliff and Paula Haughey
144 Maplewood Drive
East Lansing, Michigan 48823
Telephone (517) 337-1527