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To the Graduate Council:

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Leander F. Johnson

Leander F. Johnson, Major Professor

We have read this thesis
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THE USE OF *TRICHODERMA* SPP. TO REDUCE SEEDLING DISEASE SEVERITY IN COTTON

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Cipta Ginting

December 1988

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DEDICATION

This thesis is dedicated to the Plant Pathology Division at the University of Lampung.

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ABSTRACT

Seedling disease complex of cotton caused by pathogens including *Pythium* spp., *R. solani*, *T. basicola* and *Fusarium* spp. is important throughout the U.S. cotton belt. The use of fungicides to control seedling disease is expensive, has negative effects to the environment, and is not always successful, particularly when weather conditions favor disease development. Biological control is an alternative for controlling seedling disease. In this study, several cold-tolerant strains of *Trichoderma* spp. were tested for their efficacy to control the disease.

Trichoderma applied in wheat bran-peat moss preparation was added to raw soil at a concentration of 10^6 colony forming units per gram of soil and the soil was then placed in plastic pots. After 1 week of incubation, 6 Stoneville 213 cotton seeds were planted per pot. Data on plant height and disease severity were taken 8 and 18 days after planting, respectively. Potential pathogens of seedling disease were isolated from diseased hypocotyls.

Forty isolates comprising 4 species of *Trichoderma* were tested in a screening procedure. Eight of these plus a new T-8 isolate were retested for further study. Significant reductions in disease severity were obtained with isolates of *T. viride*, *T. harzianum*, *T. koningii*, and *T. pseudokoningii*. In the initial testing, a few isolates enhanced plant height. However, when retested, none of the isolates increased plant height significantly. Pathogens isolated from diseased

hypocotyls were *Pythium* spp., *R. solani* and *Fusarium* spp. *Pythium* and *R. solani* were isolated more frequently from severely diseased hypocotyls. *Fusarium* was isolated mostly from hypocotyls that were less severely diseased. Evidence was obtained that isolates of *Trichoderma* that significantly reduced disease severity were equally effective for controlling seedling disease caused by *R. solani* and by *Pythium* spp.

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INTRODUCTION

Seedling disease complex of cotton (Gossypium hirsutum L.) has been considered important since 1892 when Atkinson (3) described it for the first time. Pathogens inducing seedling disease include Pythium species including P. ultimum Trow., P. aphanidermatum (Edson) Fitzp., P. irregulare Buisman, P. sylvaticum Campbell and Hendrix, and P. heterothallicum Campbell and Hendrix, Rhizoctonia solani Kuhn, Thielaviopsis basicola (Berk. & Br.) Ferr., and Fusarium species including F. moniliforme Sheld, F. oxysporum Schlect and F. roseum (Lk.) amend Snyd and Hans. (13,21,36,37,38,53,55). The first two genera of fungi contain the most important pathogens (21,36,38,53). The symptoms of the disease includes seed decay, preemergence damping off, post-emergence damping off and hypocotyl or stem lesions (60).

Seedling disease has largely been controlled chemically by using fungicides applied to either soil or to seeds (27). This is expensive and may affect the environment (5). In addition, fungicides may be unsuccessful under conditions that favor disease development such as low temperatures and high humidity (38).

Some biocontrol agents such as Trichoderma have been used to suppress the casual organisms of seedling disease (14,17,48,58). However, no information is available concerning the use of cold-tolerant strains of Trichoderma to control the disease. The purpose of this study was to investigate if cold-tolerant strains of Trichoderma isolated from Alaska and Tennessee soils and other isolates from New York soil could control seedling disease of cotton.

CHAPTER I

LITERATURE REVIEW

Seedling Disease Complex of Cotton

The loss of cotton due to the seedling disease complex throughout the U.S. from 1971 to 1982 was about 2.8% per year (60). The loss in 1986 throughout the U.S. was 2.2% (237,970 bales) and that in Tennessee was 4.0% (13,478 bales) (22). The syndrome of the disease includes: rotted seed before germination; seedling decay before emergence (pre-emergence damping off); collapsed emerged seedlings (post-emergence damping off) and lesions on emerged seedling stems (sore shin), usually near the soil surface (60).

Several workers have identified the pathogens of seedling disease of cotton. According to Fulton and Bollenbacher (21) in Arkansas, the predominant pathogen of the seedling disease complex of cotton was *R. solani* followed by *P. ultimum*, *T. basicola*, *Aspergillus flavus* Link. and *Fusarium* spp. *Fusarium* spp., however, were not important pathogens. In addition, pre-emergence rot was caused primarily by *R. solani* and *P. ultimum*.

In Mississippi, Ranney (53) found that *R. solani* (most important), *Pythium* spp., *Fusarium* spp., and *Glomerella gossypii* Edg. were pathogens of cotton seedlings. Other fungi isolated from diseased cotton were *Alternaria*, *Aspergillus*, *Penicillium* and *Thielaviopsis*. Roncadori and McCarter (55) isolated *Pythium* spp., *R. solani* and

Fusarium spp. from diseased cotton roots in Georgia. In several studies (36,37,48), Johnson et al. isolated Pythium spp., R. solani, T. basicola and Fusarium spp. from diseased hypocotyls of cotton in Tennessee.

Rhizoctonia solani, P. ultimum, each alone or in combination with other pathogens, can initiate the seedling disease complex syndrome and cause all the symptoms (60). In California, P. ultimum was the most commonly isolated pathogen from diseased cotton seedlings, and seedling disease severity was positively related to the concentration of P. ultimum propagules in soil (13). Some isolates of P. ultimum and P. aphanidermatum were the most virulent. P. irregulare, P. acanthicum Drechsler and unidentified Pythium species as well as some isolates of P. ultimum were not pathogenic (12). However, P. irregulare isolates from other states (39,55) have been found to be pathogenic to cotton.

In Tennessee, species of Pythium isolated from diseased hypocotyls in decreasing order of frequency were P. ultimum, P. sylvaticum, P. irregulare and P. heterothallium. In addition, there were many Pythium-like isolates that did not produce sexual structures (38). Pythium spp. were most prevalent when postplanting soil temperatures averaged 16 C or lower and when the average of soil moisture for 30 days after planting was 17% or more (36). Therefore, Pythium spp. as seedling pathogens are particularly important in the northern part of the U.S. cotton belt (60). Rhizoctonia solani was most often isolated from cotton when the mean minimum temperature range was between 14 and 24 C; however, when the temperature was 13 C or lower, R. solani rarely

attacked the plant. When soil moisture was high, *Pythium* caused more disease than did *R. solani* (36).

Thielaviopsis basicola and *Fusarium* were also associated with the cotton seedling disease complex (21,37,38,53,60). *Thielaviopsis basicola* caused seedling disease of cotton in Arizona, but the disease usually was not severe (40). *Fusarium* spp. including *F. moniliforme*, *F. oxysporum* and *F. roseum* produced all the seedling disease syndrome (60). Johnson et al. (36,38) found, however, that *Fusarium* spp. were the least pathogenic among the pathogens isolated from diseased hypocotyls in Tennessee.

Minton and Garber (46) stressed the importance of seed quality in preventing the disease. They also suggested that cotton should be planted when temperature and moisture of the soil are favorable for cotton, seed should be planted to the depth necessary to provide enough moisture for seed germination and growth, and that rotation would reduce inoculum of the pathogens in soil.

Bird (7) selected cotton with multi-resistant genes that could influence the plant to resist or escape six major diseases including seedling disease as well as influence some desirable agronomic traits. Seedling disease severity can be reduced with chemical fungicides. Soil fungicides such as Ridomil and Rizolex, and seed-treatment fungicides such as Apron, Epic and Imazalil have been found to be effective (27). These fungicides should be used in combination depending on pathogens occurring in the field (27,46).

The Use of Trichoderma spp. as Biocontrol Agents

Rifai (54) clarified the taxonomy of *Trichoderma* by proposing a classification system based on microscopic characters. According to his scheme, there are nine species aggregates in the genus of *Trichoderma*.

Species of *Trichoderma* are distributed throughout the world and can be found in almost any soil and other natural habitats especially if organic matter is available. They grow most abundantly in acidic habitats (51). Danielson and Davey (11) found that different species within the genus have slightly different distributions. *Trichoderma koningii* Oud. and *T. hamatum* (Bon.) Bain. are most widely distributed, whereas *T. pseudokoningii* Rifai can rarely be found but is dominant under excessive moisture. *Trichoderma viride* Pers. ex S. F. Gray was found only in cool temperature regions, whereas *T. harzianum* Rifai was found more frequently in warmer regions. Some strains of *T. harzianum* as well as *T. pseudokoningii*, however, tolerate low temperature (10 or 12 C) (39). Recently, *T. viride* was isolated from eight different locations in Alaska (39).

Trichoderma asexual spores include conidia on phialides (phialospores) and chlamydospores that are commonly intercalary or rarely terminal (54). The surviving propagules in soils are chlamydospores, conidia and hyphae (51). In soil, conidia are more sensitive to soil fungistasis than chlamydospores. Thus, conidia germinated less frequently than chlamydospores in soil (6).

Weindling was the first to notice the antagonistic ability of Trichoderma when, in 1932, he observed that T. lignorum parasitized R. solani, Pythium spp., Phytophthora spp., S. rolfsii and Rhizopus spp. (61). Since that time, many workers in many parts of the world have studied this characteristic in laboratories and greenhouses, as well as in field conditions.

Wells et al. (62) were the first to report that T. harzianum was an effective biocontrol agent under field conditions. Working in Georgia, they used ryegrass-sandy loam preparation to grow T. harzianum isolated from diseased sclerotia of S. rolfsii Sacc. and found that this preparation reduced S. rolfsii disease of blue lupine, tomato and peanut under greenhouse conditions and of tomato under field conditions. However, it was necessary to apply the preparation in large quantities, 4,200 kg per hectare. Three years later Backmann and Rodriquez-Kabana in Alabama (4) found a more efficient carrier composed of diatomaceous earth impregnated with a 10% molasses solution and applied at only 140 kg per hectare. This preparation reduced peanut disease caused by S. rolfsii in field experiments and increased yields over a 3 year period. In addition, Trichoderma has also been applied in other delivery systems. For example, T. harzianum applied in wheat bran-peat preparation reduced Fusarium disease of cotton and tomato under greenhouse and field conditions (58,59). Other delivery systems effective for controlling certain diseases are seed coatings (58,59), conidial suspensions (25,47), dry granular suspensions of sand-cornmeal-bran-modified gliotoxin fermentation medium (47),

fluid-drilling gels (10) and wheat bran-sawdust preparations (20). Papavizas et al. (50) produced biomass samples of T. viride, T. harzianum, T. hamatum and other biocontrol agents in liquid fermentation composed of molasses and brewer's yeast. When these preparations were added to soil, Trichoderma proliferated and their CFU's increased from 5 x 10^3 to 2-6 x 10^6.

The reduction of Rhizoctonia disease of several plants by Trichoderma under greenhouse and/or field conditions has been well documented. Control of R. solani in cucumber (41), bean (14,23), cotton (14,47), radish (25,47), pea (25), potato (20), tomato and eggplant (23) and strawberry (15) has been demonstrated. Pythium diseases have also been found to be reduced by the antagonists; for example, Pythium diseases of radish (25,47), pea (24,25,44,48), and bean (24).

Trichoderma has also been tested in combination with other measures to control some plant diseases. The combination of T. harzianum as a wheat bran-sawdust preparation plus solar heating by mulching the field with polyethylene controlled R. solani disease of potato better than did either method alone (20). When Trichoderma was applied in plots that had been plowed to a depth of 20-25 cm (instead of disking to the depth of 5-7 cm commonly practiced in the area), the reduction of Rhizoctonia fruit rot of cucumber was significantly greater than those obtained with either the use of Trichoderma or plowing alone (41). Trichoderma has been combined with PCNB (pentachloronitrobenzene) to control R. solani and S. rolfsii (9), and

with methyl bromide to control S. rolfsii of peanut and tomato (19) and potato (20).

When applied in a wheat bran-peat preparation, T. harzianum reduced Fusarium wilt of cotton in three successive cotton plantings (58). Another long term effect was found when T. hamatum was applied to control seedling diseases of radish and pea caused by Pythium spp. or R. solani in the greenhouse (25). The antagonist protected the crop in two successive plantings and its population increased about 100 times. Trichoderma harzianum was also found to prevent the reinfestation of fumigated soil by S. rolfsii when compared to fumigated soil that had not been treated with T. harzianum (19).

There are three possible mechanisms for Trichoderma actions against other fungi: competition, antibiosis and mycoparasitism (5,44). Sivan et al. (59) found that T. harzianum proliferated well in the root zone without significantly affecting the population of the pathogen. In the same experiment, when they coated tomato seed with conidia of T. harzianum, they found that after 20 weeks, the highest numbers of the antagonist on the root segments were in the root tips where the pathogens were rare. From these data, they suggested that competition for nutrients might have occurred. Lifshitz et al. (44) found that competition was not an important mechanism in their experiments. They found that when they added d-glucose (carbon source) and l-asparagine (nitrogen source), the biocontrol of Pythium damping off by Trichoderma was not affected. In addition, they found that the germination of chlamydospores of F. oxysporium f. sp. cucumerinum Snyd.

& Hans. and sporangia of Pythium were not affected by seed treatment with conidia of Trichoderma. They suggested that the reduction of disease was due to toxic metabolites produced by Trichoderma on seed coats (44). In 1971 Dennis and Webster (12) found that many isolates of Trichoderma including T. koningii, T. harzianum, T. viride and T. pseudokoningii produced non-volatile chloroform and peptide antibiotics. In addition, some isolates of T. viride, T. koningii and T. hamatum produced volatile antibiotics.

Mycoparasitism is also an important mechanism at least in some experiments. Using a cellophane membrane technique on water agar, Elad et al. (18) observed the interaction between Trichoderma and S. rolfsii or R. solani. The antagonists parasitized the pathogens and attached to the hosts by hyphal coils, hooks, or appressoria. In addition, mycoparasitism was often observed associated with the production of hydrolitic enzymes such as glucanase and chitinase (9,16,18,23,25). It was found that isolates of T. harzianum differ in their production level of hydrolitic enzymes (16). This characteristic was positively correlated with their ability to control soil-borne disease under greenhouse conditions. Thus, isolates that produce high level of B-1,3-glucanase and chitinase were shown to be able to control S. rolfsii whose cell walls contain B-1,3-glucan and chitin (16).

The Use of Trichoderma as Plant Growth Hormone Producers

Some isolates of Trichoderma have been found to induce plant growth (8,14,63). Elad et al. (14) found that when applied as wheat-bran preparation into non-infested soil, T. harzianum increased the

growth of bean. Using either conidial suspensions or peat-bran preparation in either natural or steamed soil, Chang et al. (8) found that T. harzianum increased fresh and dry weight and height, improved flowering and earlier germination in certain plants. Windham et al. (63) obtained increased growth of tomato, tobacco, and radish with Trichoderma. They suggested that the ability of Trichoderma to increase plant growth was due to its ability to produce a "growth-regulating factor."

Biological Control of Seedling Disease of Cotton

Howell (28) in Texas, treated cotton seeds with L-sorbose at a concentration of 10 mg/seed and obtained emergence increases from 37% to 87% in soil infested with R. solani. This sugar suppressed hyphal extension of R. solani and gave saprophytic microorganisms such as Fusarium spp. a competitive advantage. He also used Pseudomonas fluorescens Migula to control cotton seedling from P. ultimum or R. solani attack (29,30). When liquid cultures of the bacterium or pyrrolnitrin, an antibiotic produced by the bacterium, was used as a seed treatment, cotton was protected from R. solani attack in soil infested with the pathogen. The antibiotic also inhibited other fungi such as T. basicola, Alternaria, sp., and Verticillium dahliae. However, P. ultimum was not affected (29). The same bacterium, or pyoluteorin, another antibiotic produced by the bacterium, applied with the same methods, protected cotton in P. ultimum-infested soil. In vitro, this antibiotic inhibited P. ultimum, but not R. solani (30).

Ichielevich-Auster et al. (35) sowed cotton seeds in soil infested with nonpathogenic isolate of R. solani, and 3 days later infested the soil with a virulent strain of R. solani. After 14 days, they observed that the nonpathogenic strain suppressed seedling disease caused by the virulent strain. The disease reduction also occurred when 3-day-old cotton seedlings were transferred to soil infested with the virulent strain.

Species of Trichoderma have been used to control cotton seedling disease (14,17,48,58). Elad et al. (17) found that seedling disease of cotton caused by R. solani was reduced by coating cotton seed with spore suspensions of Trichoderma under greenhouse and field conditions. In the field trials the results were not significantly different from those obtained by treatment with PCNB. The use of T. hamatum gave better results at 20 C than did the T. harzianum. But T. harzianum gave better results than did T. hamatum at 27 C. Elad et al. (14) applied T. harzianum in a wheat-bran preparation and obtained reduction of cotton seedling disease caused by R. solani. A new strain of T. harzianum that tolerated benomyl gave better control than did the wild strain (48). Cotton wilt caused by F. oxysporium f. sp. vasinfectum (Atk.) Snyd. & Hans. was also controlled by T. harzianum (58).

Howell (31,32) found that when applied as "in-furrow treatment," Gliocladium virens Miller, Giddens & Foster controlled damping-off of cotton in soil infested with P. ultimum or R. solani in growth chambers. In other experiments, he observed that G. virens encircled and penetrated R. solani hypha. However, mutants of G. virens produced

by UV radiation lost their mycoparasitic ability but still controlled the disease (33,34). The loss of mycoparasitic activity was not accompanied by the loss of antibiotic production. Thus the parents and mycoparasitic-deficient mutants produced similar levels of viridin and gliotoxin as determined by thin-layer chromatography (34).

Beagle-Ristaino (6) found that *T. viride*, *T. harzianum* and *G. virens* proliferated in cotton rhizosphere and nonrhizosphere soils when added as fermentor biomass. The substrates used to prepare this biomass were composed of molasses and brewer's yeast. The biomass contained mycelial fragments, chlamydospores, conidia, and nutrients (50).

CHAPTER II

MATERIALS AND METHODS

Sources of Cultures

Forty isolates of *Trichoderma* were tested for their efficacy to control seedling disease of cotton. Twenty-one of the isolates were *T. viride* from Alaska; 12 isolates of *T. harzianum* and 5 isolates of *T. pseudokoningii* from Tennessee (37), all obtained from Dr. L. F. Johnson, The University of Tennessee, Knoxville. Isolates T-8 (*T. koningii*) and T-12 (*T. harzianum*) (24) were obtained from Dr. G. E. Harman, New York Agricultural Station at Geneva, NY.

Preparation of Inoculum

Inoculum was prepared by culturing the isolates in wheat bran-peat moss-distilled water media (57). Peat moss was passed through a 2-mm sieve, and then 100 ml of each component was placed in a 0.95-liter jar which was plugged with a cotton plug. The media were autoclaved for 1 hour on 2 successive days. Sterilized media were seeded with isolates of *Trichoderma* and incubated at room temperature for 2 weeks. After the fourth or the fifth day of incubation, the jars were shaken daily. The inoculum was air dried for 3 days after which it was passed through a 2-mm sieve. After each isolate was sieved, the sieve was washed with tap water, rinsed with 70% alcohol and then rinsed with distilled water.

Inoculum density for the various isolates was determined on a selective V-8 medium (49). The inocula were diluted, and 1 ml each of the 10^7 and 10^8 dilution was spread each over the surface of the V-8 agar in Petri dishes, and incubated at room temperature for 4 or 5 days. Colony forming units (CFU's) per g of inoculum were determined from five Petri-dish replications of each isolate based on numbers of colonies observed in the culture plates.

Soil Infestation and Testing for Biocontrol

Naturely infested dexter silt loam soil known to produce seedling disease was obtained from The Jackson Experiment Station, West Tennessee. Sufficient *Trichoderma* inoculum was added to soil to obtain a *Trichoderma* population of 10^6 CFU/g of soil. The soil was then placed in 10-cm-diameter plastic pots. A randomized complete block design with three pot replications, and with two sets of controls, was used for all screening tests. The pots were then incubated in a growth chamber with a constant temperature of 24 C and continuous light. After 1 week, 6 acid-delinted Stoneville 213 cotton seeds were planted in each pot. After planting, the temperature inside each incubator was maintained at 27 C for 8 days, at which time height of the seedlings was measured. Then the temperature was lowered to 17 C. Ten days later the seedlings were carefully removed and washed in tap water. Disease severity for each seedling was rated according to the following criteria (47) (Figure 1):

0 = no disease symptoms;

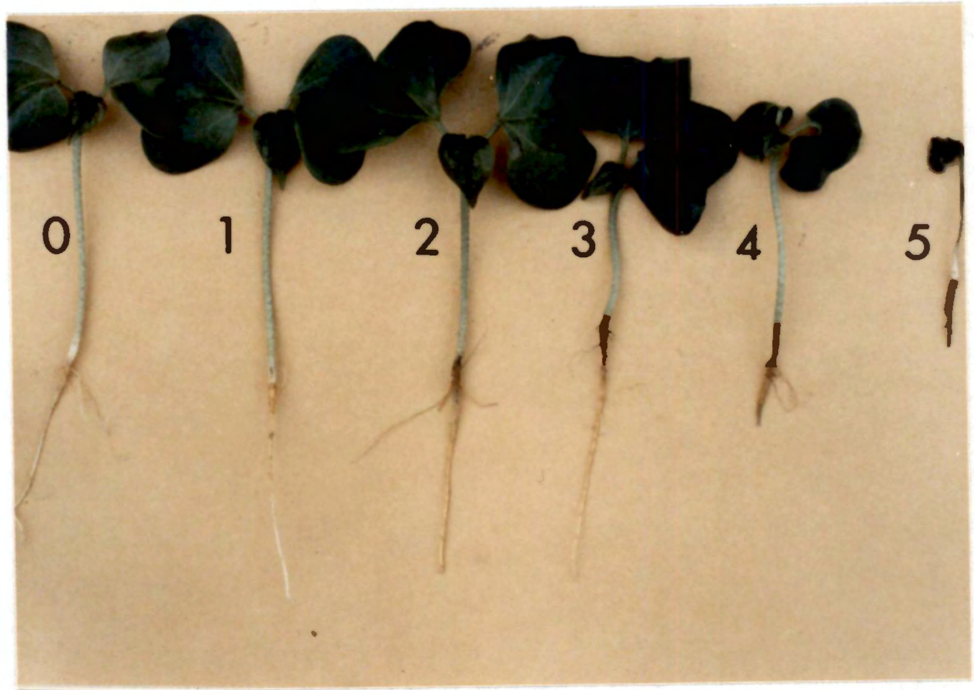

Figure 1. Criteria used in rating cotton seedlings. Healthy plant on the far left was rated 0, and the plant on the far right was 5. Unemerged seedling was rated 6 (from Palmer, 1981) (47).

1 = trace, small pinpoint dark areas, or a diffused tan area on the hypocotyl;

2 = definite necrotic lesion on the hypocotyl, less than 0.5 cm in length, might be sunken;

3 = the lesion equals or was more than 0.5 cm in length;

4 = seedling wilted (cotyledon was drooping);

5 = plant dead;

6 = plant not emerged.

Disease indices were the means of three pot replications, and six seeds were planted in each pot. Overall disease index is a measure of disease severity based on a rating of 0-6, where 0 = no symptoms, 5 = plant was dead, and 6 = preemergence mortality. In post emergence disease index, preemergence mortality was not included.

Of 40 isolates tested, 9 which were most effective in reducing seedling disease were retested. The procedures were the same as those described above except for the experimental design and soil type used. Here, a completely randomized factorial design and eight replications and a loring silt loam were used.

Isolation of the Pathogens from Diseased Cotton Seedlings

Pathogens were isolated from the seedlings in the experiment in which nine of the isolates were retested. After the seedlings were rated to determine the disease index, 0.5 - 1 cm of the hypocotyls with discolored or necrotic areas were cut and placed in 250-ml flasks containing 100 ml of distilled water. One drop of Tween-20 was added to each flask which was shaken periodically for 3 hours. Hypocotyl

segments were transferred to other flasks containing the same components, and then the segments were washed through three changes of distilled water, blotted with sterile paper and placed on the surface of 2% water agar in Petri dishes. Dishes were incubated at 24 C. After 2 days the plates were examined for fungi growing from the hypocotyls. If no Pythium or R. solani grew, the plates were then incubated for a few more days and isolated fungi were identified to genus.

To determine the relationship between the disease index rating and fungal genus isolated, isolates were grouped into three categories based on the post-emergence disease index they caused on cotton seedlings. Isolates causing post-emergence disease index between 0 and 1.0 belonged to the first category; 1.1 and 1.5 the second category; and 1.6 plus the last category. Then, means of isolation frequency of Pythium and Rhizoctonia were determined for each group.

Mycelial Compatibility of Isolates of Rhizoctonia

Anastomosis of hyphae among 13 isolates of Rhizoctonia was determined. Rhizoctonia growing from diseased hypocotyls was transferred to 2% water agar. The anastomosis tests were conducted in 2% water agar in Petri dishes, where two isolates were tested in each dish. The growing hyphae tips of the two isolates were plated in a Petri dish so that these isolates opposed each other (52). After 2 days of incubation, the dish was observed under a light microscope in low magnification (100 X), and anastomosis was confirmed with high power (400 x).

CHAPTER III

RESULTS

Screening Tests

Seventy-six isolates of Trichoderma were available; however, only 40 isolates produced the required number of CFU's for the initial tests (Appendix, Table 4). Most isolates reduced disease severity; however, only isolates T-12 and 15-12-9 (T. harzianum) gave statistically significant control of overall seedling disease. Isolates A521-10-4 and A345-10-7 (T. viride), and 15-12-10 and 15-12-12 (T. harzianum) increased seedling height significantly.

Disease Severity and Growth Enhancement of Cotton Plants

The eight best isolates for disease reduction and high number of CFU's in the screening tests were retested. A new isolate of T-8 (T. koningii) obtained from Dr. M. T. Windham was also included (Appendix, Table 5). All isolates reduced overall seedling disease significantly except isolates 1-12-1 (T. pseudokoningii) and 15-12-9 (T. harzianum) (Table 1). Four (A345-10-7, A23-10-1, A345-10-1, and A521-10-4, all T. viride) of the nine isolates tested reduced post-emergence seedling disease significantly. Best control of both pre- and post-emergence seedling disease was obtained with two Alaskan isolates of T. viride (A23-10-1 and A345-10-7). Eight-day-old cotton seedlings grown in soil infested with each of the isolates of Trichoderma, except isolates 1-12-1 and AM4-10-1 (T. pseudokoningii), were taller than those in the

Table 1. Seedling disease severity of cotton in raw soil infested with isolates of <u>Trichoderma.</u>

Isolate	CFU/g[a]	Plant Height[b] (cm)	Disease Index[c] Overall	Post-emergence
Control 2	-	7.1v	3.8v	2.0vw
Control 1	-	6.9v	3.7v	1.7vwxy
1-12-1	1.3×10^9	6.5v	3.5v	1.8vwx
15-12-9	1.2×10^{10}	7.1v	3.3vw	2.2v
T-12	3.6×10^9	7.3v	2.5wx	1.2wxyz
A521-10-4	9.2×10^8	7.8v	2.5wx	0.8z
T-8	6.6×10^8	7.8v	2.4wx	1.3wxyz
AM4-10-1	1.2×10^9	6.9v	2.3wx	1.2wxyz
A345-10-1	1.4×10^9	7.4v	2.3wx	1.2wxyz
A23-10-1	1.3×10^9	8.0v	1.7xy	0.9yz
A345-10-7	4.4×10^8	7.7v	1.2y	0.8z

[a]Colony Forming Units in peat-bran inoculum prior to soil infestation.

[b]Distance from soil to apical meristem 8 days after planting.

[c]Overall disease index is a measure of disease severity based on a rating of 0-6, where 0 = no symptoms and 6 = preemergence mortality. Post-emergence disease index is a measure of disease severity based on rating of 0-5, where 0 = no symptoms and 5 = post-emergence mortality. The disease indices were the means of 8 pot replications, and 6 seeds were planted in each pot. Index values within each column followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

control soils, but these differences were not statistically significant (Table 1).

Fungi Isolated

Fungi isolated from diseased hypocotyls and the frequency of isolation of each fungus are in Table 2. Fungi isolated were *Pythium*, *Rhizoctonia* and *Fusarium*. In addition, bacteria and nematodes were found to be associated with some diseased hypocotyls. *Pythium* and *Rhizoctonia* were rarely isolated from seedlings grown in soil infested with *T. viride* (A345-10-7, A521-10-4, A23-10-1) and *T. koningii* (T-8). These three isolates of *T. viride* caused the lowest post-emergence disease index rating. There was a progressive increase in frequency of isolation of *Pythium* and *Rhizoctonia* with increase in post-emergence disease index (Figure 2).

Based on anastomosis tests among 13 isolates of *R. solani* isolated from diseased hypocotyls, there were more than one anastomosis groups (AG's) present. Isolates 1, 2, 7, and 12 belonged to the same AG. Other isolates (3, 4, 5, 6, 8, 9, 10, 11, and 13) were in other AG(s) (Table 3).

Table 2. Genera of fungi isolated from diseased hypocotyl of emerged cotton seedlings grown in soils infested with isolates of *Trichoderma*.

Isolate	No. of Hypocotyls Cultured	Fungi Isolated (%)[a]			
		Pythium	Rhizoctonia	Fusarium	None[b]
Control-2	21	14	10	43	33
Control-1	23	26	4	48	22
1-12-1	24	29	25	42	4
15-12-9	31	19	29	48	3
T-12	25	8	20	56	16
A521-10-4	21	0	0	81	19
T-8	26	4	4	88	4
AM4-10-1	18	17	28	44	11
A345-10-1	28	14	7	71	7
A23-10-1	27	4	7	74	15
A345-10-7	23	0	0	96	4

[a]Percent of diseased hypocotyls from which each genus was isolated.

[b]Fungi did not emerge. In some, bacteria and/or nematodes were observed.

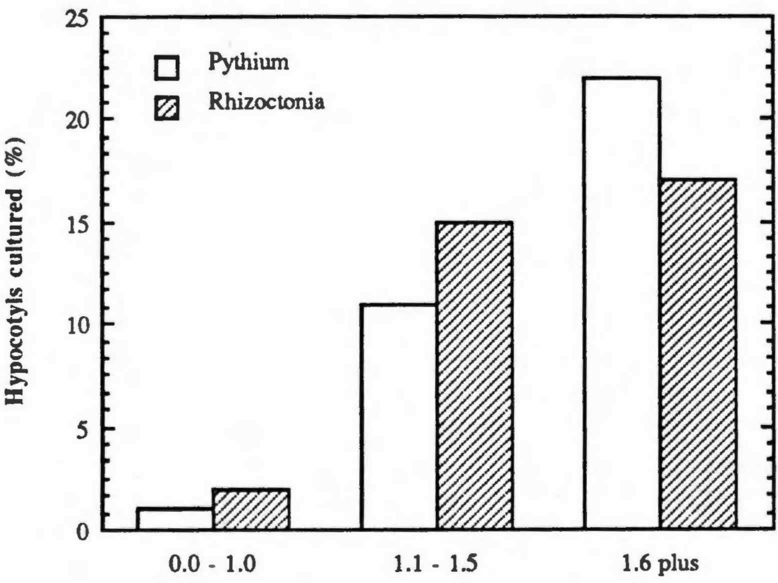

Figure 2. Isolation of Pythium and Rhizoctonia from discolored or necrotic hypocotyls of cotton seedlings grown in soils infested with isolates of Trichoderma. Controls are included in the 1.6 + category.

Table 3. Hyphal anastomosis among isolates of R. solani isolated from diseased portions of hypocotyls of cotton seedlings.

Isolates Tested	Fusion Between the Two Hypha
1 and 2	yes
1 and 3	no
1 and 4	no
1 and 5	no
1 and 6	no
1 and 7	yes
1 and 8	no
1 and 9	no
1 and 10	no
1 and 11	no
1 and 12	yes
1 and 13	no
2 and 12	yes
2 and 13	no

CHAPTER IV

DISCUSSION

Reduction of cotton seedling disease by Trichoderma amendment has been documented (14,17,47,58). The primary objective of this study was to determine if cold tolerant Trichoderma isolates (38) could control seedling disease at low temperature. Four isolates of T. viride from three different Alaska soils and one of T. pseudokoningii from Tennessee that grew at 10 C significantly reduced disease severity in growth chambers (Table 1). In addition, isolates T-8 (T. koningii) and T-12 (T. harzianum) from New York soil controlled seedling disease of cotton significantly (Table 1). T-12 was the only isolate that significantly reduced overall seedling disease in both the screening and retesting tests (Table 1 and Appendix, Table 4). T-12 was not cold tolerant (24); therefore, the antagonism of seedling disease pathogens might have occurred before the temperature was lowered to 17 C. Other effective isolates could also have reduced inoculum density of pathogens in the first 15 days when the growth chamber temperature was 24 or 27 C. Indeed, few seeds or seedlings became diseased before the temperature was lowered.

In the present study, after planting cotton seeds, the pots of soil were incubated at a growth chamber temperature of 27 C to permit germination of seeds and establish seedling growth. Then, the growth chamber temperature was reduced to 17 C to mimic cold temperatures

commonly found after spring plantings in cotton areas in Western Tennessee (36). Future studies with the isolates applied in this biocontrol test should be conducted with some modification of temperatures to determine whether these cold-tolerant strains could control seedling disease. Growth chamber temperatures should be lowered as early as possible, and other plants such as pea that grows at lower temperatures (24) can be used.

Significant control of seedling disease was not obtained with many of the isolates in the screening tests, yet these same isolates were effective when retested (Table 1; Appendix, Table 4). This may be explained by the differences in pathogen population densities in the raw soils. Johnson et al. (38) found fewer diseased hypocotyls on cotton grown in soils collected in February than in soils collected during warmer months. Soil for the screening tests was collected in 1987 in winter when pathogen concentration was low, and soil for the retesting experiment was collected from the same area during July 1988 when pathogen concentration was high. In fact, the average of overall disease index of control plants in the screening tests (3.4) was lower than that in the second test (3.8). Superior isolates such as A345-10-7 could have been more effective in soils with high concentrations of pathogen propagules (Table 2). Harman et al. (25) found that *T. hamatum* density was higher in soil infested with both *Pythium* and *R. solani* than that in soil infested with only *Pythium* after two successive plantings. Lifshitz et al. (44) showed that *Pythium*, not *R. solani*, growth was inhibited by toxic metabolites produced by

Trichoderma spp., and when the antagonists reached *Pythium* (mycoparasitism), the latter was no longer viable. Therefore, higher *R. solani* density in retesting experiment caused more proliferation of *Trichoderma* that would be more effective in both producing toxic metabolites, particularly to inhibit *Pythium*, and mycoparasitism, especially to reduce *R. solani*. Other possible reasons for the discrepancy between the screening and second tests could be the fact that different soil types used (a dexter silt loam in the screening tests and a loring silt loam in the second test), and less replications in the screening tests. Different soil types contain different proportions of soil components (organic matter, clay, silt and sand) that could influence the pathogen population and effectivity of *Trichoderma*. Less replications used in the screening tests could cause the statistic test to become less sensitive.

Only 40 isolates were tested for biocontrol agents because the other 36 exhibited only whitish hyphae, no green sporulating areas on the media. Such inocula might contain dominantly chlamydospores and hyphal fragments. These inocula, of course, could be used as inocula to infest soil; however, after counting the CFU's of seven of such isolates, they did not reach the required number of CFU's. There might be several reasons for this; first, the jars used to culture the isolates might be too small, so that the peat bran quickly dried. The temperature range for incubation of the peat-bran cultures (room temperature) may not have been within the optimum temperature range of growth for all isolates since the isolates were selected for their

ability to grow at 10 or 12 C. Finally, the 36 isolates might not produce a lot of conidia in peat bran mixture regardless of incubation conditions. However, this is unexpected because other workers (42) showed that T. harzianum produced higher conidia/chlamydospores ratio in solid media such as wheat bran than they did in liquid media. In addition, Trichoderma spp. have been shown to produce high CFU in peat bran medium (57,58,59), and Sivan et al. (57) found that wheat bran/peat was the most effective (out of six media tested) for growing Trichoderma in that it produced the highest CFU, the propagules persisted for the longest time and the low pH of medium so that bacterial contamination could be prevented easily. In future, the 36 isolates should be cultured in longer containers or at different temperatures or by different methods (50).

Wheat bran/peat preparation of Trichoderma has been shown to be an effective delivery system and reduced plant disease (58,59). Even though washed conidia usually do not germinate in soil (51) and are more sensitive to soil fungistasis (6), unwashed conidia with food base probably germinate and proliferate in soil (43,51). Papavizas et al. (48) even added aqueous suspensions of conidia of Trichoderma to soil, and seedling disease of cotton and radish caused by R. solani was reduced significantly by several UV-induced biotypes, and nonsignificantly by wild strain of T. harzianum. In this study, wheat bran/peat preparation was applied.

Trichoderma has been used with limited success at low temperatures in controlling seedling disease of pea (24,26). When applied as a seed

treatment, T. hamatum controlled damping off of pea due to Pythium spp. at 17 to 30 C, but not at 12 C. The antagonist was unable to grow well at 12 C (26). Studies in fields at Phelps and Geneva, NY, revealed that T-8 (T. koningii) grew better than T-12 (T. harzianum) at 10 to 20 C and protected pea consistently; whereas, T-12 protected pea in Geneva, but not in the cooler temperature at Phelps (24). Therefore, Trichoderma isolates that grow and control plant diseases at low temperature would be very useful.

This study showed that different isolates of the same species of Trichoderma behaved differently in sporulation, ability to enhance plant growth and efficacy as biocontrol agents. The reason is that the concept of "species aggregate" is used in the genus Trichoderma; therefore, there are probably several species in a "species aggregate" (54). Strains within a species can physiologically differ in their biocontrol activity (16). Thus, different isolates of T. harzianum produced different levels of B-1,3-glucanase, chitinase and cellulase when grown on mycelium of P. aphanidermatum, R. solani and S. rolfsii in soil. The ability of these isolates to secrete the enzymes was correlated with their efficacy to control the respective pathogens (16).

Fusarium was the most frequently isolated fungus from the diseased hypocotyls. However, its contribution to disease severity was minimal. Diseased hypocotyls from treatments that had the highest disease index (control, 1-12-1 and 15-12-9) produced less Fusarium than did the hypocotyls from treatments that had lower disease indices. This

supports the results of previous research (36,38) that *Fusarium* isolated from diseased hypocotyls is a weak parasite. The major pathogens of cotton seedlings in most cotton-growing regions including Tennessee (36), Arkansas (21), Mississippi (53) and California (13) are species of *Pythium* and *Rhizoctonia*. Isolation of these two fungi was directly related to seedling disease severity (Figure 2). There did not appear to be significant difference in isolation frequency between the two genera. Apparently the isolates of *Trichoderma* used were equally effective for controlling seedling disease caused by *Pythium* and by *Rhizoctonia*.

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LITERATURE CITED

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APPENDIX

APPENDIX

Table 4. Screening tests of 76 isolates of Trichoderma for control of seedling disease of cotton.[a]

Isolate (1)	CFU/g[b] (2)	Variation from Respective Control[c]: Plant Height[d] (cm) (3)	Disease Index[e]: Overall (4)	Disease Index[e]: Post-emergence (5)	Retested[f] (6)
A352-10-1	6.6 x 10^7	...	...	...	...
A26-10-1	0	...	...	...	...
A354-10-1	9.2 x 10^7	...	...	...	...
T-8	7.6 x 10^7	...	...	...	yes
T-12	5.7 x 10^9	0.4	-1.4	-0.6	yes
AM4-10-1	5.4 x 10^8	0.7	-1.0	-0.2	yes
15-12-1	7.1 x 10^9	-0.6	-0.5	-0.5	no
A123-10-2	1.0 x 10^7	...	...	...	...
A521-10-1	0.4 x 10^7	...	...	...	...
A23-10-1	1.5 x 10^9	0.1	-1.0	-0.7	yes
15-12-2	4.8 x 10^9	-0.0	-0.3	-0.9	no
A286-10-1	3.3 x 10^9	-0.1	-0.8	-1.2	no
A258-10-1	6.0 x 10^9	-0.1	-0.5	-0.6	no
A175-10-1	5.5 x 10^9	-0.1	-0.6	-1.0	no
A23-10-2	0	...	...	...	...
15-12-3	5x1 x 10^9	0.1	-0.8	-1.1	no
A345-10-1	3.3 x 10^9	0.1	-0.7	-0.9	yes
15-12-4	8.0 x 10^9	0.5	-0.6	0.0	no
15-12-5	6.4 x 10^9	0.3	-0.4	0.0	no
15-12-6	1.1 x 10^{10}	0.5	-0.5	0.1	no
A175-10-3	1.1 x 10^{10}	0.1	-1.1	-0.5	no
A521-10-4	5.6 x 10^8	1.0*	-0.9	-0.7	yes
A123-10-3	low	...	...	...	...
A123-10-3	low	...	...	...	...
A23-10-2	low	...	...	...	...
A175-10-2	low	...	...	...	...
A345-10-2	low	...	...	...	...
A345-10-3	low	...	...	...	...
A521-10-2	low	...	...	...	...
A345-10-5	3.0 x 10^8	0.5	-0.7	-0.1	no
A2310-4	3.6 x 10^9	0.0	-0.2	0.4	no
A521-10-13	low	...	...	...	...
A345-10-6	5.2 x 10^8	0.2	-0.3	-0.1	no
A23-10-5	5.0 x 10^8	0.0	-0.9	-0.3	no
A345-10-4	low	...	...	...	...
A521-10-8	low	...	...	...	...
A521-10-9	low	...	...	...	...

Table 4 (Continued)

Isolate (1)	CFU/g[b] (2)	Variation from Respective Control[c]: Plant Height[d] (cm) (3)	Disease Index[e]: Overall (4)	Disease Index[e]: Post-emergence (5)	Retested[f] (6)
A521-10-10	low	...	...	...	...
A521-10-11	low	...	...	...	...
A521-10-12	low	...	...	...	...
A521-10-14	low	...	...	...	...
15-12-7	2.6×10^{10}	-0.1	-0.7	-0.5	no
15-12-8	2.6×10^{10}	0.4	-0.7	-0.5	no
15-12-9	3.4×10^{10}	0.4	-1.3*	-1.0	yes
15-12-10	2.9×10^{10}	0.5*	0.0	0.0	no
15-12-11	3.1×10^{10}	-0.7	0.2	-0.7	no
15-12-12	2.7×10^{10}	0.5*	-0.3	0.0	no
A26-10-2	low	...	...	...	...
A26-10-3	low	...	...	...	...
A26-10-4	low	...	...	...	...
A26-10-5	low	...	...	...	...
A26-10-6	low	...	...	...	...
A26-10-7	low	...	...	...	...
A23-10-8	5.2×10^{8}	-0.5	-0.8	-0.4	no
A123-10-6	1.5×10^{9}	-0.3	-0.3	0.7	no
AM4-10-3	1.6×10^{8}	0.6	-0.5	0.1	no
A123-10-5	1.0×10^{8}	-0.3	0.6	0.1	no
A345-10-7	1.8×10^{8}	1.3*	-1.1	-0.1	yes
A123-10-7	2.0×10^{8}	0.0	-0.4	-0.5	no
A26-10-6	low	...	...	...	...
A175-10-6	low	...	...	...	...
A175-10-8	low	...	...	...	...
A175-10-8	low	...	...	...	...
A175-10-9	low	...	...	...	...
A175-10-10	low	...	...	...	...
AM4-10-2	5.8×10^{9}	-0.6	0.1	0.0	no
A23-10-6	6.6×10^{8}	-0.3	0.2	0.3	no
A26-10-6	9.0×10^{7}	-2.2	1.4	0.4	no
A521-10-5	3.7×10^{8}	-0.6	0.6	0.2	no
A23-10-7	2.6×10^{8}	-0.3	0.5	0.4	no
A23-10-3	2.0×10^{8}	-1.4	1.0	0.3	no
1-12-2	1.1×10^{9}	0.2	-0.4	-0.5	no
1-12-1	4.2×10^{9}	-0.1	-0.4	-0.5	yes
A175-10-4	low	...	...	...	...
A175-10-5	low	...	...	...	...
A175-10-11	low	...	...	...	...

Table 4 (Continued)

[a]Peat-bran cultures of the isolates were used to infest soil in pots to a final concentration of 10^6 CFU/g of soil. Seven screening tests were used, each with non-infested pots as controls.

[b]Colony Forming Units in peat-bran medium prior to soil infestation. Isolates designated "low" exhibited only whitish hyphae and no green sporulating areas on the medium.

[c]Starred values were statistically different from the control (P = 0.05).

[d]Distance from soil to apical meristem 8 days after planting.

[e]Overall disease index is a measure of disease severity based on a rating of 0-6, where 0 = no symptoms and 6 = preemergence mortality. Post-emergence disease index is a measure of disease severity based on a rating of 0-5, where 0 = no symptoms and 5 = post-emergence mortality. The disease indices were the means of 8 pot replications, and 6 seeds were planted in each pot.

[f]Another isolate of T-8 was retested.

Table 5. Identification and sources of isolates of *Trichoderma* that were retested.

Isolate	Soil Location	Species
1-12-1	Ames Plantation, Grand Junction, TN	*T. pseudokoningii*
15-12-9	Agricultural Experiment Station, Jackson, TN	*T. harzianum*
A23-10-1	Palmer, AK	*T. viride*
A345-10-1	Kenai Peninsula, AK	*T. viride*
A345-10-7	Kenai Peninsula, AK	*T. viride*
A521-10-4	Prudoe Bay, AK	*T. viride*
AM4-10-1	Ames Plantation, Grand Junction, TN	*T. pseudokoningii*
T-8	Arkport, NY	*T. koningii*
T-12	Arkport, NY	*T. harzianum*

VITA

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In 1984, he served as site manager in Agricultural Development Project in The Transmigration Area Air Sugihan, West Sumatra. The next year he joined the University of Lampung as an instructor. In January, 1987, he began study toward a Master's degree and received a Master of Science degree in the College of Agriculture with a major in Plant Pathology in December, 1988.

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