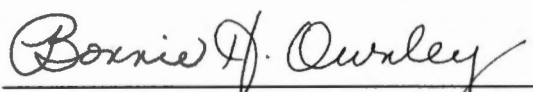


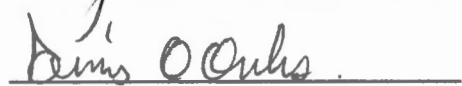
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**BIOLOGICAL AND CHEMICAL CONTROL
OF TARGET SPOT OF FLOAT-GROWN
DARK FIRE-CURED TOBACCO SEEDLINGS**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Robert L. Ashby, Jr.

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ABSTRACT

Tobacco is an important crop in Tennessee and the Southeastern United States. Farmers received \$34 million in revenue in 1991 from dark tobacco alone. Increasingly, tobacco seedlings are produced in float beds in the greenhouse. Float beds have several advantages over conventional seed beds; however, conditions in the greenhouse are often conducive to the spread of plant disease. Target spot (*Rhizoctonia* leaf spot) is a recently-described disease of float-grown tobacco and is caused by the soilborne fungus *Thanatephorus cucumeris* (anamorph *Rhizoctonia solani*). In Tennessee, significant losses in tobacco transplants grown in float systems have resulted from target spot. There also has been an increase in the number of reports of target spot on field plants, which may be related to the increase in production of transplants by the float system. Currently there are no control measures recommended for target spot on float plants except for proper sanitation and cultural practices. In this study the effect of four chemicals and four biological control agents for control of target spot on two dark-fire tobacco cultivars (DF485 and TR Madole), planted by direct-seeding or seeding-and-transferring, was evaluated. In 1993, one biological control agent, *Bacillus* sp. strain BA55, and two fungicides, fluazinam, and Dithane®, applied as foliar sprays, provided substantial control of target spot on cultivars DF485 and TR Madole, across both planting methods. In 1994, several biologicals and chemicals were tested

for control of target spot on direct-seeded plants of cultivars DF485 and TR Madole. Three fungicides, two formulations of fluazinam, and Dithane® significantly reduced target spot severity on both cultivars. The fungicide dimethomorph and the biological *Bacillus* sp. BA55 provided control only on one cultivar, DF485. Another fungicide, Rovral, also provided control of target spot; however, phytotoxicity was observed also in both years.

In 1993, direct-seeded plants of both dark-fire cultivars treated with BA55 and fluazinam, or receiving no treatment, were transplanted to the field and evaluated for further development of target spot, and the effect of disease control treatment on crop index, grade index, yield, and revenue. Environmental conditions were not conducive for further target spot development in 1993. In general, cultivar DF485 yielded more pounds per acre than TR Madole. Across both cultivars, there were no differences in crop index, grade index, yield, and revenue in plants treated with BA55 or fluazinam, or untreated.

The effects of host age, cultivar, and BA55 on target spot severity was studied also. Across five age groups, ranging from 2 to 6 wk, BA55 significantly controlled target spot. The seedlings treated at two weeks had significantly less target spot than the other age groups.

The effect of fluazinam concentration on target spot severity was studied also. Different concentrations of fluazinam, ranging from 200 to 1000 ppm, were applied weekly as foliar sprays to seed-and-transfer tobacco seedlings.

Fluazinam concentration had no effect on percent diseased foliage due to low disease pressure.

Based on the results from these experiments, fluazinam and Dithane® are potential candidates for control of target spot in float beds. The biological agent, BA55, has potential as for controlling target spot if the consistency of its performance can be improved.

A leaf disk assay was designed to prescreen bacteria for biocontrol of target spot. One hundred forty-three bacterial isolates from long-term tobacco field soils were tested in this assay. Inoculum of *Rhizoctonia solani* was placed on sterile, moistened Terralite™ horticultural mix in multi-well tissue culture plates. Disks (12 mm) were cut with a cork borer from newly emerged tobacco leaves, surface-sterilized, dipped into bacterial suspensions, and placed in the tissue culture wells. The prescreening assay was optimized for several parameters including form and age of *R. solani* inoculum, and length of assay incubation time. Using *Bacillus* sp. BA55 as a test organism, growth medium and incubation time for the bacterial test strains were optimized also. The optimized assay consisted of oat inoculum of *Rhizoctonia solani* placed on the surface of the Terralite™, and incubated for 1 day. *Bacillus* sp. BA55 was grown for 2 days in Nutrient Broth Yeast Extract (NBY). The 143 bacteria isolated from tobacco field soils at Greeneville, TN, were evaluated for their ability to inhibit colonization of *R. solani* on tobacco leaf disks. *Bacillus* isolates BA22, BA27, BA93, BA122, and BA139 significantly reduced colonization of

the leaf disks by *R. solani* compared to a sterile Tween-20 control. All isolates were tested also with *in vitro* antibiosis assays against *Geotrichum candidum*, a nonpathogenic soil fungus, and *Rhizoctonia solani*. Bacteria were spotted onto Potato Dextrose Agar (PDA) and NBY plates, and oversprayed with *G. candidum*. Five isolates (BA27, BA29, BA57, BA83, and BA125) produced inhibition zones against *G. candidum* $\geq$ 5 mm on PDA while 12 isolates (BA8, BA27, BA28, BA29, BA40, BA44, BA91, BA95, BA96, BA118, BA120, BA121) produced similar results on NBY. In the inhibition assay against *R. solani*, each bacterium was streaked also across the center of three NBY plates, and two agar plugs of 2-wk-old *R. solani* were placed on either side of the streak. Eight isolates (BA4, BA8, BA19, BA27, BA29, BA58, BA96, BA111) substantially inhibited *R. solani* (inhibition zone $\geq$ 5 mm).

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CHAPTER 1: LITERATURE REVIEW

TOBACCO PRODUCTION

Tobacco (*Nicotiana tabacum* L.) is one of the most economically important crops grown in the Southeastern United States. In 1991, farmers in nine Middle Tennessee and two West Tennessee counties received \$34.4 million for sale of dark tobacco alone (22). Three types of tobacco are grown in Tennessee. These include burley, dark air-cured, Eastern dark fire-cured and Western dark fire-cured (C.H. Hadden, personal communication).

Traditionally, tobacco is seeded in Tennessee around March 1, since tobacco seeds do not germinate at temperatures below 50°F (22), and soil temperatures usually do not reach 50°F before March 1. Ideally, tobacco beds should have a southeastern exposure and be well-drained (22, 70). Clay soil should be avoided because of its tendency to compact (22). After the seed bed has been fumigated, usually with methyl bromide, the soil is fertilized and the seeds are sowed. Often, growers will cover the beds with wheat straw and nylon canvas to protect germinating seeds (70). It is vital that the seedlings be watered frequently during dry periods with clean water.

The Environmental Protection Agency is gradually discontinuing the use of methyl bromide as a fumigant because of concerns about human toxicity and its contribution to ozone depletion. Tobacco growers, therefore, are looking for alternative methods of producing tobacco seedlings. An increasingly popular

method is to grow tobacco in styrofoam, or "speedling," trays. The trays usually contain 200 cells (20 x 10), each filled with a soilless potting mix, and are placed in float beds. Float beds are hydroponic systems which contain nitrogen-rich liquid fertilizer. The advantages of float bed systems as compared to traditional planting methods are: 1) float beds can house more seedlings than conventional systems; 2) float beds allow greater control of tobacco growth, since the seedlings are less affected by poor weather conditions when they are inside a greenhouse; 3) plant survival rate is higher; 4) weed control is unnecessary; 5) plant beds cannot fail because of dry conditions; and 6) plants can be returned to the float beds if the transplanting is delayed due to rain (13). One of the drawbacks to using float beds is that the soilless mix is not sterilized before use. It is important, therefore, to practice proper sanitation so as not to introduce any more potential pathogens than may exist already in the mix. Also, few chemicals for disease control have been registered for use in these systems, making it more difficult to control pathogens which may have been introduced. These chemicals, if introduced into the system, may also select for pesticide-resistant strains of the pathogen (51). The primary disadvantage of the float bed system is that it may cost up to 40 percent more than planting in conventional beds (13). In spite of this disadvantage, approximately one-third of Tennessee tobacco farmers produced tobacco seedlings in speedling trays in 1993, and this percentage increased to approximately 50% in 1994 (Darrell Hensley, personal communication).

By early May, the seedlings are usually sufficiently mature to transplant from the beds into the field. Seedlings are planted in rows of four by "setting machines," which allow for quicker planting (22, 70). The wheels of the setter compact the soil and provide a firm foundation for the plants. By late June, most of the transplanting has been completed. Two to three weeks after planting, the soil around the plants is tilled to create air pockets to facilitate growth (70). Nitrogen fertilizer is applied regularly until the tobacco is too large to make fertilizer application practical (22, 70).

Blooms appear in July, and soon afterward, "topping" begins. This process entails breaking off the bloom, leaving 14 to 18 leaves on the stalk, and removing suckers (22, 70). Suckers are lateral buds from the leaf joints which deprive the main shoots of the plant of nutrients. In late summer, the tobacco stalks are split to within six to eight inches of the base and cut. The plants are laid in rows on the ground beside sticks for "hanging," which allows for easier transport to the curing barn. Curing, which takes place from September to November, may occur either naturally with dark air-cured tobacco, or artificially by heat with dark fire-cured tobacco (22, 70). When the tobacco is housed and begins to take on a golden color, growers calculate the heat necessary to cure. This calculation is precise; too much heat can burn down the barn, and too little heat causes the tobacco to mold (70).

TARGET SPOT OF TOBACCO

All the above assumes that everything goes smoothly; that no pests reduce the yield of tobacco, and that weather stays favorable throughout the growing season. In practice, this is not the case. Weather has remained a problem, especially during the seedling phase, although this has been reduced with the advent of float bed systems in controlled environments. The serious problem involves pathogens. In the greenhouse, many pathogens which need high humidity in order to grow and reproduce will be favorably selected if proper cultural practices are not maintained. Pathogens are more difficult to control without either proper sanitation practices or, in many instances, disease-free seed and/or resistant cultivars. However, a trade-off may exist between disease resistance and quality. For example, cultivar DF485 is resistant to both black shank, caused by *Phytophthora parasitica* var. *nicotianae* (Breda de Haan) Tucker, and black root rot, caused by *Thielaviopsis basicola* (Berk. & Broome) Ferraris. Cultivar TR Madole is resistant to neither (22). In general, the germination rate of DF485 is lower than that of TR Madole (22). Leaf quality of DF485 is poorer than that of TR Madole; however, the yield of DF485 is higher (22). Despite the disease resistance and higher yields of DF485, many farmers continue to grow TR Madole.

Although disease severity is effectively reduced by using disease-free stock and proper sanitation, chemicals may be used also to control disease, at least in conventional seed beds. Many diseases can be controlled with the proper

chemical treatment; however, chemical control measures for some diseases have not been established. Some of these diseases have only recently been described; therefore, insufficient time has been devoted to developing control measures for the pathogens. One such disease is target spot of tobacco, caused by a basidiomycete, *Thanatephorus cucumeris* (A.B. Frank) Donk (anamorph *Rhizoctonia solani* Kühn). Target spot may be referred to as Rhizoctonia leaf spot, also. This disease was first reported in 1948 in Brazil (60, 61). It did not pose serious threats in the United States until 1983-84 (60, 61). *T. cucumeris*, in addition to causing target spot, may cause damping-off of seedlings in conventional beds (22, 60) and sore shin of tobacco through its anamorph, *Rhizoctonia solani* (60). These diseases usually are not serious (losses to sore shin seldom rise above 5%) (60); however, all types of tobacco are susceptible to both of these diseases. Although target spot rarely causes serious crop loss overall, under optimum conditions, losses can exceed one half of a given field (60). Target spot was first described in North Carolina in 1983-84, and since then, it has been a major problem for tobacco growers in the Southeastern United States. In addition to tobacco, *T. cucumeris* has been reported on other solanaceous crops such as potato (38) and tomato (26). It has been reported also on non-solanaceous hosts such as cabbage (1), wheat (50), corn (67), jute (71), cowpea, snap bean, and lima bean (66).

Target spot begins as small water-soaked lesions, which are caused by basidiospores discharged to the leaf. The lesions appear net-like on seedlings,

and under favorable environmental conditions, they expand quickly and become almost transparent. The lesions are roughly circular and are surrounded by a chlorotic halo (60). Sometimes the lesions grow more slowly and develop a pattern of concentric rings, from which target spot derives its name. These symptoms make the disease at first difficult to distinguish from *Alternaria* brown spot (60). The necrotic lesion may eventually drop out and leave a shot-hole effect (60). *T. cucumeris* mycelium is often found near the margins of the lesion, and the hymenium, a fertile layer which contains the basidia, are formed on both upper and lower leaf surfaces. Hymenia are resupinate, cream to grayish-colored, and loosely attached to the substrate (60).

RHIZOCTONIA SOLANI (ANAMORPH OF THANATEPHORUS CUCUMERIS)

Rhizoctonia solani is capable of living saprophytically in the soil, either in organic matter as hyphae or overwintering as sclerotia. Root exudates stimulate germination of the sclerotia, and once the hyphae come into contact with a root or stem, they branch profusely to form an infection cushion. From this structure, *R. solani* will enter a host cell. One study suggests that *R. solani* produces phytotoxins, phenylacetic acid (PAA), and its hydroxylated derivatives (37). PAA effectively inhibited the growth of tomato seedlings. The optimum temperature range for germination is 24-28°C, although rapid growth occurs between 20-30°C (60). This fungus has no asexual spores, and only rarely does it form the sexual stage (11). Basidiospores are formed abundantly during

extended periods of high relative humidity (99 - 100%) and moderate temperatures (20-30°C) (60), and the fungus needs 16-20 days to complete the life cycle in a favorable environment. Basidiospores are hyaline, smooth, and oblong to ellipsoid, averaging $9 \times 5.5 \mu\text{m}$ (60). Hyphae are distinguishable by branching at right angles and an absence of clamp connections.

Rhizoctonia solani, as a species, is divided into several groups based on their ability to fuse with one another, which results in mixing of cellular contents (11). Isolates usually fuse only within their own anastomosis group (AG). For example, AG-1 isolates will only fuse with other AG-1 isolates. Parmeter et al. originally described four AGs (54), and over the years, successive AGs have been added. Currently, eleven AGs are recognized, with several subtypes, although there has been a report of a possible twelfth anastomosis group (58). Some of the AGs are of minor importance and have restricted geographical and host ranges, while others are pathogenic on economically important solanaceous crops (11, 60, 61) and nonsolanaceous crops (11). Anastomosis groups pathogenic on tobacco include AG-1, AG-2-2, AG-3, and AG-4 (11, 60). Isolates from all four AGs cause damping-off and sore shin, but only isolates from AG-2-2 and AG-3 cause target spot (60).

In the laboratory, it is difficult to induce fructification and sporulation of the perfect stage of *Rhizoctonia solani* on culture media. Adams and Butler (3) studied the influence of nutrition on basidial formation. They characterized several strains of six different anastomosis groups, including AG-1, AG-2-1,

AG-2-2, AG-3, AG-4, and AG-5. Each isolate was transferred to a subdivided Petri dish, with one half containing a nutrient rich medium, and the other half containing a nutrient-poor medium. They discovered that the induction of sporulation for each AG is dependent on the amount of nitrate and glucose in the nutrient-rich medium. In their experiment, only isolates in AG-1, AG-4, and AG-5 sporulated on the medium. The anastomosis groups which caused target spot were incapable of sporulating on this medium (3). They also demonstrated that *T. cucumeris* sporulated when the flow rate of air (99% relative humidity) of 45 L per h, and that carbon dioxide concentrations above 0.03% for AG-4 and 0.06% for AG-1 inhibited basidial formation (4). At 85% relative humidity, *T. cucumeris* was incapable of sporulation. The mechanism of basidial stage inhibition is not known; it may be due to either a buildup of carbon dioxide in a poorly ventilated area, or production of ethylene or other volatiles, or low relative humidity (4).

CHEMICAL CONTROL OF TARGET SPOT AND OTHER TOBACCO DISEASES

Although *Rhizoctonia* leaf spot is usually not a serious disease of tobacco, it can be devastating given the proper environmental conditions. Infected plants may not die from infection, but the economic value of a given farmer's crop may be reduced in consequence. Because of this, tobacco farmers are searching for ways to control target spot. The only method recommended to date is practicing proper sanitation. This includes cleaning all

instruments between use, reducing leaf wetness, and not introducing soil into tobacco float beds. In the field, cultural practices such as crop rotation are not recommended for control of *Rhizoctonia solani* because it has a very wide host range and may endure sustained periods of poor environmental conditions.

Chemical control, a staple of pathogen control, has been used to some degree of success with tobacco. Cole and Zvenyika (14) used the fungicide triademinol to control sore shin of tobacco. In a study conducted from 1982-84, they applied triademinol (Baytan 15% wettable powder) at rates of 0.5 and 1 g/m² to tobacco seedlings, transplanting seedlings after two days to clay and sandy soils. They discovered that triademinol residues were beneficial to seedlings; both seedling growth and control of *R. solani* were significantly improved compared to untreated controls in both soils (14). They also found that control of sore shin was greatest at the highest level of fungicide application (1 g/m²). Although triademinol is effective against *R. solani*, it was only tested in conventional seed beds, and it was never registered in the United States.

Other chemicals have been used to control tobacco pathogens other than *R. solani*. For example, Shoemaker and Main (C.E. Main, personal communication) tested dimethomorph (CME 151) and metalaxyl (Ridomil 2E) against blue mold (*Peronospora tabacina* D.B. Adam). Both chemicals were effective against strains of *Peronospora* sensitive to metalaxyl; however, more dimethomorph was needed to obtain the level of control provided by metalaxyl

(C.E. Main, personal communication). Dimethomorph also significantly reduced infection by metalaxyl-resistant strains of *P. tabacina*. In field tests, Shoemaker and Main demonstrated that dimethomorph, applied as a foliar spray, was more effective than either metalaxyl or mancozeb (C.E. Main, personal communication). So far as is known, dimethomorph has only been tested against oomycetes, and it has never been tested in the float-bed system.

Fluazinam, a recently-developed fungicide produced by ISK Biosciences, has been tested against *Rhizoctonia* stem rot on poinsettia. Plants treated with one drench of fluazinam showed no stem rot compared to 65% stems rotted in the control (8). It is available in dry flowable powders, wettable powders, and flowable formulations. Fluazinam has a broad spectrum; not only does it act against *Rhizoctonia solani*, it has demonstrated effectiveness in control of Botrytis blight of poinsettia, *Thielaviopsis basicola*, and many species of *Pythium* and *Phytophthora* on ornamental crops, also (8).

BIOLOGICAL CONTROL

Biological control is another alternative for reducing disease. Cook and Baker (17) defined biological control as pathogen control by any organism other than man, and includes the use of antagonistic microorganisms, the host itself, or hypovirulent strains of the pathogen. It offers a more environmentally sound approach to disease control; no synthetic chemicals are released to the environment. However, chemicals that may be released by the potential biocontrol organism must be studied to determine if they are harmful either to

nontarget organisms or to the host itself. Also, it is more difficult to maintain a biocontrol agent than to store a fungicide; biocontrols may be genetically unstable in culture. However, with an adequate storage system for bacteria or fungi (freeze-dried or stored at -80°C), this potential problem is reduced.

MODES OF ACTION FOR BIOCONTROL AGENTS

Biocontrol organisms may act in one or more ways: they may 1) release metabolites which are inhibitory to pathogens such as *Rhizoctonia*; 2) parasitize the pathogen; 3) stimulate host defense mechanisms (induced resistance); or 4) compete more aggressively for available resources (including carbon, oxygen, water, and space) (74).

Antibiotic production is one of the most-studied means of biocontrol. Antibiotics are low molecular weight organic compounds which are harmful to the growth of other organisms (77). Weller and Thomashow list five supporting pieces of evidence that antibiotics play a vital role in biological control: 1) many biocontrol microorganisms produce antibiotics *in vitro*; 2) antibiotic production often correlates with biological control activity; 3) purified antibiotics alone duplicate the effects of the whole agent; 4) mutants which are antibiotic-deficient are less suppressive of disease than parent strains, and genetic complementation restores suppressiveness; and 5) some antibiotics can be isolated from natural habitats (77). One problem with antibiotics is that they

may be toxic to the host itself. Also, antibiotics may disrupt the soil microfloral populations by killing non-target organisms.

Some biocontrol fungi act on pathogens by "parasitizing" them (*Gliocladium* and *Trichoderma* are examples of mycoparasites). However, parasitism has not been demonstrated conclusively for many fungi that supposedly employ this method (18). Deacon and Berry point out in a review that many "aggressive" parasites like *Trichoderma* spp. have not been shown to gain nutrients from their hosts, and may antagonize while growing as saprotrophs (18). Mycoparasites, according to studies on *Trichoderma*, show pronounced tropism toward their hosts, determine specificity for interactions by host surface recognition, and produce enzymes which lyse host cell walls (18).

An area of biological control which recently has aroused interest is induced resistance. Inoculating a host plant with either an avirulent or hypovirulent strain of a particular pathogen, a pathogen of another host, or a nonpathogen may induce a resistance response to the pathogen in question (17). Small local lesions appear on the inoculated area. Resistance is induced either locally or systemically within the host. Many organisms, such as *Bacillus thuringiensis* Berliner, are capable of inducing resistance (45), and this method is effective against a broad range of pathogens. Induced resistance is nonspecific as to pathogen races; thus, they may be highly durable in soils with

high pathogen density. Resistance is active locally for 1-2 weeks and 3-6 weeks systemically (45).

Siderophores act on disease suppression by uptaking the ferric iron and limiting its availability to the pathogen (42). Suppression is higher in alkaline soils than in acid soils because ferric iron becomes more available as soil pH decreases. Fungal pathogens may or may not produce their own siderophores; if they do, their siderophores have a much lower affinity for ferric iron than bacterial siderophores. If they do not, they are also incapable of using the bacterial siderophore to take up iron (74).

Antagonists also may control pathogens by competing for available space around the roots. Root cell junctions and points of emergence of lateral roots are readily colonized by bacteria because root exudates are prevalent there. Inoculating planting material with bacterial antagonists may reduce establishment by pathogens at those sites (68). Also, the antagonists may simply block infection sites (57).

Vesicular-arbuscular mycorrhizae (VAM) may increase plant resistance by physical blockage also. Mostly, however, VAM increase a plant's uptake of phosphorus in soils where it is limiting. Better growth resulting from improved plant nutrition may account for the effects of VAM against plant pathogens (17).

Biocontrol organisms also may compete for available nutrients such as carbon and nitrogen. High nutrient availability enhances pathogen growth.

When a given nutrient is limiting in the soil, competition for that nutrient intensifies, especially around the root surface. If the biocontrol organism can colonize the root surface and absorb the nutrients before the pathogen can, fewer nutrients will be available to the pathogen, and pathogen growth will be limited.

Most biocontrol agents use a combination of methods to suppress pathogens. For example, a study by Weindling (reviewed in 77) on antagonism of *R. solani* by *Trichoderma viride* Pers. ex S.F. Gray suggested that an antifungal compound, gliotoxin, was involved in the suppression of *R. solani*. *Trichoderma* spp. are also known parasites of *Rhizoctonia* (46). Some *Pseudomonas* spp. produce antibiotics such as pyoluteorin and pyrrolnitrin, effective against damping-off of cotton, caused by *R. solani* (35, 36). *Pseudomonas* spp. also produce siderophores, which limit growth of the pathogen by absorbing the available iron in the soil (74). Although many microorganisms have the potential to control pathogens, this review will be limited to four groups: *Bacillus* spp., *Pseudomonas* spp., *Trichoderma* spp., and *Gliocladium* spp.

BACTERIAL BIOCONTROL AGENTS: *BACILLUS* AND *PSEUDOMONAS* SPP.

Bacillus spp. are Gram-positive, rod-shaped bacteria. Members of this genus have been a focus of interest in biological control because they possess endospores that are tolerant to heat and desiccation. *Bacillus* is known to

produce many antibiotics, such as bacitracin and gramicidin (81), which aid in the suppression of disease. *Bacillus subtilis* Cohn emend. Prazm. has been used successfully to control a rust fungus (*Uromyces appendiculatus* (Pers.) Unger) on bean (*Phaseolus vulgaris* L.) (7). Recently, it has been used in a study targeting take-all of wheat, caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici*, an ascomycete (74). Although *Bacillus* has been used extensively as a biocontrol agent, attempts to examine the genetic basis of biocontrol of plant pathogens have been incomplete because of the complexity of *Bacillus* genetics. In the last twenty years, three strains of *Bacillus* spp. have been described as effective in control of diseases of many crops. One, designated *B. subtilis* A13, was isolated in 1971 in Australia by Broadbent et al. (9) from lysed mycelia of *Sclerotium rolfsii* Sacc. and used against *Rhizoctonia solani* on cereals and carrots. Suspensions of 10^6 - 10^7 cells/ml were applied as seed treatments. *B. subtilis* A13 significantly improved yield on cereals compared to the controls (807 g/row vs. 631 g/row for the dry control). Similar results also were found on carrots. From another experiment with *B. subtilis* A13, Merriman et al. concluded that the increased growth response is not specific to soil and plant types (48). They suggested that the growth response may not be due solely to biological control of the root pathogens. It appeared to improve plant growth by suppressing pathogens and probably by stimulating plant growth. Since 1983, *B. subtilis* A13 has been

sold as a treatment for peanut under the name Quantum-4000 (Gustafson, Dallas, TX) (74).

In 1977, Fravel and Spurr isolated a bacterium, *Bacillus cereus* subsp. *mycoides* Flügge strain B23 from washings of tobacco plants. This bacterium was highly effective against *Alternaria* brown spot of tobacco (23) in a controlled environment. It significantly reduced germ tube length and the number of appressoria per conidium in greenhouse experiments. Fewer bacteria of *B. cereus* subsp. *mycoides* B23 were needed to control *Alternaria* brown spot compared to other bacteria tested in the study. Evidence suggested that B23 was producing a toxic metabolite because *Alternaria* conidia did not germinate in its presence *in vitro*.

Another strain that has been shown to be effective is *Bacillus cereus* Frankland and Frankland strain UW85 (25, 30, 31, 32, 33, 63). *B. cereus* UW85 has been shown to control oomycetes on a variety of crops, including soybeans (25, 30), tobacco (32), cucumber (63), and alfalfa (33). On cucumber, UW85 effectively controlled *Pythium aphanidermatum* (Edson) Fitzp., the cause of cottony leak. Treatment with UW85 resulted in a significant reduction of disease and of the number of wound sites rotted per plant compared to the water control (0.93 vs. 0.07 for the control and UW85, respectively). In further experiments with soybeans, Handelsman et al. demonstrated that UW85, applied as a seed treatment, colonized the radicle, spread on the roots, and persisted until harvest at densities of 10^4 colony

forming units per root segment (30). Strain UW85 germinates within 1 day after planting, which may be important in preventing disease and modifying rhizosphere microfloral populations. UW85 produces antimicrobial compounds and creates an environment which lyses zoospores (30). These compounds inhibit growth of Gram-negative bacteria and germinating zoospores *in vitro*, but it has not been determined whether or not this occurs in the rhizosphere (30).

Two molecules, or "activities," were identified as factors in disease suppression by UW85 in alfalfa. A molecule (<500 da) suppressed disease by lysing zoospores of *Phytophthora megasperma* Drechs. f. sp. *medicaginis* Kuan and Erwin. *B. cereus* UW85 also acts by chelating calcium with dipicolinic acid in the endospore. Addition of 5 mM calcium to the culture medium blocked zoospore lysis. The second activity, a 600 da peptide, is responsible for most of the *Phytophthora* suppression. This peptide also appears to be unique to UW85, because other bacilli that lysed zoospores did not suppress alfalfa damping-off (31).

A study (33) suggests that the culture medium on which *B. cereus* UW85 was grown greatly affected its ability to control *P. megasperma* f. sp. *medicaginis*. *B. cereus* UW85 cells were grown in Tryptic Soy Broth (TSB), Minimal Medium C (Min 3C), and Casamino acids-Glucose broth (CB). The bacteria grown in either Min 3C or CB increased alfalfa survival rate to 81% compared to 43% survival with bacteria grown in TSB and 1% survival in the untreated control. They also found that the greater the proportion of

endospores to vegetative cells, the greater the control of UW85 against *P. megasperma* f. sp. *medicaginis*.

There is also some evidence that a metabolite produced by isolates of *B. cereus* may be responsible for a condition in tobacco known as "frenching." (60) Frenched tobacco leaves are spindle-shaped and light green with a dark green venous system. Often, plants are severely stunted. Frenching is also associated with soils of high pH and high moisture. Usually only one or a few plants in a given field are affected. Unlike other organisms which cause diseases, *B. cereus* is not associated with tobacco tissue and is not a tobacco pathogen. In spite of this, many other *Bacillus* isolates show great potential as biocontrol agents.

Pseudomonas spp. have been studied also as biocontrol agents, although work on tobacco is limited. Stutz et al. (65) reported that a strain of *P. fluorescens* (Trevisan) Migula, CHA0, suppressed black root rot of tobacco, caused by *Thielaviopsis basicola*, in 36 disease conducive field soils. Strain CHA0 was isolated from soil in which tobacco was grown. The tobacco seedlings were grown in the greenhouse. According to a study by Ahl et al., black root rot is suppressed by a combination of iron-chelating siderophores, hydrogen cyanide, and antibiotics (5). In later studies, Défago et al. identified the antibiotic which suppressed black root rot as 2,4-diacetylphloroglucinol (Phl) (19, 39). Phl does not influence survival of *T. basicola*; rather, it stimulates plant growth (19). Although Phl is phytotoxic, *P. fluorescens* CHA0

does not produce the antibiotic in sufficient quantities to damage the plant (39). Phl-deficient mutants of CHA0 did not suppress black root rot as effectively as the wild-type strain (39), but it did inhibit *T. basicola* to some degree compared to an untreated control (39).

Siderophores produced by CHA0 may play a prominent role in disease suppression in iron-limiting soils (40). *P. fluorescens* CHA0 needs sufficient iron to suppress black root rot (19, 40); however, iron competition does not seem to be the primary mechanism for black root rot suppression (40).

Interest in *Pseudomonas* spp. as biocontrol agents was initiated in 1978 (71) when Burr et al. reported that strains of *P. fluorescens* and *P. putida* Migula increased potato growth (10). Later studies showed that pseudomonads also increased growth of sugarbeet (69) and radish (43). Fluorescent pseudomonads (*Pseudomonas* spp. which produce fluorescent siderophores) suppress pathogens on potato (10, 16, 44, 57), peanut (24), cotton (35, 36), wheat (75, 76), carnation (80), and poinsettia (12) by production of siderophores and antibiotics. Two studies demonstrated that *Pseudomonas fluorescens* Pf-5 increased cotton seedling survival from 30 to 79% against *R. solani* in infested soils (35, 36). An antibiotic, pyrrolnitrin, was identified as the substance that inhibited *R. solani*. Pyrrolnitrin was inhibitory against *Alternaria*, *Verticillium dahliae* Kleb., and *T. basicola*. It was less effective against *Fusarium* spp., and ineffective against *Pythium ultimum* Trow (36).

On poinsettia, a *Pseudomonas* spp. was effective at suppression of stem rot by *R. solani*, compared with the controls and *Gliocladium virens* Miller, Gliddens, and Foster (12). These bacteria also have demonstrated enormous potential as biocontrol agents on a wide range of crops, from wheat to ornamental plants.

TRICHODERMA SPP. AND GLIOCLADIUM SPP.

Both *Trichoderma* and *Gliocladium* are fungi that belong to the class Deuteromycetes. They have been studied for their biocontrol potential for many years. Many species of *Trichoderma*, such as *T. hamatum* (Bon) Bain, and *T. harzianum* Rifai, have been shown to be efficient biocontrol agents against *Rhizoctonia* diseases. *Trichoderma* spp. produce chitinases and fungal cell-wall degrading enzymes which would allow for penetration into the hyphae of fungal pathogens. Recently, Lorito and colleagues (47) have demonstrated antifungal activity of *T. harzianum* by production of the enzymes endochitinase and chitobiosidase. Complete inhibition for many pathogens occurred at 200-300 mg/ μ l (20, 47). Similar enzymes have been isolated from *Gliocladium virens*, and in combination with the antifungal compound gliotoxin from *G. virens*, disease incidence of *Botrytis cinerea* Pers. was reduced by as much as 95%, compared to 0-20% for either the enzyme or the antibiotic alone (20). These studies provided evidence that chitinolytic enzymes are necessary for control by *T. harzianum* and *G. virens*. Suppression of *R. solani* was

accomplished by both seed and soil treatments (47). In 1986, Windham et al. demonstrated that *T. harzianum* T-95 and T-12 produced growth factors that increased seed germination rate and dry shoot weights in tomato and tobacco seedlings (78). More recently, experiments demonstrated that *T. harzianum* significantly reduced disease incidence of *R. solani* on cotton from 100% to approximately 30% (2). In another experiment on beans, two isolates of *T. harzianum* reduced root rot of bean, caused by *R. solani*, from 71% to 2% when the two isolates were combined, compared to 40-60% for each isolate alone (2). This suggests that one organism alone may not be sufficient to reduce disease significantly on a given crop, and that a mixture of organisms, or even a biocontrol organism and a fungicide, might be more effective. One such study was conducted by Cole and Zvenyika in 1988. They compared tobacco fields in which both triademinol and *T. harzianum* were introduced. Both the *T. harzianum* and triademinol treatments reduced disease incidence separately, but in combination, disease incidence was almost zero (15). Tobacco yield also was increased significantly when both treatments were applied (3000 kg/ha with both treatments, and 2600 kg/ha with triademinol only) (15).

Papavizas (53) described experiments involving both *Trichoderma* and *Gliocladium* against damping-off of cotton seedlings by *R. solani*. Isolates of *G. virens*, *T. hamatum*, *T. harzianum*, and *T. viride* were tested over a four-year span. All isolates suppressed activity of *R. solani*; however, *G. virens* GL-21

gave the greatest inhibition against *R. solani*. As a result of this experiment, *G. virens* strain GL-21 became the first registered biological control agent for diseases of bedding plants caused by *Pythium ultimum* and *Rhizoctonia solani*. It has not yet been approved for use in float-beds. Now, however, one experiment suggests that *G. virens* is moderately pathogenic on some corn varieties (72).

CHAPTER 2: BIOLOGICAL AND CHEMICAL CONTROL OF TARGET SPOT OF FLOAT-GROWN TOBACCO SEEDLINGS

INTRODUCTION

Target spot (*Rhizoctonia* leaf spot) of tobacco is caused by the soilborne fungus *Thanatephorus cucumeris* (A.B. Frank) Donk (anamorph *Rhizoctonia solani* Kühn). At temperatures between 20 and 30°C and 99 to 100% relative humidity, basidiospores of *T. cucumeris* are shot toward the leaves. Target spot appears first as small necrotic lesions. The lesions expand outward and form concentric rings (60, 61). The lesions may often drop out, leaving a shot-hole effect (60). Hymenia on the outer edge of the lesion may produce secondary basidiospore inoculum, which is dispersed by air to healthy tobacco tissue. Although losses from target spot rarely exceed 5%, under optimum environmental conditions, crop losses may exceed 50% (60, 61). In Tennessee, significant losses in tobacco transplants grown in float systems have resulted from target spot. There also has been an increase in the number of reports of target spot on field plants, which may be related to the increase in production of transplants by the float system. Currently there are no control measures recommended for target spot on float plants except for proper sanitation. Chemicals such as dimethomorph have been tested against tobacco blue mold (C.E. Main, personal communication) with some success, although it has only been tested on oomycetes. Other chemicals such as fluazinam have

not been tested on tobacco, but have shown potential for control of *Rhizoctonia* bedding plant diseases in the greenhouse (8). Biocontrol agents such as *Bacillus* spp. and *Pseudomonas* spp. offer an alternative to chemical control. Bacteria in these genera produce a wide range of antibiotics (19, 77, 81) and have been shown to control tobacco diseases (5, 19, 22, 32, 39, 40, 65). The objectives of this study were 1) to test the efficacy of four chemicals and four biological control agents for control of target spot on dark tobacco transplants in the float system; 2) to examine the effects of two dark tobacco cultivars, DF485 and TR Madole, two planting methods, direct-seeding and seed-and-transfer, and five host ages (2 - 6 wk) on severity of target spot on float-grown tobacco seedlings; and 3) to determine if any treatments would continue to provide control of target spot in the field, and if these treatments would affect overall yield, leaf quality, and revenue.

MATERIALS AND METHODS

Pathogen inoculum. A culture of *Rhizoctonia solani* originally isolated from a target spot lesion on a tobacco leaf was provided by Darrell Hensley. Cultures of *R. solani* were maintained on 1/5 Potato Dextrose Agar (1/5 PDA, 4.8 g Potato Dextrose Broth, Difco Laboratories, Detroit, MI, and 15 g agar, Sigma Chemical Company, St. Louis, MO, in 1 L deionized water). Whole oat media was prepared by autoclaving 75 cm³ oats and 62.5 ml deionized water in 500 ml Erlenmeyer flasks for 90 min on two successive days. Twenty-three

plates of *Rhizoctonia solani* on Potato Dextrose Agar (PDA, 24 g Potato Dextrose Broth, Difco Laboratories, Detroit, MI, and 15 g agar, Sigma Chemical Company, St. Louis, MO, in 1 L deionized water) were transferred to 36 flasks of oats, five agar plugs per flask. All cultures of *R. solani* were made from the initial culture. The inoculated oat media were incubated for three wk at 25°C.

Isolation of potential biological control organisms. Soil samples were collected from three fields at both the Tobacco Experiment Station at Greeneville, TN, and the Highland Rim Experiment Station at Springfield, TN. The samples were taken from fields where tobacco had been grown for breeding or production for several years. A sample (5 g) of each soil type was placed into a sterile screw cap tube and diluted with 40 ml of sterile phosphate buffered saline (PBS). The tubes were shaken for 15 min at 250 rpm. An aliquot (1 ml) of the shaken sample was pipetted into each of 3 tubes of sterile PBS. The tubes were vortexed, and serial dilutions to 10^{-8} were prepared. Aliquots (0.1 ml) of the 10^1 - 10^5 dilutions were plated onto *Trichoderma* Medium E (TME) (52), and aliquots (0.1 ml) of the 10^4 - 10^8 suspensions were plated onto Fluorescent Pseudomonad Medium (FPM) (62). The 10^2 - 10^6 suspensions were heated in a water bath at 85 C for 20 min to kill all vegetative cells. One tenth of a ml of these suspensions was plated onto Aerobic Spore Formers Medium (ASFM) (79). Plates were checked for growth after 1 to 7 days. From the Greeneville soil, 143 bacterial colonies, designated BA1 to BA143, were isolated; 118 bacteria were selected from ASFM, and 25

bacteria were selected from FPM. Thirty-eight fungal isolates were selected from TME.

Procedures similar to the ones described above were used to isolate bacteria from the Springfield soils. TME was not used for these isolations. Isolations were made on Tryptic Soy Agar (TSA, 30 g Tryptic Soy Broth, Difco Laboratories, Detroit, MI, and 15 g agar, Sigma Chemical Company, St. Louis, MO, in 1 L deionized water) and *Arthrobacter* spp. Medium (ASM) (29), FPM, and ASFM. Three hundred forty-two isolates were selected. All isolates were streaked onto individual Nutrient Broth Yeast Extract (NBY, 73) plates. Because of time constraints, only the 143 bacteria isolated from the Greeneville soils were tested further.

Preliminary in vitro screening of bacteria from Greeneville, TN. Thirty plates of 1/5 PDA and 60 plates of 1/5 PDA with six wells cut with a #4 cork borer were prepared for this experiment. Of the 60 plates with the wells, 30 were filled with sterile Terralite™ (Grace Sierra, Milpitas, CA), and 30 were filled with sterile soil. The soil used was the soil of origin of each bacterium tested. Terralite™ is a soilless horticultural mix in which tobacco is grown. Five bacteria were inoculated onto each plate, one per well. The sixth well was mock-inoculated with 30 μ l sterile NBY broth. The bacteria were incubated for 3 days, after which, a plug of *R. solani* was placed onto the center of the plate. Zones of inhibition were measured after 7, 14, and 21 days. Bacteria that

g/cm³. Fungicide formulations were prepared 1 day before the first application. Formulations were prepared at 4X strength, and diluted.

Greenhouse experiment - 1993. In addition to the effect of disease control treatment, the effects of two dark tobacco cultivars, DF485 and TR Madole, and two planting methods, direct-seeding and seed-and-transfer were studied. In direct-seeding, tobacco seed are planted directly into cells of the float trays. In seed-and-transfer, many seedlings are grown in a container and seedlings are transferred to the cells of the styrofoam trays.

In the greenhouse provided by the Highland Rim Experiment Station (HRES) at Springfield, TN, four float beds with ten compartments each were constructed. Each float bed represented one replicate. Each individual compartment represented one disease control treatment and held four styrofoam trays. These four trays represented the two cultivars and the two planting methods. Each float tray held a maximum of 80 seedlings. The experiment was a 10x2x2 factorial in a split plot. The main plot was disease control treatment. The four trays representing two cultivars and two seeding methods were randomly placed within each compartment representing the disease control treatment. Peters Excel 21-5-20 fertilizer was added to each individual compartment at 7 ppm. Four humidifiers were placed in the walkways among the four replicates (Fig. 1).

Two of the fungicides, dimethomorph and Rovral, were applied every other week as foliar sprays. The three bacteria, fluazinam, and Dithane® were



Figure 1. Float beds and humidifiers in the greenhouse experiment for target spot control.

applied weekly as foliar sprays. Float trays were separated by treatment, moved outside the greenhouse into shade, sprayed until runoff, dried outside, then transported back to the greenhouse and placed in the proper float bed. One day after the first treatment, 8 cm³ (0.5 g) of 2-wk-old oat inoculum of *Rhizoctonia solani* was added to two locations in each tray. After 3 wk, approximately half of the seedlings in three direct-seeded treatments (BA55, fluazinam F, and the untreated check) were designated for transplanting to the field at HRES. Disease free checks, provided by HRES, were included also. Thirty plants per disease control treatment, per cultivar, were planted in 1 row per replicate. Two outside guard rows and four disease-free check rows were planted per replicate. After harvesting and curing, plants were assessed for crop index (21), grade index (49), yield, and revenue. The remainder of the plants were assessed for disease with the Horsfall-Barratt rating scale (34). Rating values were converted to percent diseased foliage for statistical analysis (56). The main effects of control treatment, cultivar, planting method, and the interactions of these factors were analyzed for significance with the General Linear Models procedure and F-protected LSD tests of the Statistical Analysis System® (59).

Greenhouse experiment - 1994. The same general procedures were followed in 1994, with these exceptions: only one planting method, direct seeding, was included; neither the disease-free check nor the GL-21 treatment was included; two new treatments, a bacterium, *Bacillus* sp. BA4, and fluazinam

DF (ISK Biosciences, 1.25 g/L) were tested. *Rhizoctonia solani* was introduced into the system as rice inoculum (8 cm³, 2.5 g) 6 days after the first treatment, instead of 1 day. *R. solani* was cultured in flasks containing 25 g long-grain rice and 18 ml sterile deionized water (autoclaved on two consecutive days) for 2 wk at 30°C.

Host age study. In June 1994, an experiment was performed to test the effects of host age, disease control treatment, and cultivar on target spot severity in the greenhouse. Five host ages, ranging from 2 to 6 wk, 2 dark cultivars (TR Madole and DF485), and two disease control treatments, *Bacillus* sp. BA55 and an untreated check, were included.

Suspensions of BA55 and pathogen inoculum were prepared as described in the 1993 and 1994 greenhouse experiments, respectively. The experiment was designed as a split plot, in a 5x2x2 factorial with disease control treatment as the main plot and host age as the subplot. Sixteen 80-cell float trays (four replicates, two cultivars, two control treatments) were direct-seeded each week starting at 6 wk and continuing to 2 wk prior to the start of the experiment for a total of 80 float trays.

Suspensions of BA55 were diluted to 4 L and applied as a foliar spray as described previously once per week for 3 wk. Six days after the first treatment, the float trays were inoculated with 8 cm³ of rice grains colonized by *R. solani*.

After 3 wk, all plants were removed from the trays, and separated by age, cultivar, and treatment. The plants were assessed for diseased foliage using the Horsfall-Barratt rating scale (34). The rating values were converted to percent diseased foliage (56), and data were analyzed for significance with the General Linear Models procedure and F-protected LSD tests of the Statistical Analysis System® (59).

Fluazinam rate study. An experiment was prepared to test the effects of five different concentrations of fluazinam F (200, 400, 600, 800, and 1000 ppm) on disease severity of target spot on 6-wk-old float-grown tobacco seedlings. The cultivar, TR Madole, was seeded-and-transferred into 15-cell trays. There were four replicates with five fluazinam rate treatments and an untreated control. The formulations were prepared in 400 ml of sterile deionized water 1 day before treatment and applied 6 days before addition of 8 cm³ of rice grains colonized by *R. solani*. The treatments were applied once per week for 3 wk. Plants were assessed for percent diseased severity with the Horsfall-Barratt scale (34), and rating were converted to percent diseased foliage for data analysis (56). The main effect of fluazinam concentration was analyzed for significance using the General Linear Models procedure and an F-protected LSD test of the Statistical Analysis System® (59).

RESULTS

Greenhouse experiment - 1993. Data from only nine disease control treatments were analyzed in 1993. Since a disease-free check could not be maintained, the data for this treatment was eliminated from the analysis. Regardless of planting method or cultivar, control of target spot on tobacco was greatest on seedlings treated with BA-55, fluazinam, Rovral, Dithane®, (Table 1, Figs. 2-4), and intermediate on dimethomorph-treated seedlings (Table 1, Figs. 2, 3, 5) when compared to the control (Fig. 6). Although Rovral provided control of target spot, phytotoxicity was also evident on seedlings (Fig. 7). No disease control was observed on plants treated with BA39, BA56, and GL-21 (Figs. 2, 3). In fact, more diseased foliage was found on direct-seeded plants treated with BA56, and on cultivar DF485 treated with BA56, than on the untreated control (Figs. 2, 3).

Greenhouse experiment - 1994. In 1994, both formulations of fluazinam, Rovral, and Dithane® significantly reduced target spot (Table 2, Figure 8), regardless of cultivar. On cultivar DF485, control was high with dimethomorph, and intermediate with BA55, BA56, and BA4. On cultivar TR Madole, no control was observed with either dimethomorph or any of the four bacteria (Fig. 8).

In both years, the main effect of cultivar was significant (Tables 1, 2). The percent diseased foliage was higher in DF485 than in TR Madole. However in 1993, the difference was not as great as in 1994. For example, in the

Table 1. Analysis of variance for the effects of control treatment (CT), cultivar (CV), and planting method (PM) on percent diseased foliage of float-grown tobacco plants with target spot in 1993

Source of variation	df	Sum of Squares	Mean Square	F value	<i>P</i> > F
Replicate	3	58.079	19.360	1.12	0.3450
Control treatment	8	5477.286	684.661	10.27	0.0001
REP x CT	24	1599.745	66.656	3.86	0.0001
Planting method	1	571.377	571.377	33.12	0.0001
CT x PM	8	610.074	76.259	4.42	0.0002
Cultivar	1	193.450	193.450	11.21	0.0012
CT x CV	8	593.664	74.208	4.30	0.0002
PM x CV	1	61.959	61.959	3.59	0.0616
CT x PM x CV	8	103.035	12.879	0.75	0.6502
Error	81	1397.261	17.250		
Corrected Total	143	10665.930			

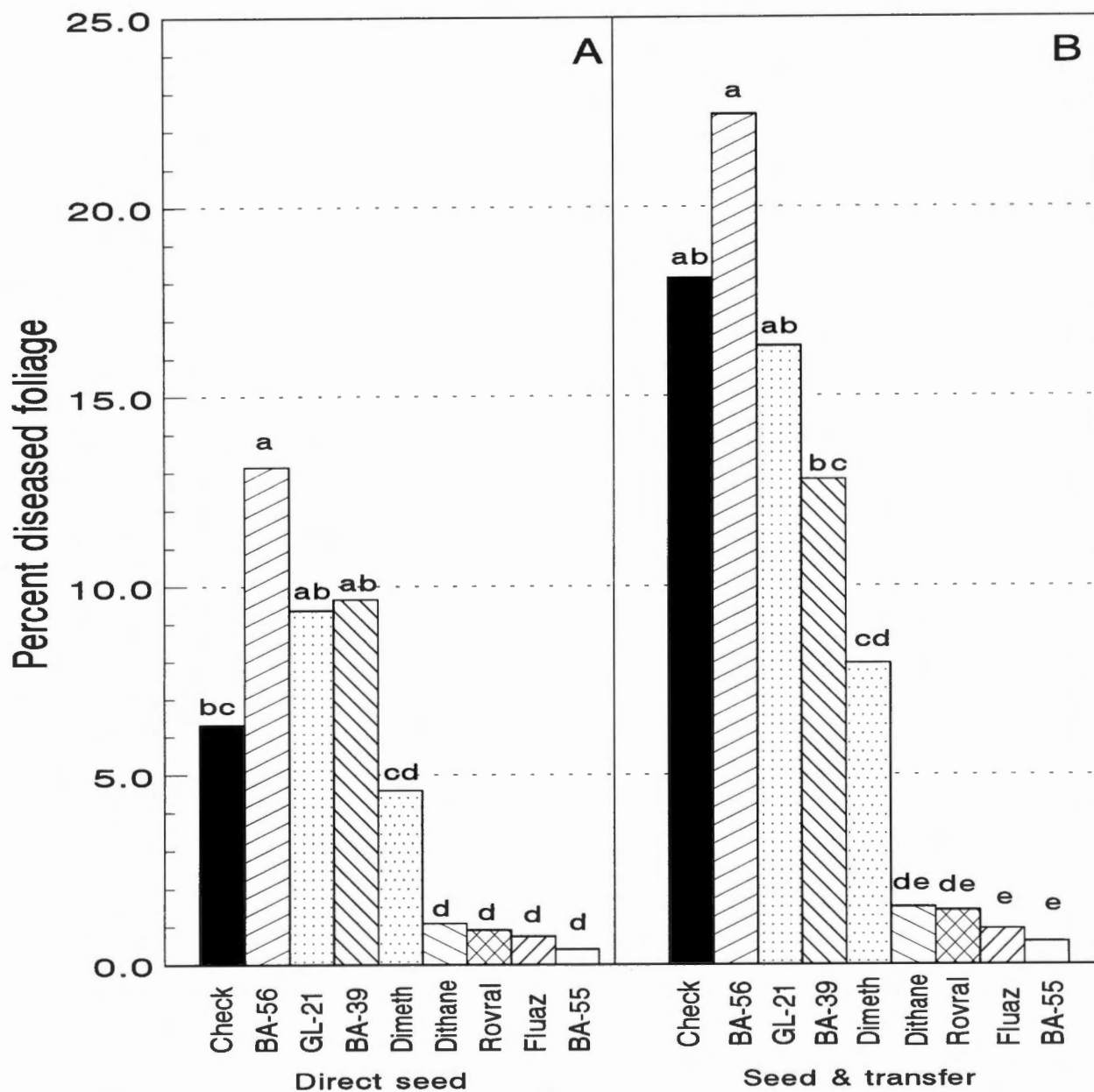


Figure 2. Effect of the interaction of disease control treatment and planting method on percent diseased foliage of tobacco plants in 1993. (A) Direct-seed, (B) Seed-and-transfer. Within each planting method, differences between bars with the same letters are not significant ($P=0.05$).

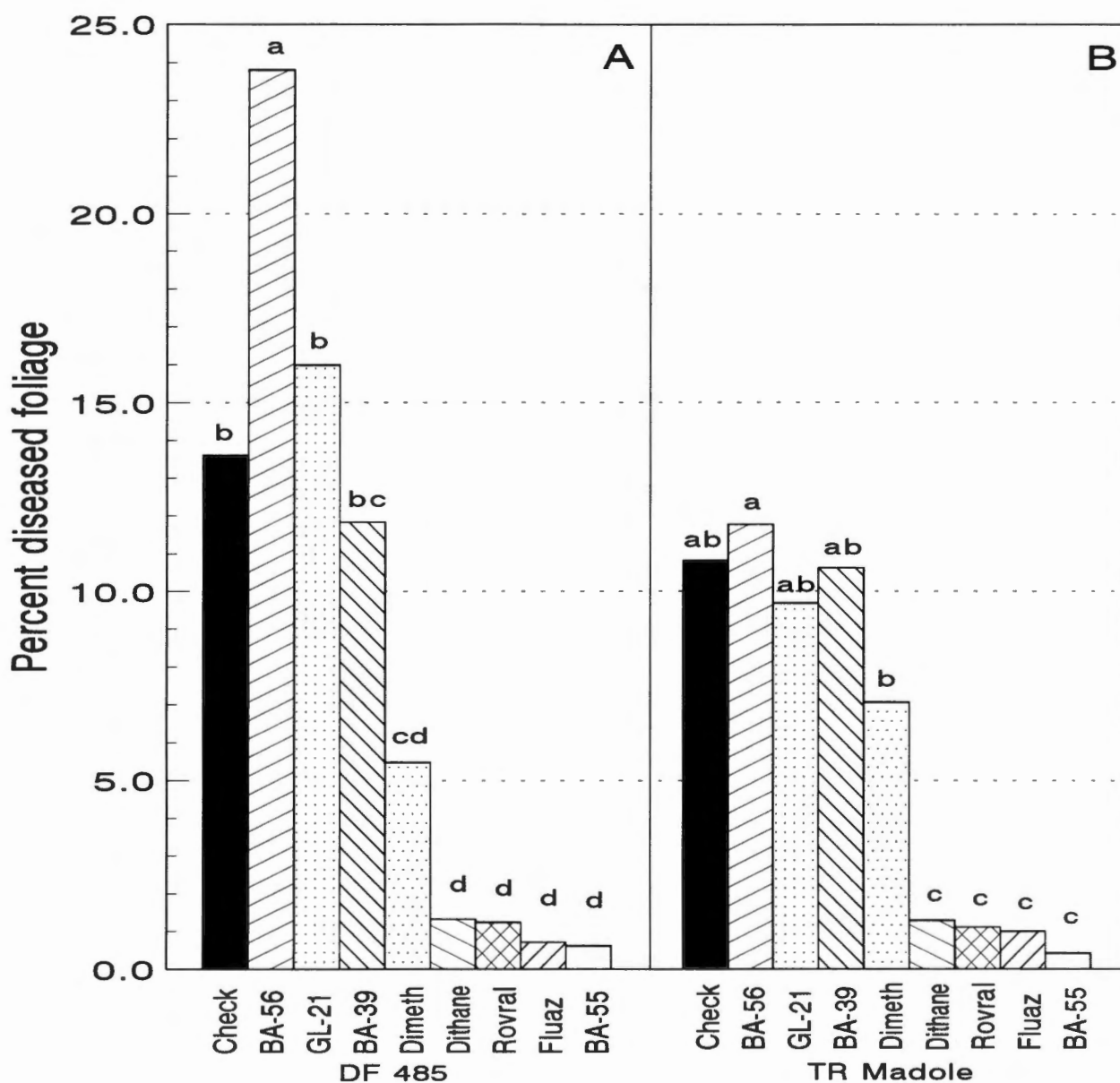


Figure 3. Effect of the interaction of disease control treatment and cultivar on percent diseased foliage of tobacco seedlings in 1993. (A) DF485, (B) TR Madole. Within each cultivar, differences between bars with the same letters are not significant ($P=0.05$).



Figure 4. Tobacco leaves treated with *Bacillus* sp. BA55 had few target spot lesions. Leaves treated with fluazinam and Dithane® were similar in appearance.



Figure 5. Untreated leaves inoculated with the pathogen had typical target spot lesions.



Figure 6. Tobacco leaves treated with dimethomorph had typical target spot lesions.



Figure 7. Tobacco seedlings treated with Rovral exhibited phytotoxicity.

Table 2. Analysis of variance for the effects of control treatment (CT) and cultivar (CV) on percent diseased foliage of float-grown tobacco plants caused by *Thanatephorus cucumeris* in 1994

Source of variation	df	Sum of Squares	Mean Square	F value	$P > F$
Replicate	3	594.002	198.000	6.42	0.0017
Control treatment	9	5455.360	606.151	19.66	0.0001
REP x CT	27	1078.277	39.936	1.30	0.2448
Cultivar	1	263.800	263.800	8.56	0.0065
CT x CV	9	792.662	88.074	2.86	0.0147
Error	30	924.878	30.829		
Corrected Total	79	9108.980			

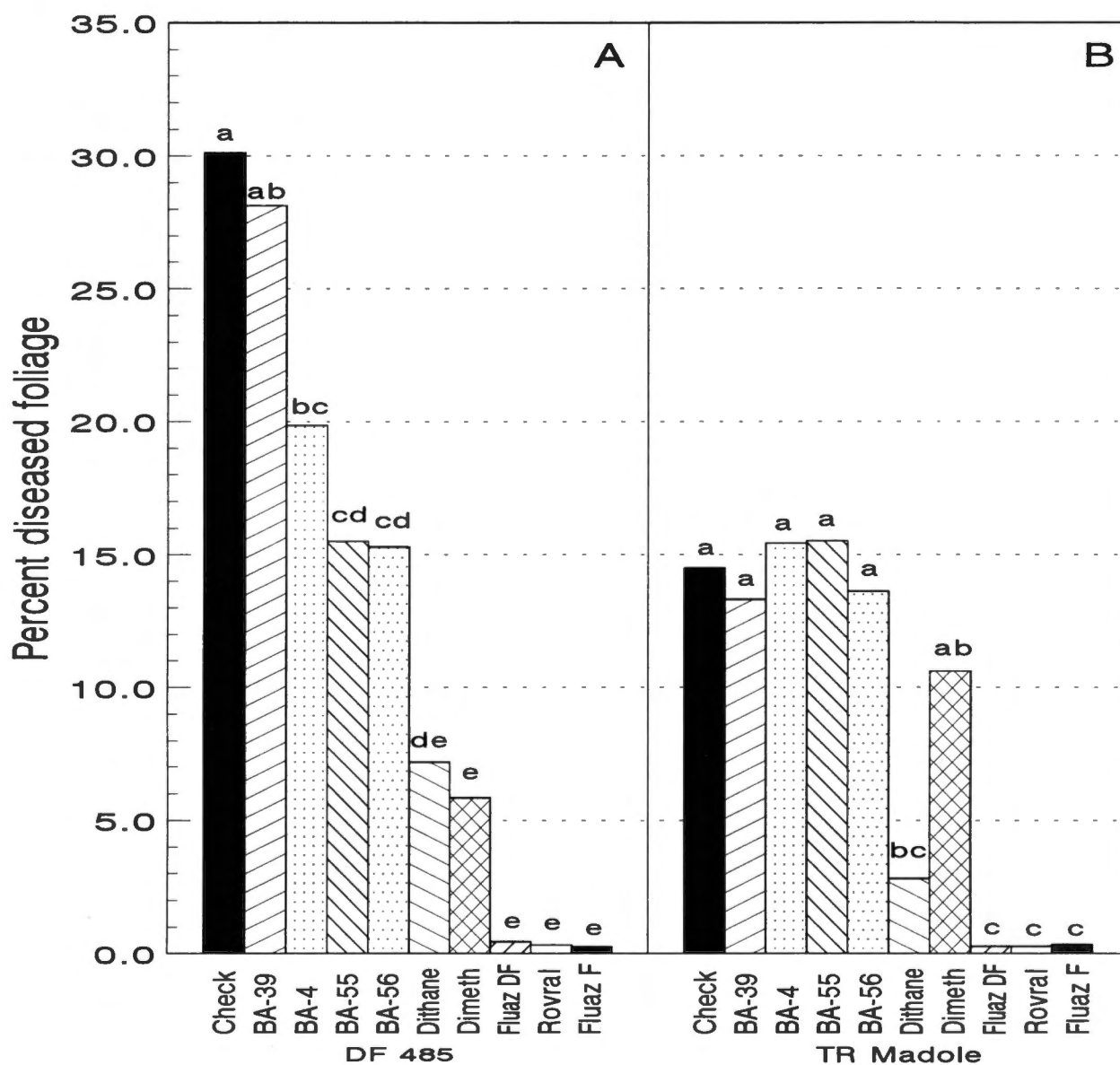


Figure 8. Effect of the interaction of disease control treatment and cultivar on percent diseased foliage of tobacco seedlings in 1994. (A) DF485, (B) TR Madole. Within each cultivar, differences between bars with the same letters are not significant ($P=0.05$).

untreated check, the percent diseased foliage was 14 vs. 11% in 1993 and 30 vs. 14% in 1994 for DF485 and TR Madole, respectively (Figs. 3, 8).

Field experiment. In 1993, there was no further development of target spot on seedlings from the greenhouse experiment that were transplanted to the field. The effect of disease control treatment was not significant for crop index, grade index, yield, or revenue (Tables 3, 4, 5). However, the effect of cultivar was significant for yield (Table 4). DF485 yielded more lbs/acre than TR Madole (almost 200 lbs/A).

Host age study. The effect of host age ($P=0.0001$) and disease control treatment ($P=0.0031$) were significant for percent diseased foliage (Table 6). Among the five different ages of the seedlings, there was less diseased foliage ($P=0.05$) on the youngest seedlings (1.959%) than on the other four age groups, which ranged from 15 to 20% (Fig. 9). Isolate BA55 significantly reduced disease compared to the control (12.5% vs. 17%, respectively). However, there was no significant difference between the two cultivars.

Fluazinam rate study. Symptoms of target spot were evident on the untreated checks from only two replicates. Therefore, data from these replicates were eliminated from the analysis. Although percent diseased foliage was lower for plants treated with fluazinam concentrations greater than 600 ppm, there were no significant differences due to low disease pressure (Table 7, Fig. 10).

Table 3. Analysis of variance for the effects of control treatment (CT) and cultivar (CV) on crop index and grade index of field-grown tobacco plants in 1993

Source of variation	df	Sum of Squares	Mean Square	F value	$P > F$
<u>Crop Index</u>					
Replicate	3	2285.348	761.783	2.05	0.1612
Control treatment	3	1737.963	579.321	1.27	0.3432
REP x CT	9	4117.776	457.531	1.23	0.3618
Cultivar	1	397.268	397.268	1.07	0.3221
CT x CV	3	462.271	154.090	0.41	0.7462
Error	12	4468.990	372.416		
Corrected Total	31	13469.616			
<u>Grade Index</u>					
Replicate	3	56.637	18.879	1.71	0.2188
Control Treatment	3	77.676	25.892	2.41	0.1343
REP x CT	9	96.693	10.744	0.97	0.5067
CV	1	25.740	25.740	2.33	0.1532
CT x CV	3	16.968	5.656	0.51	0.6823
Error	12	132.839	11.070		
Corrected Total	31	406.554			

Table 4. Analysis of variance for the effects of control treatment (CT) and cultivar (CV) on yield and revenue of field-grown tobacco plants in 1993

Source of variation	df	Sum of Squares	Mean Square	F value	$P > F$
<u>Yield</u>					
Replicate	3	862478.531	287492.844	5.78	0.0110
Control treatment	3	101538.594	33846.198	0.57	0.6466
REP x CT	9	531199.594	59022.177	1.19	0.3822
Cultivar	1	258300.781	258300.781	5.20	0.0417
CT x CV	3	33611.344	11203.781	0.23	0.8769
Error	12	596518.125	49709.844		
Corrected Total	31	2383646.969			
<u>Revenue</u>					
Replicate	3	1999881.836	666627.279	4.33	0.0276
Control Treatment	3	638672.336	212890.779	0.92	0.4687
REP x CT	9	2078669.133	230963.237	1.50	0.2520
CV	1	418269.445	418269.445	2.72	0.1253
CT x CV	3	113459.711	37819.904	0.25	0.8629
Error	12	1848342.969	154028.581		
Corrected Total	31	7097295.430			

Table 5. Effect of disease control treatment on grade index, crop index, yield and revenue of tobacco in field plots in 1993

Treatment ^u	Mean Grade Index ^{v,w}	Mean Crop Index ^x	Mean Yield ^y	Mean Revenue ^z
Check +	66.69	195.59	2934.8	4718.3
BA55	64.20	178.65	2782.5	4395.1
Check-	63.63	182.43	2873.9	4465.1
Fluazinam	62.41	176.67	2827.5	4354.8

^u - Check + = Disease free check provided by HRES, Check- = Untreated seedlings with the pathogen.

^v - The effect of disease control treatment on grade index, crop index, yield, and revenue was not significant ($P=0.05$) according to Fisher's protected Least Significant Difference test.

^w - An index of leaf quality, graded on a scale from 1 to 100, 100 being the highest.

^x - The product of grade index and yield.

^y - In lbs/acre.

^z - In total dollars.

Table 6. Analysis of variance for the effects of control treatment (CT), cultivar (CV), and host age (HA) on percent diseased foliage of float-grown tobacco plants with target spot

Source of variation	df	Sum of Squares	Mean Square	F value	$P > F$
Replicate	3	459.284	153.095	5.33	0.0027
Control Treatment (CT)	1	272.117	272.117	78.04	0.0031
REP x CT	3	10.461	3.487	0.12	0.9471
Host Age (HA)	4	3215.924	803.981	28.01	0.0001
HA x CT	4	103.403	25.851	0.90	0.4702
Cultivar (CV)	1	39.111	39.111	1.36	0.2482
HA x CV	4	98.601	24.650	0.86	0.4947
CT x CV	1	11.620	11.620	0.40	0.5273
HA x CT x CV	4	164.202	41.051	1.43	0.2366
Error	54	1550.083	28.705		
Corrected Total	79	5924.805			

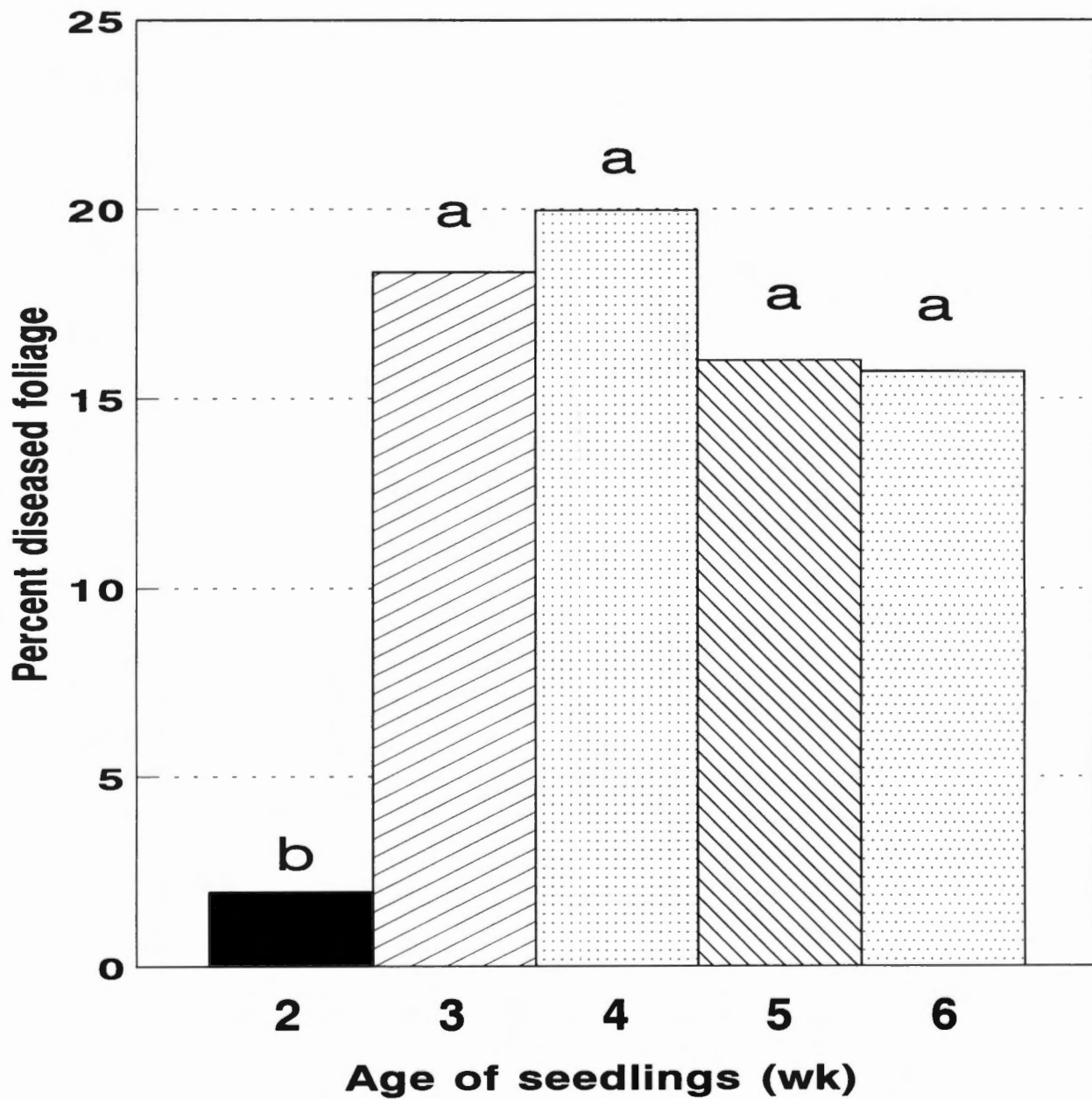


Figure 9. Effect of host age on percent diseased foliage of tobacco seedlings with target spot. Differences between bars with the same letters are not significant ($P=0.05$)

Table 7. Analysis of variance for the effect of Fluazinam rate (FR) on percent diseased foliage of float-grown tobacco seedlings

Source of variation	DF	Sum of Squares	Mean Square	F value	<i>P</i> > F
REP	1	0.970	0.970	0.81	0.4082
FR	5	17.584	3.517	2.95	0.1300
Error	5	5.959	1.192		
Corrected Total	11	24.514			

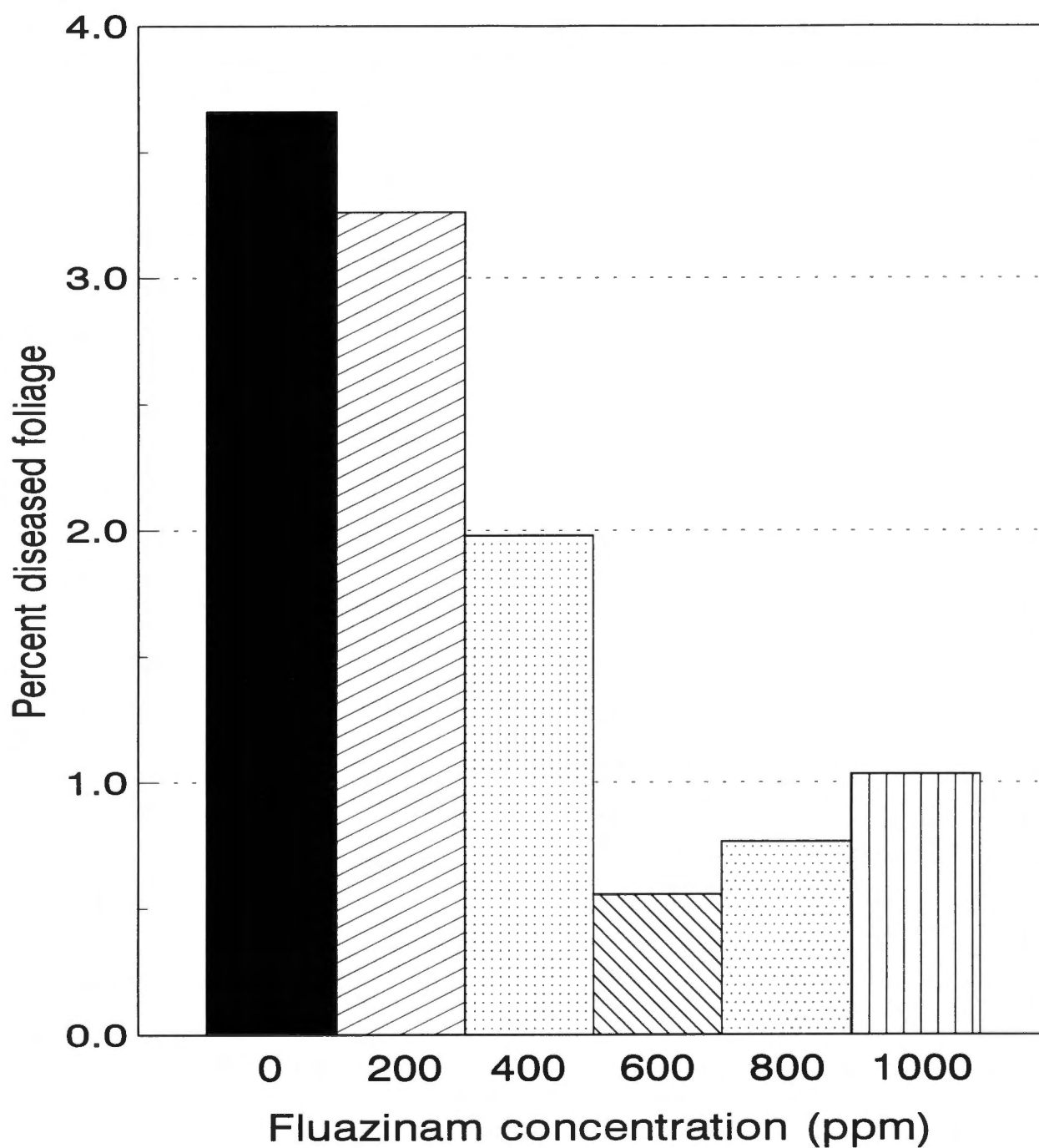


Figure 10. Effect of fluazinam F concentration on percent diseased foliage of float-grown tobacco with target spot. Differences were not significant ($P=0.05$).

DISCUSSION

Overall, disease severity was greater in seed-and-transfer plants than in direct-seeded plants. This difference is likely to be an indirect result of physiological shock associated with transplant. After the shock of transfer, the seed-and-transfer plants were more susceptible to target spot. Although DF485 is a line resistant to many diseases and TR Madole is not, target spot severity was greater on DF485 than TR Madole. It is possible that the physical differences between the cultivars may make a difference. For example, if the canopy of cultivar DF485 was more closed than that of TR Madole, the relative humidity under the canopy may have been closer to the optimum for development of *T. cucumeris* basidiospores.

Bacillus sp. BA55 controlled target spot substantially in 1993, across both cultivars and both planting methods. In 1994, BA55 reduced target spot severity on cultivar DF485 but not on TR Madole. Two factors could explain why BA55 did not perform as well in 1994 as it did in 1993. One is any nutritional or cultural difference in growing the bacteria between the two years. There is evidence to suggest that the medium used to culture *Bacillus* affects its disease control ability (32). In a study by Handelsman et al., *Bacillus cereus* UW85 cells grown on minimal media increased alfalfa survival rate against *Phytophthora megasperma* f. sp. *medicaginis* from 1% to 81% compared to 41% survival for bacteria grown on a nutrient-rich medium, and 0% for a diseased control. They discovered more free spores in minimal media than in

nutrient-rich media. Under nutrient-limiting conditions, *Bacillus* will sporulate often within minutes. Any cultural or nutritional condition which leads to increased free spore production over vegetative cell production may result in greater disease control. Although there were no known differences in culture procedures, it is possible that BA55 culture in 1993 was produced at a higher temperature than the 1994 BA55 inoculum.

The other factor is any difference in the virulence of the pathogen, and timing of treatment and pathogen inoculation between the two years. Although the volume of inoculum (8 cm³) was the same in both years, the mass was different. In 1993, 0.5 g of *R. solani* grown on oat grains was introduced 1 day after the first treatment. In 1994, 2.5 g of the fungus grown on with rice grains was introduced 6 days after the first treatment. An experiment which will be discussed in chapter 3 indicated that rice inoculum of *R. solani* is more virulent than oat inoculum.

Thanatephorus cucumeris normally needs 21 days to complete its life cycle. The results from the first experiment were consistent with this fact; however, in the second study, target spot first appeared 14 days after inoculation. The one-week difference in target spot appearance could have resulted if *T. cucumeris* basidiospores are produced more easily in rice than they are in oats.

Gliocladium virens GL-21 was applied as a soil treatment in an attempt to reduce *R. solani* inoculum before it formed the *T. cucumeris* stage. This

strategy would provide no protection after basidiospore formation. No control of target spot was observed on tobacco seedlings treated with GL-21 in the only year tested, 1993. Target spot severity was significantly greater also on direct-seeded plants and on DF485 seedlings treated with *Bacillus* sp. BA56, compared to the untreated control. This suggested that BA56 was producing phytotoxic metabolites; however, these differences were not observed in 1994, and on DF485 seedlings, BA56 significantly reduced percent diseased foliage compared to the untreated control.

In the field, tobacco seedlings grew up with hardly any signs of target spot, even on the lower leaves. The only damage on the field plants was due to lightning damage. In 1993, the weather was dry and hot, which is not conducive for the spread of target spot. Leaves with lesions were abscised, and the disease did not spread any further.

In the host age study, 2-wk old seedlings had significantly less disease than the other four age groups. These seedlings were smaller than those in the other groups. Canopy cover was insufficient to retain the high humidity necessary for basidiospore production. Even if basidiospores were to form, the probability of a spore hitting a leaf would be less for the youngest seedlings than on older, larger seedlings. Although the reduction in percent diseased foliage of plants treated with BA55 was not as pronounced as it was in the main study, it was still significantly less than that of the untreated plants.

Two replicates were eliminated from the analysis in the fluazinam rate study, because no disease was present, even in the untreated control. These two replicates were the closest to an air conditioner which was used to provide temperatures more conducive to the spread of target spot. Near the air conditioner, temperatures may have been too cool and relative humidity too low for target spot to develop and spread.

Both formulations of fluazinam (flowable and dry flowable) significantly reduced disease incidence on tobacco seedlings without producing phytotoxicity. Fluazinam and Dithane® are potential candidates for controlling target spot on float-grown tobacco seedlings. *Bacillus* sp. BA55 could be a potential biological control of target spot if the consistency of its performance can be improved; perhaps by the production of endospores as opposed to vegetative cells in foliar spray formulations.

CHAPTER 3: DEVELOPMENT OF A LEAF DISK ASSAY TO PRESCREEN BACTERIA FOR BIOCONTROL OF TARGET SPOT ON TOBACCO

INTRODUCTION

Researchers often obtain a collection of potential biocontrol isolates before conducting experiments in biological control of plant diseases. With a large collection of strains, it is not practical to test each individual strain on a given host. The time and labor involved would not justify the results. Prescreening the test strains, often by *in vitro* antibiosis assays, is more rapid and less labor-intensive. However, quick *in vitro* assays omit one critical factor: the host. An *in vivo* assay is better suited to prescreen potential biocontrol agents against a particular disease. *In vitro* assays select only for strains that control disease by either antibiosis or siderophore production. These assays would not select for any potential biocontrol organisms which may use other methods, such as induced resistance, parasitism, or competition to control a disease (41). *In vivo* assays, which incorporate the host in their design, may select biocontrol organisms which control plant diseases by any of the above methods. Some approaches to this problem have involved agar plates (41) and microtiter plates (28, 33). The main objective of this study was to develop a quick and consistent *in vivo* leaf disk assay to prescreen bacteria for potential biological control of target spot on tobacco. Two *in vitro* assays were used

also as comparisons: antibiosis assays against both *Geotrichum candidum* Link, a nonpathogenic soil fungus and *Rhizoctonia solani*.

MATERIALS AND METHODS

***Geotrichum candidum* antibiosis assays.** The 143 bacterial isolates from agricultural soils near Greeneville, TN, were each inoculated into tubes containing 5 ml Nutrient Broth Yeast Extract (NBY, 73) and incubated on a rotary shaker (100 rpm) for 3 days at 25°C (100 rpm). Cultures were then vortexed, and 25 μ l of each culture was spotted in the center of three plates each of Potato Dextrose Agar (PDA, 24 g Potato Dextrose Broth, Difco Laboratories, Detroit, MI, and 15 g agar, Sigma Chemical Company, St. Louis, MO, per liter H₂O). Plates were incubated at room temperature for 2 days then oversprayed with a chromatography sprayer containing a 1:5 dilution of *Geotrichum candidum* grown overnight in 200 ml NBY. The plates were sprayed until moist, then incubated at room temperature. Zones of inhibition (if present) were measured the following day. The experiment was repeated with three NBY agar plates per isolate. Each plate represented one replicate. Both experiments were repeated.

***Rhizoctonia solani* inhibition assay.** The 143 Greeneville isolates were inoculated into 5 ml NBY each and incubated for 2 days at 25°C on a rotary shaker (100 rpm). The cultures were vortexed, and 25 μ l of the suspension was pipetted across the center of the plate. The plates were incubated at room

Data analysis. The effect of bacterial treatment was analyzed for significance with the General Linear Models Procedure and F-protected LSD tests of the Statistical Analysis System® (59). The data sets were split into two groups because of their sizes.

RESULTS

***Geotrichum candidum* assay.** Of the 143 bacterial isolates, only 81 (56.6%) grew on PDA. Isolates that did not grow on PDA were eliminated from the analysis. Of the remaining isolates, 12 (14.8%) produced inhibition zones significantly different from zero (Table 8, Table 9, Fig. 11). Five isolates, BA27, BA29, BA57, BA83, and BA125, showed an inhibition zone greater than 5 mm on PDA (Table 9).

All of the bacteria grew on NBY, and 54 of the isolates (37.8%) significantly inhibited *G. candidum* (Table 10). Twelve isolates (8.4%) produced large inhibition zones (≥ 5 mm) against *G. candidum* on NBY. These were BA8, BA27, BA28, BA29, BA40, BA44, BA91, BA95, BA96, BA118, BA120, and BA121 (Table 9).

***Rhizoctonia solani* assay.** As in the previous assay, all of the 143 bacterial isolates grew on NBY, and 98 isolates (68.5%) produced inhibition zones against *R. solani* significantly greater than zero (Table 11). Eight isolates (5.6%) of the 143 isolates produced an inhibition zone ≥ 5 mm. These isolates were BA4, BA8, BA19, BA27 (Fig. 12), BA29, BA58, BA96, and

Table 8. Analysis of variance for the effect of bacterial isolate on inhibition of growth of *Geotrichum candidum* on PDA

Source of variation	df ^z	Sum of Squares	Mean Square	F value	<i>P</i> > F
ISOLATE	81	1462.085	18.050	31.12	0.0001
REP	2	0.024	0.012	0.02	0.9792
Error	162	93.976	0.580		
Corrected Total	245	1556.085			

^z - Only 81 bacterial isolates grew on 1/5 PDA. The isolates that did not grow were eliminated from this analysis.

Table 9. Summary of the inhibition of the 143 Greeneville isolates in the *in vitro* and *in vivo* prescreening assays for potential biocontrol activity

ISOLATE	Inhibition of Gc (PDA) ^{x,y,z}	Inhibition of Gc (NBY) ^{x,y,z}	Inhibition of Rs (NBY) ^{x,y}	Leaf disk assay (1 day) ^x	Leaf disk assay (2 days) ^x
BA1	NG	+	+	-	-
BA2	NG	-	-	-	-
BA3	-	+	-	-	-
BA4	NG	-	++	-	-
BA5	-	+	-	-	-
BA6	NG	-	+	-	-
BA7	NG	-	+	-	+
BA8	-	++	++	-	-
BA9	NG	-	+	-	-
BA10	NG	-	+	-	+
BA11	-	-	-	-	-
BA12	-	+	+	-	-
BA13	NG	-	+	-	-
BA14	-	-	+	-	-
BA15	NG	-	+	-	-
BA16	NG	-	+	-	-
BA17	-	-	-	-	-
BA18	NG	-	+	-	-
BA19	NG	-	++	-	-
BA20	-	-	-	-	-
BA21	NG	-	+	-	-
BA22	-	-	+	+	+
BA23	-	-	+	-	-
BA24	-	-	+	-	-
BA25	-	-	-	-	-

ISOLATE	Inhibition of Gc (PDA) ^{x,y,z}	Inhibition of Gc (NBY) ^{x,y,z}	Inhibition of Rs (NBY) ^{x,y}	Leaf disk assay (1 day) ^x	Leaf disk assay (2 days) ^x
BA26	-	+	-	-	-
BA27	++	++	++	+	+
BA28	NG	++	+	-	-
BA29	++	++	++	-	-
BA30	-	-	-	--	-
BA31	-	-	+	-	-
BA32	-	-	-	-	-
BA33	NG	-	+	-	-
BA34	-	+	+	-	-
BA35	NG	+	-	-	-
BA36	+	+	-	-	-
BA37	-	-	-	-	-
BA38	NG	-	-	-	-
BA39	-	-	+	-	-
BA40	NG	++	-	-	+
BA41	NG	-	+	-	-
BA42	+	+	+	-	-
BA43	-	+	+	-	-
BA44	NG	++	+	-	-
BA45	+	+	+	-	-
BA46	NG	-	+	-	-
BA47	+	-	+	-	-
BA48	-	-	+	-	-
BA49	NG	-	+	-	-
BA50	-	-	+	--	-
BA51	-	+	+	-	-

ISOLATE	Inhibition of Gc (PDA) ^{x,y,z}	Inhibition of Gc (NBY) ^{x,y,z}	Inhibition of Rs (NBY) ^{x,y}	Leaf disk assay (1 day) ^x	Leaf disk assay (2 days) ^x
BA52	-	+	+	-	-
BA53	-	-	+	-	-
BA54	-	-	+	-	-
BA55	-	-	+	-	-
BA56	NG	-	-	-	-
BA57	++	-	+	--	-
BA58	-	-	++	-	-
BA59	+	-	+	-	-
BA60	NG	+	+	-	-
BA61	-	-	+	-	-
BA62	NG	-	-	-	-
BA63	-	-	-	-	-
BA64	NG	-	+	-	-
BA65	NG	-	+	-	-
BA66	NG	-	-	-	-
BA67	NG	-	+	-	-
BA68	-	-	-	-	-
BA69	NG	-	-	-	-
BA70	-	-	+	-	-
BA71	NG	+	-	-	-
BA72	-	-	-	-	-
BA73	-	-	-	-	-
BA74	-	-	-	-	-
BA75	NG	+	-	--	-
BA76	NG	-	-	-	-
BA77	NG	-	-	-	-

ISOLATE	Inhibition of Gc (PDA) ^{x,y,z}	Inhibition of Gc (NBY) ^{x,y,z}	Inhibition of Rs (NBY) ^{x,y}	Leaf disk assay (1 day) ^x	Leaf disk assay (2 days) ^x
BA78	-	+	+	+	-
BA79	-	-	-	-	-
BA80	-	+	+	-	-
BA81	-	-	-	-	-
BA82	NG	-	+	-	-
BA83	++	-	+	-	-
BA84	-	-	+	-	-
BA85	-	-	+	-	-
BA86	NG	+	-	-	-
BA87	-	-	-	-	-
BA88	NG	-	+	-	-
BA89	NG	+	+	-	-
BA90	-	-	-	-	-
BA91	NG	++	+	-	-
BA92	NG	+	+	-	-
BA93	-	-	+	+	+
BA94	NG	+	+	-	-
BA95	NG	++	+	-	-
BA96	+	++	++	-	-
BA97	-	+	+	-	-
BA98	-	-	+	-	-
BA99	-	-	+	-	-
BA100	-	-	+	-	-
BA101	NG	+	-	-	-
BA102	NG	-	+	-	-
BA103	-	+	+	-	-

ISOLATE	Inhibition of Gc (PDA) ^{x,y,z}	Inhibition of Gc (NBY) ^{x,y,z}	Inhibition of Rs (NBY) ^{x,y}	Leaf disk assay (1 day) ^x	Leaf disk assay (2 days) ^x
BA104	-	-	+	-	-
BA105	NG	-	+	-	-
BA106	NG	+	+	-	-
BA107	-	-	+	-	-
BA108	NG	-	-	-	-
BA109	NG	-	+	-	-
BA110	NG	-	+	-	-
BA111	-	+	++	-	-
BA112	-	+	+	-	-
BA113	-	-	+	-	-
BA114	NG	-	-	-	-
BA115	-	-	-	-	-
BA116	-	-	-	-	-
BA117	-	+	+	-	-
BA118	NG	++	+	-	-
BA119	NG	+	+	-	-
BA120	-	++	+	-	-
BA121	NG	++	+	-	-
BA122	-	+	+	+	+
BA123	NG	-	+	-	-
BA124	NG	+	+	-	-
BA125	++	-	-	-	-
BA126	-	+	+	-	-
BA127	-	-	+	-	-
BA128	-	-	+	-	-
BA129	-	+	-	-	-

ISOLATE	Inhibition of Gc (PDA) ^{x,y,z}	Inhibition of Gc (NBY) ^{x,y,z}	Inhibition of Rs (NBY) ^{x,y}	Leaf disk assay (1 day) ^x	Leaf disk assay (2 days) ^x
BA130	NG	-	+	-	-
BA131	NG	-	-	-	-
BA132	NG	+	-	-	-
BA133	NG	+	+	+	-
BA134	-	+	+	-	-
BA135	NG	+	+	-	-
BA136	-	-	-	-	+
BA137	-	-	+	-	-
BA138	NG	+	-	-	-
BA139	+	-	+	+	+
BA140	-	+	+	-	-
BA141	-	+	+	-	-
BA142	-	+	-	-	-
BA143	NG	-	+	-	-

^x - ++: inhibition zone $\geq$ 5 mm, +: significantly greater than control, -: not significantly different from control, -- leaf disk colonization by *R. solani* was significantly greater than the nonbacterized control.

^y - Gc = *Geotrichum candidum*, Rs = *Rhizoctonia solani*

^z - NG - isolate did not grow on PDA



Figure 11. PDA plates with bacteria showing different levels of inhibition against *Geotrichum candidum*.

Table 10. Analysis of variance for the effect of bacterial isolate on inhibition of growth of *G. candidum* on NBY

Source of variation	df	Sum of Squares	Mean Square	F value	<i>P</i> > F
<u>BA1 to BA70</u>					
ISOLATE	69	1601.029	23.203	9.74	0.0001
REP	2	4.067	2.033	0.85	0.4280
Error	138	328.600	2.381		
Corrected Total	209	1933.696			
<u>BA71 to BA143</u>					
ISOLATE	72	978.813	13.595	13.16	0.0001
REP	2	0.612	0.305	0.30	0.7441
Error	144	148.721	1.032		
Corrected Total	218	1128.146			

Table 11. Analysis of variance for the effect of bacterial isolate on inhibition of growth of *R. solani* on NBY.

Source of variation	df	Sum of Squares	Mean Square	F value	<i>P</i> value
<u>Isolates BA1 to BA70</u>					
ISOLATE	69	384.189	5.568	27.57	0.0001
REP	1	0.471	0.471	2.33	0.1316
Error	67	13.529	0.202		
Corrected Total	137	398.189			
<u>Isolates BA71 to BA143</u>					
ISOLATE	72	284.753	3.954	16.59	0.0001
REP	1	0.336	0.336	1.41	0.2393
Error	73	17.164	0.238		
Corrected Total	146	302.253			



Figure 12. *Bacillus* sp. BA27 inhibiting growth of two isolates of *Rhizoctonia solani* *in vitro* on NBY.

BA111 (Table 9).

Optimization of leaf disk assay. The optimum disease conditions for the leaf disk assay were placing a grain of oat inoculum of *R. solani* on the surface of the Terralite™, and incubating it for 1 day before adding the leaf disks. Leaf disks were surface sterilized with 70% ethanol and treated with 2-day-old bacterial cultures.

Leaf disk assay. Most of the treatments did not inhibit colonization of the leaf disk by *R. solani* on either day of measurement. After the first day, only seven isolates (4.9%) significantly inhibited colonization: BA22, BA27, BA78, BA93, BA122, BA133, and BA139 (Table 9, Table 12). Treatment of leaf disks with four isolates (2.8%) resulted in significantly greater colonization by *R. solani* than the nonbacterized control. These four isolates were BA30, BA50, BA57, and BA75 (Table 9).

On the second day, leaf disks treated with nine isolates (6.3%) had significantly lower colonization than the nonbacterized control (Table 9, Table 13). These isolates were BA7, BA10, BA22, BA27, BA40, BA93, BA122, BA136, and BA139. A comparison of leaf disks immediately after placement on Terralite™ infested with *R. solani* and after two days is shown in Fig. 13.

All of the isolates mentioned above, as well as the four bacteria described in Chapter 2 were typed to species using the Biolog Bacterial Identification System™. Most isolates were identified as *Bacillus thuringiensis/cereus*, although there were some exceptions (Table 14).

Table 12. Analysis of variance for the effect of bacterial isolate on inhibition of leaf disk colonization by *R. solani* after 1 day incubation

Source of variation	df	Sum of Squares	Mean Square	F value	<i>P</i> > F
<u>BA1 to BA70^z</u>					
ISOLATE	70	36.523	0.522	2.43	0.0001
REP	2	0.184	0.092	0.43	0.6525
Error	140	30.025	0.214		
Corrected Total	212	66.732			
<u>BA71 to BA143</u>					
ISOLATE	73	24.667	0.337	1.21	0.0001
REP	2	0.362	0.181	2.25	0.3019
Error	146	21.888	0.149		
Corrected Total	221	46.917			

^z - Data set was split into two sections for analysis.

Table 13. Analysis of variance for the effect of bacterial isolate on inhibition of leaf disk colonization by *R. solani* after 2 days incubation

Source of variation	df	Sum of Squares	Mean Square	F value	<i>P</i> value
<u>BA1 to BA70^z</u>					
ISOLATE	70	29.868	0.427	2.18	0.0001
REP	2	0.982	0.491	2.52	0.0844
Error	140	27.350	0.195		
Corrected Total	212	58.201			
<u>BA71 to BA143</u>					
ISOLATE	73	28.289	0.388	1.28	0.1026
REP	2	0.173	0.086	0.29	0.7515
Error	146	44.077	0.302		
Corrected Total	221	72.539			

^z - Data set was split into two sections for analysis.

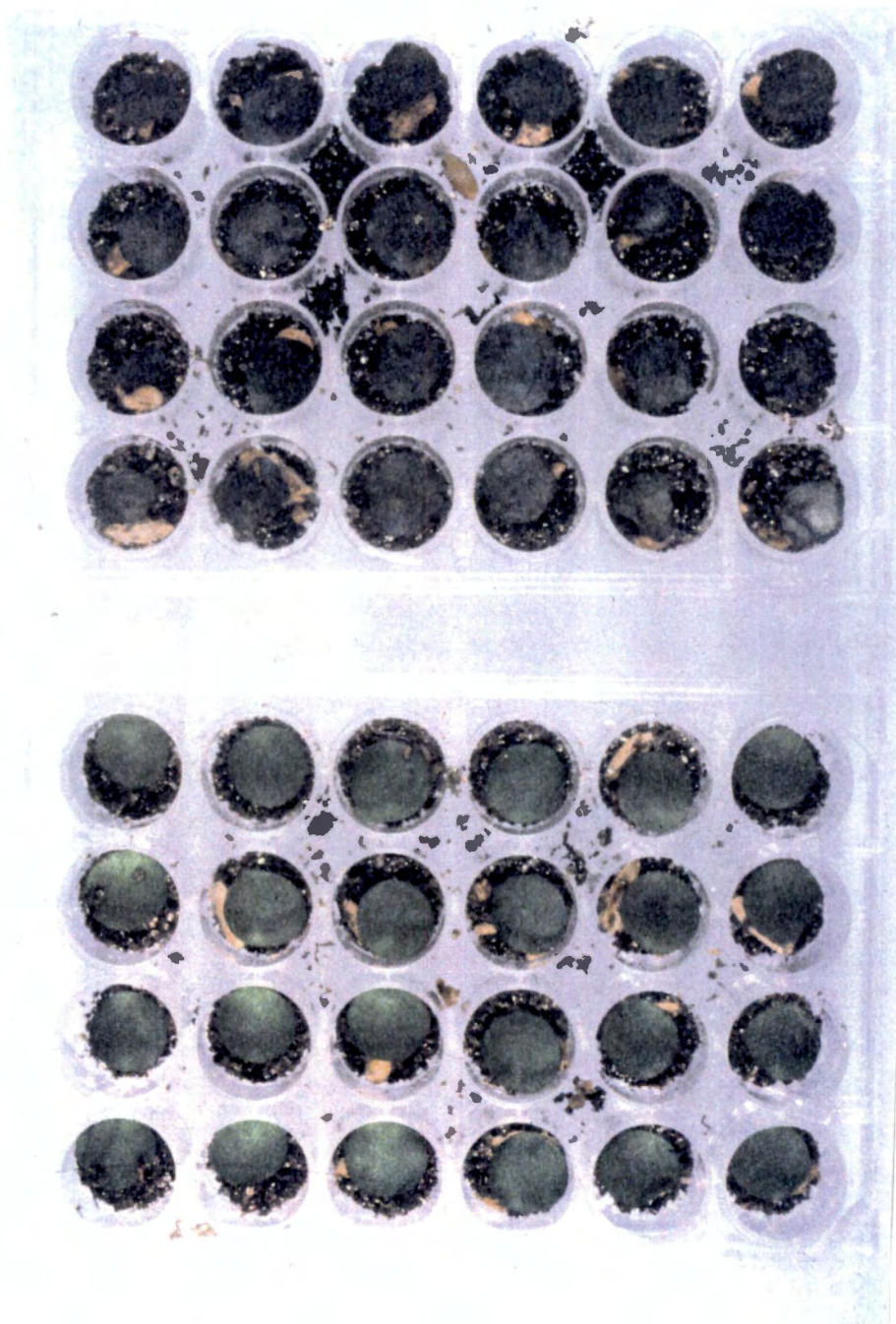


Figure 13. Multiwell microtiter plates containing Terralite™ infested with *R. solani* immediately following placement of leaf disks (right) and after 2 days (left).

Table 14. Preliminary identifications of selected Greeneville bacterial isolates

ISOLATE	IDENTIFICATION
BA4	<i>Bacillus sphaericus</i>
BA7	<i>Bacillus thuringiensis/cereus</i>
BA8	<i>Bacillus thuringiensis/cereus</i>
BA10	<i>Bacillus thuringiensis/cereus</i>
BA22	<i>Bacillus cereus/thuringiensis</i>
BA27	<i>Bacillus thuringiensis/cereus</i>
BA28	<i>Bacillus thuringiensis/cereus</i>
BA29	<i>Bacillus thuringiensis/cereus</i>
BA30	<i>Bacillus cereus/thuringiensis</i>
BA39	<i>Bacillus thuringiensis/cereus</i>
BA40	<i>Bacillus thuringiensis/cereus</i>
BA50	<i>Bacillus thuringiensis/cereus</i>
BA55	<i>Bacillus mycoides</i>
BA56	<i>Bacillus thuringiensis/cereus</i>
BA57	<i>Bacillus cereus/thuringiensis</i>
BA75	<i>Bacillus thuringiensis/cereus</i>
BA78	<i>Bacillus thuringiensis/cereus</i>
BA83	<i>Bacillus thuringiensis/cereus</i>
BA93	<i>Bacillus thuringiensis/cereus</i>
BA122	<i>Bacillus thuringiensis/cereus</i>
BA125	<i>Bacillus cereus/thuringiensis</i>
BA133	<i>Bacillus thuringiensis/cereus</i>
BA136	<i>Bacillus cereus/thuringiensis</i>
BA139	<i>Bacillus subtilis</i> var. <i>globigii</i>

DISCUSSION

Although some results corresponded in both the *in vitro* and *in vivo* assays, differences also existed between the tests. Two such examples are isolates BA22 and BA93. These isolates performed poorly in both the *Geotrichum* assay and the *Rhizoctonia* assay, but did well in the leaf disk assay. Both of these isolates are *Bacillus* sp. Although antibiotic production is the primary means of biological control in *Bacillus*, it is not the only one. Strains of *Bacillus* may act either by competition, parasitism, or induced resistance.

Bacillus sp. BA55 did not inhibit either *G. candidum* or *R. solani* significantly greater than zero on either NBY or PDA. BA55 also did not reduce colonization of leaf disks by *R. solani*. Since BA55 was incubated for 2 days instead of 3 days as described in Chapter 2, more vegetative cells than free spores may have been present in the culture medium. There is evidence to suggest that a greater proportion of free spores to vegetative cells in a *Bacillus* culture will result in greater control of plant diseases (33).

Although all of the bacteria grew on NBY and not on PDA, NBY may not be the best medium for culturing potential biocontrol bacteria. Work by Handelsman et al. (25, 30, 31, 32, 33) have demonstrated that a *Bacillus* strain, *B. cereus* UW85, incubated on nutrient-poor media significantly reduced disease incidence of *Phytophthora megasperma* f. sp. *medicaginis* on alfalfa compared to bacteria grown on a nutrient-rich medium (33).

Although the leaf disk assay is quick to complete (about 6 days, beginning to end), it is still not perfected. The main problem encountered was that basidiospores of *Thanatephorus cucumeris* could not be produced reliably in culture. The cruder method of using oat grains inoculated with *Rhizoctonia solani* was used. There also was not a way of keeping the air humid enough beyond leaving an open Petri dish with water in the crisper box containing the microtiter plates. Once these difficulties are overcome, this assay may be used to screen bacteria for biocontrol of other tobacco diseases.

Bacillus is a difficult genus to work with in the Biolog™ system because of its endospore. If a significant proportion of the cells have sporulated, a false-positive reaction will occur (that is, the well will turn violet when it should not). If the culture medium is sufficiently rich, most of the cells will be vegetative, although they will sporulate within minutes if nutrients are not diffusing into the cells.

Most of the identifications are tentative and have not been repeated. On one occasion, a second test of an organism yielded a different identification. Isolate BA55 was identified first as *B. thuringiensis/cereus*; however, on a second trial, it was identified as *B. mycoides* (= *B. cereus* subsp. *mycoides*). The Biolog Bacterial Identification System™ cannot separate *B. cereus* and *B. thuringiensis*. Both species at one time were in the genus *B. cereus*, and they share many of the same characteristics (64). *B. mycoides* is closely related also to both of these species and share many characteristics. *B. mycoides* cells

are usually shorter than *B. cereus* cells, and colonies appear grayish on agar media (64).

The *B. cereus* group, which includes *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis*, is a taxonomically confusing area of the genus *Bacillus*. Gordon et al. (27) considered *B. cereus* to be the parent species, and relegated *B. thuringiensis*, *B. mycoides*, and *B. anthracis* to subspecies status. Presently, all four taxa are separate species (55). The major difference between *B. cereus* and *B. thuringiensis* is that *B. thuringiensis* produces a crystal protein that is toxic to many insects. Otherwise, these two species are identical (64).

B. anthracis and *B. cereus* may be separated by sensitivity to penicillin (*B. anthracis* is sensitive to penicillin) and ability to decompose tyrosine (*B. anthracis* cannot decompose tyrosine). *B. anthracis* is also nonmotile. Little is known about *B. mycoides*. Like *B. anthracis*, it is nonmotile, but *B. mycoides* has a distinct rhizoidal phenotype. Gordon (27) considered these characteristics insufficient to warrant species status, but currently, *B. mycoides* is recognized as a separate species.

SUMMARY

Tobacco is an economically important crop in Tennessee. Among tobacco farmers, producing tobacco seedlings in float bed systems is becoming increasingly popular. There are several advantages to float-bed systems compared to traditional seed beds; however, in the greenhouse, the crowded conditions and high relative humidity are conducive to the spread of disease. A recently-described disease of float-grown tobacco is target spot (*Rhizoctonia* leaf spot), caused by *Thanatephorus cucumeris*. No chemicals or biological controls are registered for use against target spot. The main focus of this research was to determine whether any biological or chemical treatments could reduce severity of target spot in the greenhouse. These treatments were tested on two cultivars (DF485 and TR Madole) planted by two methods (direct-seeded and seed-and-transfer). Seedlings receiving two treatments, *Bacillus* sp. BA55 and fluazinam, which significantly controlled target spot in the greenhouse, were transplanted to the field to determine the effect of the treatment on yield and leaf quality, and on ability to continue to inhibit target spot.

In 1993, Dithane® DF, fluazinam F, and *Bacillus* sp. BA55 provided the greatest control of target spot in the float bed system in the greenhouse. Seedlings from direct-seeded plants in both cultivars treated with fluazinam F and BA55, and the diseased check, were transplanted to the field. Further colonization of target spot on field plants was not observed. Fluazinam F and

BA55 had no effect on either leaf quality or yield, although the effect of cultivar was significant on yield.

In 1994, both formulations of fluazinam (F and DF), Rovral, and Dithane® provided the greatest control of target spot in the greenhouse. *Bacillus* sp. BA55 provided intermediate control of target spot on cultivar DF485, and provided no control of target spot on cultivar TR Madole.

Target spot was less severe on 2-wk-old seedlings than on the other age groups (3, 4, 5, and 6 wk). The effect of disease control treatment was significant; *Bacillus* sp. BA55 reduced target spot severity across all age groups and both cultivars (DF 485 and TR Madole) compared to a diseased check.

Five concentrations of fluazinam F were applied weekly to tobacco seedlings. Although less disease was evident on seedlings treated with concentrations greater than 600 ppm, the effect of fluazinam concentration was not significant due to low disease pressure.

In vitro antibiosis assays against *Geotrichum candidum* and *Rhizoctonia solani* and a leaf disk assay were developed to prescreen bacteria for potential biocontrol activity against *R. solani*.

Five isolates tested in all of the assays are the best candidates for further testing of biocontrol of target spot on whole plants. These isolates are BA22, BA27, BA93, BA122, and BA139. These isolates reduced colonization of leaf disks by *R. solani* on both days. BA22, BA93, BA122, and BA139 produced small zones of inhibition against *R. solani* while BA27 produced a zone of

inhibition $\geq$ 5 mm in the *in vitro* assay. BA22 and BA93 did not inhibit the growth of *G. candidum* as did the remaining isolates.

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