To the Graduate Council:

I am submitting herewith a thesis written by Joseph P. McMahan entitled, "Biological Control of Target Spot and Sore Shin of Tobacco Seedlings with Biological Seed Treatments and Foliar Sprays." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Bonnie H. Ownley, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council

Associate Vice Chancellor
and Dean of the Graduate School

BIOLOGICAL CONTROL OF TARGET SPOT AND SORE SHIN OF TOBACCO SEEDLINGS WITH BIOLOGICAL SEED TREATMENTS AND FOLIAR SPRAYS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Joseph P. McMahan
August, 1996

AG-VET-MED.
Thesis
96
.M25

ACKNOWLEDGEMENTS

The author would like to acknowledge the following:

Dr. Bonnie Ownley for serving as my major professor, for hiring me as a research assistant, and for her knowledge and guidance. I am greatly indebted to her.

Drs. Ernest Bernard, Robert Miller, and Mark Windham for serving on my committee.

Ben Eggett, Rob Ellis, and the staff of Highland Rim Experiment Station for their outstanding assistance during my experiments.

Mary Dee, Robert Reeder, Patrice Smith, Bendik Clark, and Sarah Douglas for their help in rating the tobacco plants from the Highland Rim Experiment Station.

Dr. Carroll Southards for graciously accepting me into the Department of Entomology and Plant Pathology.

To all of the students, staff, and faculty in the Deapartment of Entomology and Plant Pathology for making the graduate school experience an enjoyable one.

The Burley Stabilization Board and U.S. Tobacco Co. for providing funds to support my research.

To my parents, Joseph Lee McMahan and Betty McMahan, whose unending love, encouragement, and support helped make it all worthwhile.

And finally, to my loving wife, Phyllis McMahan and our four sons: Titus, Jermaine, Isaac, and Joseph Quentin, whose unending love, encouragement, and support kept me going through the good times and the bad.

ABSTRACT

Target spot is a leaf spot disease of tobacco initiated by basidiospores of *Thanatephorus cucumeris*, the teleomorph stage of *Rhizoctonia solani*. Symptoms of target spot begin as small, circular, water-soaked spots about 2-3 mm in diameter. Under conditions of high relative humidity and moderate temperature, lesions enlarge rapidly, becoming light green and almost transparent, with irregular margins and chlorotic halos. Sore shin is caused by *R. solani* or *T. cucumeris*. The disease usually appears as a brown discoloration of the stem near or below the soil line. The stalk of an affected plant becomes brittle and may break under pressure.

The focus of this research was biological control of target spot and sore shin of tobacco seedlings. Recent studies have shown that applications of antagonistic bacteria can be an effective control measure against target spot.

The first goal of the present study was to optimize the biocontrol activity of *Bacillus* sp. BA55 against *T. cucumeris* and *R. solani* on tobacco by manipulating cultural conditions for growth of the bacterium. The specific objectives were: 1) to determine the effect of culture medium on biocontrol activity of *Bacillus* sp. BA55 against target spot and sore shin; 2) to determine the effect of time period between foliar application of *Bacillus* sp. BA55 and introduction of *R. solani* on severity of target spot and sore shin, and; 3) to determine the effect of solid and liquid minimal 3C medium (Min 3C) and cultural incubation temperature on biocontrol activity of BA55 against target spot and sore shin.

The effect of culture medium and time of introduction of *R. solani* on biocontrol activity of BA55 was examined by applying foliar sprays of BA55 cultured in either Min 3C broth or nutrient broth yeast extract (NBY). An untreated control was included. The foliar treatments were applied to 8-week-old plants of TN 86 tobacco one day or six days before *R. solani* was added. In trial 1, disease rating was significantly lower in plants that received the foliar treatment of BA55 cultured in Min 3C compared to plants that did not receive any treatment. Plants treated with BA55 cultured in NBY were intermediate in disease rating. In trial 2, the effect of culture medium was not significant; however, plants that were inoculated with *R. solani* six days after foliar treatments with BA55 had a significantly lower disease rating and higher shoot fresh weight than plants inoculated one day after foliar treatment.

The effects of foliar treatment with BA55 cultured on either solid or liquid Min 3C medium at 25° or 30°C, also was examined. Foliar treatment with BA55 did not affect disease rating; however, shoot fresh weight of plants treated with BA55 cultured in solid or liquid Min 3C at 25°C was significantly greater than the untreated control. Disease pressure was low in this experiment and probably contributed to the lack of significant differences in disease rating.

The second goal of the present study was to reduce target spot and sore shin with biological seed treatments and optimized foliar sprays of BA55. The specific objectives were: 1) to determine the effect of BA55 seed treatment and concentration of BA55 in foliar spray treatments on target spot and sore shin of tobacco seedlings in the float bed

system; 2) to determine the effect of *Trichoderma* seed treatment and foliar sprays of *Bacillus* BA55 on target spot and sore shin of tobacco seedlings in the float bed system; and 3) to determine the effect of seed treatment with BA55 or *Trichoderma* on sore shin.

The rate of foliar application of BA55 had a significant effect on fresh weight in one of two trials. In an experiment to test the effect of BA55 seed treatment and various concentrations of BA55 in foliar sprays, TN 90 tobacco seedlings receiving 6 log or 7 log colony-forming units (cfu) per ml had significantly greater fresh weight than untreated controls. Also, TN 90 seedlings from seed treated with BA55 had greater fresh weight than seedlings from untreated seed.

In an experiment to test the effect of a fungal agent, *Trichoderma* 22-3, and foliar application of BA55 on severity of target spot and sore shin, KY 14 tobacco plants from seed treated with *Trichoderma* 22-3 had a significantly greater fresh weight than plants from untreated seed. In the second trial of this experiment, two cultivars (KY 14 and TN 90) were included. For KY 14, there was a significant interaction between seed treatment and foliar spray application on percent diseased foliage. There was no effect of seed treatment or foliar spray on TN 90 plants.

The effect of BA55 and *Trichoderma* seed treatments on biocontrol of sore shin on two burley cultivars was examined also. The seed treatments for KY 14 tobacco were untreated, *Trichoderma* 22-3 and *Trichoderma* 22-4. The seed treatments for TN 90 tobacco were untreated, *Trichoderma* 22-1, *Trichoderma* Test, *Trichoderma* 22-2. Also, TN 90 seed was treated with *Bacillus* sp. BA55, and a separate control for this treatment

was included. The effect of seed treatment was not significant for sore shin disease rating or shoot height of KY 14. For TN 90, plants from seed treated with *Trichoderma* 22-2 had a significantly greater shoot height than the control, but there was no effect on disease rating. Treatment of seed with BA55 had no effect on disease rating or shoot height of TN 90 plants.

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CHAPTER 1: LITERATURE REVIEW

PRODUCTION OF TOBACCO SEEDLINGS IN CONVENTIONAL PLANT BEDS

Tobacco transplants can be produced successfully in conventional outdoor plant beds if attention to the following practices are observed: 1) The site selection of the plant bed should be near a source of water; 2) Soil should be well-drained, high in moisture-holding capacity, loamy, and high in organic matter. 3) The plant bed site should be sloped gently (usually in the 3 to 5 percent range) to the south or southeast as soils facing this direction warm up more quickly in the early spring. 4) Windbreaks should be present on the north and northwest sides. Windbreaks such as trees can reduce drying of the plant bed soil, but the trees should not be so close that they shade the bed or compete with the tobacco plants for moisture and nutrients (20, 21, 52).

Many tobacco growers have problems with weeds and soilborne plant pathogens in conventional plant beds. The preferred method of control is fumigation with methyl bromide (CH_2Br), a soil fumigant which kills plant pathogens and most weed seed but not clover and prickly sida. It is recommended that methyl bromide be applied at the rate of 9 lbs. per 100 square yard bed. Soil temperature and soil moisture are critical factors for good results, thus proper application is essential. Methyl bromide is a restricted-use pesticide that is a liquid under high pressure in metal cans at room temperature (20, 21, 52). When the cans are punctured in the applicator tray, the liquid immediately volatilizes to a highly poisonous gas with a vapor pressure about three times that of ordinary air.

Methyl bromide is now thought to contribute to ozone layer depletion (7). As a

result, the Environmental Protection Agency has banned this fumigant in the U.S. after January, 2001.

FLOAT-BED TRANSPLANT PRODUCTION SYSTEMS

There are currently three hydroponic transplant production systems in wide use in the southeastern U.S. These include seed-and-transfer, plug-and-transfer, and direct seed. With these methods, seedlings are produced in outdoor floatbeds, greenhouse floatbeds, and a combination of greenhouse and outdoor floatbeds (52).

Seedlings produced with each method have several characteristics in common, including preparation and construction of the floatbed. Site preparation is an important step in ensuring proper water levels and efficient fertilizer distribution within the bed. The ground under the floatbed should be smooth, level, and preferably soft to reduce the risk of puncturing the plastic liner that holds the water (52). When soft soils are unavailable at the desired construction site, sand, limestone, or a similar material can be used to create a soft foundation (52).

These systems also share heating and ventilation characteristics. Supplemental heating can be accomplished with two 350-watt, thermostat-controlled waterbed heaters placed under the plastic liner (52). Ventilation is controlled manually as needed by placing bricks or similar spacers between the frame of the floatbed and the plastic cover at each corner to allow natural air movement throughout the bed (52).

The float tray is the device that actually holds the transplants. The float tray is filled with a soilless horticultural medium made for the production of tobacco transplants.

With the direct seed method, the float tray is seeded. Seedlings or plugs are transferred to the float tray with the other two outdoor production methods.

The plug-and-transfer production system involves the purchase of three to four-week-old seedlings in mini-trays. At this age, the seedlings are between 1 and 1½ inches tall (52). The plugs are transferred to the float trays and placed on the floatbed, which is fertilized with water-soluble 20-10-20 at a rate of 4 to 6 ounces per 100 gallons of water (52). The transplants are ready for transplanting about four weeks after transfer.

The seed-and-transfer method is not widely used due to higher labor requirements and risk of newly transferred tender seedlings being injured or killed by sunlight and/or wind when first exposed to the outside environment. The major difference between the seed-and-transfer system and the plug-and-transfer system is that the grower produces the seedlings with the former instead of purchasing them as in the latter case (52). Another major difference is that the seed-and-transfer seedlings are bare-rooted when transferred, whereas in the plug and transfer system, seedlings have root balls when they are transferred to the float tray (21, 52). Since the seed-and-transfer seedling does not have a root ball, holes or dibbles are made in the potting mix contained in the float cell trays before the transfer is made.

For use in the direct seed system, the extremely small tobacco seeds undergo a coating process. The seeds are coated with clay to a diameter of approximately one-sixteenth of an inch (1.58 mm) so that they can be more easily planted in the float trays with a seeding device (52). In the direct seed system, the transplant is grown from the coated seed to transplant size in a float tray (52). Because the seedling is produced from

seed in a floatbed, the need to transfer the seedling is eliminated.

Once seeded, the float tray is placed on a floatbed. A favorable environment for growth is crucial. With one seed per cell in the float tray, differences in rate of germination and number of seedlings that germinate can have a significant effect on the number of usable transplants produced and the uniformity of the transplants (52). The environmental conditions favorable for seed germination usually occur when the average air temperature at the tray level is within the 18 to 21°C range (20, 21, 52).

The greenhouse floatbed method for transplant production shares the underlying principles of the outdoor methods, but the environment in which the transplants grow is more controllable. In the greenhouse, float trays can be planted by direct seed, seed-and-transfer, or plug-and-transfer with the trays remaining in the greenhouse until they are ready for transplanting. For the greenhouse floatbed system to work, the greenhouse should be equipped with a vented heating system, proper ventilation equipment, and shadecloth to cover each side of the greenhouse.

DISEASES OF FLOAT-GROWN TOBACCO

There are several root, stem, and foliar diseases caused by fungi and bacteria that can affect float-grown tobacco. The pathogens involved include *Rhizoctonia solani* Kühn, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Botrytis cinerea* Pers. ex Fr., *Pythium ultimum* Trow var. *ultimum* and *Erwinia carotovora*. These pathogens may be moved to the field on infected transplants.

Collar rot is caused by the fungus *S. sclerotiorum*. Tobacco seedlings may be

attacked at any time and infection is noticeable as a small, sharply defined black-brown lesion at the base of the stem (38). Often, affected stems develop a brown soft rot that spreads into the leaves, with rapid destruction of seedlings. During periods of high relative humidity, white, cottony mycelium grows over the rotted plants (38).

Gray mold and dead-blossom leaf spot are diseases caused by the fungus *B. cinerea*. Both diseases may occur on seedlings and mature plants. Gray mold usually appears about the time the tobacco seedlings are large enough to transplant. Symptoms appear first on the lower leaves as they senesce (1, 40). If the plants dry after infection, brown spots with a light yellow border develop, but the pathogen does not continue to grow (40). During rainy periods, wet-rot lesions form and their surfaces become covered with a gray, downy coating of the fungus. Occasionally, black sclerotia may be seen adhering to the plant tissue (1, 8, 40). Dead-blossom leaf spot occurs on field plants that are not topped. After flowers mature, the corollas detach and adhere to the leaves. As the flowers rot, especially in wet weather, large dead spots rapidly develop under and around the fallen blossoms (40). The spots spread until they involve most of the leaf. Frequently, the midribs are rotted and leaves hang limp and yellow (40).

Stem rot occurs on float plants and on tobacco transplants. The causal organism, *Pythium ultimum*, attacks the stems of young plants, causing brown, watery lesions to develop near the soil line (1, 8, 57). As this fungal disease develops, stem tissues collapse and disintegrate, causing death of the plant. Stem rot usually appears as a watery soft rot. A cottony fungal mass also may be present (8).

Bacterial soft rot is caused by the bacterium *E. carotovora* subsp. *carotovora*.

Symptoms include wilting and drooping of the top leaves. Eventually the leaves fall leaving the stalk bare (41). Decayed areas usually turn black.

RHIZOCTONIA SOLANI

Rhizoctonia solani is a soil fungus that infects the root tissue and causes maceration with a consequent inhibition of plant growth. The fungus is classified as a Deuteromycete since it was initially thought that it did not possess a teleomorph; however, later it became evident that the sexual stage was rare in nature but did exist (58, 59). The teleomorph is *Thanatephorus cucumeris* (A. B. Frank) Donk, which produces basidiospores that are hyaline, smooth, and oblong to ellipsoid with an average size of 9×5.5 μm (58).

The disease cycle of damping-off, sore shin, and target spot of tobacco caused by *R. solani* or *T. cucumeris* begins with overwintering of mycelia and sclerotia of *R. solani* in soil and debris (59). Mycelial growth of *R. solani* leads to formation of an infection cushion that penetrates the stem of the host. Subsequently, the stem is colonized by the fungus. At this point, either the stem will rot and the plant will die (damping-off) or a stem canker (sore shin) will form that weakens the stem.

Overwintering of mycelia and sclerotia in soil and debris may also lead to development of the sexual stage (*T. cucumeris*) and production of basidiospores. When environmental conditions are conducive, basidiospores are formed and dispersed by forcible discharge. A basidiospore landing on a suitable host will form an appressorium, which allows for invasion of the fungus into the host. Development of primary lesions

and lesion expansion follow, resulting in necrotic spots with yellow halos (target spot). Secondary inoculum develops on the leaf surface and the cycle repeats itself.

Virtually all plant species are susceptible to infection by *R. solani*, and the fungus is worldwide in distribution (17, 31, 43). Isolates of *R. solani* have tremendous variation in morphology, pathogenicity, and host specificity (17, 31, 43). For the purpose of classification, these isolates have been grouped into anastomosis groups (AG). Isolates from the same group undergo hyphal fusion with each other, but not with isolates from other groups (17, 31, 43). A more recent concept has been to classify isolates on the basis of the pectic enzymes produced during growth on pectin (43). According to this system, isolates are divided into pectic zymogram groups (ZG). This system agrees with the division based on anastomosis behavior, except that some of the AG can be further subdivided into different ZG (43).

TARGET SPOT

Target spot was reported first from Brazil in 1948 (58, 59). In the United States the disease was first observed in North Carolina in 1983 and now occurs each year in flue-cured tobacco-growing regions of North Carolina and Virginia (58, 59). Since 1989, target spot has been reported from most other tobacco-producing states (47, 58, 59) and has been observed in Zimbabwe and Bulgaria (44, 58, 59).

Symptoms of target spot begin as small, circular, water-soaked spots about 2-3 mm in diameter. On seedlings, these initial lesions are netlike in appearance when leaves are held up to a light source. On field plants, these small spots remain visible whether

or not the lesion continues to expand, whereas on small seedlings, lesions may continue to expand. Under conditions of high relative humidity and moderate temperature, lesions enlarge rapidly, becoming light green and almost transparent, with irregular margins and chlorotic halos (1, 58, 59). In less humid conditions, lesions expand more slowly and often develop a pattern of concentric rings (1). Mycelium of the fungus is often present at the margins of lesions, on the upper and lower leaf surfaces (1, 58). Target spot is more severe when levels of nitrogen in the leaf are low (58, 59).

SORE SHIN

Sore shin usually appears first in the plant bed, but infections may occur at any growth stage (1). Sore shin appears as a brown discoloration of the stem near or below the soil line. The affected area may increase in size after the plant is set in the field, sometimes extending several inches up the stalk (1, 62). The stalk of an affected plant becomes brittle and may break easily under pressure. Lower leaves of older plants may turn yellow and the plant is often stunted. Plants may wilt during the heat of the day. Diseased plants usually appear scattered in the field. In advanced stages of sore shin, the interior of large stalks may be dry and brown. Stalks with only slight infections produce near normal growth and yields unless the plants become stressed (62).

DAMPING-OFF

Damping-off can affect tobacco at any stage during seedling growth. The disease first appears as irregular areas of wilted, yellowed, stunted, or dead plants (8). These

symptoms are the result of girdling of the lower stem of the tobacco plants by *R. solani*.

The fungus causes decayed, sunken areas on the lower stem of tobacco (8). Lesions are frequently reddish brown and under favorable conditions these lesions increase in size, girdle the plants, and cause them to fall over (8).

CHEMICAL AND CULTURAL CONTROL MEASURES FOR TOBACCO SEEDLING DISEASES

Fungicides such as iprodione 4F (35, 58) can be used to control target spot in float beds in states with a section 18 exemption. However, there are concerns about residues from fungicide applications close to harvest (58), that may limit fungicide use in the field. In Tennessee, mancozeb, under a section 18 exemption, is recommended for target spot on float plants. Sanitation practices are also recommended to prevent the introduction of inoculum into seedling production beds or greenhouses (58, 59).

Collar rot inoculum can be eliminated with fumigation of conventional seedbed sites (38). Sanitation to remove all infected plant material or infested soil is critical to prevent disease development (38). Providing adequate ventilation and avoiding dense plant stands aid in maintaining an environment unfavorable for the germination and growth of the fungus. Fungicides are not recommended for the disease (38).

To help prevent gray mold, it is recommended to locate conventional seedbeds on a slight slope with well-drained soil, and to fumigate with methyl bromide (40). Overseeding should be avoided and adequate air flow should be provided across the bed (40). Weekly application of suitable fungicides reduces infection (40). No method for

preventing dead-blossom leaf spot is known (40).

Pythium stem rot of young tobacco plants can be controlled to a certain extent by fumigation of conventional seedbeds with methyl bromide or chloropicrin, or steam in greenhouses (57). Application of metalaxyl to the seedbed can also help control this disease (57). Excessively wet soils should not be used as seedbed sites and dense plant stands should be avoided.

There are no control measures for bacterial soft rot other than reducing humid conditions if the plants are wet, and providing adequate ventilation (39).

BIOLOGICAL CONTROL

Biological control is an environmentally sound way to implement total or partial destruction of pathogen populations by beneficial organisms. Cook and Baker (13) 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.

Biocontrol organisms may act in one or more ways: they may 1) release metabolites (antibiotics) 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, iron, water, and space) (4, 13).

There are some potential disadvantages in using biocontrol methods, such as the storage and maintenance of a biocontrol agent compared with storage and maintenance

of a fungicide. This problem can be alleviated somewhat by freeze-drying or storing the bacterial or fungal biocontrol agents at -80°C. Another disadvantage with biocontrol agents can be associated with antibiotic production if the antibiotic is toxic to the host plant or disrupts potentially beneficial soil microbial populations by killing nontarget organisms (69).

Despite these potential disadvantages, biocontrol is considered to be less harmful than conventional chemical treatments to the environment. Development of effective biocontrol strategies will enable growers to move toward more sustainable agricultural production.

BIOLOGICAL CONTROL OF TOBACCO DISEASES

Black Root Rot

Uneven growth of tobacco in the field is indicative of black root rot caused by *Thielaviopsis basicola* (Berk. and Broome) Ferraris. Affected plants often become chlorotic and are smaller than neighboring healthy plants. Stunting is more evident during the early part of the growing season and during cool, wet seasons. On hot days, leaves of diseased plants wilt more rapidly than those of healthy plants, but recover during the night. Plants usually grow out of the problem as soil temperatures rise, unless the cultivar is highly susceptible to the fungus and cool, wet soil conditions prevail. The most distinctive symptoms of black root rot are that the entire root system is greatly reduced with smaller roots exhibiting a typical brown to black discoloration of their tips, and larger roots having brown or black spots on their surface (8). The outer root tissue

may slough easily (1, 8).

Recent studies have demonstrated the ability of a gram-negative bacterium, *Pseudomonas fluorescens* strain CHA0, to control black root rot of tobacco (2). Strain CHA0 produces the antibiotics pyoluteorin and 2,4-diacetyphloroglucinol as well as the biocide hydrogen cyanide (HCN). Suppression of black root rot is mediated primarily by production of HCN (2).

Southern Wilt of Tobacco

Southern bacterial wilt of tobacco, also known as Granville wilt, is caused by the bacterial pathogen *Pseudomonas solanacearum.* The disease is present in the tropics and in the warmer climates throughout the world (1, 9, 10). In the United States the disease is most severe in the southeastern states where it is favored by the warm, humid climate (1). Symptoms of bacterial wilt on tobacco appear as a rather sudden wilt. Infected young plants die rapidly. Older plants may first show leaf drooping and discoloration, leaf drop, or one-sided wilting and stunting before the plants wilt permanently and die (1). *Pseudomonas solanacearum* overwinters in diseased plants or plant debris, propagative organs such as potato tubers and banana rhizomes, on the seed of some crops, and in the soil. Injured or decaying infected tissues release bacteria into the soil (1, 8). The bacteria are spread through soil water, infected or contaminated seeds, rhizomes, or transplants by contaminated knives used for pruning suckers and in some instances, by insects. The bacteria enter plants through wounds made in roots by cultivating equipment, nematodes, insects, and at natural cracks where secondary roots

emerge (1, 9). The bacteria reach the large xylem vessels, then spread throughout the plant. Along the vessels they escape into the intercellular spaces of the parenchyma cells in the cortex and pith, dissolve the cell walls, and create cavities filled with slimy masses of bacteria and cellular debris.

The root systems of tobacco dipped in bacterial suspensions of avirulent bacteriocin-producing strains (ABPS) of pathogenic *P. solanacearum* were found to be more effectively protected against bacterial wilt caused by *P. solanacearum* than those dipped in suspensions of an avirulent nonbacteriocin-producing strain (10). Minimum colonization for wilt induction in susceptible tobacco plants by *P. solanacearum* was approximately 5×10^5 colony forming units (cfu)/g of root under glasshouse conditions (10). The rate of colonization by the pathogen was reduced but not completely prevented by ABPS (10). The threshold population needed for disease development was eventually reached so that the ABPS treatment only delayed appearance of symptoms and reduced disease severity (10). Although dipping roots of young seedlings in a suspension of ABPS can protect the treated roots from bacterial wilt, it is not an effective protectant of new root growth because APBS cells do not readily move over the root system, thus the present strains of APBS need to be improved before they can be used effectively for control of bacterial wilt (9, 10).

Tobacco Black Shank

Tobacco black shank is caused by *Phytophthora parasitica* var. *nicotianae* Breda de Haan and is primarily a disease of the roots and lower stalk of tobacco. The

symptoms of black shank vary with weather conditions, age of plants, stage of disease development, degree of plant resistance, and virulence of the soilborne fungal pathogen. Stunting is often the first symptom of black shank, followed by wilting. Wilted plants usually will recover in the evening hours, but later become permanently wilted, with the leaves turning yellow and hanging down the stalk (1, 8).

A simple, rapid, small-scale assay for screening bacteria for biocontrol activity against *P. parasitica* var. *nicotianae* has been developed (27). In this assay, one seven-day-old tobacco seedling was placed in each well of a 96-well microtiter plate and inoculated with 500 zoospores of *P. parasitica* var. *nicotianae* (27). After 72 h, all of the inoculated seedlings of the susceptible cultivar KY14 were infected, and the pathogen had produced sporangia that were visible on the surfaces of the seedlings. Sporangia did not develop on seedlings that were inoculated simultaneously with zoospores and either 1 μg/ml of the chemical fungicide metalaxyl or 5 μl of filtrate of a sporulated culture of the bacterial biocontrol agent, *Bacillus cereus* strain UW85.

Blue Mold

Blue mold, caused by *Peronospora tabacina* D. B. Adam, can be devastating to tobacco plants. After penetration through the epidermis, hyphae of the blue mold fungus grow in intercellular spaces (8, 65). The fungus then penetrates cell walls and produces a haustorium to establish a successful infection (65). Usually, the first symptom of blue mold in a seedling bed is an area of yellowed plants (1, 8). The leaves of affected plants have a cupped appearance, and on the undersides of the leaves, blue-gray downy fungal

growth appears. Older plants can be deformed and twisted, and the main growing point eventually is killed (1, 8). Veins in affected areas of twisted leaves are reddish-brown and can become necrotic. On older plants, definite yellow spots with bluish fungal growth on the underside of leaves can occur. During weather favorable for blue mold (cool, cloudy, and wet), the disease can develop rapidly, causing numerous leaf lesions that result in leaf blighting and severe leaf loss. The disease may develop during hot, humid periods if cool nights occur (1).

Tuzun et al. (65) found that plants of *Nicotiana tabacum* L. cv. Jaltepec stem-injected with sporangiospores of metalaxyl-tolerant strains of *P. tabacina*, developed fewer and smaller lesions compared to plants treated with metalaxyl or compared to noninjected stem controls, regardless of metalaxyl application. The technique utilized for stem injection with *P. tabacina* introduces inoculum specifically into the stem tissue external to the xylem (65). Stem-inoculated plants overcame stunting and became immune, with increased growth. Thus, biological control was implemented in this study by using a modified, hypovirulent strain of the pathogen.

Tobacco Brown Spot

Tobacco brown spot, or *Alternaria* leaf spot, is a major foliar disease of tobacco. The causal organism is *Alternaria alternata* (Fr. ex Fr.) Keissl (64). The symptoms of brown spot first appear on the lower leaves (8). Spots are brown with sharply defined margins and may be surrounded by yellow halos (8). Brown spot is distinguished by the dark concentric rings within the spot that give it a target-like appearance (8). With

magnification, reproductive structures of the causal agent are seen as tufts of black fungal spores or conidia (8, 64). Under severe conditions, spots are numerous and may coalesce, giving the leaf a ragged appearance as the infected tissue falls out (8, 64).

Fravel and Spurr (23) demonstrated biocontrol of tobacco brown spot by screening bacterial isolates in vitro for effects on spore germination and germ tube development of three *A. alternata* isolates. One isolate, identified as *Bacillus cereus* subsp. *mycoides* strain B23, effectively controlled tobacco brown spot lesion development in a controlled environment when B23 was inoculated simultaneously with the pathogen (23).

Target Spot

Recent studies (4) have shown that applications of antagonistic bacteria can be an effective control measure against target spot caused by *T. cucumeris*. In this work, foliar applications of *Bacillus* sp. strain BA55 provided substantial control of target spot on two dark fire-cured tobacco cultivars in greenhouse experiments (3, 4). However, variability in performance of BA55 in subsequent tests was noted. In 1993, percent diseased foliage was approximately 1% in plants treated with BA55 and 18% in untreated plants. In 1994, percent diseased foliage was 15% in plants treated with BA55 and 30% in untreated plants. One difference between the two tests was that the plants were inoculated with *R. solani* one day after they had been sprayed with BA55 in 1993, and six days after BA55 had been applied in 1994. In both years, inoculum of BA55 was prepared by culturing the bacterium in nutrient broth yeast extract (NBY; 66). In a related study (P. Smith and B. Ownley, *Unpublished*) antibiosis by *Bacillus* sp. BA55

against *R. solani* was greater on minimal 3C medium (Min 3C; 28) than on NBY agar.

OBJECTIVES OF THIS STUDY

The objectives of this research were 1) to determine the effect of culture medium on biocontrol activity of *Bacillus* sp. BA55 against *R. solani* on tobacco; 2) to determine the effect of time period between foliar application of *Bacillus* sp. BA55 and introduction of *R. solani* on severity of target spot and sore shin; 3) to determine the effect of solid and liquid culture medium on biocontrol activity of *Bacillus* sp. BA55 against *R. solani* on tobacco; 4) to determine the effect of cultural growth temperature on subsequent biocontrol activity of *Bacillus* sp. BA55; 5) to determine the effect of BA55 seed treatment and concentration of BA55 in foliar spray treatments on biocontrol of target spot and sore shin of tobacco seedlings in the float bed system; 6) to determine the effect of *Trichoderma* seed treatment, foliar sprays of BA55, and cultivar on biocontrol of target spot and sore shin of tobacco seedlings in the float bed system; 7) to determine the effect of biological seed treatment (*Bacillus* sp. BA55 or *Trichoderma* spp.) and cultivar on sore shin caused by *R. solani* on tobacco seedlings.

CHAPTER 2

EFFECT OF CULTURAL CONDITIONS ON EFFICACY OF *BACILLUS* SP. BA55 AS A BIOCONTROL FOR *RHIZOCTONIA* DISEASES OF TOBACCO

Many gram-positive bacilli form endospores and one of the most common is *Bacillus*. The process of bacterial sporulation begins when rapid growth ceases due to nutritional deprivation, which causes the cells to divide asymmetrically. Cultural factors that affect sporulation include nutrient and mineral composition, pH, temperature, and aeration of the medium (18). During sporulation, characteristics unique to spores such as heat and chemical resistance develop and dipicolinic acid (DPA) and calcium accumulate (18).

Spores of all bacterial species have the same basic structure, which includes a central core (protoplast) surrounded, in turn, by a plasma membrane, germ cell wall, cortex, coat(s), and in some species an exosporium (outermost layer) (17). The core, plasma membrane, and germ cell wall essentially represent a modified vegetative cell which is protected by the cortex, coat(s), and exosporium (18).

The molecular contents of the core are similar to the contents of vegetative cells with a few key exceptions. Spores contain a complement of enzymes, ribosomes, DNA, and lipids not significantly different from vegetative cells of the same species (18). A unique feature of the core is the presence of dipicolinate anions, at least some of which are chelated 1:1 with Ca^{2+} ions (18). Spores for the most part appear to be in a relatively dry state although they are water-permeable (18). The cortex consists primarily of peptidoglycan only slightly modified from the peptidoglycan present in vegetative cells walls and is relatively uniform among spores of various species. Spore

dormancy can be broken by degradative enzymes termed spore lytic enzymes that are associated with peptidoglycan, the cortex and germ cell wall (18).

Handelsman et al. found that *Bacillus cereus* UW85 grown in minimal medium 3C (Min 3C), compared to cells grown in tryptic soy broth (TSB) or casamino acids glucose broth (CB), provided greater biological control of damping-off of alfalfa seedlings caused by *Phytophthora megasperma* f. sp. *medicaginis* (28). In this study, suppression of damping-off by culture filtrates of UW85 during various stages of sporulation and spore release was examined. In both TSB and Min 3C, as the cultures aged, the proportion of endospores released from the cells increased. As the proportion of released endospores increased, the survival rate of seedlings treated with filtrates of these cultures and *Phytophthora megasperma* f. sp. *medicaginis* also increased (28). When seedlings were treated with *Phytophthora megasperma* f. sp. *medicaginis* and UW85 cultured in TSB, CB or Min 3C, the survival rate of seedlings was significantly greater with UW85 grown in Min 3C or CB. The authors suggested that a biologically active peptide or antibiotic was responsible for biocontrol activity and was released concurrently with the release of endospores. They concluded that biocontrol activity was not due to a product of sporulation (such as dipicolinic acid).

Bacillus sp. BA55 inhibits growth of *Rhizoctonia solani* in culture by antibiosis (4). BA55 can be effective against target spot of tobacco caused by *Thanatephorus cucumeris*, the teleomorph of *R. solani* (3, 4). However, variability in performance of BA55 has been noted (4). Ashby et al. (4) found differences in the level of biocontrol by *Bacillus* sp. BA55 against target spot in greenhouse tests conducted in 1993 and 1994.

In 1993, BA55 controlled target spot substantially across two cultivars (DF 485 and TR Madole) and two planting methods (direct-seeding and seed-and-transfer) (4). In 1994, BA55 reduced target spot severity on cultivar DF 485 but not on TR Madole (4). It was reasoned that the difference in control may have been related to the time of inoculation of *R. solani*. During 1993, *R. solani* was introduced one day after the foliar treatments of BA55, whereas *R. solani* was introduced six days after the foliar treatments during 1994 (4).

In both years, BA55 was cultured in nutrient broth yeast extract (NBY; [66]). In subsequent tests, antibiosis by BA55 against *R. solani* in vitro was more pronounced on Min 3C agar than on NBY (P. Smith and B. Ownley, *Unpublished*). Knowledge of the effects of cultural variables on biocontrol of BA55 is lacking and may lead to development of protocols that result in more consistent biocontrol performance.

In this study the objectives were: 1) to determine the effect of culture medium on biocontrol activity of *Bacillus* BA55 against *R. solani* and *T. cucumeris* on tobacco; 2) to determine the effect of time period between foliar application of *Bacillus* BA55 and introduction of *R. solani* on severity of target spot and sore shin and; 3) to determine the effects of solid and liquid medium, and cultural temperature (25, 30°C) on biocontrol activity of *Bacillus* sp. BA55.

MATERIALS AND METHODS

Culture media. *Bacillus* BA55 was cultured in Min 3C or in NBY. Min 3C broth consists of 2.0 g ammonium sulfate, 6.0 g monobasic potassium phosphate, 14.0

g dibasic potassium phosphate, 0.2 g magnesium sulfate heptahydrate, 0.00025 g manganese sulfate monohydrate, 1.0 g trisodium citrate dihydrate, 0.01 g thiamine hydrochloride, 2.0 g L-glutamic acid, and 5.0 g acid-hydrolyzed casein dissolved in 1 liter deionized water. After autoclaving, 10 ml of a sterile 50% (w/v) glucose solution (50 g glucose + 100 ml deionized water) and 10 ml of sterile ferric chloride hexahydrate solution (0.4 g ferric chloride hexahydrate + 100 ml deionized water) were added. NBY consists of 8.0 g Bacto-nutrient broth, 2.0 g Bacto-yeast extract, 2.0 g potassium monohydrogen phosphate, 0.5 g potassium dihydrogen phosphate, 0.25 g magnesium sulfate heptahydrate, and 5.0 g dextrose, in 1 liter deionized water.

Preparation of *Bacillus* sp. BA55 as a foliar spray. Strain BA55 was streaked on NBY agar plates and incubated at 25°C for 24 h. The NBY cultures were used to inoculate four tubes containing 5 ml of Min 3C broth or 5 ml of NBY with *Bacillus* BA55. The inoculated tubes were incubated on a rotary shaker (100 rpm) for 24 h at 28°C. Ten-ml aliquots of the broth cultures were used to inoculate 250 ml of Min 3C or NBY broth in two 2800-ml culture flasks. The inoculated flasks were incubated for 72 h on a rotary shaker (100 rpm) at 28°C. The bacterial suspensions were then centrifuged at 5000 rpm for 10 min in a Sorvall RC-SB refrigerated superspeed centrifuge. The supernatant was then decanted and the pellets were resuspended in 500 ml of sterile deionized water. The bacterial cell suspension was stirred for approximately 20 min before applying to tobacco plants as a foliar spray. The Min 3C preparation of BA55 had approximately 4.5 to 5.1 log cfu/ml while the NBY preparations had 6.1 to 7.3 log cfu/ml.

Preparation of *Bacillus* sp. BA55 for testing the effects of solid and liquid culture medium and cultural temperature on biocontrol activity. In order to test the effects of solid and liquid medium, and cultural temperature on the biocontrol activity of BA55, *Bacillus* sp. BA55 was cultured on solid Min 3C agar or in Min 3C broth which lacked the agar component. For preparation of broth cultures, four tubes containing 10 ml of Min 3C broth were inoculated with BA55. Two tubes were incubated at 25°C and two were incubated at 30°C. All four tubes were incubated on a rotary shaker (150 rpm) for 24 h. Ten-ml aliquots of these cultures were used to inoculate 500 ml of Min 3C broth in four 2000-ml culture flasks. The inoculated flasks were incubated for 72 h on rotary shakers (150 rpm) at either 25°C or 30°C. The contents were then centrifuged at 3500 rpm for 10 min and the pellets were resuspended in 500 ml of sterile deionized water for use as a foliar spray.

Cultures of BA55 on solid medium were prepared by streaking the bacterium onto 12 NBY agar plates. Six of these plates were incubated at 25°C and the remaining six were incubated at 30°C. After 24 h, the 12 NBY plates were flooded with sterile deionized water. The bacterial lawn was suspended in the water and transferred to Min 3C agar plates which were then incubated at either 25°C or 30°C for 72 h. The Min 3C agar plates were then flooded with 2 ml of deionized water, and the bacteria were suspended in the aqueous solution. The suspensions from the 25°C and 30°C plates were further diluted with 200 ml of deionized water for use as a foliar spray. The concentration of BA55 in the four different Min 3C preparations was adjusted to 6 log cfu/ml.

Pathogen inoculum. Three isolates of *R. solani*, designated TN-1, 1600, and 1700 were used in this study. Isolate TN-1 was obtained from D. Hensley, University of Tennessee, Knoxville, TN. Isolates 1600 and 1700 were obtained from D. Shew, North Carolina State University, Raleigh, NC. Agar plugs of *R. solani*, cultured on full-strength potato dextrose agar (PDA; 24 g potato dextrose broth, Difco, Detroit, MI, and 20 g agar, Sigma Chem. Co., St. Louis, MO, in 1 liter deionized water), that had been incubated for one week at 25°C were used to inoculate 500-ml Erlenmeyer flasks containing whole rice medium (100 g of rice grains per 72 ml of deionized water, autoclaved twice for 60 min on two successive days). For production of pathogen inoculum, the inoculated flasks were incubated at 28°C for 7 days.

Greenhouse assay to test the effect of cultural medium of BA55 and time period between application of BA55 and introduction of *R. solani* on biocontrol activity of BA55. Eight-week-old TN 86 burley tobacco plants were sprayed with an aqueous solution of either BA55 grown in Min 3C or BA55 grown in NBY until run-off, or received no treatment. Plants were fertilized with Peters Excel 21-5-20. Two grams of rice grain inoculum of *R. solani* TN-1 were placed on the soil surface under the canopy of each plant, either 1 day or 6 days after foliar treatment. All plants were air-dried for approximately 4 h, then each plant was enclosed in a plastic bag to maintain high relative humidity. Two weeks later plants were sprayed again with an aqueous solution of either BA55 grown in Min 3C or BA55 grown in NBY until run-off, or received no treatment. After an additional 4 weeks, plants were assessed for disease severity.

The experimental design was a 2 × 3 factorial with two time periods between pathogen introduction and foliar treatment, and three foliar treatments. There were four replicates per treatment arranged in a randomized complete block design. The experiment was repeated with eight replicates per treatment.

Greenhouse assay to test the effect of cultural conditions (solid and liquid medium, and cultural temperature) on biocontrol activity of BA55. TN 86 Burley tobacco plants (eight weeks old) were sprayed with an aqueous solution of BA55 inoculum cultured at either 25°C or 30°C in liquid Min 3C broth or on solid Min 3C agar, until run-off, or received no treatment. All plants were air-dried for approximately 4 h, then each plant was enclosed in a plastic bag to maintain high relative humidity. A mixture of two grams of rice grain inoculum of *R. solani* isolates 1600 and 1700 were placed on the soil surface under the canopy of each plant, 6 days after foliar treatment. All plants were fertilized with Peters Excel 21-5-20. Two weeks later, the appropriate foliar treatments were reapplied to all plants. After an additional 4 weeks, plants were assessed for disease severity.

The experimental design was a randomized complete block with five foliar treatments. The foliar treatments were BA55 cultured on either solid Min 3C at 25°C, in liquid Min 3C at 25°C, on solid Min 3C at 30°C, or in liquid Min 3C at 30°C, and a no treatment control. There were 16 replicates for the control and 8 replicates for other treatments.

Assessment of BA55 populations in foliar sprays. A micro-dilution plating technique (15) was employed to determine population counts of BA55 in the foliar

sprays. Briefly, a 3 × 3 grid was drawn on the bottom of Min 3C and NBY agar plates. A dilution series, from 10^{10} to 10^{0}, of the foliar sprays was prepared and three 10-μl droplets of each dilution were placed in a row on either Min 3C or NBY agar and allowed to dry. There were three droplets (replicates) per dilution. The plates were incubated at 25°C for 24 h. The number of colony forming units per ml (cfu/ml) were counted in each droplet. The highest dilution with discrete colonies in each replicate droplet was selected. Counts were averaged across the three droplets and the resulting number was multiplied by 100 and the dilution factor from which it was derived to yield the final cfu/ml.

Disease Assessment. Plants were assessed for disease severity with a rating scale of 0 - 7, where, 0 = healthy; 1 = a few target spot lesions, 0 - 5% chlorosis, no stem canker; 2 = target spot lesions present, 5 - 25% chlorosis; 3 = target spot lesions present, 25 - 50 % chlorosis; 4 = small stem canker, not encircling the stem; 5 = large canker, encircling stem; 6 = large canker, hymenium of fungus evident on the crown of the plant; 7 = dead. Shoot fresh weight was determined also.

Data Analysis. The main effects of foliar treatments and pathogen introduction time, and the interactions of these factors on disease rating and shoot fresh weight were analyzed for significance with the General Linear Models procedure and F-protected LSD tests of the PC-Statistical Analysis System®. Similarly, the effects of BA55 cultural conditions on disease rating and shoot fresh weight were analyzed.

RESULTS

Effect of cultural medium of BA55 and time period between application of BA55 and introduction of *R. solani* on biocontrol by BA55. In the first trial, the interaction of culture medium and pathogen introduction time was not significant for either disease rating or shoot fresh weight. However, the main effect of culture medium was significant ($P = 0.0134$) for disease rating (Table 2-1, Fig. 2-1).

The disease rating was 46% less in plants treated with BA55 cultured in Min 3C broth compared to the untreated plants (Figs. 2-1, 2-2). Although plants treated with BA55-Min 3C were larger than either untreated plants or plants treated with BA55 cultured in NBY broth (by 36% and 15%, respectively) the differences were not significant (Table 2-1, Fig. 2-3).

In the first trial, the main effect of time period between foliar treatment and introduction of *R. solani* was not significant for either disease rating or shoot fresh weight, regardless of whether the pathogen was introduced 1 day or 6 days after foliar treatments (Table 2-1, Fig. 2-2). More importantly, BA55-Min 3C was effective in reducing disease severity for both time period treatments (Figs. 2-1, 2-3).

Overall, the level of disease was low in the second trial. In contrast to the first trial, the effect of time of introduction was significant for both disease rating ($P = 0.0418$) and shoot fresh weight ($P = 0.0119$) (Table 2-2, Figs. 2-4, 2-5). Plants treated with BA55 6 days before introduction of *R. solani* had a lower disease rating than plants treated 1 day before pathogen introduction.

Table 2-1. Analysis of variance for the effects of culture medium of *Bacillus* sp. BA55 and day of introduction of *Rhizoctonia solani* on disease rating and shoot fresh weight of tobacco seedlings in trial 1.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Disease rating					
Replicate (REP)	3	5.458	1.819	0.93	0.4496
Medium (MED)	2	22.750	11.375	5.83	0.0134
Day	1	0.375	0.375	0.19	0.6675
MED x Day	2	0.750	0.375	0.19	0.8273
Error	15	29.292	1.953		
Corrected Total	23	58.625			
Shoot fresh weight					
REP	3	42.833	14.278	0.13	0.9420
MED	2	232.583	116.292	1.04	0.3765
Day	1	16.667	16.667	0.15	0.7044
MED x Day	2	243.583	121.792	1.09	0.3606
Error	15	1672.167	111.478		
Corrected Total	23	2207.833			

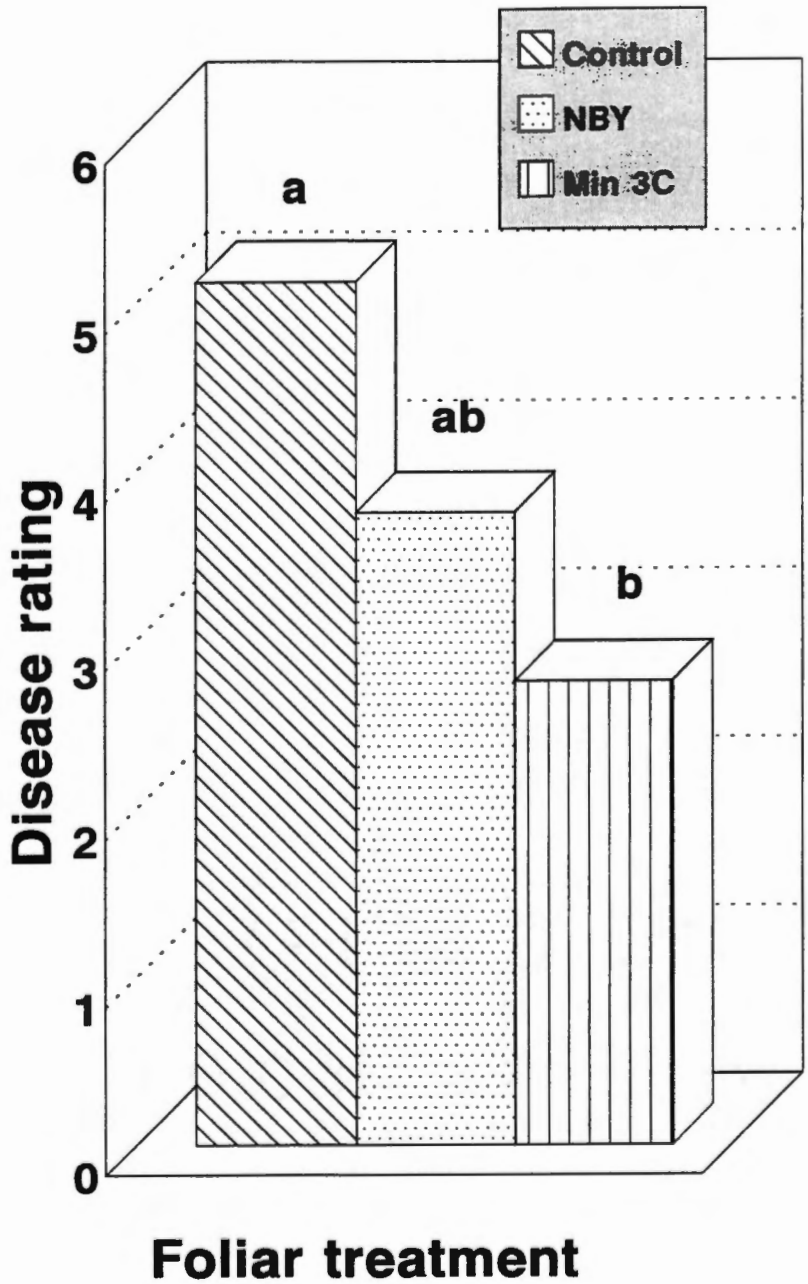


Fig. 2-1. Effect of foliar treatment on disease rating of target spot and sore shin, caused by *T. cucumeris*, on tobacco. Foliar treatments were *Bacillus* BA55 cultured in Min 3C broth, BA55 grown in NBY broth, or untreated. Bars with the same letter are not significantly different according to a F-protected Least Significant Difference test (P = 0.05). Trial 1.



Fig. 2-2. Appearance of plants receiving foliar applications of *Bacillus* BA55 cultured in either Min 3C broth or NBY broth, or untreated. Plants were inoculated with *R. solani* 1 day (A) or 6 days (B) after foliar treatment. The photographs were taken four weeks after the inoculation with *R. solani*. Trial 1.

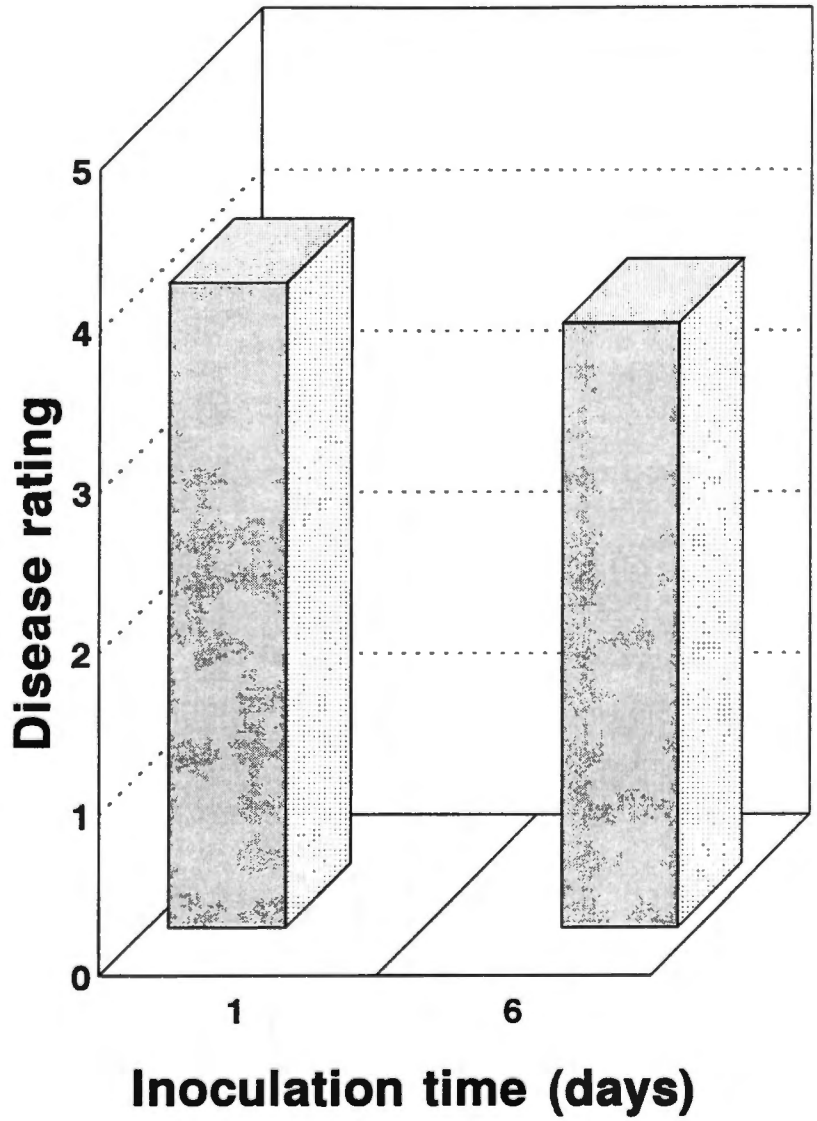


Fig. 2-3. Effect of time of introduction of *R. solani* on disease rating of tobacco with target spot and sore shin in trial 1. The TN 86 plants were inoculated either 1 day or 6 days after foliar treatments with *Bacillus* BA55 cultured in either Min 3C or NBY, or untreated. Bars are not significantly different according to a F-protected Least Significant Difference test ($P = 0.05$).

Table 2-2. Analysis of variance for the effects of culture medium of *Bacillus* sp. BA55 and day of introduction of *Rhizoctonia solani* on disease rating and shoot fresh weight of tobacco seedlings in trial 2.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Disease rating					
Replicate (REP)	7	24.405	3.486	0.83	0.5694
Medium (MED)	2	11.264	5.632	1.34	0.2746
Day	1	18.741	18.741	4.46	0.0418
MED x Day	2	0.137	0.068	0.02	0.9838
Error	35	146.970	4.199		
Corrected Total	47	201.979			
Shoot fresh weight					
REP	7	1312.010	187.430	1.54	0.1860
MED	2	248.974	124.487	1.02	0.3698
Day	1	856.754	856.754	7.04	0.0119
MED x Day	2	241.614	120.807	0.99	0.3806
Error	35	4256.990	121.628		
Corrected Total	47	7126.667			

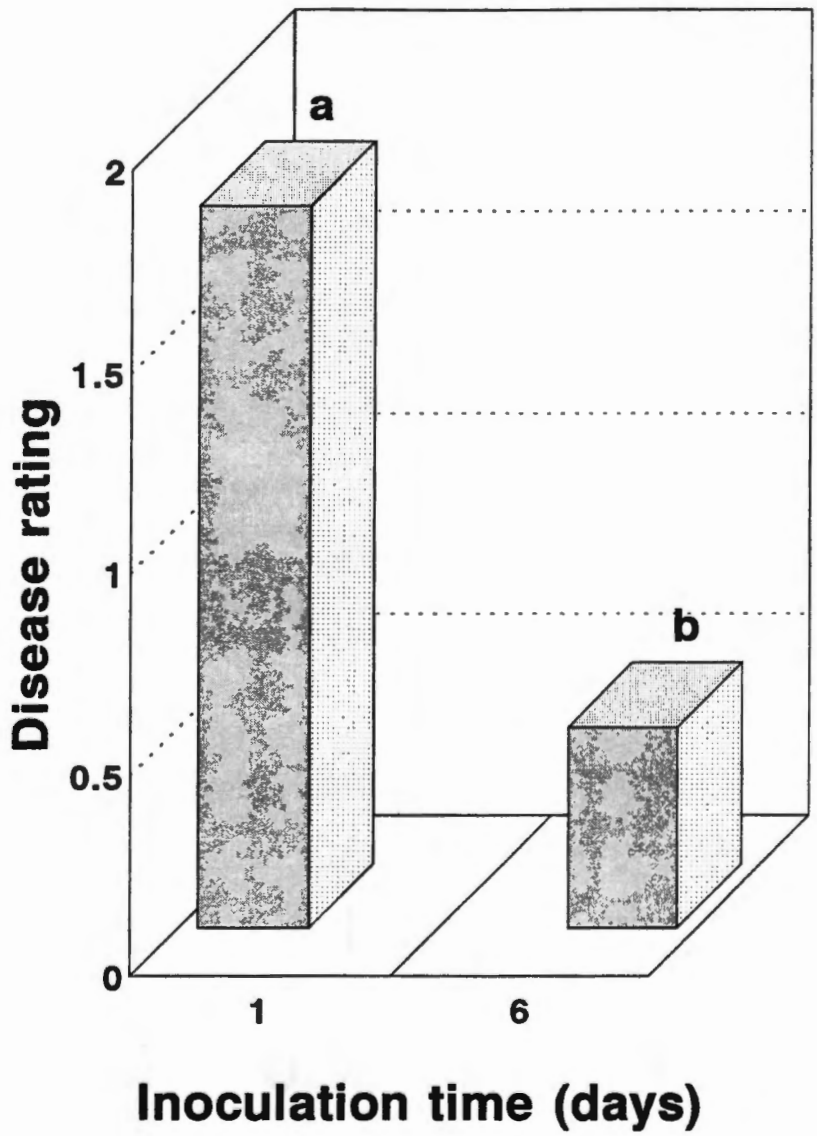


Fig. 2-4. Effect of time of introduction of *R. solani* on disease rating of tobacco with target spot and sore shin in trial 2. The TN 86 plants were inoculated either 1 day or 6 days after foliar treatments with BA55 cultured in either Min 3C or NBY, or untreated. Bars with the same letters are not significantly different according to a F-protected Least Significant Difference test ($P = 0.05$).

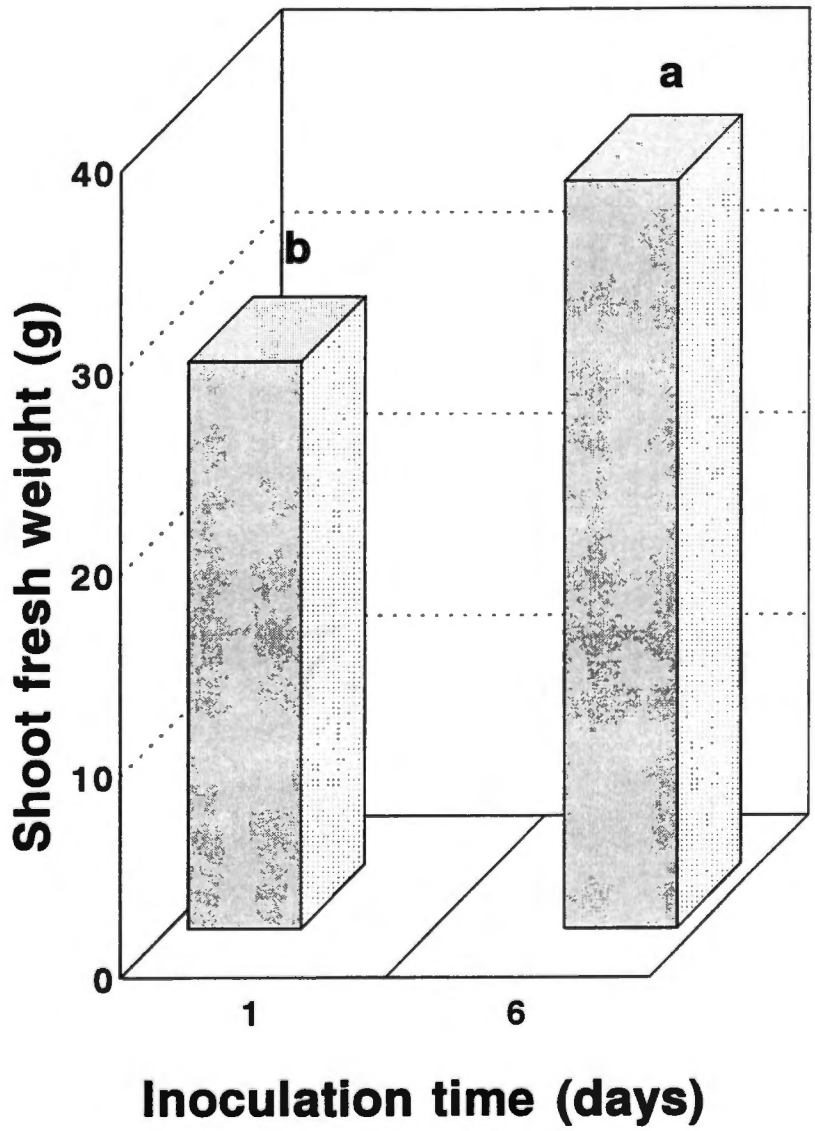


Fig. 2-5. Effect of time of introduction of *R. solani* on shoot fresh weight of tobacco with target spot and sore shin in trial 2. The TN 86 plants were inoculated either 1 day or 6 days after foliar treatments with BA55 cultured in either Min 3C or NBY, or untreated. Bars with the same letter are not significantly different according to a F-protected Least Significant Difference test ($P = 0.05$).

Effect of cultural conditions (temperature and solid or liquid culture medium) on biocontrol activity of BA55 against target spot and sore shin. Overall, the level of disease was very low in this test. The effect of cultural conditions was not significant for disease rating (Table 2-3). However, the effect was significant ($P = 0.0292$) for shoot fresh weight (Table 2-3, 2-4). Foliar treatment with BA55 cultured in solid or liquid Min 3C at 25°C resulted in plants with greater shoot fresh weights than the untreated control.

DISCUSSION

Bacillus sp. BA55 cultured in Min 3C, applied as a foliar spray significantly reduced disease rating due to target spot and sore shin by 46% compared to untreated control plants in one of two trials. The disease rating of plants treated with BA55 cultured in NBY was intermediate, but not different from the untreated plants or plants treated with BA55-Min 3C. NBY is a relatively nutrient-rich medium compared to Min 3C.

The results of the BA55-Min 3C study are similar to a report by Handelsman et al. (28), in which *Bacillus cereus* UW85, incubated on nutrient-poor media (Min 3C and CB), significantly reduced incidence of damping-off caused by *Phytophthora megasperma* f. sp. *medicaginis* on alfalfa seedlings, compared to bacteria grown on a nutrient-rich medium (TSB). In their study, sterile filtrates of cultures containing vegetative cells, cultures containing spores in the parent cell, and cultures containing predominantly released spores were tested for the ability to suppress damping-off of alfalfa in a bioassay

Table 2-3. Analysis of variance for the effects of cultural conditions of *Bacillus* sp. BA55 on disease rating of target spot and sore shin and shoot fresh weight of tobacco seedlings.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Disease rating					
Replicate (REP)	7	6.313	0.902	1.03	0.4285
Medium (MED)	4	1.604	0.401	0.46	0.7664
Error	36	31.563	0.877		
Corrected Total	47	39.479			
Shoot fresh weight					
REP	7	132.917	18.988	0.16	0.9917
MED	4	1460.854	365.214	3.05	0.0292
Error	21	4314.146	119.837		
Corrected Total	31	5907.917			

Table 2-4. Effect of cultural conditions on biocontrol activity of *Bacillus* sp. BA55 against target spot and sore shin assessed by disease rating and shoot fresh weight.

Treatment[1]	Disease rating	Shoot fresh weight (g)
Solid - 25°C	0.6	54.1 a
Liquid - 25°C	0.3	53.5 a
Liquid - 30°C	0.8	51.4 a
Solid - 30°C	0.5	48.6 ab
Untreated control	0.8	40.8 b

[1]Plants were sprayed with *Bacillus* sp. BA55 that had been cultured in either solid or liquid Min 3C Medium and incubated at 25° or 30°C, or untreated.

(28). There was little activity in the filtrates of vegetative cultures, significantly more activity in filtrates of cultures that contained a combination of free spores and spores remaining in the parent cell, and much biocontrol activity in both the culture and filtrate of fully released spore cultures. Nutrient-poor media (Min 3C and CB) promoted production of free endospores, as opposed to vegetative cells. Sterile filtrates of 3-day-old cultures reduced seedling mortality as effectively as did the entire culture (28). The authors suggested that an extracellular product was responsible for the biocontrol activity since many *Bacillus* spp. produce extracellular antibiotics during sporulation. Cultures with less than 10% free spores showed little biocontrol activity and it was noted that efforts to detect intracellular activity in the vegetative and endospore cultures have been unsucessful to date.

The culture filtrates of sporulating Bacillaceae contain many peptides with biological activities, e.g. antibiotics, enzymes, and exotoxins (18, 19, 29, 67). Most of the peptides are antibacterial, and a few are known to be antifungal, antitumor, cytotoxic, fibrinolysis-promoting, or immunosuppressive (29, 67). In a study by Hodgson (29), it was noted that peptides have an ability to modify membrane permeability which in turn aids the accumulation of dipicolinic acid and Ca^{2+} ions in the cortex of the spore, removal of water from and contraction of the prespore, and the formation of the spore coat layer. It is thought that these processes in conjunction with antibiotic production are required for, or aid in the sporulation process. Antibiotic production has frequently been described as a function or early event of spore formation since cultural conditions which suppressed sporulation often reduced antibiotic production (36). If this hypothesis is

correct, then this may help explain why *Bacillus* sp. BA55 cultured in minimal medium had greater biocontrol activity than the same isolate cultured in a nutrient-rich medium. This result agrees with observations by Smith and Ownley (*Unpublished data*) that BA55 exhibited greater antibiosis activity against *R. solani* in Min 3C culture than in NBY.

The second trial on the effect of foliar treatments and time of introduction of *R. solani* had extremely low disease pressure. In that trial, the effect of foliar treatment was not significant, but the effect of pathogen introduction was significant for disease rating. The 6-day-inoculated plants had significantly less disease and greater shoot fresh weight than the 1-day-inoculated plants. Although the differences were not significant, the 6-day-inoculated plants in trial 1 had numerically lower disease ratings and greater shoot weights than the 1-day-inoculated plants. This trend suggests that the additional time may have enabled *Bacillus* sp. BA55 to better colonize the foliage and displace the indigenous microbial community. Also, the additional time may be important in the mechanism(s) of biocontrol utilized by BA55. It has been established that BA55 produces antibiotics in culture. It is also possible that induced resistance may be operating in this system. A lag time of 4-7 days is thought to be required for activation of defense mechanisms in plants (54). This test could have been improved by adjusting the vegetative cell counts of BA55 cultured in Min 3C or NBY to the same values in the foliar treatments and determining the number of endospores and released spores in both treatments.

The results of this study have implications for future studies. According to Ashby (4), 2-week-old plants were significantly less suspectible to target spot than 3-week-old

plants. This was probably due to development of a canopy by the 3-week-old plants that was large enough to maintain relative humidity high enough for basidiospore production by *T. cucumeris*. Based on the present study, sprays of BA55 should be applied when the plants are two weeks old. This would allow one week for BA55 to become established before the plants canopy is large enough for disease conducive conditions to be established.

The low disease incidence in the second trial may be explained by the presence of fungus gnats which feed on fungi and were observed in high numbers on the *R. solani* rice grain inoculum. Another explanation could be that different greenhouses were utilized for the two trials. The temperature of the greenhouse for trial 2 was at least 5 to 8°C higher than the first greenhouse utilized due to mechanical problems and this could have led to conditions that were not conducive to disease development.

The effect of foliar treatments of BA55 cultured in liquid or solid Min 3C, and incubated at 25 or 30 C, was not significant for disease rating but was significant for shoot fresh weight. Overall, disease pressure was low in this test and this factor probably contributed to the lack of significance in disease rating. The increased shoot fresh weight may be due to a growth enhancement effect by BA55 or a subtle reduction in disease that was not detected by the disease rating scale employed. As for the low disease pressure in this test, in addition to environmental conditions that may not have been conducive to disease, it was noted that the *R. solani* rice grain inoculum was colonized by *Trichoderma* at the end of the test. It is conceivable that *R. solani* was parasitized by *Trichoderma*, which would result in low disease pressure.

Many *Bacillus* spp. can perform biocontrol of many fungal and bacterial diseases. Biocontrol of postharvest diseases of apple (63), bacterial wilt of tomato (45), damping-off diseases of alfalfa and tobacco (27, 28), and seedling damping-off caused by *Botrytis cinerea* (36) are just a few examples. *Bacillus* sp. BA55 could be a potential biological control agent of *Rhizoctonia* diseases of tobacco if the consistency of its performance can be improved, possibly by enhancing production of free endospores rather than vegetative cells in foliar spray formulations.

CHAPTER 3

EFFECT OF BIOLOGICAL SEED TREATMENT AND *BACILLUS* SP. BA55 CONCENTRATION IN FOLIAR SPRAYS ON TARGET SPOT AND SORE SHIN OF TOBACCO SEEDLINGS

Target 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 forcibly ejected. Target spot appears first as small necrotic lesions. The lesions expand outward and form concentric rings (58, 59). The lesions may drop out, leaving a shot-hole effect (1, 32, 58, 59). Hymenia on the outer edge of the lesion may produce secondary basidiospore inoculum, which is dispersed by air to healthy tobacco tissue.

Fungicides such as iprodione 4F (35, 58) or mancozeb can be used to control target spot on seedlings in float beds in states with a section 18 exemption. On mature plants, there are concerns about residues from fungicide applications close to harvest (58), which limits fungicide use in the field. Sanitation practices are recommended also to prevent the introduction of inoculum into seedling production beds or greenhouses (58, 59).

Rhizoctonia damping-off can be very destructive to tobacco seedlings. Poor germination of seeds or poor emergence of seedlings is the result of damping-off in the preemergence stage (1). Older plants are seldom killed when infected with the damping-off pathogen, but they develop root and stem lesions (sore shin) or root rots, which retard their growth and reduce yield (1). In the field, certain cultural practices can be helpful in reducing infection, including good soil drainage, improvement of heavy soils,

and air circulation (1).

Biological control offers an alternative approach to controlling these diseases. Biocontrol of plant diseases includes the use of antagonistic microorganisms, the host itself, or hypovirulent strains of the pathogen (13).

Biocontrol organisms may act in one or more ways including the release of secondary metabolites (antibiotics) which are inhibitory to pathogens such as *Rhizoctonia* (4, 13). Bacteria in the genus *Bacillus* produce a wide range of antibiotics (13, 23, 27, 29, 67, 69,) and have been shown to control tobacco diseases (2, 13). Ashby (4) found significant control of target spot on tobacco seedlings treated with foliar applications of *Bacillus* sp. BA55 at the rate of 6 log colony-forming units/ml (cfu/ml) of tap water. Theoretically, the earlier a biological agent can be applied to plants or seeds, the more protection afforded the plant from the plant pathogen.

An excellent example of biological control is use of the antagonistic fungus *Trichoderma*. Species of *Trichoderma* have demonstrated the ability to be effective against *R. solani* isolates by competition and parasitism (11, 12, 37). *Trichoderma* spp. belong to the subdivision Deuteromycotina, class Hyphomycetes.

The objectives of this study were 1) to determine the effect of *Bacillus* sp. BA55 bacterial seed treatment on tobacco seedlings in the float bed system; 2) to determine the optimum concentration of bacteria in foliar spray applications of *Bacillus* sp. BA55 for control of target spot and sore shin of tobacco seedlings in the float bed system; 3) to determine the effect of *Trichoderma* seed treatment and foliar sprays of *Bacillus* BA55 on biocontrol of target spot and sore shin of tobacco seedlings in the float bed system;

and 4) to determine the effect of treating seed with BA55 or *Trichoderma* isolates on biocontrol of *Rhizoctonia* sore shin of tobacco seedlings.

MATERIALS AND METHODS

Seed treatment and tobacco cultivars. All seed were bioprimed or primed and pelletized by Rickard Seed Co., Winchester, KY. Seed of the cultivar TN 90 were treated with *Bacillus* sp. BA55, *Trichoderma* 22-1, *Trichoderma* 22-2, *Trichoderma* Test (ABC) or *Bacillus* sp. BA55. Seeds of the cultivar KY14 were bioprimed with *Trichoderma* T-22, *Trichoderma* 22-3, or *Trichoderma* 22-4. Primed and pelletized seed of each cultivar with no microorganisms added served as controls.

Culture media and microorganisms. Inoculum of *Bacillus* sp. BA55 was produced by inoculating sterile culture tubes containing 10-ml aliquots of Min 3C broth with BA55. The tubes were incubated for 24 h at 28°C on a rotary shaker at 100 rpm. The contents of the tubes were added to four 2800-ml culture flasks containing 500 ml Min 3C broth. The four 2800-ml culture flasks were incubated at 28°C on a rotary shaker at 100 rpm for 72 h. The cultures were then centrifuged at an angular velocity of 3500 or 5000 rpm for 10 min. The supernatant was decanted and the pellets were resuspended in 1 liter of tap water. The colony forming units/ml of BA55 were determined by a microdilution plating technique (15) and tap water was added to obtain final bacterial concentrations of 4 log, 5 log, 6 log, 7 log, or 8 log cfu/ml BA55.

Similarly, for a separate experiment, an 8 log cfu/ml preparation of BA55 was produced by inoculating two culture tubes with 10-ml aliquots of Min 3C broth with

BA55. The tubes were incubated for 24 h at 28°C on a rotary shaker at 150 rpm. The contents of the two tubes were added to two 1000-ml culture flasks containing 250 ml of Min 3C broth. The culture flasks were incubated for 72 h on a rotary shaker at 100 rpm and 28°C.

Two isolates of *R. solani*, 1600 and 1708, were cultured on full-strength potato dextrose agar (PDA; 24 g potato dextrose broth, Difco Laboratories, Detroit, MI., and 20 g agar, Sigma Chemical Co., St. Louis, MO, in 1 L deionized water). Agar plugs from the PDA cultures were used to inoculate 500-ml Erlenmeyer flasks containing sterile rice grains (125 g of rice and 90 ml of deionized water were autoclaved on two successive days for 1 h). The inoculated rice cultures were incubated at 28°C for 7 days prior to inoculation of plants.

Effect of BA55 seed treatment and concentration of BA55 in foliar applications on target spot and sore shin. The effects of bacterial seed treatments and concentration of bacteria in foliar spray treatments on biocontrol of target spot and sore shin were tested in the greenhouse. The experiment was designed as a 2 × 6 factorial in a split plot with two seed treatments and six foliar spray concentrations of bacteria. The main plot was seed treatment and the sub-plot was the foliar spray concentration of BA55. The seed treatments were bioprimed with *Bacillus* sp. BA55 or primed with no microorganism. The foliar spray concentrations of BA55 were 4 log, 5 log, 6 log, 7 log, 8 log cfu/ml or a tap water control. There were four replicates with all treatment combinations represented in each replicate. Each treatment combination consisted of one 80-cell direct-seeded styrofoam float tray per replicate. Plants were fertilized with Peters

Professional 20-10-20.

Five-week-old TN 90 burley tobacco plants were sprayed with an aqueous solution of the appropriate foliar spray until run-off. Two grams of rice grain inoculum of *Rhizoctonia solani* were placed on the soil surface under the canopy of each plant 2 days after the first foliar treatment. All plants were air-dried for approximately 2 h. After two weeks, the tobacco seedlings were sprayed again with the appropriate foliar spray. Two weeks (trial 1) or four weeks (trial 2) later, plants were assessed for severity of target spot or sore shin, and fresh weight (shoot and roots) was determined.

Effect of BA55 or *Trichoderma* seed treatments and foliar application of BA55 on target spot and sore shin of TN 90 and KY 14 seedlings. The effects of biological seed treatment and foliar spray of BA55 were evaluated in float-grown tobacco seedlings in the greenhouse. In the first trial, the experiment was designed as a 2 × 2 factorial in a randomized complete block. The treatments were pelleted KY 14 tobacco seed bioprimed with *Trichoderma* 22-3 or pelleted, primed seed without *Trichoderma* 22-3. The foliar treatments were either 8 log cfu/ml *Bacillus* sp. BA55 foliar spray or control (sprayed with tap water only).

In the second trial, two cultivars, KY 14 and TN 90, were tested. The biological treatments were different on the two cultivars. For KY 14, the seed treatments were pelleted, primed seed and pelleted seed bioprimed with *Trichoderma* 22-3. For TN 90, the seed treatments were pelleted, primed seed and pelleted seed bioprimed with *Trichoderma* 22-1. The foliar treatments for both cultivars were either 8 log cfu/ml *Bacillus* sp. BA55 foliar spray or control (sprayed with tap water only). For cultivars

the tests were designed as 2 × 2 factorials in a randomized complete block design. In both trials, there were four replicates with all treatment combinations represented in each replicate. Each treatment combination consisted of one direct-seeded 80-cell styrofoam float tray per replicate. Plants were fertilized with Peters Professional 20-10-20.

Burley tobacco KY 14 and TN 90 were sprayed until run-off with an aqueous solution of either 8 log cfu/ml of BA55 or tap water. Two grams of rice grain inoculum of *R. solani* were placed on the soil surface under the canopy of each plant 2 days after the first foliar treatment. All plants were air-dried for approximately 2 h. Two weeks later, the plants were sprayed again with the appropriate foliar spray. After two (trial 1) or four (trial 2) additional weeks plants were assessed for severity of target spot and sore shin. Fresh weight (roots and shoot) also was determined also.

Effect of biological seed treatment on severity of sore shin of tobacco seedlings. The tobacco cultivars and biological seed treatments are listed in Table 3-1. Seed of each treatment were planted in 6-inch clay pots filled with Terralite™. The pots were bagged to promote seedling germination. Eight-week-old seedlings were transplanted into rectangular trays lined with plastic insect screen to cover the drainage holes, and filled with Terralite™. Five seedlings were planted in two rows and two grams of rice inoculum of *R. solani* were placed in a furrow between the row of plants. There were four or five replicates per treatment. Plants were assessed for severity of sore shin, and shoot height was determined after two weeks.

Disease Assessment. Plants were assessed for percent diseased foliage due to target spot with the Horsfall-Barratt rating scale (30). In this scale, 0 = healthy (no

Table 3-1. Seed treatments for the sore shin test.

KY 14 Tobacco	Designation Code	TN 90 Tobacco	Designation Code
Control	CON	Control	CON
Trichoderma 22-3	T22-3	*Trichoderma* 22-1	T22-1
Trichoderma 22-4	T22-4	*Trichoderma* Test	TTEST
		Trichoderma 22-2	T22-2
		with BA55	+BA55
		without BA55	-BA55

lesions), 1 = 1 - 2%, 2 = 3 - 6%, 3 = 7 - 12%, 4 = 13 - 25%, 5 = 26 - 50%, 6 = 51 - 75%, 7 = 76 - 88%, 8 = 89 -94%, 9 = 95 - 97%, 10 = 98 - 99%, and 11 = dead. The rating scores were converted to percent diseased foliage for data analysis (50).

The severity of sore shin was assessed on a scale of 1 to 7, where 0 = healthy, no lesion on hypocotyl; 1 = lesion < 2.5 mm long; 2 = lesion 2.5 to 5.0 mm long; 3 = lesion > 5.0 mm long; 4 = lesion girdling stem, 5 = lesion girdling and plant stem constricted (< 75%), 6 = lesion girdling and stem constricted (> 75%), and 7 = seedling dead.

Data Analysis. For each cultivar, the main effects of foliar treatment and seed treatment, and the interactions of these factors were analyzed for significance with the General Linear Models procedure and F-protected LSD tests of the PC-Statistical Analysis System®.

RESULTS

Effect of BA55 seed treatment and concentration of BA55 in foliar applications on target spot and sore shin. The interaction of bacterial seed treatment and rate of foliar spray of BA55, and the main effects of seed treatment and foliar treatment rate were not significant for percent diseased foliage (Table 3-2). However, the main effects of seed treatment (P = 0.0518) and rate of foliar treatment of BA55 were significant (P = 0.0322) for fresh weight (Table 3-2, Fig. 3-1). Across all foliar treatments, seed treated with BA55 resulted in plants with a fresh weight of 36.083 g

Table 3-2. Analysis of variance for the effects of seed treatment and bacterial foliar treatments on biocontrol of target spot and sore shin of tobacco with *Bacillus* sp. BA55 in float beds in trial 1.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Percent diseased foliage					
Replicate (REP)	3	157.478	52.493	0.40	0.7523
Seed Treatment (STRT)	1	29.491	29.491	0.23	0.6379
Bacteria Rate (RATE)	5	625.197	125.039	0.96	0.4585
STRT x RATE	5	346.042	69.208	0.53	0.7513
REP x STRT	3	79.571	26.524	0.20	0.8933
Error	30	3913.314	130.444		
Corrected Total	47	5151.093			
Fresh weight					
REP	3	269.726	89.909	2.95	0.0487
STRT	1	186.188	186.188	6.11	0.0518
RATE	5	433.922	86.784	2.85	0.0322
STRT x RATE	5	206.796	41.359	1.36	0.2685
REP x STRT	3	56.783	18.928	0.62	0.6071
Error	30	914.922	30.497		
Corrected Total	47	2068.336			

while plants from untreated seed had a fresh weight of 32.144 g. Plants treated with 6 log and 7 log cfu/ml of BA55 had the greatest fresh weights with 37.7 g and 37.709 g, respectively. The lowest fresh weights were found for the control plants and plants treated with 8 log cfu/ml with 29.631 g and 31.595 g, respectively (Fig. 3-1).

In the second trial, there were no significant effects on any of the variables measured from the foliar and seed treatments applied (Table 3-3).

Effect of BA55 or *Trichoderma* seed treatment and foliar application of BA55 on target spot and sore shin of TN 90 and KY 14 seedlings. In trial 1, with KY 14 seed only, the interaction of *Trichoderma* seed treatment and BA55 foliar spray treatment was not significant for percent diseased foliage or fresh weight (Table 3-4). The main effects of seed treatment and foliar spray also were not significant for percent diseased foliage. However, the main effect of seed treatment was significant ($P = 0.0753$) for fresh weight (Table 3-4, Fig. 3-2). Seed treated with *Trichoderma* 22-3 resulted in plants with greater shoot weight than from untreated seed (Fig. 3-2).

In the second trial, both TN 90 and KY 14 seed were tested. Since the seed treatments were different for the two cultivars, the data were analyzed separately. For KY 14, there was a significant interaction ($P = 0.0341$) for seed treatment and foliar spray (Table 3-5). For untreated seed, percent diseased foliage was similar for both the control and BA55-foliar spray treatment (Table 3-6). Likewise for plants sprayed with

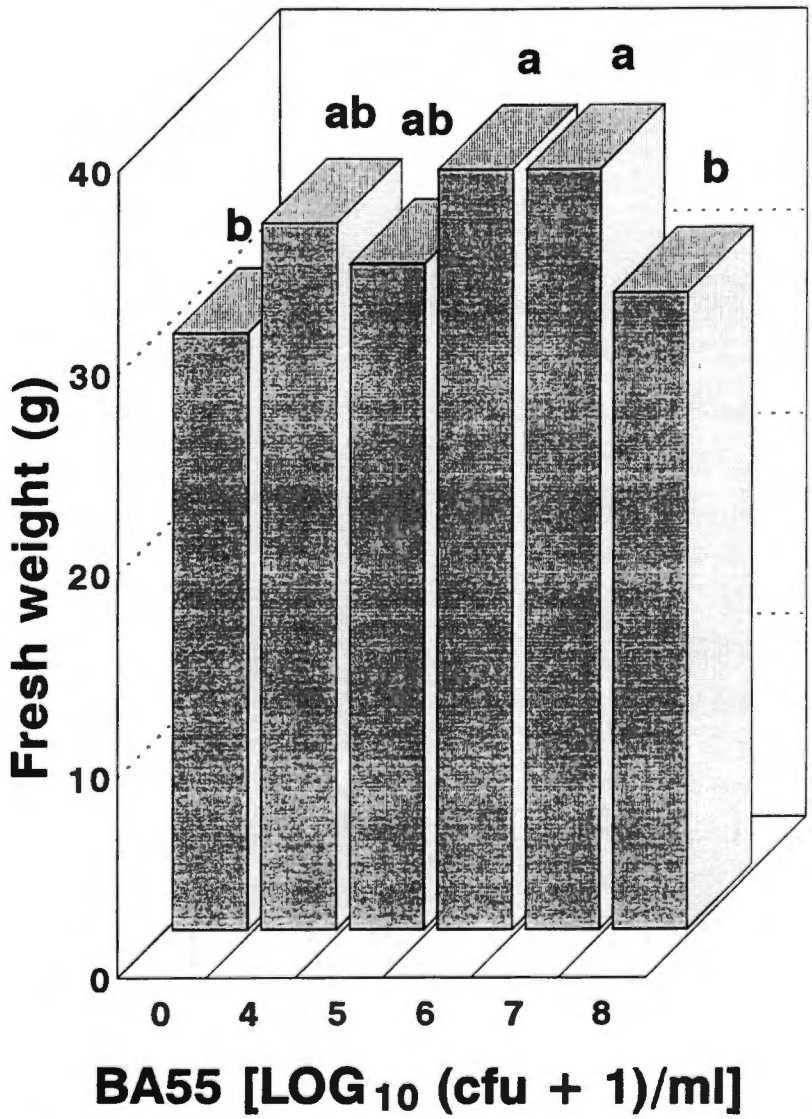


Fig. 3-1. Effect of seed treatment and foliar spray concentrations on fresh weight of TN 90 tobacco in float beds in trial 1. Seed treatments were pelleted, primed TN 90 seed with no bacteria and pelleted, TN 90 seed bioprimed with *Bacillus* sp. BA55. The foliar spray concentrations were the control (tap water only), 4 log, 5 log, 6 log, 7 log, or 8 log cfu *Bacillus* sp. BA55 per ml tap water. Bars with the same letter are not significantly different according to a F-protected Least Significant Difference test (P = 0.05).

Table 3-3. Analysis of variance for the effects of seed treatment and bacterial foliar treatment on biocontrol of target spot and sore shin of tobacco with Bacillus sp. BA55 in float beds in trial 2.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Percent diseased foliage					
Replicate (REP)	3	1031.012	343.671	2.01	0.1342
Seed Treatment (STRT)	1	0.162	0.162	0.00	0.9558
Bacterial Rate (RATE)	5	194.687	38.937	0.23	0.9473
STRT x RATE	5	1034.011	206.802	1.21	0.3289
REP x STRT	3	134.561	44.854	0.26	0.8517
Error	29	4952.573	170.778		
Corrected Total	46	7357.359			
Fresh weight					
REP	3	1093.433	364.478	1.50	0.2356
STRT	1	12.728	12.728	0.05	0.8206
RATE	5	596.022	119.204	0.49	0.7807
STRT x RATE	5	297.825	59.565	0.24	0.9389
REP x STRT	3	293.559	97.853	0.40	0.7523
Error	29	7050.743	243.129		
Corrected Total	46	9381.509			

Table 3-4. Analysis of variance for the effect of *Trichoderma* seed treatment and BA55 foliar treatment on target spot and sore shin of KY 14 tobacco seedlings in float beds in trial 1.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Percent diseased foliage					
Replicate (REP)	3	194.725	64.908	0.92	0.4757
Seed Treatment (STRT)	1	8.992	8.992	0.13	0.7310
Foliar Spray (SPRAY)	1	18.286	18.286	0.26	0.6253
STRT x SPRAY	1	220.850	220.850	3.11	0.1156
Error	8	567.247	70.906		
Corrected Total	14	1058.214			
Fresh weight					
REP	3	1022.382	340.794	11.39	0.0029
STRT	1	124.924	124.924	4.17	0.0753
SPRAY	1	0.723	0.723	0.02	0.8803
STRT x SPRAY	1	69.147	69.147	2.31	0.1670
Error	8	239.441	29.930		
Corrected Total	14	1545.441			

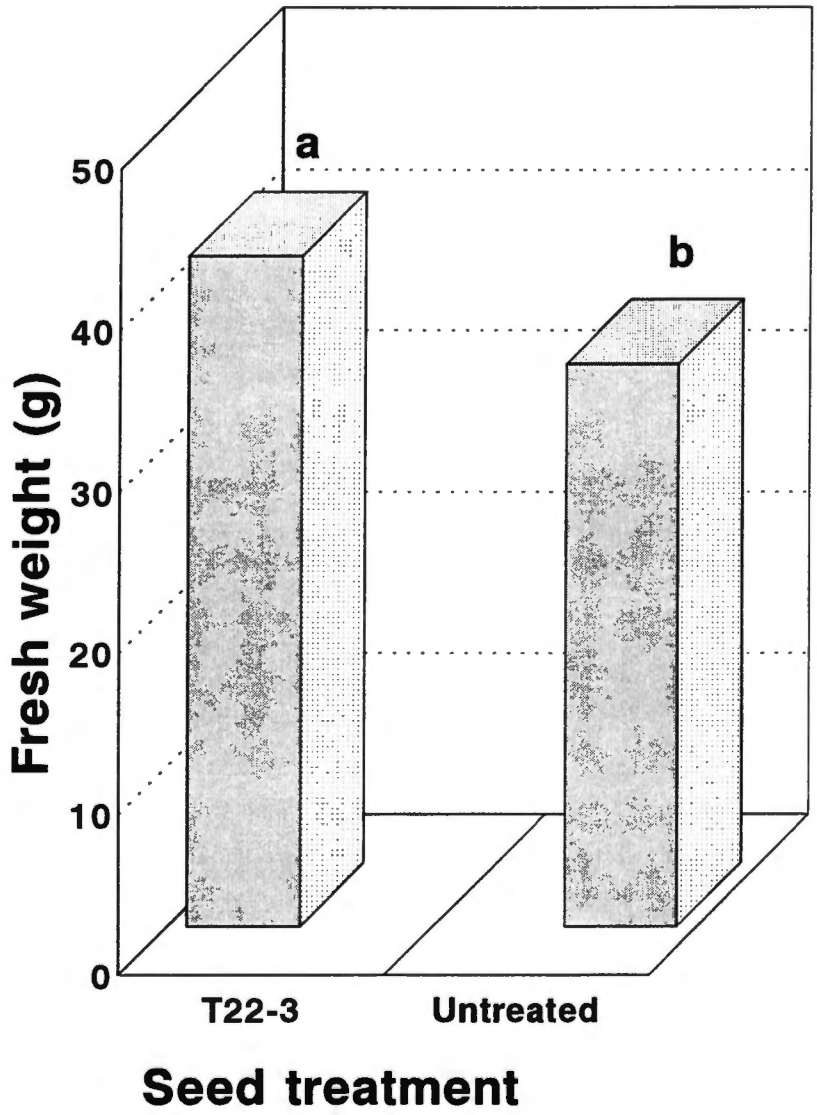


Fig. 3-2. Effect of treatment of seed with *Trichoderma* 22-3 on fresh weight of KY 14 tobacco in float beds in trial 1. Bars with the same letter are not significantly different according to a F-protected Least Significant Difference test ($P = 0.0753$).

Table 3-5. Analysis of variance for the effect of *Trichoderma* seed treatment and BA55 foliar treatment on target spot and sore shin of KY 14 tobacco seedlings in float beds in trial 2.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Percent diseased foliage					
Replicate (REP)	3	978.654	326.218	4.29	0.0387
Seed treatment (STRT)	1	153.130	153.130	2.01	0.1895
Foliar spray (SPRAY)	1	147.867	147.867	1.95	0.1965
STRT × SPRAY	1	473.375	473.375	6.23	0.0341
Error	9	683.993	75.999		
Corrected Total	15	2437.020			
Fresh weight					
REP	3	137.450	45.817	0.78	0.5348
STRT	1	30.269	30.269	0.51	0.4913
SPRAY	1	0.892	0.892	0.02	0.9047
STRT × SPRAY	1	38.359	38.359	0.65	0.4402
Error	9	529.340	58.816		
Corrected Total	15	736.310			

Table 3-6. Effect of seed treatment and foliar spray on percent diseased foliage of KY 14 tobacco seedlings in float beds in trial 2.

Trichoderma 22-3	
Control	41.3
BA55	24.6
Untreated seed	
Control	24.5
BA55	29.3
BA55 spray	
Trichoderma 22-3 seed	24.6
Untreated seed	29.3
Control (no spray)	
Trichoderma	41.5
Untreated	24.5

BA55, the percent diseased foliage was not different for treated and untreated seed. However, for plants that did not receive a foliar spray of BA55, seed treated with Trichoderma 22-3 resulted in plants with 41.5 percent diseased foliage while plants from untreated seed had 24.5 percent diseased foliage (Table 3-6). In plants from seed treated with *Trichoderma* 22-3, percent diseased foliage was 41.3 and 24.6 in plants sprayed with BA55 (Table 3-6). For TN 90 seed, there were no significant effects (Table 3-7).

Effect of biological seed treatment on severity of sore shin of tobacco seedlings. For KY14 seed treated with *Trichoderma* isolates or the appropriate control, the effect of seed treatment was not significant for sore shin disease rating or shoot height (Table 3-8). For TN 90 seed treated with *Trichoderma* isolates, seed treatment was significant for shoot height ($P = 0.0532$) (Table 3-9, Fig. 3-3). Seed treated with *Trichoderma* 22-2 had the greatest height, those treated with 22-1 or Test were intermediate and plants from untreated seed were the shortest. Treatment of TN 90 seed with BA55 had no effect on disease rating or shoot height (Table 3-10).

DISCUSSION

In the first trial of the experiment that examined the effect of BA55 seed treatment and concentration of BA55 in foliar sprays on target spot and sore shin, the rate of foliar application of BA55 had a significant effect on fresh weight. Plants receiving 6 log or 7 log cfu/ml had the greatest weight. The noticeable decrease in fresh weight of the plants receiving 8 log cfu/ml is in agreement with prior research (26, 36, 63) with *Bacillus* spp. in other biocontrol systems. In addition, Ashby's (4) success with BA55

Table 3-7. Analysis of variance for the effect of *Trichoderma* seed treatment and BA55 foliar treatment on target spot and sore shin of TN 90 tobacco seedlings in float beds.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Percent diseased foliage					
Replicate (REP)	3	953.897	317.966	1.96	0.1902
Seed treatment (STRT)	1	46.980	46.980	0.29	0.6033
Foliar spray (SPRAY)	1	19.862	19.862	0.12	0.7343
STRT × SPRAY	1	3.976	3.976	0.02	0.8790
Error	9	1458.014	162.002		
Corrected Total	15	2482.729			
Fresh weight					
REP	3	228.085	76.028	1.99	0.1857
STRT	1	2.586	2.586	0.07	0.8005
SPRAY	1	118.621	118.621	3.11	0.1117
STRT × SPRAY	1	29.090	29.090	0.76	0.4052
Error	9	343.363	38.151		
Corrected Total	15	721.745			

Table 3-8. Analysis of variance for the effect of KY 14 seed treated with *Trichoderma* on severity of sore shin and shoot height of tobacco seedlings.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Disease rating					
Replicate (REP)	4	7.157	1.789	4.19	0.0404
Treatment (TRT)	2	0.672	0.336	0.79	0.4872
Error	8	3.415	0.427		
Corrected Total	14	11.245			
Shoot height					
REP	4	581.678	145.419	1.43	0.3077
TRT	2	439.006	219.503	2.16	0.1776
Error	8	812.201	101.525		
Corrected Total	14	1832.885			

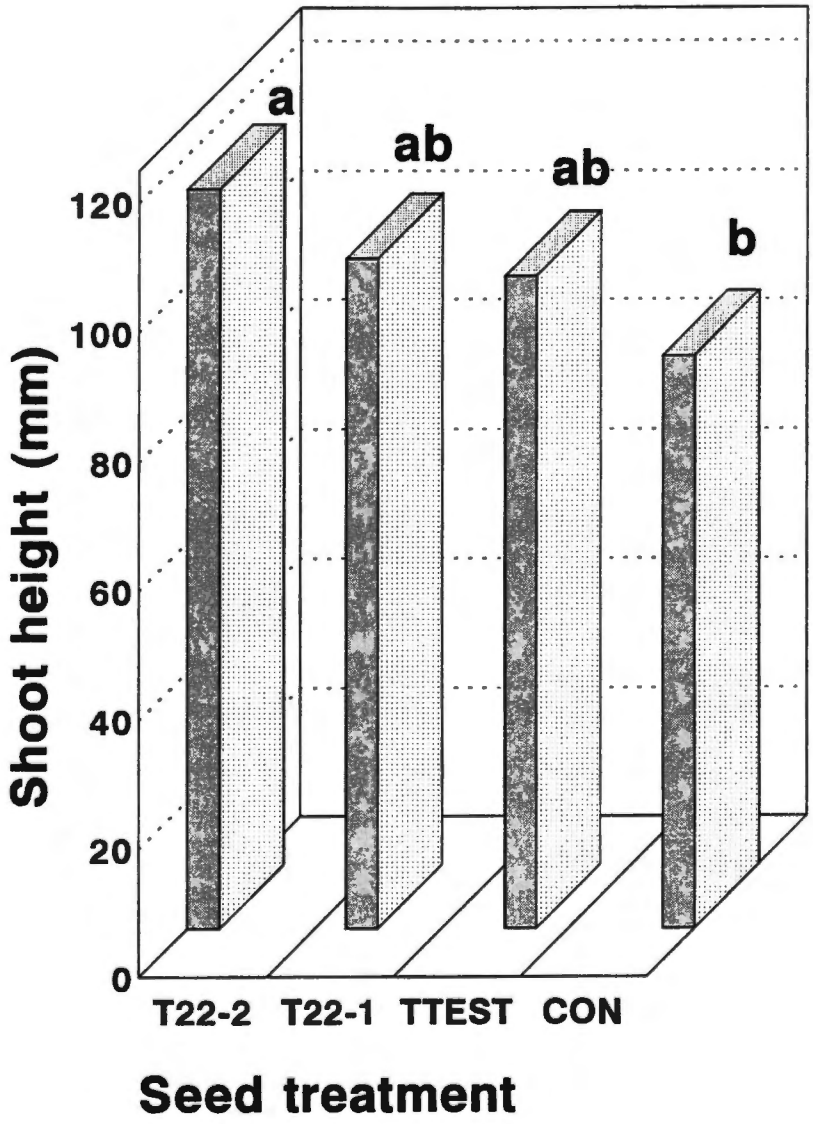


Fig. 3-3. Effect of *Trichoderma* seed treatments on shoot height of TN 90 tobacco. The seed treatments were *Trichoderma* Test, *Trichoderma* 22-1, *Trichoderma* 22-2, and the untreated control. Bars with the same letter are not significantly different according to a F-protected Least Significant Difference test ($P = 0.05$).

Table 3-9. Analysis of variance for the effect of TN 90 seed treated with *Trichoderma* on severity of sore shin and shoot height of tobacco seedlings.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Disease rating					
Replicate (REP)	4	7.105	1.856	5.01	0.0177
Treatment (TRT)	3	2.701	0.900	2.54	0.1153
Error	10	3.542	0.354		
Corrected Total	17	13.668			
Shoot height					
REP	4	295.012	73.753	0.55	0.7011
TRT	3	1444.023	481.341	3.61	0.0532
Error	10	1331.867	133.187		
Corrected Total	17	3149.709			

Table 3-10. Analysis of variance for the effect of *Bacillus* sp. BA55 seed treatment on severity of sore shin and shoot height of tobacco seedlings.

Source of variation	df	Sum of squares	Mean square	F value	$P > F$
Disease rating					
Replicate (REP)	4	2.002	0.500	1.01	0.4954
Treatment (TRT)	1	0.00876	0.00876	0.02	0.9005
Error	4	1.977	0.494		
Corrected Total	9	3.988			
Shoot height					
REP	4	2077.900	519.475	1.99	0.2612
TRT	1	541.698	541.698	2.07	0.2234
Error	4	1045.657	261.414		
Corrected Total	9	3665.255			

as a biocontrol of target spot when applied as a foliar spray, was conducted with 6 log cfu/ml.

The increased fresh weight of plants from seed treated with BA55 was not unexpected since it is known that beneficial microorganisms, including *Bacillus* spp., can enhance growth of various plants (3, 4, 28). The enhanced growth may also be related to a subtle reduction in disease or control of minor pathogens in the peat mix since the Terralite™ was not autoclaved.

In the second trial of this experiment, the foliar sprays and seed treatments did not affect any of the variables measured. This result can partly be explained by very low disease pressure. The greenhouse was kept at a temperature range that was optimal for target spot development during both trials (1, 58, 59, 60). However, humidifiers were placed in the greenhouse during the first trial and not in the second. The increased relative humidity in the first trial probably enhanced disease development. Also, the second trial was initiated in early October as opposed to late April for the first trial. Differences in light intensity may have had an effect on disease development.

In the first trial of the experiment that examined the effect of BA55 foliar spray and *Trichoderma* seed treatment on target spot and sore shin of KY 14 seedlings, the main effect of *Trichoderma* seed treatment was significant for fresh weight. Growth enhancement of various plants by *Trichoderma* spp. is well documented (3, 4, 11, 12, 28, 37). The failure of an 8 log cfu/ml BA55 spray to control target spot and sore shin is not surprising given the results of the test involving a range of concentrations of BA55 in foliar sprays. In that study the 8 log cfu/ml spray was not different from the tap water

control in its effect on disease rating and fresh weight. Foliar applications of 6 log or 7 log cfu/ml BA55 would have been a better choice for this experiment.

In the second trial, there was a significant interaction between seed treatment and foliar spray for KY 14 seed on percent diseased foliage. Interestingly, for seed treated with *Trichoderma* 22-3, plants sprayed with BA55 had much less disease than plants sprayed with water. This was not expected given the high concentration of BA55 (8 log cfu/ml) in the foliar spray.

The effect of seed treatment with *Trichoderma* to enhance growth of seedlings was observed again in the sore shin test. Shoot height of TN 90 plants was enhanced by *Trichoderma* seed treatments. Unlike previous tests, *Trichoderma* seed treatments did not enhance height of KY 14 seedlings and BA55 did not enhance growth of TN 90. The inconsistencies are partially related to the varying levels of disease in the different tests. Also, there may be specific cultivar-microbial interactions that have not yet been defined. For example, the growth and/or biocontrol responses may be different depending on the cultivar and beneficial microorganism involved.

In general, these studies suggest that applications of *Trichoderma* spp. and BA55 can enhance growth of tobacco and reduce *Rhizoctonia* diseases, although the latter response is variable. Growth enhancement may be due to reduction of *Rhizoctonia* diseases, reduction of minor root pathogens that were indigenous to the horticultural mix, or production of plant growth hormones. Testing these seed treatments for control of *Pythium* and other seedling pathogens may be warranted.

Given a more environmentally-conscious society around the globe, increasing

emphasis will be placed on environmentally friendly methods of plant pathogen control. With future improvements in culturing, storing, and handling *Bacillus* sp. BA55 or similar strains, along with combinations of other biocontrol agents such as *Trichoderma*, an effective biocontrol of *Rhizoctonia* or other tobacco pathogens may be developed for use in the float bed system.

SUMMARY

Tobacco is an economically important crop in Tennessee. Many tobacco farmers have found it to be advantageous to produce tobacco seedlings in the float bed system rather than in the traditional seed bed. The high plant density and high relative humidity that can occur in the greenhouse translates into conditions conducive for development of target spot and sore shin. Target spot is a leaf spot disease of tobacco that is caused by *Thanatephorus cucumeris*, the teleomorph stage of *Rhizoctonia solani*. Sore shin refers to stem cankers and can be caused by *T. cucumeris* or *R. solani*.

Control options for target spot on float-grown seedlings are limited. Past research has shown that *Bacillus* sp. BA55 has potential as a biocontrol of target spot. The first goal of this study was to determine whether manipulating cultural conditions of *Bacillus* sp. BA55 and time of introduction of *R. solani* after foliar sprays with BA55 would optimize biocontrol activity of BA55 against target spot and sore shin of burley tobacco seedlings.

An experiment was conducted to test the effect of culture medium of BA55 and the effect of different time periods between application of BA55 and introduction of the

pathogen on disease rating and shoot fresh weight. Disease rating was significantly lower in plants that received the foliar treatment of BA55 cultured in Min 3C, a minimal medium, compared to plants that did not receive any treatment in trial 1. Plants that received BA55 cultured in NBY, a nutrient rich medium, were intermediate in disease rating. In trial 2, disease pressure was low and the effect of culture medium was not significant; however, plants that were inoculated with *R. solani* six days after foliar treatments had a significantly lower disease rating and higher shoot fresh weight than plants inoculated one day after foliar treatment.

The effects of foliar treatment with BA55 cultured on either solid or liquid Min 3C medium and incubated at 25°C or 30°C or no treatment were not significant for disease rating. However, the shoot fresh weight of plants treated with BA55 cultured in solid or liquid Min 3C incubated at 25°C was significantly greater than the untreated control. Overall, disease pressure was low in this experiment and this factor probably contributed to the lack of significance in disease rating.

The second goal of this study was to reduce target spot and sore shin with biological seed treatments and optimized foliar sprays of BA55. In an experiment to test the effect of BA55 seed treatment and various concentrations of BA55 in foliar sprays, TN 90 tobacco that received 6 log or 7 log cfu of BA55 per ml of tap water had significantly greater fresh weights than controls sprayed with tap water only.

In an experiment to test the effect of a fungal biocontrol agent, *Trichoderma* 22-3, and foliar application of BA55 on severity of target spot and sore shin, plants from seed treated with *Trichoderma* 22-3 had a significantly higher fresh weight than untreated

seed. In the second trial of this experiment, two cultivars KY 14 and TN 90 were utilized. For KY 14 there was a significant interaction between seed treatment with *Trichoderma* 22-3 and foliar spray application on percent diseased foliage. For *Trichoderma*-treated seed, plants sprayed with BA55 had less disease than plants sprayed with tap water only. For plants that did not receive BA55 foliar spray, there was more disease among plants from *Trichoderma*-treated seed than from untreated seed. There were no significant effects of TN 90 from seed treated with *Trichoderma* 22-1 and receiving foliar sprays of BA55.

The effect of BA55 and *Trichoderma* seed treatments on biocontrol of sore shin on two burley cultivars were tested also. The effect of seed treatment was not significant for sore shin disease rating or shoot height of KY 14 plants treated with various isolates of *Trichoderma*. For TN 90, seed treatment with *Trichoderma* 22-2 resulted in plants with significantly greater shoot height than the control, however there was no effect on disease rating. On TN 90, seed treatment with BA55 had no effect on sore shin disease rating or shoot height.

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