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FIELD EFFICACY, PERSISTENCE, AND
ANTIBIOTIC PRODUCTION OF PSEUDOMONAS AUREOFACIENS

presented by

Philip Joseph Dwyer, Jr.

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Botany & Plant Path.

Major professor

Date 5 May 1999

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FIELD EFFICACY, PERSISTENCE, AND ANTIBIOTIC PRODUCTION OF *PSEUDOMONAS AUREOFACIENS*

By

Philip Joseph Dwyer Jr.

A DISSERTATION

Submitted to
Michigan State University
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ABSTRACT

FIELD EFFICACY, PERSISTENCE, AND ANTIBIOTIC PRODUCTION OF *PSEUDOMONAS AUREOFACIENS*

By

Philip Joseph Dwyer Jr.

Pseudomonas aureofaciens strain Tx-1 is used as an effective biological control bacterium for control of certain turfgrass diseases. The field efficacy and survivability of Tx-1 upon application to turf was studied. A bioactive mutant (Tn-1A) deficient in production of the primary antibiotic, phenazine-1- carboxylic acid (PCA), was used to determine if antibiotics other than PCA could be identified and correlated with control. In separate studies, Tx-1 was applied to turf at various concentrations and in combination with fungicides to control the diseases dollar spot (*Sclerotinia homoeocarpa*) and pink snow mold (*Microdochium nivale*). Significant control of both dollar spot and pink snow mold was achieved by applying Tx-1 at specific concentrations and intervals. Survivability of Tx-1 in turf was determined by monitoring populations over a two-year period. Results showed Tx-1 can overwinter and survive throughout the year in the foliage, thatch, and soil of a turfgrass system. Strain Tn-1A was used in *in vitro* bioassays and in the field to study its ability to control dollar spot. Tn-1A effectively controlled dollar spot in both bioassays and in the field. The active compound from Tn-1A fermentation broth was extracted, purified using various chromatographic techniques, and identified using nuclear magnetic resonance (NMR) and mass spectral (MS) experiments. The structural analysis of the active compound revealed the molecule to be $C_{10}H_6N_2O_2Cl_2$ (Pyrrolnitrin).

I dedicate this thesis to the lives of Bradley, Nicole, and Kevin Mohammad, may their spirits live on by our actions today. God bless them, their families, and friends.

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INTRODUCTION

Biological control can be defined as using the disease suppressive properties of an organism to improve plant health. One mechanism of biological control is antibiosis in which an organism produces antibiotics as their means of suppressing disease (15). *Pseudomonas aureofaciens* is one such organism whose control mechanism is antibiosis. Biological control occurs during a complex interaction between the plant, pathogen, biological control organism, surrounding microbial community, and environment (15). It is the understanding of this complex interaction in the rhizosphere, signal interchange, and antibiotic expression which have led to *P. aureofaciens* being one of the best understood biological control organisms (33). As the understanding of *P. aureofaciens* increases in specific agricultural systems, the use of this organism for control will be possible, as it has been in turfgrass disease management. The focus of this thesis research is to increase our understanding of *P. aureofaciens* strain Tx-1 in order to optimize the effectiveness of its use as a tool in managing disease in turfgrass systems.

In recent times, commonly used disease management strategies involving fungicides have come under question by both regulatory issues and public perception. This has led to the elimination of certain fungicides and the possibility of losing others. Another threat to current disease management techniques that is related specifically to the turfgrass pathogen dollar spot (*Sclerotinia homoeocarpa*) is the occurrence of fungicide resistance (44). Of the systemic fungicides used to control dollar spot, resistance to the benzimidazoles, dicarboxymides, and demethylation inhibitor (DMI) classes has been

reported (45)(9)(12). The loss of these important classes, in some cases, and a proactive response to environmental concerns by the turfgrass industry has led researchers to examine alternative disease management strategies.

The introduction of *P. aureofaciens* strain Tx-1 for use in managing dollar spot has been a successful addition for management of dollar spot on golf courses. A commercial fermentation and delivery system was developed specifically for application of this organism through the pre-existing irrigation system on a golf course. Turfgrass is ideally suited for this type of application because delivery of Tx-1 through irrigation water allows the organism to be applied to all desired areas on a routine basis. Application of Tx-1 to turf will improve as research continues to discover ways of optimizing its use.

These studies were done to investigate responses in the field using Tx-1 and various treatment combinations, determine Tx-1's ability to persist in a turfgrass system, and identify the effect of a mutant with altered antibiotic production capabilities. The field studies were done over two years in which Tx-1 was used to control naturally occurring dollar spot on a fairway height stand of annual bluegrass (*Poa annua*). A Tx-1 population study was conducted in the field over two years to monitor the organism's ability to persist in a turfgrass system at various times of the year. The results of this will show population changes over time, and may also expand future research into root diseases by demonstrating that threshold bacterial populations can be achieved. It is important for sufficient populations of a biological control organism to exist at the site of pathogen infection to control disease, and also to persist in the environment where released (47). The experiments done with a mutant strain of Tx-1 sought to identify

production of an antibiotic other than PCA and correlate this antibiotic to disease suppression in both the field and *in vitro*.

Chapter 1

LITERATURE REVIEW

Pseudomonas aureofaciens

Kluver first described the bacterium *Pseudomonas aureofaciens* in 1956 (20). *P. aureofaciens* was isolated from clay soils that had been soaked in kerosene (3). The bacterium is a flagellated root colonizer, aerobic, and a member of the fluorescent Pseudomonads (3). The name "aureofaciens" was given to it due to its ability to produce yellow and orange pigments that are "made golden" (3). Haynes characterized these pigmented antibiotics produced by *P. aureofaciens* into the phenazine class of antibiotics in 1956 (16). Phenazines are heterocyclic,-N-containing molecules produced by bacteria as secondary metabolites.

Phenazine producing strains of *P. aureofaciens* are found worldwide and are associated with various plants and soils (33). These antibiotics have biological activity against fungi, bacteria, and nematodes (33). The shikimic acid pathway has been described as the likely site of phenazine synthesis (47). Pierson also found the product of one phenazine biosynthesis gene shares homology with products from the shikimic acid pathway (31). The primary antibiotics shown to be responsible for fungal inhibition are phenazine-1- carboxylic acid (PCA), 2-hydroxy-phenazine carboxylic acid (2OHPCA), 2-hydroxy phenazine (2PZ), 2,4-diacetylphloroglucinol (Phl), and pyrrolnitrin (Prn)(33). Hydrogen cyanide and siderophores are also produced by *P. aureofaciens* (33). Several

hypotheses have been proposed to describe the primary mode of action by phenazines in inhibiting fugal growth. These include disruption of normal membrane functions such as active transport, inhibition of RNA synthesis, DNA replication and transcription processes, and the uncoupling of electron transport and energy production (32).

Two decades after the discovery of *P. aureofaciens*, Cook and Rovira reported, that a similar fluorescent Pseudomonad, *Pseudomonas fluorescens*, was possibly the cause of a naturally occurring biological control of a fungal disease in wheat (7). They distinguished general and specific antagonism as two types of disease suppressive soils. General antagonism was characterized as a type of suppressiveness present in all soils, not affected by moist heat, able to survive fumigation, and not transferable. Specific antagonism was defined as disease suppressiveness, which was eliminated by moist heat or fumigation, and this property was transferable from soil to soil. In defining a clear distinction between properties of these two soil types, Cook and Rovira were then able to correlate the presence and role of *P. fluorescens* with suppression. Certain soils were found to become suppressive to the pathogen *Gaeumannomyces graminis* var. *tritici* that causes the disease take-all in wheat (7). The phenomenon of "take-all decline" (TAD) was noted when, after several years of wheat monocropping and occurrence of take-all, natural suppression of the disease occurred. In searching for a factor that caused these suppressive soils to have a specific antagonism, it was discovered that fluorescent pseudomonads were found in these soils. Over 100 isolates of soil microbes from diseased and protected wheat roots were used in bioassays to suppress take-all. Eight of these microbes were able to suppress disease and all were pseudomonads, seven of which were fluorescent. Treatments of moist heat and fumigation eliminated the specific

antagonism of these soils. This research correlated the presence of specific pseudomonads to suppression of take-all.

Work by Weller and Cook verified that certain pseudomonads were able to provide biological control of plant pathogen's (46). Thomashow and Weller demonstrated that disease control of take-all was from production of phenazine antibiotics produced by *P. fluorescens* (47). Mutants defective in phenazine production (Phz $^-$) were compared to wild type (Phz $^+$) strains to determine the effect of phenazines at inhibiting take-all on wheat roots. Phz $^-$ mutants did not inhibit take-all in vitro and were significantly less suppressive on wheat seedlings compared to the Phz $^+$ strains. Genomic DNA from the parent strain restored antibiotic biosynthesis and fungal inhibition to the previously Phz $^-$ strain.

The phenazines produced by *P. fluorescens* strain 2-79 and *P. aureofaciens* strain 30-84 were shown to be responsible for take-all control on wheat roots (43). Direct evidence of the role of phenazines was lacking because they were not recovered from natural soils where disease control occurred. Thomashow et al. coated wheat seeds with Phz $^+$ strains 2-79, 30-84, Phz $^-$ mutants of each, and untreated controls. In both growth chamber and field studies, PCA was recovered only from roots coated with Phz $^+$ strains. PCA was not detected on roots from Phz $^-$ mutant coated seeds or from the control. Roots from which PCA was recovered had significantly less disease compared to the Phz $^-$ and control treatments. In detecting phenazines from protected roots, the effectiveness of PCA was demonstrated due to the small amounts found.

Phenazines were shown to be the principal factors in disease suppression by *P. fluorescens* strain 2-79 and *P. aureofaciens* strain 30-84. It was also thought that these

compounds played a role in the competitive fitness of the organisms. Mazzola et al. introduced strains 30-84, 2-79, and Phz $^-$ mutants into natural and pasteurized soils before growing wheat seedlings from them (24). The population sizes of Phz $^-$ strains declined more rapidly than the Phz $^+$ strains in natural soils. In pasteurized soils, both Phz $^-$ and Phz $^+$ population levels were similar. It was thought that the Phz $^-$ strains could not compete with the indigenous microflora in the natural soils while the loss of competition in steamed soils allowed more Phz $^-$ bacteria to survive. Mazzola et al. concluded the production of phenazines by *Pseudomonas* spp. is a selective advantage to their survival in the rhizosphere.

Research on *P. aureofaciens* and *P. fluorescens* demonstrated the role and importance of phenazines as the mechanism by which the bacteria suppress fungal pathogens. Subsequent research focused on identifying the genetics involved in the biosynthetic pathways and regulation of production.

P. aureofaciens strain 30-84 was identified as producing PCA, 2OHPCA, and 2HZPCA (33). Mutants capable of only PCA production provided less suppression than strains producing all three phenazines. Mutants deficient in all phenazine production but maintained pyoverdin and HCN production were no different from the control. Researchers determined that production of all three phenazines as the primary mechanism of take-all suppression by strain 30-84, and the other two metabolites play no major role in suppression. A phenazine biosynthetic locus was identified in strain 30-84 by Pierson and Thomashow that restored both phenazine production and fungal inhibition to Phz $^-$ mutants (29). When this locus and a functional promoter were introduced into *E. coli*, expression of all three phenazines occurred.

Pierson et al. identified a regulator gene (*phzR*) that activates expression of phenazine biosynthesis in *P. aureofaciens* 30-84 (30). The *phzR* gene was identified as a requirement for phenazine production. Its product, PhzR, regulates phenazine production by activating a *phzB* gene involved in biosynthesis. Inactivation of *phzR* resulted in the complete loss of phenazine biosynthesis. The amino acid sequence of the PhzR protein has homology with several other bacterial transcriptional regulators suggesting it is a member of the LuxR/LuxI family of activators. These activators regulate gene expression in response to cell density signals. In recognizing PhzR as a member of this two component system, researchers sought to identify a second gene whose product would be a signal in the LuxI family of N-acyl-L-homoserine lactone synthases (N-acyl-HSL). Wood et al. discovered the *phzI* gene whose product was a diffusable signal and member of the LuxI family of N-acyl-HSL (48). Inactivation of *phzI* resulted in the loss of both signal and phenazine production. To determine if *phzI* produces a diffusable signal, cell-free supernatants from *E.coli* containing the *phzI* gene and a control were added to strain 30-84Z. Strain 30-84Z contains a promoterless *LacZ* gene fused to the phenazine biosynthesis gene, *phzb*, and cannot induce its own phenazine production. Signal production was shown when cell-free supernatants from cultures of *E.coli* containing Phz I activated *phzb* expression in strain 30-84Z. The control supernatant, which did not produce Phz I, failed to activate expression. Wood and Pierson identified a 5.7 kb region from strain 30-84 which produced phenazines in *E. coli* when inserted with a promoter (48). The nucleotide sequence of this fragment contained the open reading frames (ORF's) of five genes involved in phenazine biosynthesis. These ORF's encode enzymes found in the shikimic acid, enterochelin, and tryptophan biosynthetic pathways. Future

understanding of the specific roles of each gene will help identify how bacteria form phenazines.

Handelsman identified a minimum of three components that must be present for a biological control organism to suppress disease (14). Interactions between the host plant, infecting pathogen, and the biological control organism must occur for this phenomenon to be successful. Pierson and Pierson hypothesized a likely scenario of how *P. aureofaciens* interacts with a wheat plant and the pathogen *G. graminis* var. *tritici* to result in the decline of take-all in the field (32). The plant root provides a niche in the rhizosphere where populations of *P. aureofaciens* survive off of root exudates. The pathogen *G. graminis* var. *tritici* seeks to infect the roots in seek of food and, in doing so, causes disease and death to the plant. The penetration of the root by the pathogen causes a flush of nutrients to leak and are used by the pathogen and bacteria. The bacterial population quickly increases due to the excess nutrient availability and, in turn, results in an increase and accumulation of Phz I signals. The signals interact with the Phz R transcriptional activator proteins that then activate the *phz* antibiotic biosynthesis genes. The phenazines are produced in greater amounts and diffuse to the surroundings. The presence of the phenazines results in the suppression and biological control of the fungal pathogen and protection of the wounded plant from further infection.

The importance of and interest in PCA as a control mechanism is apparent in that it is the most studied of the many antibiotics produced by *P. aureofaciens*. The antibiotic pyrrolnitrin (Prn) was also studied due to its antifungal activity. Pyrrolnitrin was first isolated and identified by Arima et al. in 1964 (1). The mode of action of pyrrolnitrin is not fully understood, although it is thought to interfere with membrane functions of other

organisms. The addition of DL-tryptophans to *P. aureofaciens* culture increased pyrrolnitrin production.

Much of the research involving the biosynthesis and regulation of pyrrolnitrin has been done on *P. fluorescens*. Pfender et al. identified a genomic region that when cloned, restored antagonism and pyrrolnitrin production to a mutant previously deficient in pyrrolnitrin production (28). Hill et al. isolated a strain of *P. fluorescens* and identified pyrrolnitrin as its mechanism of antagonism against the pathogen *Rhizoctonia solani*. Mutants deficient in pyrrolnitrin production did not inhibit the pathogen. Parental DNA was transferred to the Prn$^-$ strains and resulted in both pyrrolnitrin production and antagonism. (17). The global regulatory mechanism used by *P. aureaofaciens* for pyrrolnitrin production was identified as LemA/GacA. Corbell et al. discovered a gene closly related to *lemA* (8). Hammer et al. identified a 6.2 Kb region containing a cluster of four genes required for pyrrolnitrin biosynthesis. When these genes were cloned by PCR, fused to a *tac* promoter, and transferred to *E. coli*, pyrrolnitrin production resulted (14).

Dollar spot

Dollar spot is a disease of turfgrass found throughout the world (41). It is considered one of the most important diseases of high maintenance turf such as the type found on golf courses (44). The causal agent of dollar spot is the fungus *Sclerotinia homoeocarpa* F.T. Bennet. This name is currently under contention due to the inability of this organism to produce sclerotia, and instead producing a flat stroma. Current work by Powell will likely result in the changing of its name to *Rutstroemia floccosum*

(unpublished). Cultures of this fungus are characterized by a mat of fast-growing, fluffy white mycelium that turns gray, brown and other colors as it ages. After 2-4 weeks of growth, planes of dark stroma can be seen within culture media (41).

Symptoms

The common name of dollar spot is derived from the symptoms it produces on turf, which resemble a silver dollar (44). The disease appears on greens and low mown turf as small, straw colored patches, less than 6 cm and commonly 1-3 cm in diameter. In taller grass, such as home lawns, these patches may reach 15 cm across. As disease severity increases, the individual spots often coalesce into irregularly shaped patches, destroying large areas of turf. Lesions on individual leaf blades are distinguished by a chlorotic to bleached water soaked band, bound by a tan or reddish-brown margin. The shape of this lesion takes on an hourglass shape across the leaf blade. During periods of leaf wetness from morning dew, the active mycelium are visible as a white, cottony or cobwebby growth (2)(41)(44).

Dollar spot is known to infect many species of turfgrass (41). In the northern U.S. it is most damaging to creeping bentgrass (*Agrostis palustris*), annual bluegrass (*Poa annua*), colonial bentgrass (*Agrostis tenuis*), and fine-leaf fescues (*Festuca*)(44). Dollar spot can occur on turf from the latter part of the spring through the end of the fall. Most epidemics occur in July and again in late August through early September (41). Conditions most favorable to dollar spot are temperatures of 15-30° C with warm humid days preceded by cool nights. Cooler night temperatures are conducive to dew formation as nutrient rich guttation fluid is exuded from the plant. Guttation fluid is an ideal food source for the fungus. Dollar spot is more severe under conditions of low nitrogen

fertility, dry soils, and/or water stress. Dissemination of the fungus on turf is by mechanical movement such as infected leaf clippings spread by mowers, foot traffic, and water. The fungus can survive as mycelia and stromata on leaf tissue when conditions for infection are unfavorable.

Managing dollar spot

Dollar spot management combines the use of cultural, chemical, and biological control techniques. Methods of cultural control are effective at reducing the level of diseased turf and can reduce chemical inputs. Adequate nitrogen fertility, when applied at times of heavy disease pressure, reduces disease (44). Maintaining proper soil moisture to avoid plant stress also contributes to disease control. One of the most important cultural control techniques is to remove dew from the leaf blades as early in the morning as possible. Doing so inhibits fungal mycelial growth and subsequent spread of the disease.

In many cases, the use of fungicides is necessary for control of dollar spot. Contact fungicides, like chlorothalonil, are effective at controlling dollar spot for shorter periods of time. Resistance to contact fungicides has not been reported and is unlikely to develop due to multi-site mode of action of these fungicides. Effective systemic fungicides include propiconazole, fenarimol, iprodione, and vinclozolin. Systemic fungicides are desired for their longer control interval when compared to contact fungicides. One drawback to systemics is there is a greater likelihood of resistance occurring amongst dollar spot populations. Resistance can be defined as reduced efficacy and shortened control interval of a previously sensitive fungal population. Since 1972, resistance has been reported to all three of the major systemic fungicide classes which

includes the benzimidazoles (45), dicarboximides (9), and demethylation inhibitors (DMI's)(12).

Pink Snow Mold

Symptoms/Epidemiology

Pink snow mold, caused by the fungal pathogen *Microdochium nivale*, is a severe disease in areas of prolonged cool (0-8 °C) and wet conditions (41). It differs from other snow molds in that it does not require snow cover for infection to occur (41). This disease is most severe in the Pacific Northwest and is present in most of the northern areas of the United States. It occurs from early fall to late spring, and can exist year round if conditions favor its development.

Pink snow mold spreads from leaf to leaf during snow and thawing cycles, and in light rain. Foot traffic and equipment can spread conidia and infected tissue. Symptoms appear as small, water-soaked, circular patches (5cm or less), and these change to a light gray as the mycelium develops. Sunlight exposure induces sporulation and also changes the mycelium to its characteristic pink color. As temperatures increase and drier conditions occur, the pathogen becomes inactive and survives in the grass plants and dead debris. Species most susceptible to infection by *M. nivale* include *Poa annua* and *Agrostis* spp.

Management techniques

Reducing fall nitrogen applications can help decrease the severity of this disease. Excess nitrogen causes the grass plants to be succulent and more prone to infection. Poor drainage, matted turf, and pockets of humidity are factors, which favor development of this disease (41). Many fungicides exist for managing pink snow mold. Contact fungicides are effective in areas without prolonged snow cover. In regions likely to experience extended snow cover, the use of systemic fungicides provides longer control intervals (44). Recent field trials have shown combinations of different fungicides to be the most successful in controlling pink snow mold.

Chapter 2

BIOLOGICAL CONTROL OF DOLLAR SPOT FIELD STUDY

Introduction

The turfgrass disease dollar spot, caused by *Sclerotinia homoeocarpa*, is considered one of the most important foliar pathogens of low cut turf on golf courses (44). The importance of this disease can be demonstrated by the fact that more money is spent in managing dollar spot than any other disease (44). The most common means of managing dollar spot has been through the use of cultural and chemical methods such as proper irrigation, adequate nitrogen fertilization, and application of fungicides. The use of biological controls, such as organic composted topdressings and antagonistic fungi, has been reported as means of controlling dollar spot (13)(22). Perhaps the most successful commercially available system for the biological control of dollar spot is the application of the soil bacterium *Pseudomonas aureofaciens* strain Tx-1. Other strains and species of fluorescent pseudomonads have been correlated with disease suppression and biological control (41). Sarniguet and Lucas correlated a higher population of antagonistic fluorescent pseudomonads to a zone of disease remission in turfgrass infected by take all patch, *Gaeumannomyces graminis* var. *avenae* (40).

The purpose of this study was to further our understanding of Tx-1's ability to control dollar spot in the field. The results will be used to optimize the current system and be able to improve this technology to control the disease in different conditions. Showing the effectiveness of Tx-1 may encourage the acceptance of using Tx-1 alone or as a supplement to controlling dollar spot by turfgrass managers.

Previous field studies using Tx-1 to control dollar spot were done on greens height ($^{5}/_{16}$") creeping bentgrass (*Agrostis palustris*). The turf chosen for this study was fairway height ($^{1}/_{2}$") annual bluegrass *Poa annua*). Fairway height annual bluegrass was chosen for two reasons. Annual bluegrass is a species of low mown turfgrass comprises large areas of low cut turf on some golf courses. The fairway height of cut accounts for the largest area of intensively mown turf on a golf course. Fairways are valued differently than greens and, in some situations, it may not be economically feasible to manage disease on fairways to the high standard of greens. By determining the effectiveness of using Tx-1 on fairway height annual bluegrass, the application of Tx-1 may make it practical to manage dollar spot in these areas. Another objective of this study was to determine if using fewer applications or lower concentrations of a fungicide in conjunction with Tx-1 could prolong the effective interval of the fungicide and allow for reduced fungicide use. Such a reduction in fungicide use would be substantial due to the large area fairways comprise.

A field study was done in August and September of 1997 and 1998 using various Tx-1 concentrations, intervals, and combinations with fungicides to answer the above questions and others related to the use of Tx-1 on fairway height annual bluegrass.

Materials and Methods

Field Conditions

A field study was done in 1997 and 1998 to test the effectiveness of *Pseudomonas aureofaciens* strain Tx-1 in controlling dollar spot (*Sclerotinia homoeocarpa*) on annual bluegrass (*Poa annua*). This study was done at Michigan State University's Hancock Turfgrass Research Center in East Lansing, Michigan. The stand of annual bluegrass was maintained by mowing three times per week at ½ of an inch. Irrigation was applied daily at varied rates to maintain healthy turf. Fertility was applied at ½ lb. of N/1000 ft^2/month during the study. The statistical error control design was a randomized complete block, containing four replications of each treatment. Individual plot sizes were 4.5 by 2 feet, separated by one foot buffer strips on all sides.

Treatments

In 1997 the study was initiated on August 15th and terminated on September 25th; the 1998 study was initiated on July 31st and terminated on October 2nd. Termination dates were determined when natural disease pressure in the control plots declined. A list of treatments used in each year's study is shown in the appendix, tables 3 and 4. In 1997 the contact fungicide Daconil Ultrex (chlorothalonil) and systemic fungicide Banner (propiconazole) were both applied once on August 19th. In 1998 the 2oz. and 0.5 oz. rates of Banner Maxx were applied once on August 3rd. Banner Maxx was reapplied on September 9th at a 1oz. rate to plots which had previously received the 0.5 oz. rate. All treatments involving Tx-1 were applied either one or five times per week at the concentrations stated in table 1. Treatments were applied using a nitrogen powered backpack sprayer calibrated to deliver rates of 2.2 gallons/1000ft^2 for biologicals and 1.1

gallons/1000ft^2 for chemicals. Tx-1 treatments were applied after 4:30 p.m. to lessen mortality of bacteria from the suns ultraviolet radiation and reduce drift.

Bacterial Fermentation

Tx-1 was grown for 24 to 30 hours before applying, the exception being the 8 hour growth treatment used in 1997. Seed cultures of 50 to 100 ml were used to inoculate 4L flasks containing 2L of Tryptic soy broth (Difco). Optimal growth was achieved by aerating the culture with a constant supply of sterile forced air that was mixed into the culture using a magnetic stir plate. Temperature was maintained at 28° C.

Tx-1 was quantified daily for precise application. A spectrophotometer was used to measure an absorbance by optical density. The absorbance value was used in a previously derived formula, based on a growth curve of Tx-1 (35).

The "heat-killed" treatment of Tx-1 was killed by bringing the culture to a rolling boil. This technique was shown to be completely effective at lysing all cells since no bacterial growth occurred when plated onto growth media. Thin layer chromatography (TLC) was used to verify that the antibiotic PCA remained active after boiling. Boiled and living cultures of Tx-1 were extracted with chloroform, spotted onto a TLC plate, and separated by a 1:1 chloroform:methanol solvent system. Similar R_f values, of 0.55 for boiled and 0.57 for non-boiled treatments, were sufficient to conclude that PCA is not destroyed by boiling.

Disease Ratings/Data Analysis

Disease ratings were taken every seven days unless delayed by inclement weather conditions. The occurrence of disease was rated by counting individual infection sites. Data was analyzed using the SAS (Statistical Analysis Software, Cary N.C.) mixed procedure slicing. Slicing allows for comparison of treatments over time and also comparison within treatments over time.

Results

1997

In 1997, the daily application of Tx-1 at a rate of $2x10^7$ CFU/cm^2 provided significant control of dollar spot as compared to the control (Figure 1). Significant control by Tx-1 applied weekly was observed on rating dates of September 2nd, 18th, and 25th. Results of Tx-1 applied daily treatment show a significant drop in the dollar spot population that correlates with Tx-1 being able to curatively control this disease. Once suppression occurred, Tx-1 continued to prevent disease for the remainder of the study. Tx-1 applied daily showed significantly greater control than Tx-1 applied one time per week. The amount of control seen when one application of Tx-1 per week was used was not acceptable for managed turf on golf courses.

Tx-1, used in combination with the systemic fungicide, Banner, provided the same amount of control as Tx-1 used alone by the September 2nd rating. After the first week, Tx-1 alone provided more control than the combination. In the second week, the combination was more effective than the contact fungicide Daconil Ultrex used alone but was not different from Tx-1 used alone. Figure 2 illustrates that the combinations of Tx-1

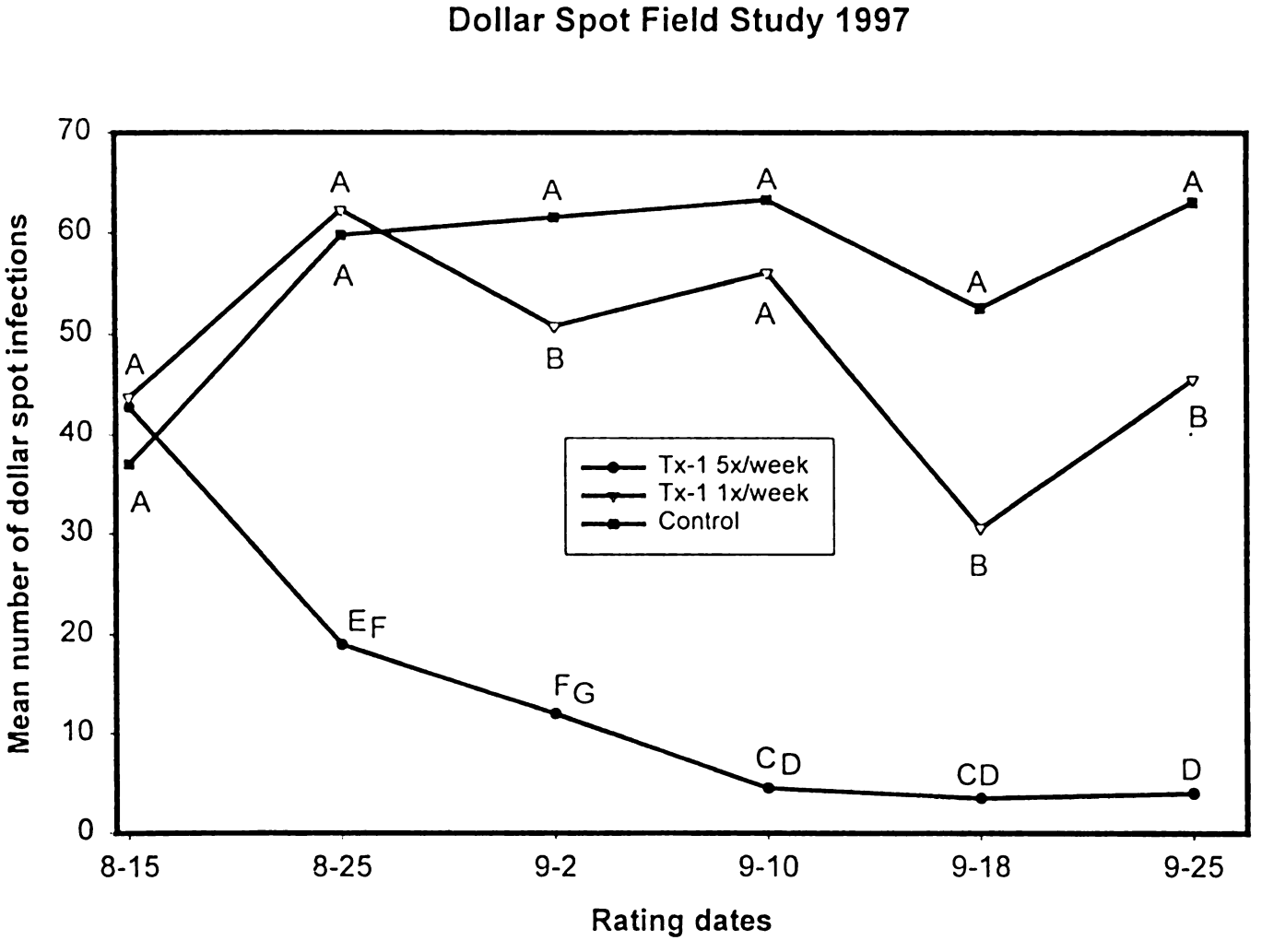

Figure 1. Comparrison amongst Tx-1 and control applied at different intervals.
-Symbols followed by the same letter are not significantly different at a given rating date at the 5% level.

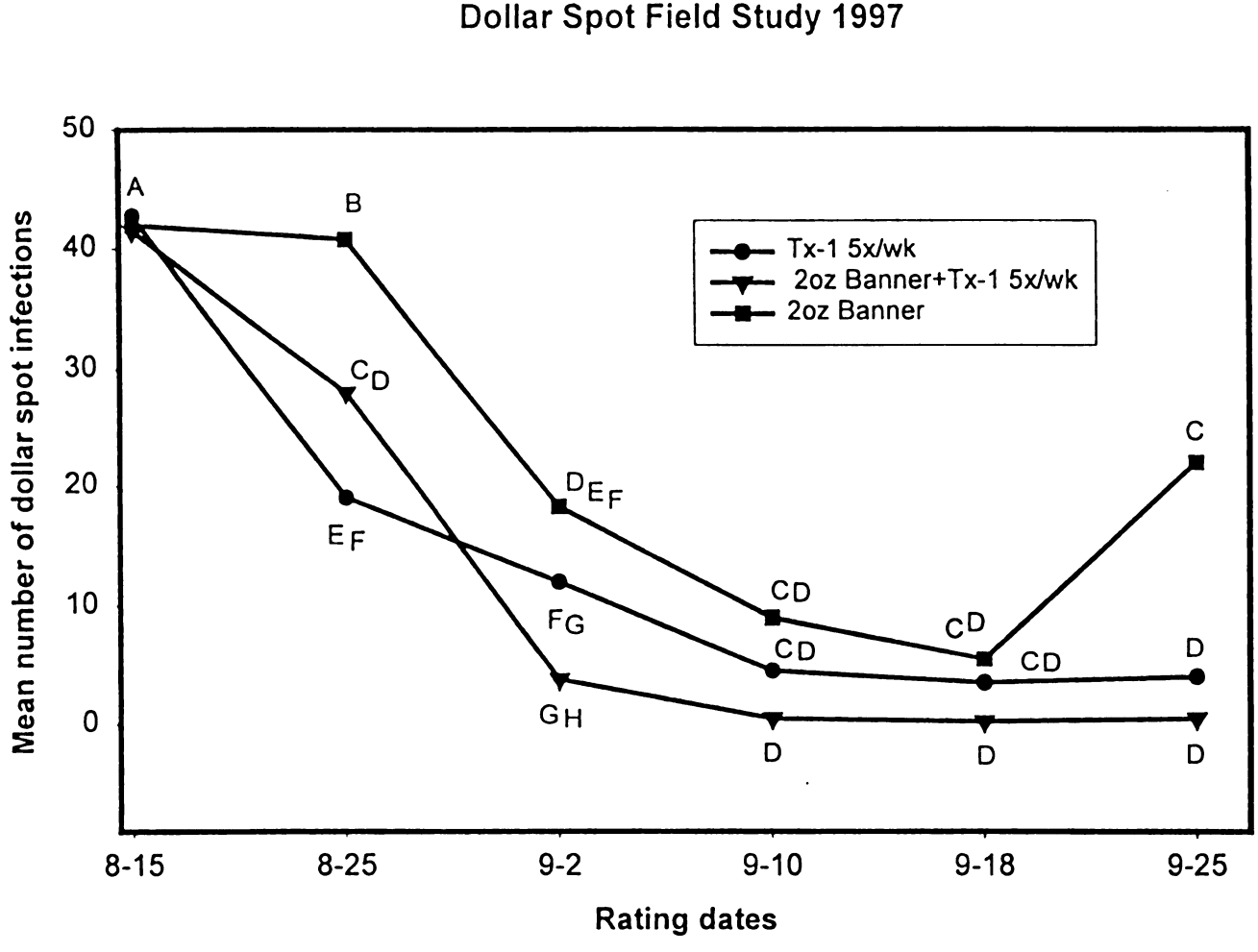

Figure 2. Comparrison amongst treatments of Tx-1 used in conjunction with Banner.
Symbols followed by the same letter are not significantly different at a given rating date at the 5% level

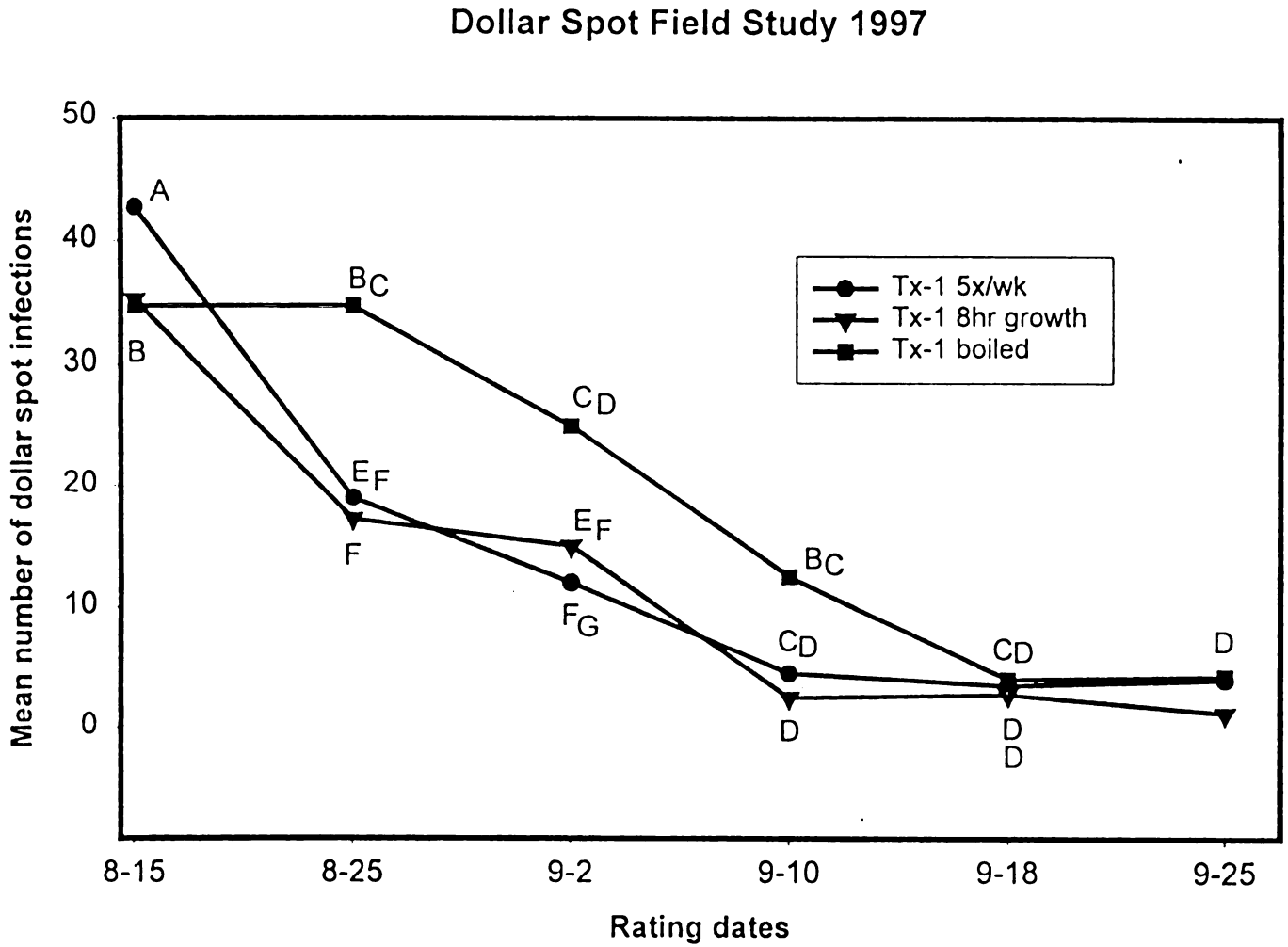

Figure 3. Comparrison of different Tx-1 growth treatments.
Symbols followed by the same letter are not significantly different at a given rating date at the 5% level

and fungicides vs. fungicides alone work better at the September 2nd rating and also after the September 9th rating. The Banner/Tx-1 combination was more effective than the Banner alone at the August 25th and September 2nd ratings.

The 8hr. growth and heat-killed treatments of Tx-1 both provided significant control by the August 25th rating (Figure 3). Compared to the Tx-1 applied daily treatment, the 8hr. treatment provided the same level of control throughout the study. The heat killed treatment provided significantly less control than the Tx-1 applied daily for the first three rating dates.

1998

Comparisons amongst the lower concentrations of Tx-1 and fungicides are shown in Figure 4. The lower rate of Tx-1 at $2x10^5$ CFU/cm^2 was similar to the control through the September 12th rating. Significant control was obtained using the low rate from September 19th through October 2nd. The low concentration of Tx-1 used in combination with the low rates (0.5 and 1 oz.) of Banner Maxx was similar to the fungicide used alone during the entire study. Treatments with a low rate of the fungicide provided significant control at the August 15thand August 21st ratings. After the fungicide was reapplied on September 3rd, control was observed from September 19th through October 2nd.

Tx-1 applied 1x/week at $2x10^7$ CFU/cm^2 provided significant control on August 15th, August 21st, and from September 19th through October 2nd. The low concentration of Tx-1 applied 5x/week at $2x10^5$ CFU/cm^2 provided significant control from September 19th through October 2nd. The heat-killed treatment of Tx-1 provided significant control from 8/15 through 10/2 and was no different than the high rate of Tx-1 at any rating date during the study.

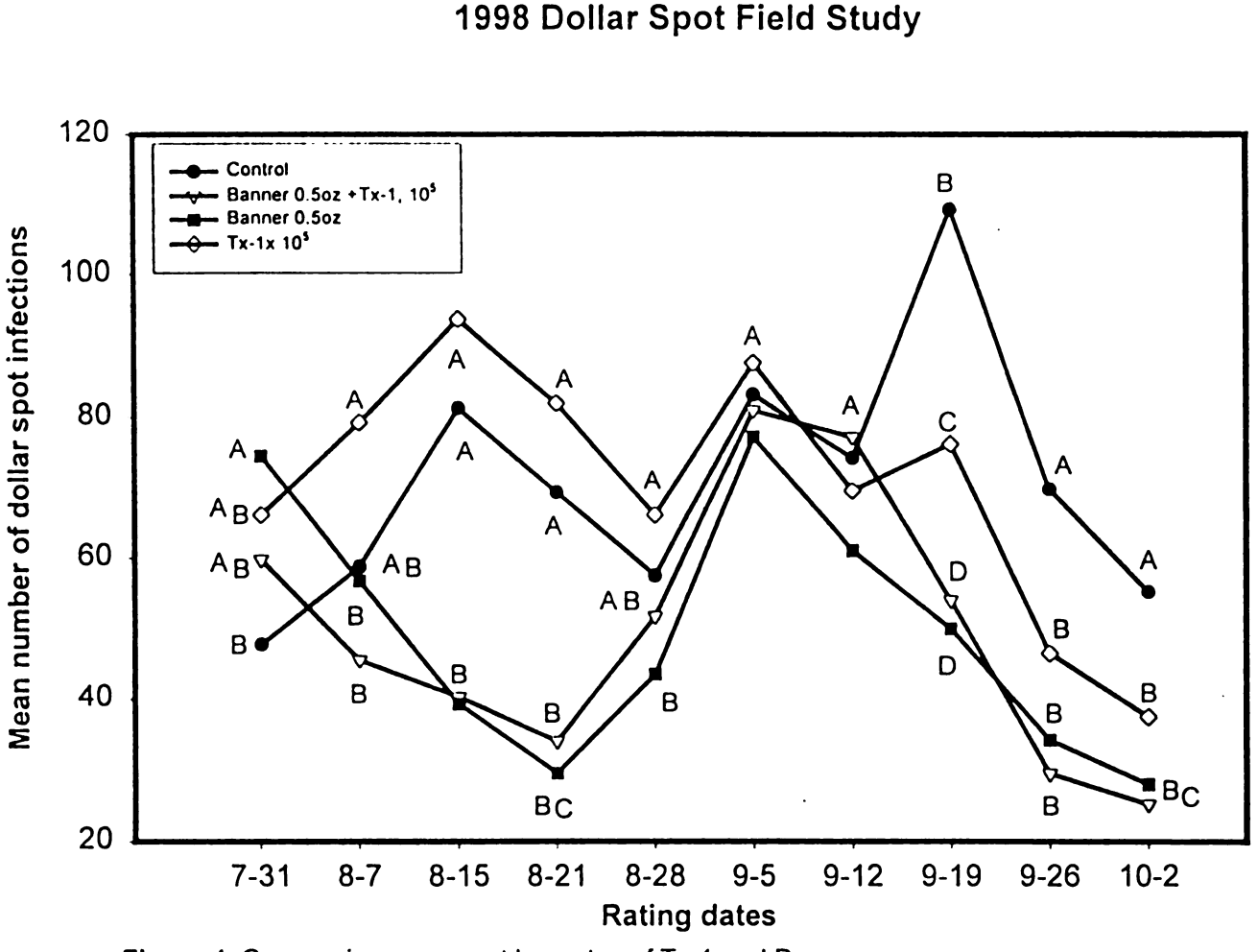

Figure 4. Comparrison amongst low rates of Tx-1 and Banner
Symbols followed by the same letter are not significantly different at a given rating date at the 5% level
Banner was reapplied on 9/2 at 1oz/1000 ft^2

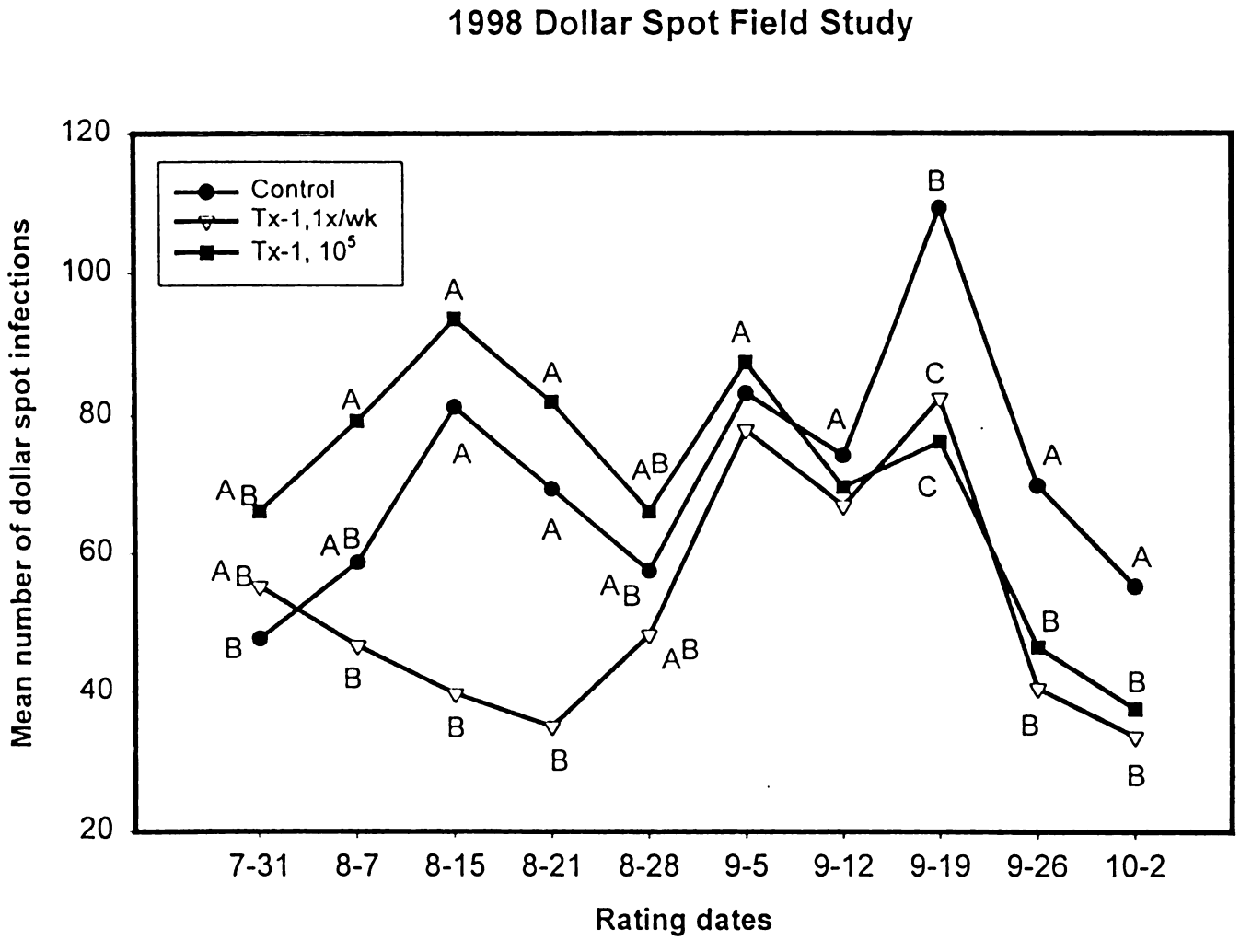

Figure 5. Comparrison amongst Tx-1 and the control.
Symbols followed by the same letter are not significantly different at a given rating date at the 5% level

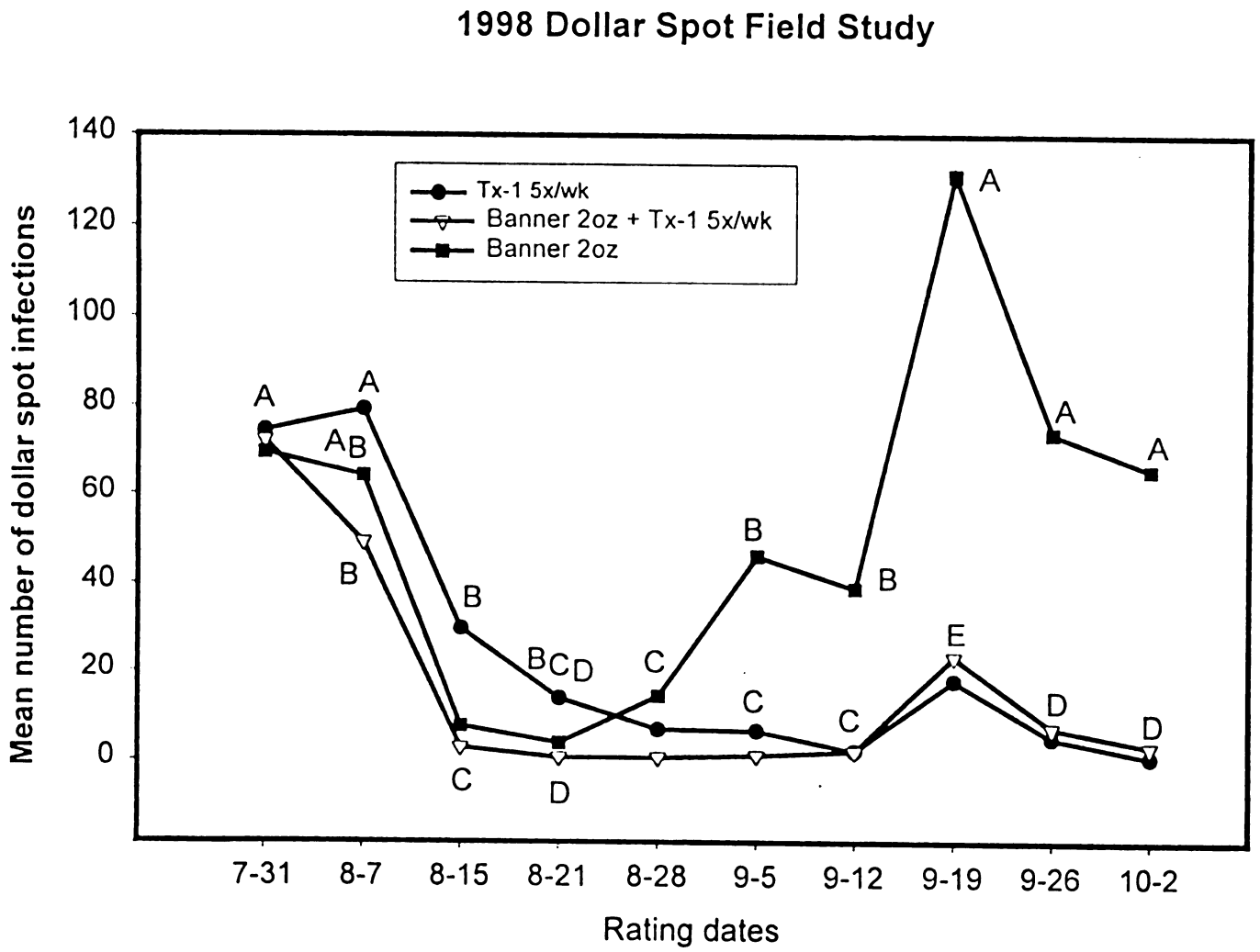

Figure 6. Comparrison amongst high rates of Tx-1 and Banner
Symbols followed by the same letter are not significantly different at a given rating date at the 5% level

Tx-1 applied 5x/wk was similar to the control after the first week and significant control was seen thereafter (Figure 6). Banner Maxx applied alone at the 2 oz. rate was more effective than Tx-1 in the beginning of the study at the August 15th rating. The combination of Banner Maxx, applied at the 2 oz. rate and Tx-1 applied 5x/week at $2x10^7$ CFU/cm^2, provided significantly greater initial control on August 7th and August 15th. After that rating, there was no difference between the combination and Tx-1 used alone, which was similar to 1997 results. All data from both 1997 and 1998 are in Tables 3 and 4 found in the appendix.

Discussion

The results of two years of field studies show that applying Tx-1 at a rate of $2x10^7$ CFU/cm^2 on a daily basis effectively controlled dollar spot on fairway height annual bluegrass. It is possible that using Tx-1 to manage dollar spot on fairways may result in substantially less fungicide inputs here. This would be so because fairways are the largest area of treated turf on golf courses.

Applying Tx-1 at a rate of $2x10^7$ CFU/cm^2, 5x/week controlled dollar spot both curatively and preventively. In both years the number of dollar spots declined significantly when using this treatment, and did not significantly increase at any rating date while Tx-1 was being applied. The initial curative action was preceded by sustained prevention of any significant increase in dollar spot, regardless of the disease pressure.

In 1997, Tx-1 grown for 8 hrs. was compared to the standard 24hr.fermentation of Tx-1 to determine any differences in efficacy. This was compared because of concern that PCA is produced in the greatest quantities during the stationary phase of growth, which occurs after eight hours of fermentation. Throughout the study, the two treatments

were similar in their ability to control dollar spot and no significant difference was seen between them. Similar results were likely due to the concentrations used. Both growth period treatments were applied at a concentration of $2x10^7$ CFU/cm^2 and therefore the growth period is not as great of a factor in control as concentration is.

To determine if less Tx-1 would be efficacious, Tx-1 at $2x10^5$ CFU/cm^2 5x/week and $2x10^7$ CFU/cm^2 1x/week were compared to $2x10^7$ CFU/cm^2 5x/week. Both $2x10^5$ CFU/cm^2 5x/week and $2x10^7$ CFU/cm^2 applied once per week provided significantly less control than the higher rate of $2x10^5$ CFU/cm^2 5x/week in 1997 and 1998. When compared to the control, the application of Tx-1 once per week at the high rate provided sustained control of dollar spot after September 10th in 1997 and again after September 12th in 1998. Similarly, the low rate of $2x10^5$ CFU/cm^2 5x/week was effective compared to the control after September 12th in 1998. Although significant disease control was achieved at these times by both treatments, the control afforded would not be acceptable on maintained turf. Two possible explanations exist for the control seen after September 12th. One reason is that a population of Tx-1 built up over the period of application which was effective at controlling the naturally declining dollar spot population. Another possible explanation is that in the latter part of year (in September) the sun is setting sooner after application. This may lessen the mortality of bacteria from ultraviolet degradation and desiccation from heat, allowing more Tx-1 to survive when applied. Because control occurred in both years near the same rating date, and 1998 had more total applications up to this time point, it is likely due to an increase in the Tx-1 population surviving from daily applications because of a decrease in mortality rather than a buildup over time. If effective control is due to greater numbers of bacteria

surviving daily then it could be recommended that Tx-1 applied at night would have a higher survivability and possibly greater efficacy.

The heat-killed Tx-1 was effective after the initial rating in both years. In 1997 the heat-killed treatment was less effective compared to the live Tx-1 treatment for the first three rating dates, and in 1998 the two treatments were similar at all rating dates. This suggests that the active compound PCA is likely the means of fungal inhibition rather than inhibition of pathogens by the living bacteria. Previous work by Powell demonstrated that applying purified PCA to turf was sufficient for control (35).

Combined treatments of Tx-1 and different fungicides and rates were tested to determine if using a combination could be more effective than the Tx-1 used alone. This question was posed to develop a strategy for times of severe disease pressure in which Tx-1 alone might not provide adequate protection. During such times, a single application of a fungicide may be used to increase control or extend the fungicide interval when used as a combination. Our findings in comparing the high rate of Tx-1 with a high rate of fungicide versus the high rate of Tx-1 alone showed the combination did not significantly extend the required interval for fungicide application. The combination provided no greater disease control than the Tx-1 alone, demonstrating that all additional control is from Tx-1. In 1998 lower concentrations of the systemic fungicide Banner Maxx were used in combination with the lower rate of Tx-1x10^5. The fungicide was applied at a rate of 0.5 oz./1000ft^2 on August 3rd and again at 1 oz./1000 ft^2 on September 2nd. At no time was the combination any different than the fungicide alone. At several rating dates the fungicide was more effective than the Tx-1x10^5 used alone, which signifies all control from the beginning of the study through September 12th was due to the action of the

fungicide. The lower rates of fungicides gave poor results as compared to the 2oz. rate. The September 2nd application of 1oz.of Banner/1000ft^2 is a rate commonly used and recommended for the curative control of dollar spot. Since this rate performed poor in comparison to what was expected, there is a possibility of fungicide resistance in this population of dollar spot. Because of this possible resistance, it can not be determined from this years results if a lower fungicide rate would reduce disease pressure enough to increase the effectiveness of Tx-1x10^5.

Chapter 3

PERSISTENCE OF TX-1 APPLIED TO TURF

Introduction

Several factors are required for a biological control organism to be effective in the field. Two such factors are the ability of the biological control agent to possess a mechanism of controlling disease and, the ability to successfully colonize and persist in the environment where control occurs (10). The ability of an introduced organism to survive and colonize is based on factors such as mobility, growth rate, competition with existing microflora, and ability to use organic acids as a food source (42). More often than not, potential biological control organisms are unsuccessful when applied in the field due to the organism's inability to establish themselves in a particular cropping system (48).

Previous studies were done involving fluorescent pseudomonads, in which the organism's success in control was determined by their ability to colonize the environment where introduced (4). This study is the first to monitor populations of the biological control organism *Pseudomonas aureofaciens* strain Tx-1 applied to turf. The purpose of this study was to determine if Tx-1 applied to turf can survive in the phylloplane and rhizosphere, and to measure these population changes in the system.

Measuring population sizes allows us to understand how a population changes over time and to determine the highest population of Tx-1 attainable in a turf system. In determining these factors, we can optimize applications to achieve successful control of various turfgrass pathogens.

In this study Tx-1 was applied daily for approximately two months in 1997 and 1998 and the populations were monitored at set intervals throughout both years. Populations were measured in the foliage, thatch, and soil at depths of 1,2,and 4cm.

Materials and Methods

This study was done at the Hancock Turfgrass Research Center in East Lansing, Michigan. The plots used were greens height (5/16 inch) creeping bentgrass (*Agrostis palustris*) cultivar Penncross, established in 1981. The soil type was a modified loamy sand composed of 83.5 % sand, 10.6 % silt, and 5.9 % clay (25). Tx-1 was applied to five plots, each of size 2 x 3 ft^2, grown in full sun with daily irrigation to maintain healthy turf. Applications were made from early August to the end of September totaling approximately 40 applications per year. Tx-1 was grown and applied five times per week at a concentration of $2x10^7$ CFU/cm^2 by methods previously described on pg.16 of this thesis. All daily applications were made after 4:30 p.m. The strain of Tx-1 used was made resistant to the antibiotic rifampicin to aid in its recovery and identification. Resistance to rifampicin was achieved by spreading Tx-1 colonies onto media amended with rifampicin. Colonies that grew were selected because they had developed resistance to the antibiotic.

Population samplings were taken at similar rating dates over two years. The first sampling was taken in May (5/28/97 and, 5/13/98), after the turf had broken dormancy. This sampling quantified the population of Tx-1 that had overwintered from the previous year's application. A sampling in late July (7/20/98) was taken to measure the population after the summer months and prior to the beginning of the year's application; the July

1997 sampling was lost due to error in sampling. A sampling was taken in late September/early October after two months of applications to measure population while Tx-1 was being applied (10/19/97 and, 9/22/98). The final sampling was made approximately one month after application had ceased (11/28/97 and, 11/5/98).

Three random samples were taken from each of the five plots at the specified sampling dates. The soil probe used for this was surface-sterilized in a 10% bleach solution and rinsed twice in dH_2O between each sample taken. Samples were stored in a 4° C refrigerator for 2-4 hours until dissection began. The samplings on (10/19/97 and, 9/22/98) were taken 24 hours after application of the bacteria.

Each of the 15 total samples was dissected into five segments of 1cm sizes comprising the foliage, thatch, 1, 2, and 4cm soil portions. Each segment was placed into a 30ml. glass test tube containing 10mls. of buffered physiological saline solution, pH 7.0. The buffered solution was made by adding 3.9ml/L of solution from a stock made from 2.8g NaH_2PO_4 in 100ml dH_2O and 6.1ml/L of solution from a stock made from 2.8g Na_2HPO_4 in100ml dH_2O. The buffered solution was added to all test tubes for dilution of samples. Dilutions were made for each sample and 100*ul* aliquots were plated out at 10^{-3}, 10^{-4}, and 10^{-5} dilutions in four replications. The media used was potato dextrose agar (PDA) (Difco, Detroit MI) amended with rifampicin at 50*ug*/ml to select for only rifampicin resistant soil organisms. Plates were stored for 2-3 days at room temperature for colony to growth. Individual colonies were counted on plates and numbers ranging from 20-200 colony forming units (CFU's) per plate were used for compiling data.

Results

This study demonstrated the ability of Tx-1 applied to turf to overwinter and persist throughout the year. Figure 7., depicts the change in Tx-1 population in foliage over two years. In comparing the 1997 time points 1,2, and 3 to 1998 points 5,6, and 7, there is no significant difference between the populations in the two years. A direct correlation of populations to times sampled is seen as the population increases when Tx-1 is applied and decreases after application. The foliage experienced the largest drop in bacterial population in the month after application stopped in both years.

The population in thatch was measured by CFU/cm^{3}and is represented by Figure 8. The May 1997 and 1998 populations were not significantly different from one another. The two fall populations differed from one another with a higher population in 1997. In both foliar and thatch layers, the largest populations of Tx-1 were in October and the lowest populations in the July sampling.

Measurements of Tx-1 populations in the soil regions at 1,2, and 4cm depths show the highest population in the 1cm region and the 4cm region has the lowest population, (Figure 9). At no time did the mean populations of a region exceed a region above it. A significant difference was observed between 1997 and 1998 at the 10/97 and 11/97 times. The populations in the three regions do not differ significantly from one another at the May 1997 and 1998, July 1998 or November 1998 time points. At 10/97, all three regions are different from one another, at November 1997 1cm and 4cm differ, and at September 1998 1cm differs from 2cm and 4cm. The population at the 4cm region

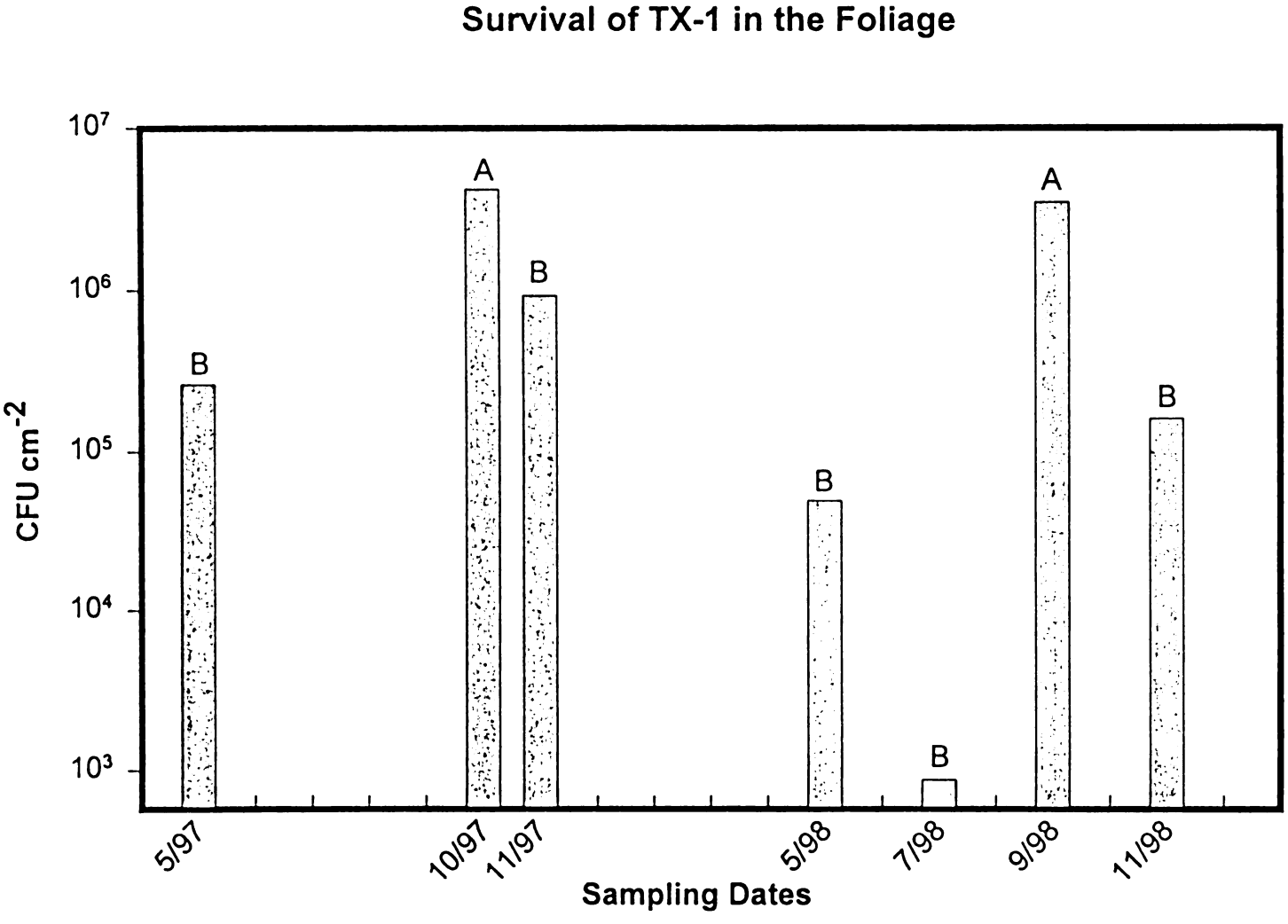

Figure 7. Survival of Tx-1 in foliage as measured in CFU cm^{-2}

Symbols followed by the same letter are not significantly different at a given sampling point at the 5% level.

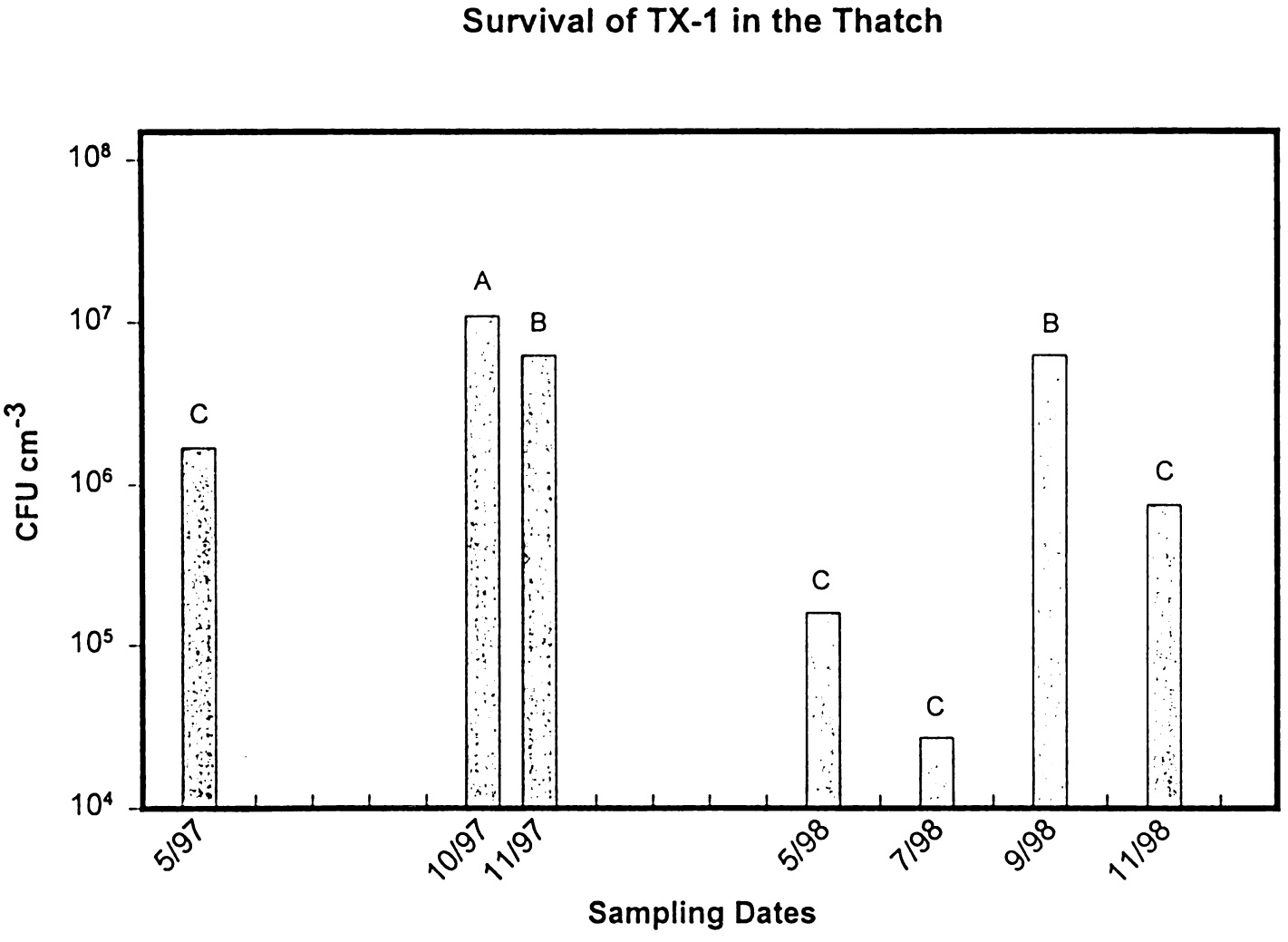

Figure 8. Survival of Tx-1 in thatch as measured in CFU cm^{-3}

Symbols followed by the same letter are not significantly different at a given sampling point at the 5% level.

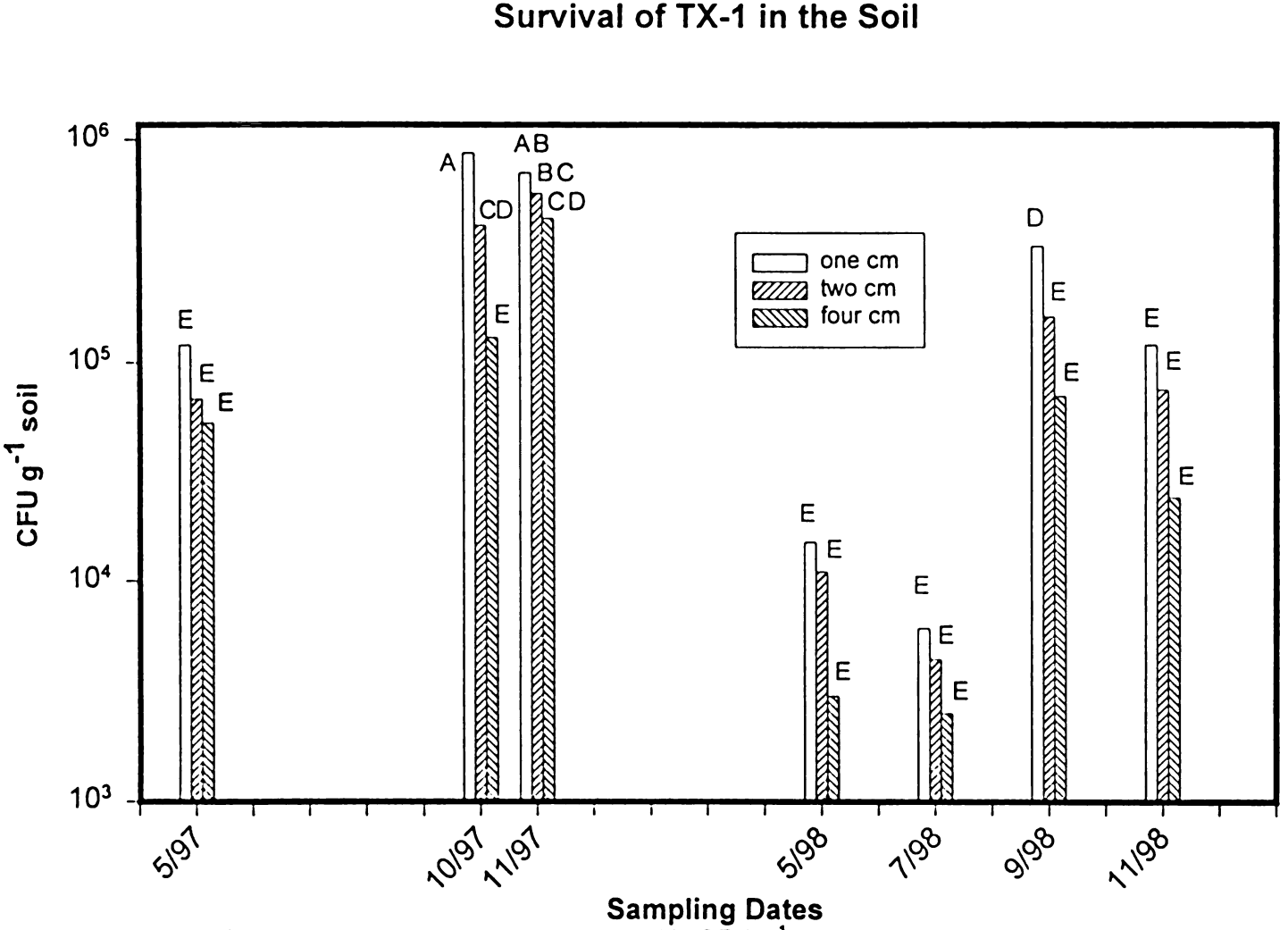

Figure 9. Survival of Tx-1 in the soil as measured in CFU g^{-1} .
Symbols followed by the same letter are not significantly different at a given sampling point at the 5% level.

increased significantly from October 1997 to November 1997, which was after application ceased. The 2cm region also increased at that time although not significantly. The population from the 1cm depth had the largest population changes throughout the two seasons. The 2 and 4cm populations were more stable and had the same pattern of significant population increases and decreases over the two years.

Discussion

One of the initial objectives of this study was to determine if Tx-1 applied to turf had the ability to overwinter and persist for long periods of time. This study has shown Tx-1 does persist in the foliage, thatch, and soil regions of turf. Tx-1 did not diminish to a population of zero at any time. It may be possible that reestablishment of population levels necessary for disease control may be achieved in less time by adding to a population already present, rather than a non-existent population was at zero each time.

The foliage and thatch regions responded similarly over time by experiencing significant increases and decreases in bacterial populations between the same rating dates. In both years, Tx-1 populations declined significantly in the fall one month after application had ceased. This suggests less stability in these areas compared to the populations in soil that did not experience as large a decline during this time.

Tx-1 was detected on the foliage in July of 1998. Tx-1 had not been applied since the previous fall. Due to the daily removal of foliage by mowing, all Tx-1 applied previously would be removed. This shows that Tx-1 can move to the phylloplane from the soil and/or thatch. Lamb et al. demonstrated that *P. aureofaciens* can move from the rhizosphere to aerial plant tissue on wheat and corn (21). The bacteria was transported

internally through guttation drops to the outside of the leaf, and externally from the plant emerging from the rhizosphere. It may be possible Tx-1 is moving by one of these mechanisms to the foliage.

Populations of Tx-1 in the soil corresponded to depth, with the largest population occurring at 1cm and the smallest population occurring at the 4cm depth. The greatest drop in soil populations occurred from November to the following May. The population remained stable from the end of the application period to one-month post-application time. During this time, a significant drop was seen only at the 1cm depth in 1998, and in 1997, a significant increase was recorded at the 4cm depth at this time. The significant increase seen after applications ceased may have occurred from the downward movement of overlying populations. Percolating water plays an essential role in the passive distribution of bacteria in the rhizosphere (27).

In this study, Tx-1 was applied to an area where significant control of the foliar pathogen dollar spot was occurring. The concentration of Tx-1 applied daily to the plots was $2x10^7$ CFU/cm^2. The threshold population measured during disease control was $4.2x10^6$ CFU/cm^2 in 1997 and $3.5x10^6$ CFU/cm^2 in 1998; the value in both years was one log factor lower than 10^7 which was being applied daily. In defining a threshold population of Tx-1 for control of dollar spot, a disease control model could be derived based on this population. This type of population monitoring could be used in future research to determine necessary Tx-1 populations to control different diseases infecting turf or being harbored in thatch and soil/roots. In identifying Tx-1 threshold populations for control of other diseases, it may be possible to predetermine a control model which

would predict the number of applications needed to preventively control disease in both turfgrass and other cropping systems.

Chapter 4

BIOLOGICAL CONTROL OF PINK SNOW MOLD

Introduction

Pink snow mold, caused by the fungus *Microdochium nivale*, is one of the most severe diseases of turf in regions where prolonged cool and wet conditions exist (44). It occurs on turf from early fall through late spring and, in some regions, it exists throughout the year (41). One difficulty in controlling this disease with fungicides is that the active infection period can be longer than the control period obtained with most fungicides. During this time, prolonged periods of snow cover can prevent reapplication of fungicides. In the past, mercury-based fungicides were used because of their effectiveness and longevity (44). These fungicides are no longer labeled for use on turf, and, since their removal, effective alternatives have been sought.

Pseudomonas aureofaciens strain Tx-1 has been used successfully in the field for controlling dollar spot (*Sclerotinia homoeocarpa*) (35). Due to the success of Tx-1 in controlling dollar spot, it has been tested for its ability to control other diseases of turf. The purpose of this study was to determine the effectiveness of Tx-1 in controlling pink snow mold. By applying Tx-1 in the fall of the year prior to infection, it was thought Tx-1 might prevent this disease. We also sought to determine if a combination of a single fungicide application used in conjunction with Tx-1 could provide greater control than either used alone.

Materials and Methods

This study was done at the Hancock Turfgrass Research Center in East Lansing MI. The grass chosen for this study was the Pennlinks cultivar of creeping bentgrass *Agrostis palustris*, mown at greens height (5/8 inch). Pennlinks was used because it is more susceptible to pink snow mold than many other cultivars. The error control design was a randomized complete block with four replications of each treatment. The seven treatments are listed in Table 1. All treatments using Tx-1 were applied a total of 17 times from 10/16/97 to 11/11/97. Tx-1 was grown and applied using the same techniques used in the dollar spot field study, pages 17-18, chap 2. The fungicide Fore (mancozeb) was applied to treatments using fungicides on 10/31 and again on 11/10 to only one treatment as noted in Table 1.

The occurrence of disease was rated as percentage of area infected, and data was recorded 104 days after termination of applications. Data was analyzed using the Statistical Analysis Software general linear model, differences in means were separated by least significant differences.

Results and Discussion

In this study it was shown that all treatments were effective at controlling pink snow mold on greens height creeping bentgrass as compared to the control. There was no significant difference in control amongst any of the effective treatments. The results from this field study are seen in table 1.

Table 1. List of treatments and comparison of means as a percent area infected by pink snow mold.

Treatment	Mean	P= .05
Control	43.75	A
Tx-1 / 10^4, daily	15.50	B
Fore / 6oz./1000ft^2 applied once	15.50	B
Tx-1 / 10^7, daily	12.50	B
Tx-1 / 10^7, daily + Fore / 6oz./1000ft^2 applied once	9.75	B
Tx-1 / 10^4, daily + Fore / 6oz./1000ft^2 applied once	2.50	B
Fore / 6oz./1000ft^2 applied weekly	2.00	B

The results of this study are the first to demonstrate Tx-1 is effective at controlling pink snow mold in the field. Because the low concentration of Tx-1 provided similar control to the high concentration, there would be no benefit from using the higher concentration to manage this disease. One possible reason for the control seen using the low 10^4 concentration is related to the time of year Tx-1 was applied. During this study, the sun would set shortly after application and a decrease in mortality due to UV radiation and dessication would be likely. This differs from the summer dollar spot study in which several hours of UV radiation and dessication could reduce the applied population.

Tx-1 provided a similar level of control as the fungicide mancozeb when it was applied at recommended rates. If future research in varying conditions confirms the effectiveness of Tx-1 in managing pink snow mold, the use of such fungicides may be reduced.

Many questions related to how Tx-1 controls pink snow mold still exist. Several possibilities might explain how Tx-1 is working. It is not known if Tx-1 remains active

throughout the season under snow cover to prevent infection when the pathogen is most active. Studies on *M. nivale* may reveal if the conidia it produces are prevented from germinating by a fungistasis-type interaction with Tx-1, or if Tx-1 kills the pathogen prior to its infection of turf. Because of the few Tx-1 applications made and low concentration used, it is possible that the bacteria affected the pathogen at a time crucial to the development of the pathogen. Once this is better understood, the use and application timing of Tx-1 may be optimized.

Chapter 5

ANTIBIOTIC PRODUCTION OF PSEUDOMONAS AUREOFACIENS

Introduction

Pseudomonas aureofaciens has the ability to control plant pathogenic organisms through production of several antibiotics. The first antibiotics reported were the phenazines in 1956 by both Haynes and Cluver (16)(20). Pierson stated the most important factors to determine the success of a biological control organism is in its ability to disseminate and survive on the host, and for the organism to inhibit the pathogens ability to cause disease. The primary factor responsible for *P. aureofaciens* control ability is its production of antibiotics (15). Much research has been done to determine how antibiotics are produced and regulated by *P. aureofaciens*. Work by Weller and Thomashow showed that production of phenazines PCA was responsible for control by the organism. Similar work has shown the antibiotics phloroglucinol and pyrrolnitrin also to be produced and responsible for inhibition (36). MS research by Powell identified the antibiotic PCA to be produced by strain Tx-1 and correlated it to control in the field. Powell was able to apply purified PCA from Tx-1 to the field as a fungicide and obtained significant control of dollar spot (35). Thomashow and Weller found that a mutant of *P. aureofaciens* deficient in PCA production to be non-inhibiting compared to the PCA producing parent strain 30-84 (42). Most work on this organism has focused on its production and regulation of the phenazine PCA.

The objective of this study was to determine the importance of PCA by creating a mutant deficient in PCA production. This mutant was evaluated in the field for control of

dollar spot and also was used for identification of other antibiotics produced by it. In creating such a mutant, we could determine if other factors or antibiotics influence control by *P. aureofaciens*. Understanding the effect of individual antibiotics may lead to optimizing antibiotic production of these organisms. In this study a PCA$^-$ mutant strain was effective in the field and the antibiotic responsible for control was identified as pyrrolnitrin.

Materials and Methods

Mutagenesis. A random transposon mutagenesis was performed on the wild type Tx-1 to obtain a PCA$^-$ mutant. The donor strain, *E.coli* carrying a mini-Tn5 transposon, was resistant to the antibiotic's kanamycin (Km) and ampicilin (Ap) and grown on Luria-Bertani (LB) agar amended with 50 *u*g/ml of Km and 100 *u*g/ml Ap. The recipient, *P. aureofaciens* strain Tx-1, was resistant to rifampicin (Rif) and grown on LB plates amended with 100 *u*g/ml Rif. Both cultures were grown in amended LB broth shake cultures overnight at 30° C. Bacteria were collected by centrifugation, and rinsed with 10mM $MgCl_2$ buffer. The two cultures were mixed onto a LB plate and allowed to grow overnight. The culture was resuspended in buffer and spread onto LB/Rif/Km plates to select for transconjugants of *P. aureofaciens*. Individual colonies were bioassayed against *Sclerotinia homoeocarpa*. Mutants with any change in color or control ability were chosen for further assay. Mutant Tn-1A was selected for use in *in vitro* bioassays, field studies, and antibiotic identification. After mutagenesis, Tn-1A had lost the characteristic orange pigment produced by Tx-1 and also inhibited *S. homoeocarpa* differently then

Tx-1. Tn-1A was verified as *P. aureofaciens* by FAME (Fatty Acid Methyl Esterase) analysis performed by Microcheck, Inc. (Northfield Falls, VT).

Plate Bioassay. Tx-1 was compared to Tn-1A in a plate bioassay. A sterile loop was used to streak a loopful of each culture lengthwise onto Potato Dextrose Agar (PDA) Petri plates (Difco, Inc. Detroit MI). On both sides of each culture, a fungal plug of *S. homoeocarpa* was placed to challenge the inhibitory action of the culture. The bioassay used two growth times of 0 and 24 hours of bacterial growth before the fungal plugs were added. Mycelial growth of the fungus towards the bacteria was measured 5 days after each group of fungal plugs were added. Eight replications of each bacterium were measured. Growth measurements were analyzed using SAS to separate means by least significant differences.

Field Study. Tn-1A was used in the 1997 and 1998 Dollar spot Field Study. All materials, methods, and statistical analysis used, were the same as described in pages 17 and 18, chapter 2, of this text.

Identification of Compound from Tn-1A

General Experimental Procedures. ^{1}H (proton) and ^{13}C (carbon) NMR (nuclear magnetic resonance) and DEPT spectra were recorded on a Varian INOVA 300 MHz spectrometer. ^{1}H NMR spectra were recorded at 300 MHz, while ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts were recorded in $CDCL_3$ and the values are in δ (ppm) on the basis of the δ residual of $CHCL_3$, 7.24, and $CDCL_3$, 77.0. Coupling constraints, *J*, are in hertz. The silica gel used for VLC was Merck Silica gel 60 (35-70 *u*m particle size). TLC plates (GF Uniplate, Analtech, Inc. Newark DE), after developing

were viewed under UV light (254 and 366 nm). All organic solvents used were ACS reagent grade (Aldrich Chemical Co., Milwaukee, WI) (36).

Fermentation. Seed cultures of Tn-1A were grown in TSB (Tryptic Soy Broth, Difco, Inc., Detroit, MI). Batch fermentation was conducted using 2 L buffered flasks containing 500 ml of autoclaved TSB. Each flask was innoculated with 5 ml seed and grown in a 27° C growth chamber for 5 days, in a 100 rpm shake culture at in the dark.

Extraction and Isolation. Fermentation broth was centrifuged on a Sorval Instruments RC 5C centrifuge at 10,000 rpm, 4 °C, for 10 minutes. Pellets were collected and added to 500 ml of a 4:1 $CHCl_3$: MEOH solution. The solution was homogenized at 2,000 rpm for 15 minutes; the chloroform soluble active fraction was separated using a separatory funnel. The chloroform fraction was dried by rotoevaporation and dessicated overnight. The dried extract was stored at –20 C. This extraction procedure yielded ~2 g of crude extract/13 L of fermentation broth. Fractionation of this extract (20.0 g) was carried out by vacuum liquid chromatography (VLC). Silica gel (240 g) was placed in a sintered glass funnel (600 mL, 10-15 *u*m mesh), hexane with increasing amounts of ethyl acetate and lastly methanol was used as the eluting solvents. Five fractions, 1-5, were collected: (1) 100% hexane, 1.05 g; (2) hexane-ethyl acetate, 8:1, 450 mg; (3) hexane-ethyl acetate, 4:1, 540 mg; (4) hexane-ethyl acetate, 1:1, 560 mg; (5) 100% methanol, 7.87 g. Fraction 3 was biologically active at 250 ppm, and inhibited all growth of *S. homoeocarpa* after seven days.

Fraction 3 was purified by prep. thin layer chromatography, (PTLC), and further purified by high pressure liquid chromatography (HPLC) using a Jai LC-20 preperative liquid chromatograph on two Jaigel S-343-15 ODS columns in series (i.d. 20 x 250 mm),

eluted with MeOH (70%) H_2O (30%) at a flow rate of 3 mL/min, and detected using UV of 210 nm. This yielded compound 1 (42 mg). Compound 1 was identified by ^1H NMR, ^{13}C NMR, and mass spectral experiments.

Compound 1. ^1H NMR: δ 6.78 (1H, m, H-2), 6.81 (1H, m, H-2), 7.42 (2H, m, H-4' and H-6') 7.51 (1H, m, H-5') 8.34 (1H, bs, exchangeable with D_2O, -NH). ^{13}C NMR: δ 111.70 (C-3), 115.24 (C-4), 116.45 (C-5)*, 117.21 (C-2)*, 124.77 (C-1'), 127.65 (C-3'), 128.55 (C-6')*, 129.30 (C-2'), 130.12 (C-4')*, 130.35 (C-5')*; * interchangeable. EI-MS: m/z 256 (M^+), 229, 210, 201, 193, 183, 175, 166, 148, 140, 138, 113, 102, 87, 75, 63, 55. Compound 1 was identified as pyrrolnitrin, $C_{10}H_6Cl_2N_2O_2$, by comparison of its ^1H, ^{13}C NMR, and mass spectral data which corresponded to literature values (5)(11)(18)(19).

Results

The plate bioassay comparing Tx-1 to Tn-1A showed no significant difference in control when bacteria were streaked onto plates at the same time fungal plugs were added. Tn-1A provided significantly greater control than Tx-1 when the bacteria were allowed to grow for 24 hours before adding the fungal plug Table 2.

Table 2. Plate bioassay, comparing Tx-1 to Tn-1A, grown for two time periods. Numeric numbers represent mean growth in cm, and letter symbols separate means at P=.05.

Time	Tx-1	Tn-1A
0 hours	8.96 A	8.09 A
24 hours	3.25 B	0.84 A

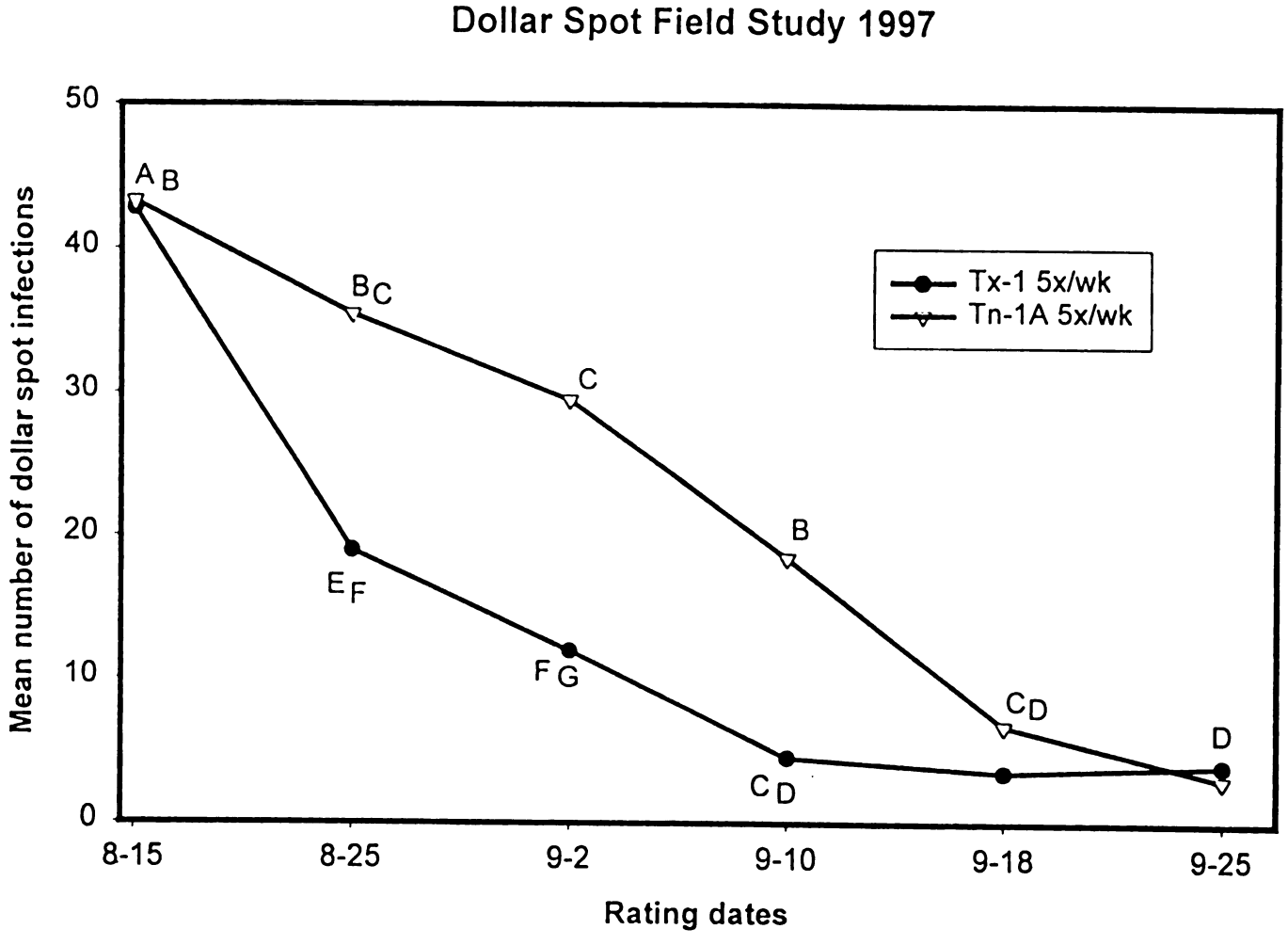

Figure 10. Comparrison of Tx-1 to Tn-1A in 1997
Symbols followed by the same letter are not significantly different at a given rating date at the 5% level

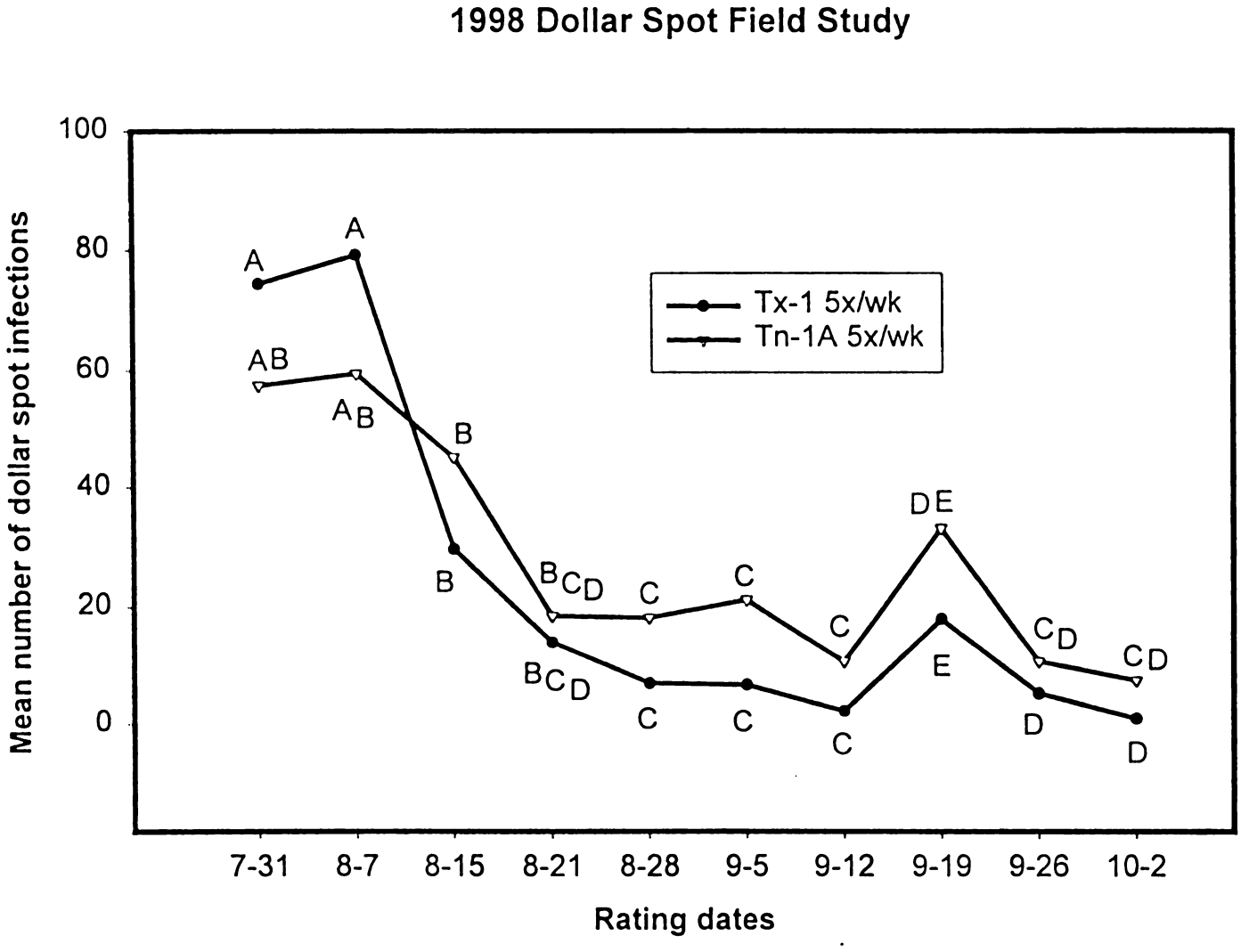

Figure 11. Comparrison of Tx-1 to Tn-1A in 1998

Symbols followed by the same letter are not significantly different at a given rating date at the 5% level

After two weeks, there was a noted difference in mycelial development. The mycelia on the Tx-1 plates appeared thin and wispy, in comparison to the normally developed mycelia on the Tn-1A plates. The *S. homoeocarpa* developed normally as noted by the stromatized tissue in the agar, behind the Tn-1A zone of inhibition. The agar in the Tx-1 plates had an orange pigmentation as compared to Tn-1A that remained clear.

The two-year field study showed significant control of dollar spot by Tn-1A as compared to the control. In 1997, Tn-1A provided significantly less control than Tx-1 in the first three ratings, (Figure 10). The last two ratings were similar. In 1998, there was no significant difference between the two treatments at any time point throughout the study, Figure 11.

Tn-1A was extracted to identify all active compounds produced by it. The primary antibiotic produced by strain Tx-1 is PCA. PCA was not produced by strain Tn-1A. A total of 42 mg of pyrrolnitrin was extracted from 20 g of crude Tn-1A extract. In plate bioassays, 250 ppm of purified pyrrolnitrin challenging a plug of *S. homoeocarpa*, completely inhibited all growth for two weeks. There after, the mycelia from the plug began to grow outward slowly.

Discussion

This study sought to identify if PCA was the only active compound or mechanism responsible for control of dollar spot by *P. aureofaciens* strain Tx-1. This question was answered by creation of a PCA$^-$ mutant for use in field and bioassay studies.

Results from the plate bioassay showed Tn-1A controls *S. homoeocarpa* similarly to Tx-1. When given 24 hours to grow, Tn-1A was more effective than Tx-1. This may

have been due to a difference in time needed for antibiotic production of each strain. The mycelium of *S. homoeocarpa* developed normally behind the zone of inhibition on the Tn-1A plates, while mycelia ceased to develop on Tx-1 plates. Normal development was evident by the production of stromatized tissue. This indicates the antibiotic from Tn-1A only inhibited growth rather than killing the organism. The type of inhibition seen correlates to literature describing pyrrolnitrin as inhibiting growth rather than killing the target organism (33).

The field studies showed greater control at some rating dates by Tx-1 in 1997 than Tn-1A, and no difference occurred between treatments in 1998. Although not always significant, Tx-1 plots maintained fewer dollar spot infections than Tn-1A. This may be due to two factors. One is that the ecological competency and survivability in the field is often reduced by manipulations to an organism (24). Another reason is in the time required of each specific antibiotic being produced. PCA production is dependent upon cell density, and PCA extracts can be produced and detected on TLC plates after a fermentation period of 24 hours. It is said that Pyrrolnitrin is produced during starvation and late into stationary growth phase. Our work showed optimal and maximum quantities of pyrrolnitrin, being produced from five days of fermentation. Since both cultures were grown for 20-24 hours, Tx-1 may have had an advantage in quantity of antibiotic produced daily.

Thin layer chromatography verified that Tn-1A did not produce PCA however it is not known if Tx-1 is producing pyrrolnitrin. Another unknown is if pyrrolnitrin production by Tn-1A has been increased in quantity as compared to quantities possibly produced by Tx-1. Work by Rodriguez and Pfender stated that an increase in one

antibiotic may cause a decrease or increase of other antibiotics (37). Salcher and Lingens used mutagenesis to produce a mutant of *P. aureofaciens* that had a 30-fold increase in pyrrolnitrin production (38). Our mutagenesis may also have affected normal pyrrolnitrin production.

In understanding the role of various antibiotics produced by *P. aureofaciens*, future research may fully utilize this organism for control of plant pathogens to improve plant health. The results of this study are the first to show that a mutant of Tx-1 producing only pyrrolnitrin was suppressive to dollar spot in the field and similar to a PCA-producing Tx-1.

Chapter 6

EPILOGUE

The four individual projects in my thesis were conducted to answer questions regarding the biological control organism *Pseudomonas aureofaciens* strain Tx-1. The answers to the questions posed by this research were used to increase the efficiency of Tx-1 as a biological control of diseases on Turf.

The dollar spot field study showed Tx-1 to be effective at controlling dollar spot on fairway height annual bluegrass. Since it will be used on golf courses, and applied at lower concentrations, it must be determined how to make such applications effective. One possible project would involve applying low rates of Tx-1 at night or closer to the time when the sun sets. This would show whether lower concentrations could be effective by increasing the likelihood of lower mortality from UV radiation and dessication. Another aspect to examine utilization of lower concentrations would be to apply Tx-1 preventively, before a large epidemic arises. In this study Tx-1 was applied after a large initial infection had occurred. Perhaps less initial disease pressure would allow greater sustained control when applying lower concentrations of Tx-1.

The use of Tx-1 for control of pink snow mold was the first report of control of this organism in the field. The effectiveness of the 10^4 CFU/cm^2 application rate of Tx-1 is very promising for turf managers because such concentrations are attainable using the current fermentation system. It may be possible that even lower concentrations of Tx-1 are also effective. Future research should determine when control is occurring. For instance, is Tx-1 actively inhibiting the pathogen during the season, or is precise

application timings inhibiting conidia before infection or normal growth of the fungi can occur? Work could also be done to determine if Tx-1 could control outbreaks in the late spring.

The persistence of Tx-1 in a turfgrass system has been shown. This is vital to rebuilding a population capable of control in the spring or beginning of a new disease epidemic. I believe the greatest applicability of this research will come in future studies to determine threshold Tx-1 populations necessary for control of different turfgrass pathogens. It is likely that specific population sizes must be achieved in different regions to control specific diseases. Sampling to determine populations present will allow for a correlation of control or the lack of when population requirements are not met. Another important study could be to determine the factor(s) most responsible for the mortality of Tx-1 over time. To do so may require a determination of population limit or carrying capacities of Tx-1 in turf. One aspect of the plating technique used for recovery and enumeration is that it gives only an estimate of recoverable viable bacteria. This estimate is not the total bacterial population. Modern molecular techniques using selective markers such as *lacZY* have been widely used for quantifying recovery of bacterial populations and perhaps such methods would prove more efficient or accurate.

The project involving mutant strain Tn-1A was important in demonstrating an antibiotic other than PCA can effectively control disease in the field. Many studies could be conducted to understand the genetics of this mutant as it compares to the wild type strain. It is not known how PCA production was disturbed from mutagenesis and what mechanisms if any have changed in the pathway regulating biosynthesis of pyrrolnitrin. Understanding these pathways could lead to means of overexpressing specific antibiotic

production. The evolutionary role of multiple antibiotic production may be linked to nutrient availability in the environment over time. Some antibiotics inhibit specific fungal pathogens differently or better than others. It would be interesting to determine if *Pseudomonas aureofaciens* has developed separate antibiotics to compete with specific groups of organisms within the microbial community, and at different times throughout the year.

The use of *Pseudomonas aureofaciens* as a biological control in the field is still in its infancy of understanding and optimally utilizing the naturally suppressive properties it possesses. Future research will expand the knowledge needed to use this and other microbes for protection of plants from phytopathogenic organisms.

APPENDICES

Table 3. Treatment means of dollar spot occurrence in 1997.

Treatments	8/15	8/25	9/2	9/10	9/18	9/25
Tx-1 10^7x 5/wk	42.75 AB[1] c[2]	19 EF b	12 FG bc	4.5 CD c	3.5 CD c	4 D c
Tx-1 10^7x 1/wk	43.75 A c	62.25 A a	50.75 B bc	56 A ab	30.5 B d	45.24 B c
Control	37 AB c	59.75 A ab	61.5 A a	63.25 A a	52.5 A b	63 A a
Daconil Ultrex 3.8 oz/1000ft^2 + Tx-1 10^7x 5/wk	36.75 AB a	24.25 DEF b	2.5 H c	0.75 D c	0.5 D c	2 D c
Daconil Ultrex 3.8 oz/1000ft^2	37 AB ab	32.5 BCD	12.75 F c	6.5 CD c	12 C c	42.75 B a
Banner 2 oz/1000ft^2 + Tx-1 10^7x 5/wk	41.5 AB a	28 CD b	3.75 GH c	0.5 D c	0.25 D c	0.5 D c
Banner 2 oz/1000ft^2	42 AB a	40.75 B a	18.25 DEF b	9 CD c	5.5 CD c	22 C b
Tx-1 10^7x 5/wk 8 hr. growth	35.25 AB	17.25 F b	15 EF b	2.5 D c	2.75 D c	1.25 D c
Tx-1 10^7x 5/wk Heat killed	34.75 B a	34.75 BC a	25 CD b	12.5 BC c	4 D c	4.25 D c
Tn-1A 10^7x 5/wk Mutant	43.25 AB a	35.5 BC ab	29.5 C b	18.5 B c	6.75 CD d	3 D d

[1] Comparison of treatments in columns at individual time points. Treatments sharing the same uppercase letter are not significantly different at P= 0.05.

[2] Individual treatments compared within rows over time. Treatments sharing the same lowercase letter are not significantly different at P= 0.05.

Table 4. Treatment means of dollar spot occurrence in 1998.

Treatments	7/31	8/7	8/15	8/21	8/28	9/5	9/12	9/19	9/26	10/2
Tx-1 10^7x 5/wk	74.5 A[1] a[2]	79.25 A a	29.75 B b	14 BCD bc	7 C c	6.75 C c	2.25 C c	18 E bc	5.25 D c	1 D c
Tx-1 10^7x 1/wk	55.25 AB bc	46.75 B bcd	39.75 B cd	35 B cd	48.25 AB bcd	78 A a	66.75 A ab	82.5 C a	40.5 B cd	33.5 B d
Control	47.75 B d	58.75 AB cd	81.25 A b	69.25 A bc	57.5 AB cd	83.25 A b	74.25 A bc	109.25 B a	69.75 A bc	55.25 A cd
Banner 0.5 oz/1000ft^2+ Tx-1 10^5x 5/wk	59.75 A bc	45.5 B cde	40.25 B cde	34 B de	51.75 AB cd	81 A a	77.25 A ab	54 D cd	29.5 BC e	25.25 BC e
Banner 0.5 oz/1000ft^2	74.5 A a	56.75 B abc	39.25 B cde	29.5 BC de	43.5 B bcde	77.25 A a	61 A ab	50 D bcd	34.25 B de	28 BC e
Banner 2 oz/1000ft^2+ Tx-1 10^7x 5/wk	72.25 A a	49.24 B b	3 C cd	0.75 D d	0.75 C d	1.22 C d	2.2 C cd	23.4 E c	7.35 D cd	3.42 D cd
Banner 2 oz/1000ft^2	69.5 A b	64.25 AB bc	7.75 C cd	4 D e	14.5 C e	46.25 B cd	39 B d	131.25 A a	74 A b	65.75 A bc
Tx-1 10^5x 5/wk	66 AB bc	79.25 A ab	93.75 A a	82 A a	66 A bc	87.75 A a	69.5 A b	76.25 C ab	46.5 B cd	37.5 B d
Tx-1 10^7x 5/wk Heat killed	48 B a	53.75 B a	40.75 B ab	11.5 CD c	5.5 C c	7 C c	4.75 C c	20.25 E bc	5.5 D c	3.5 D c
Tn-1A 10^7x 5/wk Mutant	57.5 AB a	59.5 AB a	45.25 B ab	18.5 BCD cd	18.25 C cd	21.25 C cd	10.75 C d	33.25 DE bc	10.75 CD d	7.5 CD d

[1] Comparison of treatments in columns at individual time points. Treatments sharing the same uppercase letter are not significantly different at P= 0.05.

[2] Individual treatments compared within rows over time. Treatments sharing the same lowercase letter are not significantly different at P= 0.05.

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