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FOR THE MANAGEMENT OF FUNGAL TURFGRASS DISEASES

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UTILIZATION OF BACTERIAL METABOLITES FOR THE MANAGEMENT OF FUNGAL TURFGRASS PATHOGENS

By

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ABSTRACT

UTILIZATION OF BACTERIAL METABOLITES
FOR THE MANAGEMENT OF FUNGAL TURFGRASS PATHOGENS

By

Jon Frederick Powell

Two strains of *Pseudomonas aureofaciens*, Tx-1 and Tx-2, were isolated from a turfgrass sample and exhibited strong antifungal activity toward the fungal turfgrass pathogens *Sclerotinia homoeocarpa* and *Magnaporthe poae* in *in vitro* bioassays. Application of the live bacteria as biological control agents for dollar spot disease failed to provide disease management. A single antibiotic was isolated which proved inhibitory to turfgrass fungal pathogens in laboratory bioassays. Preliminary studies of this compound by thin layer chromatography and solubility suggested that it is structurally different than other antibiotics reported from *P. aureofaciens*. Minimum inhibitory concentrations of this antibiotic to many pathogenic turfgrass fungi were between 10 to 25 μg/ml. Greenhouse evaluation proved the antibiotic to be a potential chemical treatment for the management of dollar spot. Field evaluation of the antibiotic resulted in significant reductions in the incidence of dollar spot disease on treated turf.

To my parents
Fred and Jo Powell,
for all they have done for me.

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INTRODUCTION

Techniques currently employed for the management of fungal turfgrass diseases are highly dependent on the application of chemical fungicides. A broad array of chemical fungicides are currently available for the management of these fungal pathogens. However, the development of resistance to certain classes of chemical fungicides and the failure of agrochemical companies to re-register currently used chemical fungicides with the EPA due environmental concerns have promoted research into identification of novel chemical fungicides as well as research into alternative disease management strategies. The two major alternatives to the use of chemical fungicides being examined are biological controls and organic or composted fertilizers.

Development of new chemical fungicides generally occurs through the mass screening of novel synthetic compounds. Utilization of antifungal compounds produced by microbial organisms, such as antibiotics, have been highly exploited in the development of medicinal compounds. Application of medicinal antibiotics for the management of plant diseases has been restricted due to concerns of the development of resistance to these compounds by potential human pathogens.

This study was conducted to examine the potential for applying bacterial produced antifungal agents for turfgrass disease management treatments. Several bacteria have been identified as producing a variety of classes of compounds that are antifungal in nature including enzymes, siderophores, hydrogen cyanide, ethylene, and antibiotics. Although all of these compounds have been implicated in biological control activity by bacteria, the commercial application of enzymes for plant disease management is not likely due to their sensitivity to environmental conditions. Another class of compounds that would not be feasible for study are volatile compounds such as hydrogen cyanide and ethylene. Optimally, this research will be able to identify a novel antibiotics that may have potential for commercial application in turfgrass disease management.

The use of bacteria as biological control agents is one of the fastest growing fields of research in disease management. The concept of the management of disease through the application of soilborne bacteria is attractive due to its sensitivity to environmental concerns. However, significant breakthroughs yielding biological controls that provide consistent disease management have not yet been realized.

REVIEW OF LITERATURE

Pseudomonas aureofaciens

Pseudomonas aureofaciens is a gram negative rod shaped bacterium, possessing one or more flagella, strictly aerobic, and chemoorganotrophic. *P. aureofaciens* is included in the class of fluorescent pseudomonads and was included taxonomically as a biovar of *Pseudomonas fluorescens* by Stanier et al. (43). Inclusion of *P. aureofaciens* in the group of fluorescent pseudomonads is based on the ability of most strains to produce fluorescent pigment pyoveridin (43). The name "*aureofaciens*" literally means to "make golden" which refers to its ability to turn artificial media to a orange-gold color. This color is caused by production of nonfluorescent phenazine pigments (43). Phenazine pigments reported to be produced by *P. aureofaciens* are phenazine-1-carboxylic acid (PCA), phenazines 2-oxophenazine and 2-oxophenazine-1-carboxylic acid (60). Evidence has been presented that the role of phenazine compounds produced by *P. aureofaciens* allows for the removal of excess reducing equivalents from NADH and NADPH under substrate and/or oxygen limitations.

The utilization of antibiotics produced by microorganisms such as *Penicillium* sp (penicillin),

Streptomyces griseus (streptomycin), *Streptomyces erythraeus* (erythromycin), *Cephalosporium acremonium* (cephalosporin), and several others (41) for medical uses have been well documented. The potential for application of antibiotics as chemical treatments for plant disease has recently been explored. Application of culture filtrates of *Bacillus subtilis* provided better management of bean rust than the fungicide mancozeb (1) and protected peaches from infection by *Monilinia fructicola* (44). Melvin et al., (35) demonstrated that application of the antibiotic faeriefungin produced by *Streptomyces griseus* var. *autotrophicus* provided management of summer patch disease of turfgrass caused by *Magnaporthe poae* equal to that of the chemical fungicide fenarimol.

Management of turfgrass diseases has conventionally been conducted through the employment of proper cultural practices and the application of chemical fungicides (59). One of the more recent instruments of disease management to be examined is the utilization of bacteria and fungi as biological controls. Investigations into the application of biological controls have been conducted toward the management of the turfgrass diseases, brown patch (*Rhizoctonia solani*) (7), dollar spot (*Sclerotinia homoeocarpa*) (20, 23, 38), fairy rings (many basidiomycetes) (54), necrotic ring spot (*Leptosphaeria korrae*) (36), summer patch (*Magnaporthe poae*) (36, 58), take-all (*Gaeumannomyces graminis* (68), and typhula blight (*Typhula incarnata* and

Typhula ishikariensis) (8).

Several studies in the mid 1970's correlated fluorescent pseudomonads with the occurrence of take-all decline. Evaluation of 100 bacterial strains for specific antagonism to *G. graminis* var. *tritici* in greenhouse conditions by Cook and Rovira (12), identified 8 strains which yielded suppression greater than or equal to those of natural suppressive soils. All 8 strains were *Pseudomonas* spp., 7 of which were fluorescent. Further evaluation of bacterial populations by Cook and Rovira (12) indicated suppressive soils contained 1000 times more fluorescent pseudomonads than non-suppressive soils. Simon and Ridge (48) similarly found 100 to 1000 fold increases of fluorescent pseudomonads on roots infected with *G. graminis* than on healthy roots. Agar plate tests demonstrated that over 70% of the fluorescent pseudomonads isolated from suppressive soils were antagonistic to *G. graminis*. Increases in fluorescent pseudomonad populations have also been linked with the decline of take-all (*Gaeumannomyces graminis var. avenae*) of turfgrass (46). Species of fluorescent pseudomonads that are correlated to the development of soil suppressiveness are *P. fluorescens* (65) and *P. aureofaciens* (11).

Several mechanisms of pathogen suppression by fluorescent pseudomonads have been proposed. Competition for nutrients and colonization sites has not received recent attention but plays an important role in disease

suppression. Bacteria which are capable of using a broad array of nutrients rapidly can reduce carbon and nitrogen sources available for pathogen sporulation and colonization (66). Coupled with high metabolism is the ability to undergo rapid reproduction which increases the organisms potential for dispersal and occupation of available niches (9). *Pseudomonas* spp. act in this manner as exemplified by their non-fastidious nature (43).

The argument for disease suppression by competition emphasizes the importance of colonization in the development of specific antagonistic disease suppression. The degree to which *P. fluorescens* is able to colonize wheat root tissue can be directly correlated with a reduction in the number of root lesions caused by *G. graminis* var. *tritici* (6). Colonization of plant roots by bacteria may be divided into two stages (25). Stage 1 involves the ability of the bacterium to become attached to the plant root. Stage 2 is dependent on the bacterium's ability to compete for available nutrients. The ability of a bacterium to colonize root tissue is referred to as its rhizosphere competence. Several traits which may play a role in determining a bacterium's rhizosphere competence include surface polysaccharides, presence of flagella and/or fimbriae, chemotaxis, osmotolerance, and the ability to utilize complex carbohydrates (67).

Siderophores were the first class of metabolic compounds associated with disease suppression by fluorescent

pseudomonads (29). Siderophores are "low molecular weight, high affinity iron (III) chelators" (67). Under conditions of low iron concentrations, these yellow-green fluorescent compounds are excreted by bacteria and complex with available iron. The bacterium is able to recognize and absorb this complex through membrane receptor proteins. It is believed that siderophores sequester iron thereby making it unaccessible to pathogenic fungi. Support for this mode of antagonism has come from studies in which mutants deficient in siderophore production are less suppressive than the siderophore producing parents (2). Additional evidence has come from studies indicating that the addition of the synthetic iron chelating compound Fe ethylene-diamine-di-o-hydroxphenylacetic acid (Fe EDDA) yields disease suppression. Addition of excess iron in the form of ferric-ethylenediamine-tetraacetic acid (FeEDTA) represses siderophore production and eliminates suppressiveness (66). Antibiosis by siderophore activity has been linked to antagonism toward *Pythium* spp. (2), *Fusarium oxysporum* (18), and *G. graminis* var. *tritici* (29). Recent work by Hamdan et al., (22) involving the generation of siderophore deficient mutants indicated that siderophores have no significant effect on take-all caused by *G. graminis* var. *tritici*. Although the role of siderophores in the suppression of *G. graminis* var. *tritici* is still in contention, there is little argument regarding it's role in the suppression of *Pythium* spp. in soil.

Disease suppression of soil pathogens by *Pseudomonas* spp. has been strongly attributed to the production of antibiotics. Two antibiotics have been attributed to the inhibitory nature of fluorescent pseudomonads, 2,4-diacetylphloroglucinol (DAPG) (47) and phenazine-1-carboxylic acid (PCA) (23). Strain Q2-87 of *P. aureofaciens* which produces DAPG was identified as being suppressive to take-all. DAPG was later confirmed as being one of the sources of antifungal activity in *P. aureofaciens* Q2-87 to *G. graminis* var. *tritici* (63).

The production of PCA by *P. aureofaciens* was first identified by Haynes et al. in 1956 (23). Recent work (21) has identified PCA as playing a major role in the inhibitory activity of *P. fluorescens* against *G. graminis* var. *tritici*. Pure crystals of this compound are needle shaped and yellow to green-yellow in color. PCA was shown to be inhibitory to a broad range of fungi with minimum inhibitory concentrations (MIC) to completely prevent fungal growth ranging from 1 to 40 μg/ml on in vitro tests (21). Initial reports of the structure of PCA by Gurusiddaiah et al. (21) proposed that PCA occurred as a dimeric molecule. However, this structure of the antibiotic was later revised by Brisbane et al. (1987), who showed that the antibiotic existed in a monomeric state rather than as a dimer.

Thomashow and Weller (56) demonstrated the importance of PCA in biological suppression of take-all by *P. fluorescens* 2-79 through the generation of transposon-

induced mutants deficient in PCA production. All PCA deficient mutants were unable to inhibit *G. graminis* var. *tritici* in agar plate tests and provided significantly lower levels of disease suppression in greenhouse studies. Similar studies by Pierson and Thomashow (42) illustrated similar results with *P. aureofaciens* strain 30-84. Thomashow et al. (57) were able to quantify the production of PCA by *P. aureofaciens* and *P. fluorescens* on wheat roots grown in steamed and natural soil in the greenhouse. Concentrations of PCA detected in the steamed and natural soils were up to 578 and 133 ng/g root, respectively. PCA was also recovered from the roots of seed treated plants grown in wheat fields (5-12 ng/g root) and in virgin fields (19-27 ng/g root). These results demonstrated the production of antibiotics in the environment and also confirmed that very small amounts of antibiotics delivered to the microsite by biological control agents can be effective in disease management.

DOLLAR SPOT

Dollar spot is caused by the fungal organism *Sclerotinia homoeocarpa* F. T. Bennett (3) and is one of the most prevalent diseases of turfgrasses throughout the world (51). It is also the most economically significant disease of turfgrass in the United States and parts of Canada (20, 60). Inclusion of this organism in the genus *Sclerotinia* is currently on a provisional basis. The dollar spot organism produces a flat stroma unlike the sclerotia characteristic

of the genus *Sclerotinia*. Recent work examining the protein composition and anatomy of the stroma (30) as well as the utilization of electron microscopy and immunological comparisons (40) suggested that *Sclerotinia homoeocarpa* should not be included in the genus *Sclerotinia*. However, proper classification of this organism is not possible as the fertile teliomorph stage of the life cycle is rare or no longer exists. Reexamination of data recorded from fertile teliomorphs collected by Bennett suggested that this organism would be better classified in either the genus *Lanzia* Sacc. or *Moellerodiscus* Henn.. (52)

Symptomology

Turfgrass infected with *Sclerotinia homoeocarpa* exhibits a bleached or straw-colored appearance. On golf course putting greens and fairways dollar spot lesions appear as sunken spots less than 5 cm in diameter. Under sufficient disease pressure these spots will coalesce and form larger irregular patches. Infected blades initially appear chlorotic and water soaked which later appears as bleached or tan bands traversing the width of the blade with brown margins. Under favorable growth conditions following nights with dew formation, "cobwebs" of white fuzzy mycelium of the fungus may be seen on infected turf (52, 60).

High humidity and temperatures ranging from 15°C to 30°C are favorable for dollar spot. Dew formation is also conducive to disease development. Much of dew formed is actually guttational water produced by the grass. This

guttational water contains carbohydrates and amino acids which may be used as nutrients by the pathogen promoting it to grow and spread. Disease epidemics in Michigan occur in July followed by a second outbreak in late August and early September. The presence of these two separate epidemics may suggest that there may be more than one species of dollar spot, one of which is virulent at temperatures under 20°C, and the other is active at higher temperatures with cool nights (60). Factors which may increase disease severity include low nitrogen fertility and dry soil conditions (14). Spread of the disease occurs through hyphal growth, and the transport of infected tissue by maintenance equipment and people.

Grasses susceptible to dollar spot in cool weather climates are primarily creeping bentgrass (*Agrostis palustris* Huds.) and annual bluegrass (*Poa annua* L.) although Kentucky bluegrass (*Poa pratensis* L.), perennial ryegrass (*Lolium perenne* L.) and fescues (*Festuca* spp.) may also be infected. Susceptible grasses in warm weather climates are bermudagrass (*Cynodon dactylon* (L.) Pers.), zoysiagrasses (*Zoysia* spp.), bahaigrass (*Paspalum notatum* Flugge.), centipedegrass (*Eremochloa ophiuroides* (Munro.) Hack.), and St. Augustinegrass (*Stenotaphrum secondatum* (Walt.) Kuntze) (60).

Disease Management

Cultural practices may be implemented to reduce disease severity. Maintenance of moderate to high nitrogen

fertility will reduce disease intensity and promote plant growth and recovery. Irrigation should be used to maintain adequate soil moisture. Irrigation in the early evening should be avoided in order to decrease the duration of leaf wetness. Removal of guttational water by mechanical means or through irrigation promotes rapid drying of the turf and washes guttational nutrients from the leaf surface. Resistant cultivars of susceptible turfgrass species have not yet been identified or developed. Varieties of turfgrass highly susceptible to dollar spot should not be used.

The use of chemical fungicides as preventative and curative treatments of dollar spot are significant disease management tools. Chemicals currently recommended to manage dollar spot are the contact fungicide chlorothalonil (Daconil 2787) and the systemic fungicides propiconazole (Banner), fenarimol (Rubigan), iprodione (Chipco 26019, Proturf Fungicide VI), triadimefon (Bayleton, Proturf Fungicide VII) and vinclozolin (Curlan, Touche', Vorlan) (55).

Despite the effectiveness of chemical fungicides, *S. homoeocarpa* has developed resistance to several classes of these chemicals. Resistance to fungicides is generally characterized by reduced duration to complete failure of a fungicide to manage disease. One of the first studies of resistance to fungicides by *S. homoeocarpa* was conducted by Cole et al. (10). Their findings demonstrated the presence

of strains of dollar spot which exhibited reduced levels of sensitivity to the fungicide thiram and identified strains which were 100 times less sensitive to the fungicide cadmium succinate than sensitive strains. Warren et al. (64) reported the identification of a strain of *S. homoeocarpa* which exhibited reduced sensitivity to the contact fungicide anilazine (Dyrene). These resistant strains were isolated from golf courses where these fungicides had been used extensively.

However, most of the wide spread and economically important resistance of *S. homoeocarpa* has been to the systemic fungicides. Resistance has also arisen to the classes of systemic fungicides.Warren et al. (64) reported the first case of resistance of *S. homoeocarpa* to the benzimidazole class of fungicides (benomyl, thiobendazole, and methyl and ethyl thiophanate). These resistant strains exhibited tolerance to these fungicides over 100 times that of the sensitive strains. Due to the widespread development and persistence of resistance to this class of fungicides, they are no longer recommended for the management of dollar spot (62). The next systemic fungicides developed to manage dollar spot was iprodione, which belongs to the carboxamide class of fungicides. Detweiler et al. (17) identified a strain of *S. homoeocarpa* which showed resistance to iprodione at levels 100 times that of the sensitive strains. This iprodione resistant strain also exhibited resistance to the benzimidazole class of fungicides. The most recent

class of systemic fungicides released to manage dollar spot are the demethylase inhibitors (DMI) which include the fungicides triadimefon, fenarimol, and propiconazol. Although resistance has been slow to develop, reduced sensitivity has been identified (62). All fifteen isolates resistant to the DMI fungicides examined have shown resistance to the benzimidazole class of fungicides with two of the isolates showing reduced sensitivity to iprodione. With resistance or reduced sensitivity to all three classes of systemic fungicides used on turf the only effective treatment for these strains remains the use of the contact fungicide chlorothalonil.

One alternative to chemical fungicides currently being examined is the utilization of organic sources of nitrogenous fertilizers for dollar spot disease management. Cook et al. (13) noted differences in the incidence of dollar spot with the use of different nitrogen sources. In these tests an organic form of nitrogen, activated sewage sludge, significantly reduced disease incidence in relation to urea, ammonium sulfate, and ammonium nitrate. The effectiveness of activated sewage sludge in reducing dollar spot incidence was supported by Markland et al. (34) in a study comparing 7 different nitrogen sources. Examination of several compost and organic fertilizers for suppression of dollar spot was conducted by Nelson and Craft (39). In this study fertilizers were applied as topdressings mixed with 70% sand. Two organic fertilizers (plant and animal

meal) tested provided disease management significantly equal to that provided by application of the chemical fungicide propiconazole. Suppression of disease was effective for 30 days after application.

Research into alternative management strategies of dollar spot has also been directed toward the application of biological controls. One of the initial cases of biological control of dollar spot involved application of the fungus *Gliocladium virens*, which has been used in greenhouse conditions to reduce damping off by *Pythium* spp. and *Rhizoctonia* spp. (23). Bi-weekly applications in the form of alginate pellets of *G. virens* spores resulted in reductions of disease severity ranging from 46% to 70% (23). Goodman and Burpee (20), reported significant reductions in disease intensity with top-dressing applications of a sand-cornmeal mixture amended with a fungal strain of *Fusarium heterosporum*, which was isolated from a dollar spot lesion on fine-leaved fescue (*Festuca* sp.). Significant disease reduction was evident with applications at four week intervals. Topdressing applications at one week intervals resulted in 86% to 93% reductions in disease intensities. The mode of antagonism expressed by this organism is likely to be based on the production of fungi-toxic metabolites. Nelson and Craft (38), noted significant reductions in dollar spot intensity with topdressing applications of sand-cornmeal amended with strains of the bacterium *Enterobacter cloacae*. As preventative treatments applied at a 30 day

interval, the bacterial topdressing provided a 63% reduction in disease severity which was statistically as effective as the fungicide propiconazole. Application of the bacterial amended topdressing on a curative basis was as effective as the fungicide iprodione in reducing disease severity 12 days after application. The mode of dollar spot suppression by *E. cloacae* is not understood, but may be related to the ability of the bacterium to parasitize the fungus through adherence to fungal hyphae (38).

SUMMER PATCH

Summer patch is a disease of the cool season turfgrasses Kentucky bluegrass, *Poa pratensis,* and annual bluegrass, *Poa annua* (33). The symptoms induced by this disease and necrotic ring spot were originally known as Fusarium blight (15) and later as Fusarium blight syndrome (49). Smiley and Craven Fowler (50) later identified two fungal organisms associated with Fusarium blight syndrome; *Phialophora graminicola* (Deacon) Walker and *Leptosphaeria korrae* Walker & Smith. Disease caused by the fungus *L. korrae* Walker & Smith, was renamed to necrotic ring spot by Worf et al. (69) while disease arising from *P. graminicola* (Deacon) Walker was labeled as summer patch by Smiley (49). Reexamination of the isolates identified as *P. graminicola* (Deacon) Walker by Landschoot (31) indicated that this organism was actually *Magnaporthe poae* Landschoot & Jackson. A comparison of the pathogenicity of fungi with *Phialophora*

anamorphs isolated from turf exhibiting the symptoms of summer patch by Landschoot and Jackson (33) included the organisms *M. poae*, *P. graminicola*, *Gaeumannomyces incrustans*, and *Gaeumannomyces cylindrosporus*. *M. poae* was the most pathogenic of these organisms on *P. pratensis* and *P. annua*, although the other species also reduced turf quality to lesser degrees. Field inoculation of *Poa pratensis* with the test organisms resulted in the formation of summer patch symptoms in the plots inoculated with *M. poae* while no symptoms developed in the other plots. Koch's Postulates were completed by reisolating *M. poae* from the infected turf, thereby proving *M. poae* as being the causal agent of summer patch.

Symptomology

Infection of susceptible plants by *M. poae* occurs by means of a hyphopodium which colonize tissue in the form of hyaline infection hyphae (32). *M. poae* becomes infectious in the spring when soil temperatures reach 18° C to 20° C. Optimal growth of the organism is favored by high soil temperatures and soils with high water potentials (26). Symptoms of summer patch first appear in early-to-mid summer and are induced by hot weather (30° to 35°C) accompanied by heavy rains (27, 51). The disease may be spread by the use of infected sod and by contaminated aerification and dethatching equipment (52).

Leaves of infected Kentucky bluegrass plants initially wilt, turning dark green in color. If continued heat stress

occurs the foliage will change to a bronze or red-brown color prior to becoming straw colored and dying. Patches of infected turf may range from 3 cm to 60 cm in diameter. Older patches may have a frog-eyed appearance through recolonization of the Kentucky bluegrass or through colonization of infected patches by resistant turf species (bentgrasses {*Agrostis* spp.}, ryegrasses {*Lolium* spp.}, and fescues {*Festuca* spp.}). Diagnostic characteristics of infected and dying plants are the appearance of roots, rhizomes, and crowns that are dark brown in color. Dark brown runner hyphae may be seen surrounding the root and sheath of the plant as well as inside infected tissues (32).

Management

Several cultural management practices may be employed in order to reduce the severity of summer patch. Irrigation should be applied when the first signs of stress to plants becomes evident (28). However, high soil moisture should be avoided as it promotes the growth of the pathogen *M. poae* (26). Application of daily light irrigation (syringing) during hot and dry weather helps cool infected plants to reduce heat stress. Maintenance of turf below recommended heights should also be avoided to reduce plant stress (52). Davis and Dernoeden (16) showed that maintenance of Kentucky bluegrass at 7.6 cm rather than 3.6 cm height significantly reduced severity of summer patch. Also shown in this study was that the soil temperature of turf maintained at the 7.6 cm height was between 1.3° to 1.6°C lower than turf

maintained at 3.6 cm, thereby reducing heat stress. Use of slow release fertilizers such as sulfur coated urea is also recommended as they are associated with lower disease intensities in comparison to quick release fertilizers such as urea which may increase disease severity (16). Conversion of affected turf areas to resistant species (bentgrasses {*Agrostis* spp.}, ryegrasses {*Lolium* spp.}, and fescues {*Festuca* spp.}) may also reduce disease expression.

Application of chemical fungicides is a common management practice (55). Preventative management may be obtained through applications of fenarimol, triadimefon, or propiconazole when soil temperature reaches 18°C at 5 cm depth (61). After disease symptoms have developed, curative treatments with benomyl or thiophanate-M are effective.

Little work has been done regarding the use of natural products and biological controls for summer patch management. Thompson and Clarke (58), evaluated eighteen strains of bacteria from the genera *Pseudomonas*, *Enterobacter*, and *Bacillus* as potential biological control agents for summer patch. Of those tested, five strains were effective in reducing disease severity by 25 to 36 % in field studies.

CHAPTER I

MANAGEMENT OF TURFGRASS PATHOGENS WITH BACTERIAL METABOLITES

SECTION I

MICROBIAL METABOLITE SCREENING

INTRODUCTION

Preliminary examination of soilborne bacteria for biological control of turfgrass disease and analysis of their antifungal metabolites was conducted on four strains of bacteria. These bacteria were chosen for study based on their capability to produce large zones of inhibition to fungal turfgrass pathogens when screened in antibiosis plate assays. The presence of large zones of inhibition suggested that the bacteria produces a metabolite which was able to diffuse through agar media and inhibit the growth of test organisms.

Two of these bacterial isolates, Tx-1 and Tx-2, were isolated by A. R. Detweiler, from a turfgrass sample received at the Michigan State University Turfgrass Pathology Laboratory. Identification of these bacterial strains by cellular fatty acid analysis conducted by Microcheck, Inc., Northfield, VT (April, 1990) reported a "similarity index" of 0.63 - 0.69 of Tx-2 to the type strain of *Pseudomonas aureofaciens*. A similarity index between 0.5-0.99 normally indicates a positive identification of the species. Bacterial strain Tx-1 was identified as *P. aureofaciens* with a "similarity index" of 0.216. "Similarity indexes" between 0.1 - 0.3 is considered as an

incomplete cellular fatty acid match at the species level with any of the 7000 strains of bacteria in their database.

The other two bacteria examined were isolated from the Hancock Turfgrass Research Center, East Lansing, MI by B. P. Melvin (36) from thatch and soil of turfgrass recovering from necrotic ring spot. These bacteria were identified as *P. aureofaciens* strain IV-7-T and *Bacillus pumilus* strain VI-6-S.

The purpose of this experiment was to compare the biological activity of fermentation broths of the four antagonistic bacterial isolates. The bacterial isolate(s) which exhibited the greatest activity would then be tested as a potential biological control agent against dollar spot and summer patch.

MATERIALS AND METHODS

Preparation of Bacterial Cultures

Bacterial cultures were grown in 100 ml of YMG broth media (0.4 g yeast extract, 1.0 g malt extract, and 0.4 g anhydrous dextrose per 100 ml distilled water) at 26° C on a rotary shaker at 100 rpm. 500 ml erlenmeyer flasks with four baffles were used to improve culture medium aeration. Cultures were seeded by the transfer of a single colony from a nutrient agar plate streaked 48 hours prior to seeding and incubated at 26° C. The cultures were incubated for seven days prior to further use.

Processing of the Culture Broth

After seven days of growth, cultures were centrifuged

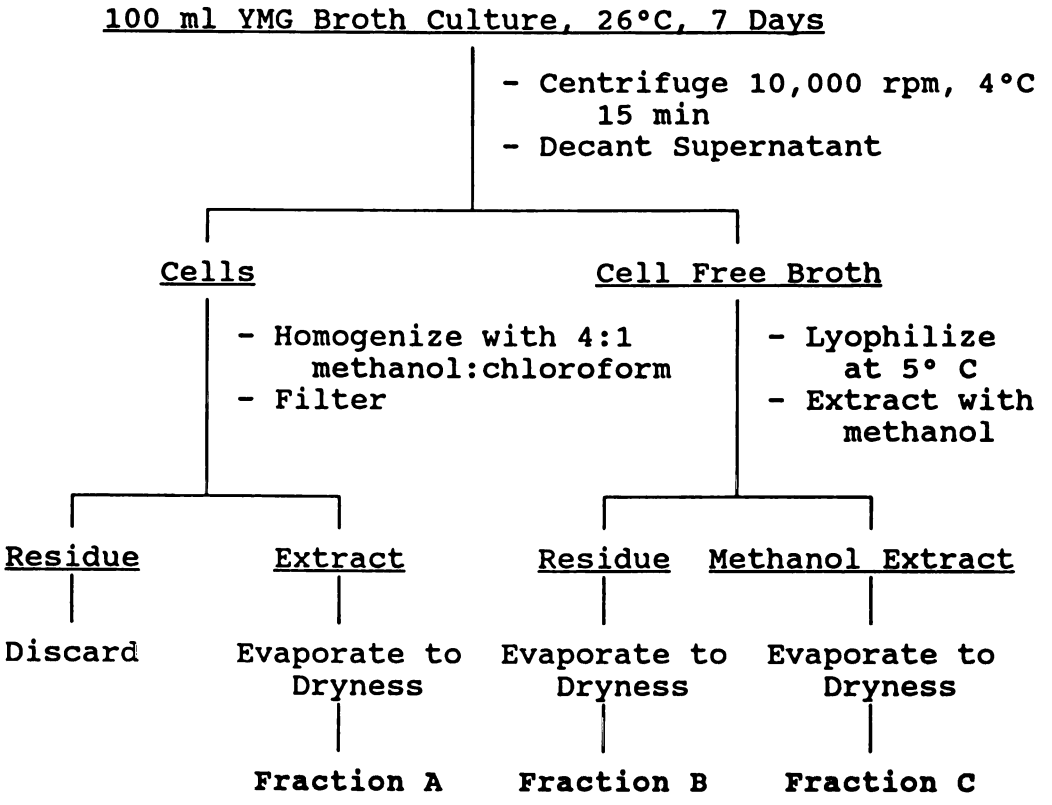
in a Sorvall centrifuge at 10,000 rpm at 4°C for 15 min. The cell free broth (supernatant) was decanted into large weigh boats and lyophilized in a Dura Top Bulk Tray Dryer (FTS Systems Inc) for 24 h at 5°C. The resulting dried powder was extracted with 100 ml of methanol through a 60 ml (10-15 Å) fritted funnel (medium). Methanol insoluble residue was dried overnight in a desiccator. Methanol soluble fraction was evaporated to dryness using a rotary evaporator at 40°C and dried overnight in a desiccator.

The packed cells resulting from centrifugation were homogenized with a mixture of 100 ml methanol and 25 ml chloroform for 2 min at 80% maximum power in a Kinematica GmbH tissue grinder (attached to a 120 volt variable autotransformer). Methanol/chloroform solvent was separated from insoluble cell matter by filtration through a fine (4.5-5 Å) fritted funnel. Insoluble cell mass was discarded and the methanol/chloroform extract was evaporated to dryness using rotary evaporation at 40°C and further dried in a desiccator for 24 h. An outline of the extraction procedure is provided in Figure 1.

Antifungal Assays of Fractions A-C

Test organisms used for this screening were *Bipolaris sorokiniana*, *Magnaporthe poae*, and *Sclerotinia homoeocarpa*. *B. sorokiniana* spores were cultured on Potato Dextrose Agar (PDA) made with (Difco) 39 g/l distilled water. The plates were incubated under light for a period of two weeks at 22° C. The spores were harvested by adding 10 ml of sterile

Figure 1. Schematic for the procedure used to prepare bacterial extract fractions.



saline solution (8.5%) and suspending the spores by gently rubbing with a bent glass rod. Spore concentration was determined by using a Levy-Hauser hemocytometer. A stock solution of 10,000 spores per ml was prepared by serial dilution. *B. sorokiniana* assay plates were conducted by spreading 0.1 ml of the stock spore solution on a 100 mm diameter petri dish of PDA.

Cultures of *M. poae* and *S. homoeocarpa* were grown in 100 ml Potato Dextrose Broth (PDB) (Difco, 27 g/l distilled water) in 250 ml erlenmeyer flasks. The cultures were incubated on a rotary shaker at 100 rpm and at 26° C for 5 days. In order to spread these cultures on agar plates to create a fungal lawn, the mycelial masses were shredded using Kinematica tissue grinder at 20% maximum power for 20 sec. Aliquots of 0.1 ml of the ground culture were transferred to a PDA agar plate and spread with a bend glass rod. Cultures were stored for up to 2 week under refrigeration at 4° C. Stock solutions containing 10,000 μg/ml of each of the fractions were prepared using dimethyl sulfoxide (DMSO) as a solvent. From each stock solution, serial dilutions of 2,000 and 1,000 μg/ml were prepared. 25 μl aliquots from each stock solution were spotted on plates inoculated with test organisms. 25 μl aliquots from the stock solutions delivered 250, 100, and 50 μg samples of the extract, respectively. Plates were set for 5 min to allow the solvent to be absorbed by the agar media. Plates were incubated for three days at 28° C.

Zones of inhibition were measured in mm from the center of the region of no growth to the point at which growth first becomes evident.

RESULTS AND DISCUSSION

YMG broth cultures of Tx-1 and Tx-2 became orange colored after 48 h of growth and became more intense through the seven day culture period. Resulting broths were processed as in Figure 1. The yields of Tx-1 fractions were 11.0, 688, and 47.4 mg, respectively. Yields from the Tx-2 broths were 11.4, 584, and 70.3 mg, respectively. Cultures of VI-6-S and IV-7-T did not exhibit color changes through incubation. Processed broth cultures of VI-6-S were respectively 12.7, 301, and 233 mg. Fractions from IV-7-T were 8.9, 689, and 34.2 mg, respectively.

Zones of inhibition were evident following three days of growth at 26°C for each of the three test pathogens assayed. The zone of inhibition measurements for fractions from the four antagonistic bacteria are provided in Table 1. The cell extracts were the only fractions which exhibited antifungal activity for each of the antagonistic bacterial strains examined. This indicated that the antifungal compound was intracellular. Zones of inhibition on plates inoculated with *B. sorokiniana* spores appeared as regions in which spore germination did not occur. Inhibitory zones on plates inoculated with *M. poae* and *S. homoeocarpa* appeared as regions in which there was no mycelial growth.

The cell extract from Tx-1 produced the largest zones

of inhibition to *B. sorokiniana* and *S. homoeocarpa*, whereas the cell extract of Tx-2 yielded the largest zones of inhibition to *M. poae*. Cell extracts of VI-6-S and IV-7-T produced zones of inhibition that were smaller than those of Tx-1 and Tx-2, but were active to some degree to all three of the fungal pathogens screened.

The purpose of this study was to identify a bacterial strain with antifungal activity to which further work could be conducted. Strains Tx-1 and Tx-2 produced zones of inhibition of comparable sizes. However, both of these strains were used in further studies until clear differences could be established between the two.

Although this study does not deal directly with the identification of the chemical nature of the antagonistic quality of the extracts prepared, certain inferences into the nature of the antibiotic can be made. It is unlikely that the source of bioactivity was due to enzymatic action as interaction of the non-polar solvent system (4:1 methanol:chloroform) with proteins, would adversely affect the hydrogen bonding involved in maintaining protein structure and thereby cause proteins to denature. Also, the antibiotic is extracted with a methanol-chloroform solvent system from the cell and of low polarity. The antibiotic is chloroform soluble and deep orange in color.

Table 1. Screening of methanol/chloroform soluble cell fractions of each bacterial isolate against target fungal pathogens *B. sorokiniana*, *S. homoeocarpa*, and *M. poae* at the different concentrations of cell fraction.

	B. sorokiniana			*S. homoeocarpa*			*M. poae*		
Isolate	**250**[a]	**100**	**50**	**250**	**100**	**50**	**250**	**100**	**50**
Tx-1	23[b]	23	21	44	32	28	24	20	14
Tx-2	22	21	16	38	22	16	28	28	20
VI-6-S	16	12	8	16	12	10	20	20	16
IV-7-T	16	10	9	17	14	10	22	20	16

[a] Concentrations listed as μg/ml.
[b] Diameter of zone of inhibition recorded in mm.

SECTION II

IMPROVEMENT OF CULTURE PARAMETERS FOR ANTIBIOTIC PRODUCTION

INTRODUCTION

To facilitate further study of the antifungal compound(s) produced by the Tx-1 and Tx-2 strains for determination of minimum inhibitory concentrations, greenhouse studies, field studies, and chemical structure identification, large amounts of cell extract would be required. The set of experiments included in this section were conducted to improve the yield and bioactivity of cell extracts. In this study, cultures grown in YMG medium, used in the initial experiment, were compared with cultures grown in A9 medium (37). The second part of this study was to examine the rate of production of antibiotic at two day intervals over an eight day period.

MATERIALS AND METHODS

Media Comparison for Improved Antibiotic Production

A comparison of media for the growth of Tx-1 and Tx-2 was conducted with YMG (section I, pg) and A9 (5 g Bacto peptone, 10 g anhydrous dextrose [Baker Analyzed], 20 g Brer Rabbit Dark molasses, 1000 ml distilled water) media. Baffled 500 ml erlenmeyer flasks with 100 ml of YMG or A9 medium were inoculated by the transfer of 1 ml of a 24 h culture of Tx-1 or Tx-2 grown on Trypticase Soy Broth BBL (Becton Dickinson) (24 g/l) from single colonies.

Inoculated flasks were placed on a rotary shaker (100 rpm) for 7 days at 26°C in the dark. After the seven days of culture, cell extracts were prepared as previously explained (Section I, pg 26) and dry weights were recorded. The production of the antibiotic was compared by TLC analysis on silica plates (250 μm, 254 fluorescence) using a solvent system of 4:1 chloroform:methanol. Developed TLC plates were viewed under UV light at 254 and 366 nm and fluorescent spots were marked. Distance of compound migration was recorded to the center of the spots. R_f values for migrating compounds were determined by using the formula:

$$R_f = \frac{spot\ migration\ (mm)}{solvent\ migration\ (mm)}$$

Solutions of the extracts were prepared at concentrations of 10,000, 2,000, and 1,000 μg/ml using dimethylsulfoxide (DMSO) as the solvent. Bioassays were conducted against *B. sorokiniana* on PDA plates inoculated with 1,000 spores/plate. 25 μl aliquots of each extract from their stock solutions were spotted onto the pre-lawned test organism plates to deliver 250, 100, and 50 μg of extract. Following treatment, plates were left undisturbed for 5 min prior to being transferred to the incubator. Zones of inhibition were recorded after 3 days of incubation at 26°C.

Time Course Study for Bacterial Growth and Antibiotic Production

Four flasks (baffled 500 ml erlenmeyer flask with 100

ml A9 medium) were inoculated with bacterial strains Tx-1 and Tx-2 respectively and were incubated at 26° C on a rotary shaker at 100 rpm. A single flask of each bacterial culture was processed at 48, 96, 144, and 192 h of incubation. Cell extracts were prepared as discussed in section I. Dry weights of cell extracts were recorded. Cell extracts were made into solutions containing 4,000 µg/ml with DMSO. 25 µl aliquots were spotted on PDA plates inoculated with 1,000 spores of *B. sorokiniana*. Plates were incubated for 3 days at 26° C, following which measurements of zones of inhibition were recorded.

RESULTS AND DISCUSSION

Media Comparison

Broth cultures of Tx-1 and Tx-2 in both YMG and A9 media exhibited orange color after one day of growth. Dry weights of the resulting cell extracts are given on Table 2.

Table 2. Comparison of cell extract dry weights of Tx-1 and Tx-2 cultures grown in YMG broth against those grown in A9 broth medium.

	YMG	A9
Tx-1	0.011	0.046
Tx-2	0.011	0.040

The bioactivity of these extracts were assayed against *B. sorokiniana*. Comparison of zones of inhibition between the two culture media are recorded for Tx-1 and Tx-2 on Table 3.

Table 3. Comparison of zones of inhibition in mm of cell extracts of Tx-1 and Tx-2 cultures grown in YMG broth media versus those grown in A9 broth medium.

Concentration in μg/ml	Tx-1 Zone of Inhibition		Tx-2 Zone of Inhibition	
	YMG	A9	YMG	A9
250	23.5	35.0	18.3	37.0
100	17.2	30.0	13.0	26.6
50	12.5	18.9	7.0	23.5

Analysis of cell extracts by TLC showed two spots which were found in both YMG and A9 broth cultures. Observation of TLC plates under UV light at 254 and 366 nm identified the presence of two discrete spots. Spots with an R_f value of 0.69 appeared dark blue on the fluorescent silica plates at 254 nm. Spots with an R_f value of 0.64 were visible at 366 nm and produced an orange glow. These spots were visible as yellow spots in visible light.

Extracts for both YMG an A-9 media gave identical spots on TLC. This information implied that compounds produced in YMG cultures examined in section I of this chapter are also produced in cultures fermented in A9 medium. Since the spots developed from cultures of Tx-1 directly matched to those arising from cultures of Tx-2, it is possible that both of these bacterial strains produced the same antibiotic. This is not completely surprising as these bacteria were isolated from the same soil sample and have been identified as the same species.

Comparison of cell extract weight resulting from cultures grown on YMG versus those grown on A9 media indicated that four times as much extract can be recovered from cultures grown on the A9 medium in comparison to that prepared from YMG. In order to determine if the increase in the amount of cell extract harvested on A9 medium is significant, the bioactivity of this extract was examined.

Comparison of the bioactivity of cell extracts from both A9 and YMG media was conducted using *B. sorokiniana* as the test organism. *B. sorokiniana* was selected since as its spores are readily harvested, may be stored for long periods without loss of viability, and are easily spread onto agar plates for bioassays. Cell extracts from Tx-1 grown in A9 produced zones of inhibition in plate assays that were 1.5 times greater than those of cell extracts derived from cultures grown in YMG medium. Tx-2 cell extract weights from cultures grown on A9 were 2 times greater than those from YMG.

The results of this study indicated that both Tx-1 and Tx-2 produced the antibiotic(s) when cultured in YMG and A9 media. Both bacterial strains produces four times more cell extract in the A9 medium than in YMG medium. In addition to providing greater quantities of cell extract, cultures in A9 media produced extracts that are 1.5 to 2.0 times more active than those prepared from YMG cultures. This confirmed that production of the antibiotic by Tx-1 and Tx-2 is greater in A9 medium than YMG. Based on this study,

future work with cell extracts and antibiotic production were conducted using bacterial strain Tx-1 in A9 medium.

Time Course Study for Bacterial Growth and Antibiotic Production

Cell cultures were processed at 48, 96, 144, and 192 h. Dry cell extract weights and zones of inhibition against *B. sorokiniana* are provided on Tables 4 and 5, respectively.

Table 4. Cell extract weights (g) of cultures of Tx-1 and Tx-2 in A9 medium over a period of 8 days.

	2 Days	4 Days	6 Days	8 Days
Tx-1	0.027	0.054	0.045	0.059
Tx-2	0.026	0.051	0.036	0.048

Table 5. Zones of inhibition (mm) of cell extracts (100 μg) from Tx-1 and Tx-2 in A9 medium taken at 2 day intervals against *B. sorokiniana*.

	2 Days	4 Days	6 Days	8 Days
Tx-1	25	34	34	37
Tx-2	25	32	34	37

Cell extract weights doubled from day 2 to day 4 and dropped slightly at day 6 with a slight increase to day 8. Bioassays of these extracts showed marked increases in activity from day 2 to day 4. Little or no change was noted from day 4 to day 6 followed by a small increase on day 8. These studies suggested that in order to achieve the greatest level of cell extract activity, cell cultures should be maintained for a period of at least 6 days.

SECTION III

LARGE SCALE PRODUCTION OF ANTIBIOTICS BY *Pseudomonas aureofaciens* Tx-1 AND Tx-2

INTRODUCTION

In order to facilitate further studies, such as minimum inhibitory concentrations (MIC), greenhouse studies, and field studies, large quantities of cell extract will be required. As stated in section II of this chapter, 0.044 g of cell extract can be isolated from a 7 day culture of Tx-1 or Tx-2 grown in 100 ml of A9 media. At this rate, 23 flasks would be required to obtain 1.0 g of crude cell extract.

Two fermentation strategies for large scale production of the cell extracts were examined. One involved the utilization of 2 L baffled erlenmeyer flasks as a scaled up approach similar to that used in section II of this chapter. The second strategy was fermentation of Tx-1 in a 100 L bio-reactor within which the bacteria would be fermented under constant conditions as batch cultures.

MATERIALS AND METHODS

Shaker Flasks

2 L erlenmeyer flasks which had four baffles in the bottom corners were used for this procedure. These flasks contained 400 ml of A9 medium described in section II. Seed cultures were prepared by inoculating two of the 2 L flasks with single colonies of Tx-1 bacteria grown on nutrient

agar. These flasks were cultured for 3 days on a rotary shaker at 100 rpm and maintained at a temperature of 26° C.

Fifteen of the 2 L flasks were prepared with 400 ml of A9 media each. These flasks were inoculated by transferring 50 ml of three day old seed cultures. Following inoculation, these flasks were incubated on a rotary shaker at 100 rpm and 26° C for a period of seven days.

At the end of the incubation period, the cells were recovered by centrifugation using a Sorvall centrifuge at 10,000 rpm for 10 min. The cells were homogenized with 2 L of 4:1 methanol:chloroform for 10 min and filtered using a medium (10-15 Å) 600 ml fritted funnel. The residue was re-extracted with another 500 ml of the same solvent system by homogenizing for 2 min. This suspension was filtered as above and the residue was discarded. The combined extracts were evaporated to dryness using a rotary evaporator. The weight of the crude cell extract was weighed and assayed for activity. The cell extract was stored at -20° C.

100 L Bio-Reactor

The 100 L bio-reactor used was a Biostat Model 100D (Braun Instruments, Bulingame CA). 100 L of A9 medium was prepared. This medium was sterilized by heating to 121° C for a period of 45 minutes. Media was stirred continuously at a rate of 50 rpm during the sterilization procedure. The medium was then allowed to cool and stabilize at 26° C. At this point the air flow was set to 50 inches of mercury and the stir rate to 100 rpm.

Preparation of starter inoculum for the use of the Biostat 100D bio-reactor was conducted by the same procedure as outlined previously for the fermentation in 2 L shake flasks. The seed cultures were combined (total volume of 6 L) and poured through a sample port in the reactor vessel with the aid of a sterile funnel and hose.

Following the addition of the starter inoculum the internal pressure of the vessel was increased to 0.7 barrs. During incubation, the culture was observed for foam accumulation every four hours. If the level of foam occupied over half of the head space within the reactor vessel 0.25 ml of autoclaved Sigma Antifoam A was added.

The fermentation was terminated after 66 h and the cells were harvested. Samples were taken every 4 h during the incubation period. Cell counts were performed on each of these samples through serial dilutions and plated on PDA agar plates to obtain the number of colony forming units (CFU) per ml of broth. To determine the rate of production of the active compound, 3 ml of broth was extracted with an equal volume of chloroform. The chloroform was removed and placed into a spectrophotometer at 510 nm from which the percent transmission was recorded. Straight chloroform was used as the blank sample. Data recorded as percent transmission was converted to absorbance using the formula provided below:

$$Absorbance = Log\left(\frac{100}{\%Transmission}\right)$$

Percent transmission was converted to absorbance values in an attempt to obtain a correlation between duration of fermentation and antibiotic production.

Successive 100 L fermentations were carried out by re-inoculating 6 L of the fermentation broth by adding to a newly prepared mixture in the bio-reactor.

The cells were harvested by connecting a hose from the bio-reactor sample port to a Sharples Model As-12 (The Sharples Company, Philadelphia) continuous flow centrifuge. Once the centrifuge reaches 15,000 rpm, broth from the reactor was allowed to flow through the centrifuge at a rate of 800 to 1,000 ml per min. Once the bio-reactor vessel is drained of culture, 2 to 4 L of distilled water are added to rinse and collect any matter which may have settled during processing. Effluent media was discarded through a common drain. The sedimented cell mass was removed from the centrifuge to a polypropylene bottle and stored on ice. Two L of effluent containing a portion of the cell mass was collected from the centrifuge discharge when the centrifuge was shut off and stored at 4° C until processing. The cells were stored at -20° C until processing.

The cells were processed by suspending them in 2 L of effluent medium from the centrifuge and suspended by a magnetic stirrer. 250 ml of this suspension was placed into a 4 L separatory funnel along with 1 L of distilled water saturated with sodium chloride. This mixture was then extracted with 1 L of chloroform. Emulsions of water and

chloroform formed during the separation was broken by passing through cheese cloth. Any emulsion which was not broken following this step was combined and re-extracted with an additional 1 L of chloroform. The aqueous layer was discarded while the chloroform fraction was evaporated to dryness using a rotary evaporator. The resulting extract was weighed and stored at 4° C.

RESULTS AND DISCUSSION

Shaker Flasks

After one day of incubation, seed flasks exhibited the characteristic orange color which remained for the rest of the fermentation period. The first extraction of recovered cells gave a deep orange colored organic extract. The second extraction produced a yellow to light orange colored extract. The weight of the dried cell extract was 2.6 g per 6 L of culture or 0.43 g extract/L of culture broth. Comparison of the cell extract recovered from this method of preparation to cell extracts recovered in section II indicated no qualitative differences. These results indicated that the use of the 2 L flasks were effective for the production of gram quantities of cell extract.

100 L Bio-Reactor

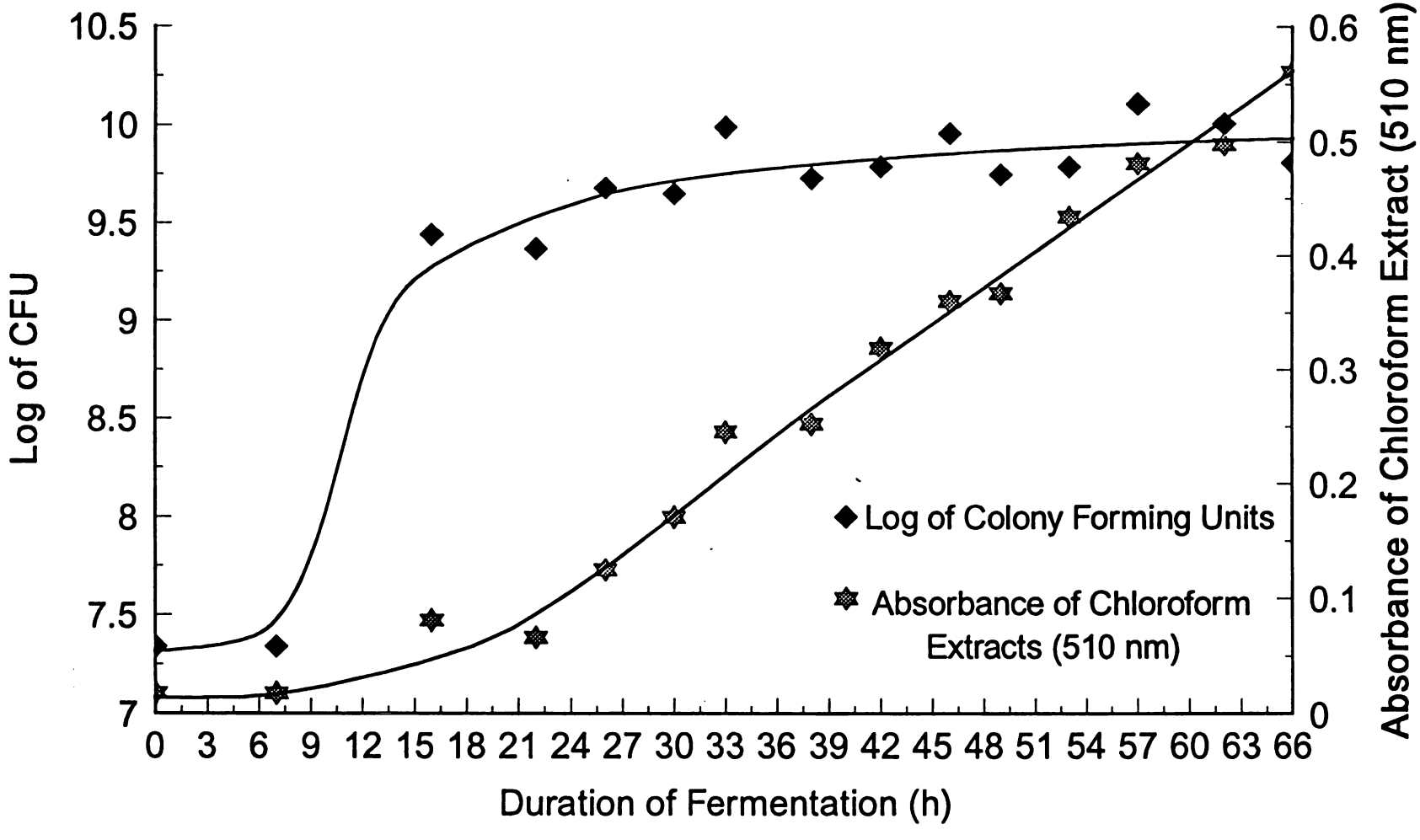
Foaming was observed immediately after the inoculation. Further foaming was not evident until the culture was 24 h old and continued to foam up to 32 h. A total of 1.0 ml of antifoam was added for the entire period of fermentation.

Samples taken during the course of the 100 L

fermentation were evaluated for antibiotic concentration by extracting with chloroform and for bacterial colony forming unit (CFU) counts. A plot of the absorption (510 nm) of the chloroform extract against the time of fermentation is shown in Figure 2. This figure demonstrated that an initial lag phase of antibiotic synthesis followed by a linear increase in the absorption over the incubation period beginning at about 20 h. Also, Figure 2 suggested that increasing the incubation period may result in an improved yield of cell extract. A plot of the log of CFU versus duration of fermentation is shown in Figure 2. The resulting curve follows the lag, exponential, and stationary phases of growth typical to bacterial growth in batch cultures. Exponential growth appears to begin at 10 h and end at about 15-20 h at which time the stationary phase begins. This curve did not represent a population decline phase. The continued stationary phase may represent the utilization of complex carbon sources following the depletion of the simple carbon sources present. Comparison of the CFU count to the absorbance of chloroform extracts correlated an increase in the extract production to the onset of stationary phase occurring at about 20 h. This information indicated that the antibiotic was produced primarily during the stationary phase of growth.

Cell mass removed from the centrifuge possessed a slimy texture and ranged from brown-orange to grey in color. When removing the cell mass from the centrifuge a bright orange

Figure 2. Plot of the Log of Colony Forming Units (CFU) and Absorbance of Chloroform Extracts vs. Fermentation Time of Tx-1



precipitate unlike the cell mass had sedimented at the bottom of the cylinder. This portion may represent precipitated antibiotic released from lysed cells. The total cell mass from the 100 L broth after centrifugation was 724 g. The cell mass was stored at -20° C until processing.

2 L of emulsion, collected during the extraction procedure which could not be broken by the cheese cloth, was extracted with an additional 2 L of chloroform. Following this procedure 2 L of emulsion still remained. Further attempts to break the emulsion were unsuccessful.

The total weight of crude cell extract recovered from the 100 L fermentation was 21.5 g. This was the equivalent to the production of 0.21 g of cell extract per L of culture media which was only half of that procured from shaker flask cultures. Although the yield was less on an equal volume basis, the overall yield was significantly higher considering that this procedure takes the same amount of time as does the shaker flasks. It was possible that yield could be increased by improving the culture parameters employed and increasing the fermentation time. Another possibility would be the isolation of strains of bacteria which overproduce the antibiotic.

SECTION IV

ISOLATION OF THE ANTIBIOTIC FROM Tx-1

INTRODUCTION

Work conducted up to this point involved the examination of the crude cell extract with regards to bio-activity. TLC chromatography of the crude extract showed the presence of at least two compounds within this fraction. The bio-active compound had to be isolated in a pure form in order to perform studies investigating the structure of this compound, determination of MIC's, and for field studies. This section outlines the procedure used to purify the bio-active compound(s) from the crude cell extract by vacuum liquid chromatography (VLC). The resulting bio-active fraction was further purified and crystallized.

Pure Tx-1 antibiotic was compared to the solubility and TLC of phenazine-1-carboxylic acid (PCA) which has been identified as being an antibiotic characteristic of *P. aureofaciens* (21,24).

MATERIALS AND METHODS

Vacuum Liquid Chromatography

The crude cell extract, obtained from 100 L bio-reactor, (3.5 g) was suspended in 50 ml of 1:1 hexane:acetone.

The chromatographic absorbent was silica gel (250 g, Analtech, 60 Å pore size and 35-75 μm particle size) which

was placed into a 600 ml (10-15 Å) fritted funnel and conditioned with 500 ml of 1:1 hexane:acetone. This was accomplished by allowing the solvent to saturate the bed, at which time air bubbles were removed through gentle stirring with a glass rod. The solvent was collected under vacuum in a side-armed erlenmeyer flask and discarded. The cell extract suspension in hexane:acetone was loaded onto the column and eluted with the same solvent system. Two fractions, I and II were collected (500 and 350 ml, respectively) using 1:1 hexane:acetone solvent. The solvent system was then changed to straight chloroform. A total volume of 1 L of solvent was eluted under vacuum as fraction III. Fraction IV was collected by the elution of 1.5 L of 4:1 chloroform:methanol solvent under vacuum. A final fraction was collected by eluting with 600 ml of straight methanol as fraction V. Solvent was removed from the fractions using a rotary evaporator, and further dried under vacuum in a desiccator for a period of 30 min. After a TLC check, fractions I and II were combined. Dry weights of the fractions were recorded. Fractions were compared by TLC chromatography on silica G plates and developed with 4:1 chloroform:methanol. Fractions that were identical under UV were combined.

All fractions were assayed for biological activity. 100 µg/ml solutions of the fractions were prepared in DMSO. 25 µl aliquots were spotted on PDA plates inoculated with 1,000 spores of *B. sorokiniana*. Zones of inhibition were

recorded after three days of incubation at 28° C.

Recrystallization

Recrystallization of the bio-active fraction was conducted by dissolving the fraction in a minimal volume of chloroform with mild heating in hot air. Crystallization was initiated by the addition of methanol. Further crystallization is promoted by cooling the solution at -4° C for a period of 24 h. The crystals were filtered off using a 30 ml (4.5-5 Å) fritted funnel, dried under vacuum in a desiccator and stored at -4° C. The crystals were bio-assayed against *B. sorokiniana* at 400 µg/ml.

Characterization of the Tx-1 Antibiotic

The solubility of the Tx-1 antibiotic was determined in various solvents such as acetone, chloroform, ethyl acetate, hexane, methanol, petroleum ether, water, 2N HCl, and 2N NaOH. Solubility was determined by transferring a small portion of antibiotic crystals to 1 ml of solvent in a test tube and mixed for 30 sec. Solubility was evident by clear solutions with orange color in the solvents.

TLC analysis of the crystalline antibiotic was conducted using silica plates (250 µm layer, 254 nm fluorescent) using 4:1 chloroform:acetone. Following development, the plates were observed under UV light at 254 and 366 nm. R_f value of the antibiotic was recorded and compared to that of phenazine-1-carboxylic acid (PCA).

RESULTS AND DISCUSSION

Vacuum Liquid Chromatography

The cell extract formed an orange layer on the surface of the silica gel during VLC. Elution of the column with hexane:acetone initiated the migration of a single band which was green in color. Most of this band eluted with the first 500 ml of 1:1 hexane:acetone. The column was eluted with an additional 350 ml of hexane:acetone until no color was seen in the effluent. As these two fractions were eluted with the same solvent system and gave similar TLC profiles, they were combined as fraction I.

Elution of the column with 1,000 ml of chloroform initiated the migration of an orange band. Much of this band eluted with chloroform resulting in a yellow-orange solution. This was labeled as fraction II. To remove the remainder of this orange band, a solvent system of higher polarity, chloroform:methanol, was used. 1.5 L of this solvent system removed the remainder of the orange band which had not eluted with 100% chloroform. A final elution was performed with methanol which afforded an orange-red solution (fraction IV).

Characteristics of these fractions following removal of the solvent were as follows:

Fraction I: Highly viscous and pale green in color.

Fraction II: Crusty and yellow-orange in color.

Fraction III: Crusty and orange in color.

Fraction IV: A film that is red-orange in color.

Dried weight of fractions and a listing of percent of the total extract weight are shown on Table 6.

Table 6. Weights of VLC fractions and as percentages of crude cell extract weight.

Fraction	Dry Weight (g)	% of Total Wt.
I	2.1942 g	42.6
III	0.5801 g	16.5
IV	1.1493 g	32.7
V	0.1620 g	4.6

The R_f values obtained from the TLC of fractions I - IV are listed in Table 7.

Table 7. R_f values for VLC fractions I - IV.

Fraction	R_f
I	0.72
II	0.67
III	0.66
IV	0.66

Fractions II, III, and IV were not discernable from one another with respect to R_f values and hence, these three fractions were combined to obtain MN-8-8A.

Bioassays were conducted using fraction I and the combined fraction of II, III, and IV. Assays of fraction I did not exhibit any zone of inhibition. The presence of distinct zones of inhibition identified MN-8-8A as being responsible for the bioactivity of the crude cell extract.

Recrystallization

MN-8-8A was used for recrystallization of pure extract. This combined fraction was readily solubilized in hot

chloroform. Methanol was added until the solution became cloudy or the first signs of precipitation were evident. After cooling in the freezer for 24 h the crystals were filtered off to yield MN-8-10A. The dried crystals were orange in color and needle-like. Bioassay of these crystals confirmed antifungal activity.

MN-8-10A was needle-shaped as in the case PCA, which have been reported to be produced by strains of *P. aureofaciens* (21,24). However, the color of the crystals isolated are orange in color in contrast to the yellow to green-yellow color of PCA.

Characterization of the Tx-1 Antibiotic

The Tx-1 antibiotic MN-8-10A was insoluble in hexane, petroleum ether, water, and 2N HCl, and exhibited solubility in ethyl acetate, chloroform, and 2N NaOH. Partial solubility was observed with acetone and methanol.

Evaluation by TLC using a mobile phase of 4:1 chloroform:acetone showed a single orange spot with an R_f value of 0.34. This value was significantly different than the reported R_f value of 0.655 (21) for PCA when analyzed under identical conditions. The Tx-1 antibiotic fluoresced orange under UV light at 366 nm and was faintly blue at 254 nm.

Solubility ratings for the Tx-1 antibiotic was similar to those of PCA with the exceptions that PCA was soluble in acetone and 2N HCl. Differences in solubility in the 2N HCl indicated a major difference between the Tx-1 antibiotic and

PCA. TLC analysis further supported the hypothesis that the Tx-1 antibiotic is not PCA by significant differences in R_f values.

Characterization of the Tx-1 antibiotic on the basis of color, solubility, and TLC chromatography indicated that this compound is different from antibiotics reported from *P. aureofaciens* or *P. fluorescens*.

SECTION V

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF THE Tx-1 ANTIBIOTIC FOR FUNGAL TURFGRASS PATHOGENS

INTRODUCTION

Screening procedures described previously in this thesis have been qualitative in nature. These tests have been based on the zone of inhibition caused by the diffusion of the compound through the medium. The antibiotic concentration at the point where the test organisms begin to grow cannot be determined by this technique. The lowest concentration of antibiotic at which no growth of the test organism occurs is the MIC. Determination of MIC's in this study was conducted using the amended plate assay, in which the inhibitory compound was incorporated to known concentrations in agar medium prior to being poured in the petri plates. This ensures uniform distribution of antibiotic throughout the medium. Test organisms were assayed on media containing increasing concentrations of the antibiotic.

The purpose of determining the MIC was to obtain the concentrations of Tx-1 antibiotic required for greenhouse and field studies. MIC's were also conducted in parallel with two commonly used fungicides used on turf. The commercial fungicides were the contact fungicide chlorothalonil and the systemic compound triadimefon. This allowed for the comparison of the bioactivity between

antibiotic produced by *P. aureofaciens* Tx-1 and Tx-2 and commercial fungicides

MATERIALS AND METHODS

Seven 500 ml erlenmeyer flasks with 175 ml of PDA were autoclaved and maintained in a water bath at 50° C. 0.0431 g of Tx-1 antibiotic crystals (prepared as described in section IV) were solubilized in 4.0 ml of DMSO. This solution was added to the flasks of media to provide antibiotic concentrations of 100, 50, 25, 10, 1.0, and 0.1 μg/ml respectively and a total of 1% DMSO. Similarly DMSO was used as a control at a concentration of 1%. Five ml aliquots of medium was poured into 30 petri dishes (60 mm × 15 mm).

Plates for MIC determination with commercial fungicides were prepared as in the case of the Tx-1 antibiotic, with triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazole-1-yl)-2-butanone) and chlorothalonil (tetrachloroisophthalonitrile). Triadimefon stock solution was prepared by dissolving 0.2 g Bayleton (commercial fungicide containing 25% triadimefon) to 5 ml of sterile distilled water. Chlorothalonil stock solution consisted of 0.1 ml of Daconil 2787 (a commercial fungicide containing 0.5 g chlorothalonil/ml). Stock solutions were amended to the PDA medium at 100, 50, 25, 10, 1.0, and 0.1 μg active ingredient/ml, respectively. Thirty petri plates containing 5 ml of media per concentration were prepared as were non-amended control plates. The plates were stored at 4° C

until used.

Turfgrass pathogens assayed were *S. homoeocarpa* (sensitive to demethylase inhibitor fungicides [DMI^S]), *S. homoeocarpa* MI-7 (resistant to demethylase inhibitor fungicides [DMI^r]), *M. poae* ATCC 66441, *Leptosphaeria korrae*, *Gaeumannomyces graminis*, *Typhula incarnata*, *Microdochium nivale*, *Bipolaris sorokiniana*, *Rhizoctonia solani*, *Colletotrichum graminicola*, and *Pythium*. Cultures of these pathogens were grown on PDA at 22° C except for *T. incarnata* which was grown at 4° C.

Plates were inoculated by transferring 4 mm diameter plugs of the fungi. Plugs were taken from the advancing margin of the fungal mycelium. Each treatment was replicated three times. Plates were incubated in the dark at 22° C with exception to *T. incarnata* which was incubated at 4° C.

Data was collected as the average of six measurements (mm) per plate of fungal growth. Ratings were taken after two days of incubation for *S. homoeocarpa* (DMI^S), *S. homoeocarpa* (DMI^r), and *R. solani*, five days for *C. graminicola* and *M. nivale*, seven days for *B. sorokiniana* and *M. poae*, ten days for *L. korrae* and *G. graminis* and fourteen days of incubation for *T. incarnata*. Rating dates were determined based on the time required for the fungal mycelium in the control treatments to approach the outer edge of the plate. Resulting measurements of radial growth were divided by the average radial growth of control plates

to yield a percentage of control. These data are listed as the average relative growth.

MIC determination was carried out twice for all organisms.

RESULTS AND DISCUSSION

The average relative growth of the test organisms for the Tx-1 antibiotic, chlorothalonil, and triadimefon are provided in Appendix A. Table 8 provides a comparative list of MICs for the Tx-1 antibiotic, chlorothalonil, and triadimefon.

The Tx-1 antibiotic yielded MIC's of less than 100 μg/ml for all test pathogens except for *M. nivale* and *R. solani*. MIC values generally ranged from 10 to 50 μg/ml. At concentrations less than the MIC, test organisms exhibited reduced growth. *B. sorokiniana* spores plated on 0.1 and 1.0 μg/ml concentrations of the antibiotic expressed increased sporulation of mycelium in comparison with untreated control plates indicating stress to the organism. MIC values of the Tx-1 antibiotic were lower than or equal to chlorothalonil or triadimefon against *B. sorokiniana*, *C. graminicola*, *G. graminis*, *L. korrae*, and *M. poae*. In direct comparison with chlorothalonil, the Tx-1 antibiotic exhibited lower MICs against *C. graminicola*, *G. graminis*, *L. korrae*, *M. poae*, *S. homoeocarpa* (DMI^s), and *S. homoeocarpa* (DMI^r). In direct comparison with triadimefon the Tx-1 antibiotic provided lower MICs against *B. sorokiniana*, *C. graminicola*, *L. korrae*, and *M. poae*.

Neither the Tx-1 antibiotic, chlorothalonil, nor triadimefon completely inhibited the growth of *M. nivale* at concentrations of less than 100 μg/ml.

These results demonstrated that the Tx-1 antibiotic provided fungicidal activity comparable to commercial fungicides.

Table 8. Comparative MIC values for the Tx-1 antibiotic, chlorothalonil and triadimefon.

Test Organism	Tx-1 Antibiotic	Chlorothalonil	Triadimefon
B. sorokiniana	10 > 25[a]	10 > 25	> 100
C. graminicola	10 > 25	50 > 100	25 > 50
G. graminis	1.0 > 10	50 > 100	50 > 100
L. korrae	10 > 25	> 100	> 100
M. poae	10 > 25	50 > 100	50 > 100
M. nivale	> 100	> 100	> 100
R. solani	> 100	> 100	10 > 25
S. homoeocarpa (DMI')	25 > 50	> 100	1.0 > 10
S. homoeocarpa (DMI')	25 > 50	50 > 100	10 > 25
T. incarnata	10 > 25	0.1 > 10	10 > 25

[a] Concentrations listed in μg/ml.

SECTION VI

GREENHOUSE EVALUATION OF THE EFFICACY OF THE Tx-1 ANTIBIOTIC FOR MANAGEMENT OF DOLLAR SPOT

INTRODUCTION

The first step in examining the potential of a bio-active compound for dollar spot management was to evaluate it on an *in-vitro* basis, such as determining MIC's. MIC evaluation has shown the Tx-1 antibiotic to be comparable in activity with the contact fungicide chlorothalonil and to the systemic fungicide triadimefon (section V).

The greenhouse investigation of dollar spot management is conducted on a smaller scale than field trials since it is not dependent on seasonal differences and environmental factors. The greenhouse study examines the potential of the Tx-1 antibiotic as a tool for management of dollar spot of bentgrass in the field. This study compares the effectiveness of the Tx-1 antibiotic with commercial fungicides at recommended rates. Another aspect to be investigated was whether the Tx-1 antibiotic acts in a contact or systemic nature. The systemic activity was determined by applying drench treatments whereas the contact activity was by spray application.

MATERIALS AND METHODS

Greenhouse turf was prepared by evenly spreading 0.25 g Penncross bentgrass (*Agrostis palustris*) seed (treated with Subdue) into 8 ounce styrofoam cups (250 ml of soil steam

sterilized for 1 hour on two consecutive days), providing an equivalent seeding rate of 11.4 lbs of seed/1000 ft^2. Seeds were covered with 20 ml of fine sifted soil and watered to maintain soil moisture. Following germination, the cups were clipped three times a week to maintain a height of 0.5 cm. The turf was fertilized with Peters 20-20-20 NPK fertilizer to provide nitrogen at a rate of 0.5 lb N/1000 ft^2 every 2 weeks. Fertilizer was withheld two weeks prior to the treatment. Cups were grown for a period of two months prior to use.

The study was set up as a completely randomized block design with four replications. Two sets of treatments were applied, one receiving *S. homoeocarpa* inoculum, and the other without the inoculum to examine the phytotoxic effects of the Tx-1 antibiotic. All treatments were applied as either foliar or drench applications. An atomizer attached to an air line was used for spray applications (2.0 ml per cup). Drench applications (10 ml per cup) were conducted by using a syringe to inject the treatment into the soil.

Two fungicide controls were included in this study as representatives of the systemic and contact classes of fungicides. The contract fungicide, chlorothalonil (tetrachloroisophthalonitrile), was used in the commercially available form as Daconil 2787 Flowable (ISK Biotech, Mentor, OH) at the recommended rate of 95.5 ml/100 m^2 (3.0 oz. per 1000 ft^2). The amount of Daconil 2787 applied to a single cup (45 cm^2) was 0.00473 g (0.00232 g AI).

The systemic fungicide used in this study was triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazole-1-yl)-2-butanone) available as Bayleton 25 (Mobay Corp., Kansas City, MO). The rate of application was 31.8 g/100 m^2 (1.0 oz./1000 ft^2), the recommended rate for dollar spot management. 0.0015 g (0.000375 g AI) of Bayleton was required to treat a single cup of grass.

As the Tx-1 antibiotic is insoluble in water, organic solvents were used in the formulation. 0.05 g of Tx-1 antibiotic was dissolved in 3.5 ml of 1:1 acetone:hexane. Tween 80 (0.07 ml)(polyxyethylene sorbitan monooleate) was added to the solution to act as an emulsifier. The antibiotic rates applied corresponded to the same AI as the commercial fungicides. The Tx-1 antibiotic was applied at the rate equal to that of Daconil 2787 Flowable by combining 0.166 ml of the antibiotic stock solution with 0.034 ml of solvent mixture. Antibiotic treatment at the same rate of active ingredient as Bayleton was prepared by combining 0.027 ml of antibiotic solution and 0.173 ml of solvent mixture. Antibiotic solutions were added to 1.8 ml of distilled water for spray applications and to 9.8 ml water for drench applications.

S. homoeocarpa inoculum was prepared by culturing it on a wheat seed medium. The wheat seed medium was prepared by autoclaving 250 g of wheat seeds with 250 ml of nutrient broth for 30 minutes in a 2 L erlenmeyer flask. Ten plugs of *S. homoeocarpa* were transferred to the wheat seeds medium

from a fresh culture grown on PDA. The wheat seed culture was incubated at 26° C for a period of two weeks, during which the culture was shaken daily to prevent the wheat seeds from clumping.

Inoculation of grass grown in cups was carried out 24 h after treatment application to allow for evaporation of solvents. The grass in cups was inoculated by placing 10 wheat seeds infested with *S. homoeocarpa* on the surface of the cups. All cups were then transferred to a humidity chamber in the greenhouse and misted daily with a spray bottle.

On the fourth day of incubation, the cups were allowed to dry and the wheat seeds were removed. The cups containing the grass were allowed to dry until the seventh day following initial inoculation at which time they were rated. Disease ratings were taken as a percentage of the area of the grass per cup showing disease symptoms. Ratings for phytotoxicity were made using a 10 to 0 scale with 10 being healthy turf and 0 being completely dead.

The experiments were replicated twice.

Data for disease management ratings was transformed (Dr. J. Gill, personal communication) using the formula:

$$Data = Log(\%\ area\ infected + 1)$$

Data transformation was conducted to normalize the distribution of ratings. Transformed data was analyzed using Tukey's test. Phytotoxicity data was not normalized and analyzed with Tukey's test.

RESULTS AND DISCUSSION

Addition of the solution of the Tx-1 antibiotic to water resulted in the formation of an emulsion when mixed. This emulsion was stable during the application period. Spray application of the Tx-1 antibiotic at the rate of 0.00232 g/cup resulted in slight discoloration of plant foliage, which may be due to the high level of orange colored Tx-1 antibiotic applied. Discoloration was less noticeable for cups treated with the low rate of the Tx-1 antibiotic.

Two days following inoculation of grass with *S. homoeocarpa*, fungal growth was evident. Cups treated with spray applications of triadimefon, chlorothalonil, and the 0.00232 g/cup rate of Tx-1 antibiotic did not exhibit mycelial growth from the wheat seed inoculum. Drench applications of all treatments showed fungal growth.

Average percentage of infection for each treatment and their transformed data are shown in Table 9. All foliar application treatments provided effective disease management. Solvent applications as spray and drench treatments did not control dollar spot. Spray application of the Tx-1 antibiotic at the rates tested provided disease management statistically equal to those of the commercial fungicides at the 95% level of confidence. The Tx-1 antibiotic at 0.00232 g/cup resulted in disease management significantly better than the untreated control.

The only drench treatment to reduce disease

significantly was that of triadimefon. This is as expected since triadimefon is a systemic fungicide, and is absorbed by plants when applied as a drench. Possible explanations for the reduced level of effectiveness of the drench is that significant amounts of triadimefon may be absorbed by the soil, or that the plants were not provided sufficient time to assimilate sufficient quantities of triadimefon to provide disease management. Drenching of chlorothalonil did not provide disease management. The fact that Tx-1 antibiotic did not express any disease reduction when applied as a drench suggested that this compound acts as a contact type fungicide.

Phytotoxicity ratings are shown in Table 9. Only spray applications of the Tx-1 antibiotic showed reduced turf quality with respect to the untreated control, while drench applications exhibited no deleterious effects. The only significant reduction in turf quality occurred with the high rate of Tx-1 antibiotic (0.00232 g/cup). Reduction in turf quality was evident after four days. Phytotoxicity was visible by the development of a "water soaked" appearance on older foliage. "Water soaked" tissues later turned brown in color. At the 0.00232 g/cup rate of application, turf loss was approximately 30%. New plant tissue was not affected and continued to grow during the course of this study. The low rate of application of the Tx-1 antibiotic (0.000375 g/cup) expressed phytotoxicity to a lesser degree. Phytotoxicity was confined to older tissues and occurred on

less than 10% of the turf.

Application of the Tx-1 antibiotic provided disease management to the same extent as the fungicides triadimefon and chlorothalonil at recommended rates. The disease management results from this study supported further investigation into the application of the Tx-1 antibiotic as a chemical treatment for the management of dollar spot. However, phytotoxic side effects raised concerns about its acceptability as a commercial fungicide.

Table 9. Dollar spot efficacy and phytotoxicity ratings for the Tx-1 antibiotic for greenhouse grown bentgrass.

		Disese Management		
Treatment	Application	Percent Infection	Transformed Data[a]	Phytotoxicity
No Treatment	-----	35.0	1.44 A[b]	10.0 A
Triadimefon	0.378 mg/cup, Spray	0.0	0.00 C	9.8 AB
Triadimefon	0.378 mg/cup, Drench	10.0	0.86 BC	10.0 A
Chlorothalonil	2.32 mg/cup, Spray	0.0	0.00 C	10.0 A
Chlorothalonil	2.32 mg/cup, Drench	31.3	1.22 AB	9.8 AB
Tx-1 Antibiotic	0.378 mg/cup, Spray	1.8	0.32 BC	9.3 B
Tx-1 Antibiotic	0.378 mg/cup, Drench	16.8	1.23 AB	9.8 AB
Tx-1 Antibiotic	2.32 mg/cup, Spray	0.0	0.00 C	7.3 C
Tx-1 Antibiotic	2.32 mg/cup, Drench	30.5	1.31 AB	10.0 A
Solvent	0.2 ml/cup, Spray	23.8	1.34 AB	9.8 AB
Solvent	0.2 ml/cup, Drench	20.5	1.29 AB	10.0 A
	Std. Deviation	6.2	0.21	0.2

[a] Data transformation performed as log(# of spots + 1)
[b] Data analyzed with Tukey's test, treatments followed by the same letter are not significantly different at the P=0.05 level

SECTION VII

FIELD EVALUATION OF THE Tx-1 ANTIBIOTIC FOR MANAGEMENT OF DOLLAR SPOT

INTRODUCTION

The ultimate test of any disease management agent is its efficacy under field conditions. *In vitro* MIC evaluation the Tx-1 antibiotic indicated that its antifungal activity was comparable to the commercial fungicide chlorothalonil and to a lesser extent to triadimefon.

Greenhouse studies were conducted with the Tx-1 antibiotic and two fungicides, chlorothalonil and triadimefon at recommended rates for dollar spot control. Greenhouse evaluation of Tx-1 antibiotic spray treatments yielded levels of disease management that were s (25% AI) at 1.02 g/plot. This was applied by dissolving 1.02 g Bayleton 25 into 250 ml distilled water and spraying the entire volume onto a single plot using a CO_2 sprayer. Tx-1 antibiotic was applied by dissolving the extract (purified by VLC) into 50 ml of DMSO. 12.5 ml of this solution was transferred to 250 ml of distilled water which was then sprayed onto a single plot with a CO_2 hand sprayer.

The second year study (1993) was set up as a randomized complete block design with four replications. Plots were reduced in size to 1.0 m × 1.2 m (3 ft × 4 ft). The first application date was July 27 and the study was continued

until mid October.

The number of rates and application intervals at which treatments were applied was expanded for this study. Fertilizer (18-5-9) was applied at a rate of 0.85 g nitrogen/m^2 (¼ lb/1000 ft^2) every 14 days.

The systemic fungicide triadimefon was applied at the recommended rate of AI applications of 0.077 g of AI/m^2 (21 day interval) and at the 0.015 g of AI/m^2 (14 day interval) rate used in the 1992 study. Chlorothalonil was the representative contact fungicide used for this study. It was applied at 10 day intervals at the recommended rate of 0.48 g of AI/m^2 and a reduced rate of 0.16 g of AI/m^2.

Tx-1 antibiotic was produced as described in section III. Applications were made using the crude cell extract. In order to apply the appropriate amount of active ingredient, the percentage of AI in the crude cell extract was determined by performing vacuum liquid chromatography as described in section III. Tx-1 antibiotic was applied at 7.5, 15, 16, and 48 g of AI/100 m^2 at the respective time intervals of 21, 14, 10, and 10 days. The formulation of the crude cell extract was made in 1:1 hexane:acetone amended with 2% Tween 80 (polyoxyethylene sorbitan monooleate). A formulation utilizing 1% Tween 80 was used in the first three weeks of the study but did not provide a sufficient emulsion to prevent the antibiotic from precipitating when used at higher concentrations. Ten ml of solvent system was applied per plot (6.25 ml/m^2).

In the 1992 preliminary study DMSO was used as a solvent for the Tx-1 antibiotic, however a solvent control treatment was not included. DMSO was included as a treatment in the 1993 study and applied to deliver the same rate over 14 day intervals.

Spray treatments for the 1993 study were applied using a CO_2 hand sprayer. Measured samples for treatment per individual plot were brought to a total volume of 150 ml with distilled water. The entire volume was then applied to a single plot.

Disease ratings were conducted on a weekly basis throughout the course of both studies. Ratings were taken by counting individual dollar spot patches per plot. Data analysis was performed using the MSTAT statistical analysis software. Due to variation in plots with higher disease incidence than those at lower disease levels, data distribution was normalized using the formula:

$$Data = Log\ (Number\ of\ Patches + 1)$$

Following normalization, data was analyzed with Tukey's test at P=0.05.

RESULTS AND DISCUSSION

Formulation of the Tx-1 antibiotic using DMSO as a solvent was problematic since the antibiotic precipitated when the solution was mixed with water. Agitation of the spray bottle helped to maintain a suspension. However, a significant portion of the antibiotic remained in the spray

bottle. The amount of compound which was retained in the spray bottle was not recorded.

Data from the 1992 preliminary study is provided on Table 10. Only two rating dates are listed as disease was not active until August 26 and disease pressure abated after the september 11 rating date. As of the first rating date both triadimefon and the Tx-1 antibiotic provided significant levels of disease management. No significant differences in the level of disease management between triadimefon and the Tx-1 antibiotic were discernable. Disease pressure increased through the second rating date. Although Tx-1 antibiotic failed to maintain levels of disease management equal to tridimefon, management was better than the untreated control.

These results suggested that the Tx-1 antibiotic is a potential fungicide for dollar spot management. Although complete disease management was not realized, management levels were not statistically different from those of triadimefon. It must also be considered that a portion of each of the biweekly treatments was not applied due to precipitation prior to application. Concerns regarding the effect of DMSO on disease incidence was addressed in the 1993 study in which no significant disease reduction was observed with biweekly application of DMSO solution.

The 1993 study examined different rates and frequencies of applications. As greenhouse studies suggested that the Tx-1 antibiotic acted as a contact type fungicide.

Therefore the Tx-1 antibiotic was compared to the contact fungicide chlorothalonil as well as to the systemic, triadimefon. The Tx-1 antibiotic was compared to chlorothalonil at application rates of 0.48 g of AI/m^2 (recommended rate for chlorothalonil) and 0.16 g of AI/m^2 at 10 day intervals. The low rate was included to examine 10 day application frequencies without concern of phytotoxicity which was observed at the high rate (0.48 g of AI/m^2) in greenhouse stuhe antibiotic remained in the spray bottle. The amount of compound which was retained in the spray bottle was not recorded.

Data from the 1992 preliminary study is provided on Table 10. Only two rating dates are listed as disease was not active until August 26 and disease pressure abated after the september 11 rating date. As of the first rating date both triadimefon and the Tx-1 antibiotic provided significant levels of disease management. No significant differences in the level of disease management between triadimefon and the Tx-1 antibiotic were discernable. Disease pressure increased through the second rating date. Although Tx-1 antibiotic failed to maintain levels of disease management equal to tridimefon, management was better than the untreated control.

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The 1993 study examined different rates and frequencies of applications. As greenhouse studies suggested that the Tx-1 antibiotic acted as a contact type fungicide. Therefore the Tx-1 antibiotic was compared to the contact fungicide chlorothalonil as well as to the systemic, triadimefon. The Tx-1 antibiotic was compared to chlorothalonil at application rates of 0.48 g of AI/m^2 (recommended rate for chlorothalonil) and 0.16 g of AI/m^2 at 10 day intervals. The low rate was included to examine 10 day application frequencies without concern of phytotoxicity which was observed at the high rate (0.48 g of AI/m^2) in greenhouse studies.

A new solvent system was developed to prevent precipitation of the antibiotic when mixed with water. Hexane:acetone was employed to solubilize the extract and Tween 80 added to form the emulsion when mixed with distilled water. This formulation proved to be effective for maintaining the antibiotic in solution. However, the formulation failed for the 10 day applications of the antibiotic. Since these applications required higher concentrations of the antibiotic (0.48 g/m^2). The three

applications of the 10 day treatments after initial application experienced problems with precipitation of antibiotic. Once the rate of Tween 80 was increased, no further problems were experienced.

Data for the ratings taken over the course of the 1993 field study are provided on Tables 11, 12, 13, and 14. Ratings prior to August 17 were not included due to lack of significant disease pressure.

The chemical fungicides chlorothalonil and triadimefon provided effective management of dollar spot when applied at or above recommended rates.

The solvent system used to formulate the Tx-1 antibiotic did not effect disease incidence throughout the course of the study. Application of the Tx-1 antibiotic at 0.077 g/m² rate on a 21 day schedule failed to yield significant disease reduction in relation to the solvent control treatments. The Tx-1 antibiotic (0.15 g/m²) applied at 14 day intervals was as effective as triadimefon at an equivalent rate of application through the month of August. Through September and October, this treatment was significantly better than those of the solvent control, reducing dollar spot incidence of 30% when compared to the solvent controls. Photographs of representative plots of the Tx-1 antibiotic at these two rates of application as of the September 28 rating are provided on Figures 3 and 4.

Use of the commercial fungicide chlorothalonil at the low recommended rate (0.48 g/m²) on a 10 day schedule

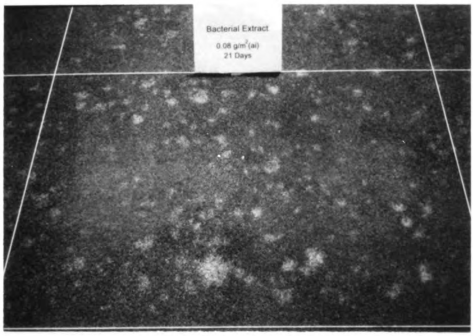

Figure 3. Plot treated with 0.077 g Tx-1 antibiotic/m^2 at 21 day intervals. (September 28, 1993)

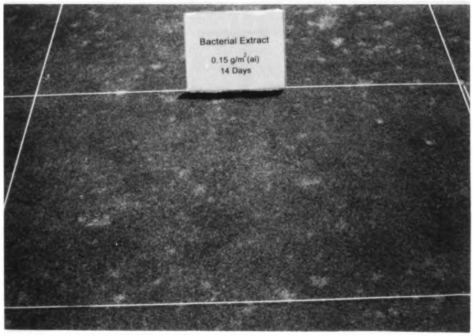

Figure 4. Plot treated with 0.15 g Tx-1 antibiotic/m^2 at 14 day intervals. (September 28, 1993)

provided dollar spot management equal to that of triadimefon at recommended rates (0.077 g/m²) for the most part of the field study. Use of chlorothalonil at ⅛ of the recommended rate (0.16 g/m)² reduced disease significantly in comparison to the untreated control but did not equal the level of control realized by that of the recommended rate.

Due to formulation difficulties experienced in the early portion of this study, the amount of Tx-1 antibiotic applied for treatments to be compared with the fungicide chlorothalonil was less than the experimental rates. Application of the antibiotic at 0.16 g/m² failed to provide a significant level of disease management until the last two rating dates. However, application of 0.15 g/m² of antibiotic at 14 day intervals managed dollar spot to the same degree as the application of chlorothalonil at 0.16 g/m² every 10 days. Although this comparison did not correlate the antibiotic is as effective as chlorothalonil at 0.16 g/m². Application of the Tx-1 antibiotic at 0.48 g/m² over 10 day intervals failed to provide dollar spot management over the first two rating dates due to formulation problems. Once the formulation technique was improved, disease management increased significantly. Photographs of plots treated with the Tx-1 antibiotic at 0.16 g/m² and 0.48 g/m² rates at 10 day intervals shown in Figures 5 and 6. However, dollar spot intensity was not reduced by the Tx-1 antibiotic to levels provided by chlorothalonil or triadimefon at recommended rates.

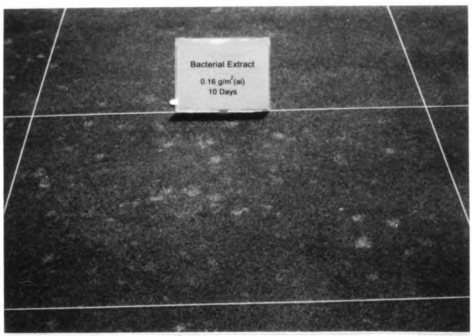

Figure 5. Plot treated with 0.16 g Tx-1 antibiotic/m² at 10 day intervals. (September 28, 1993)

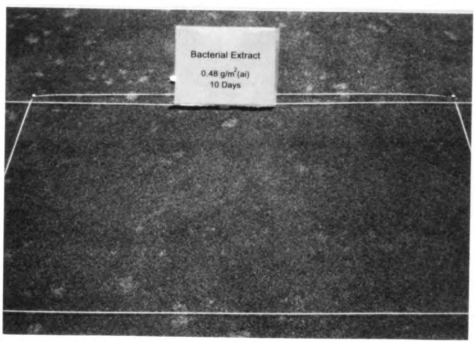

Figure 6. Plot treated with 0.48 g Tx-1 antibiotic/m² at 10 day intervals. (September 28, 1993)

This field study demonstrated that the Tx-1 antibiotic is effective at reducing dollar spot incidence under field conditions. The best disease management was exhibited at the highest rate of application, 0.48 g/m², which was made on a curative basis following disease onset rather than as a preventative due to formulation problems early in the study. This study failed to show that the antibiotic efficacy was similar to triadimefon when applied at 0.15 g/m² over 14 day intervals as demonstrated in the first years study. This may be accounted for by differences in disease intensity and weather conditions. Although the Tx-1 antibiotic was not as effective as chlorothalonil (Daconil 2787) or triadimefon (Bayleton 25), it must be considered that the commercial fungicides have been formulated to assure maximum effectiveness. Future work with the Tx-1 antibiotic requires careful formulation to assure even spray distribution, maximum foliar coverage, and product stability.

Table 10. Preliminary field evaluation of the Tx-1 antibiotic against dollar spot.

		August 28, 1992		September 11,1992	
Treatment	Rate	# of Spots	Normalized[a] Data Analysis	# of Spots	Normalized Data Analysis
18-4-10 Fertilizer	1.7 g N/m² Monthly	12.7	1.11 A[b]	73.7	1.86 A
Triadimefon	0.15 g/m² 14 Days	0.0	0.00 B	0.0	0.00 C
Bacterial Extract	0.15 g/m² 14 Days	0.3	0.10 B	13.7	1.02 B
	Std. Deviation	8.3	0.11	6.3	0.13

[a] Data transformation performed as log(# of spots + 1).
[b] Data analyzed with Tukey's test, treatments followed by the same letter are not significantly different at the P=0.05 level.

Table 11. Dollar spot ratings and data analysis of field application of the Tx-1 antibiotic.

Treatment	Rate	August 17,1993 # of Spots	August 17,1993 Normalized[a] Data Analysis	August 24,1993 # of Spots	August 24,1993 Normalized Data Analysis
18-5-9 Fertilizer	0.85 g N/m² 14 Days	29.3	1.41 A[b]	40.5	1.57 A
Triadimefon	0.077 g/m² 21 Days	5.3	0.69 ABCD	0.3	0.08 D
Triadimefon	0.15 g/m² 14 Days	0.0	0.00 D	6.0	0.35 CD
Chlorothalonil	0.16 g/m² 10 Days	8.3	0.89 ABC	10.5	0.88 ABC
Chlorothalonil	0.48 g/m² 10 Days	3.0	0.46 BCD	0.3	0.08 D
Tx-1 Antibiotic	0.077 g/m² 21 Days	17.3	1.11 AB	26.5	1.24 AB
Tx-1 Antibiotic	0.15 g/m² 14 Days	1.3	0.31 CD	4.8	0.75 BCD
Tx-1 Antibiotic	0.16 g/m² 10 Days	8.0	0.87 ABC	10.0	0.92 ABC
Tx-1 Antibiotic	0.48 g/m² 10 Days	15.8	1.14 AB	15.0	1.15 AB
Solvent	7.5 ml/m² 21 Days	8.8	0.94 ABC	17.3	1.24 AB
Solvent	7.5 ml/m² 14 Days	17.5	1.14 AB	28.8	1.39 AB
Solvent	7.5 ml/m² 10 Days	9.3	0.86 ABC	14.0	1.10 ABC
DMSO	6.5 ml/m² 14 Days	13.3	1.06 AB	19.8	1.31 AB
	Std. Deviation	8.9	0.15	5.6	0.16

[a] Data transformed as the formula log(# of spots + 1).
[b] Treatments followed by the same letter are not significantly different at the P=0.05 level using Tukey's test.

Table 12. Dollar spot ratings and data analysis of field application of the Tx-1 antibiotic.

Treatment	Rate	August 31, 1993 # of Spots	August 31, 1993 Normalized[a] Data Analysis	September 13,1993 # of Spots	September 13,1993 Normalized Data Analysis
18-5-9 Fertilizer	0.85 g N/m² 14 Days	75.5	1.71 A[b]	81.5	1.90 A
Triadimefon	0.077 g/m² 21 Days	0.0	0.00 D	1.0	0.18 D
Triadimefon	0.15 g/m² 14 Days	0.0	0.00 D	0.0	0.00 D
Chlorothalonil	0.16 g/m² 10 Days	18.3	1.03 AB	33.3	1.49 AB
Chlorothalonil	0.48 g/m² 10 Days	0.8	0.19 CD	5.5	0.77 C
Tx-1 Antibiotic	0.077 g/m² 21 Days	39.5	1.42 AB	61	1.64 AB
Tx-1 Antibiotic	0.15 g/m² 14 Days	0.8	0.23 CD	16.5	1.24 BC
Tx-1 Antibiotic	0.16 g/m² 10 Days	24.0	1.29 AB	32.0	1.49 AB
Tx-1 Antibiotic	0.48 g/m² 10 Days	9.5	0.79 BC	21.3	1.23 BC
Solvent	7.5 ml/m² 21 Days	22.3	1.36 AB	54.5	1.74 AB
Solvent	7.5 ml/m² 14 Days	41.3	1.55 A	77.3	1.87 A
Solvent	7.5 ml/m² 10 Days	25.3	1.35 AB	48.8	1.68 AB
DMSO	6.5 ml/m² 14 Days	29.8	1.48 AB	52.3	1.72 AB
	Std. Deviation	6.3	0.15	9.1	0.11

[a] Data transformed as the formula log(# of spots + 1).
[b] Treatments followed by the same letter are not significantly different at the P=0.05 level using Tukey,s Test.

Table 13. Dollar spot ratings and data analysis of field application of the Tx-1 antibiotic.

Treatment	Rate	September 22, 1993 # of Spots	September 22, 1993 Normalized[a] Data Analysis	September 28, 1993 # of Spots	September 28, 1993 Normalized Data Analysis
18-5-9 Fertilizer	0.85 g N/m² 14 Days	70.3	1.81 A[b]	108.0	2.03 A
Triadimefon	0.077 g/m² 21 Days	1.0	0.23 CD	1.5	0.38 E
Triadimefon	0.15 g/m² 14 Days	0.0	0.00 D	0.0	0.00 E
Chlorothalonil	0.16 g/m² 10 Days	23.3	1.35 AB	16.8	1.19 CD
Chlorothalonil	0.48 g/m² 10 Days	4.8	0.66 C	1.3	0.32 E
Tx-1 Antibiotic	0.077 g/m² 21 Days	45.5	1.61 AB	76.3	1.87 AB
Tx-1 Antibiotic	0.15 g/m² 14 Days	16.8	1.17 B	32.3	1.41 BC
Tx-1 Antibiotic	0.16 g/m² 10 Days	37.3	1.58 AB	45.5	1.65 ABC
Tx-1 Antibiotic	0.48 g/m² 10 Days	25.8	1.40 AB	7.8	0.92 D
Solvent	7.5 ml/m² 21 Days	49.3	1.69 A	68.5	1.83 AB
Solvent	7.5 ml/m² 14 Days	70.3	1.81 A	103.0	2.01 A
Solvent	7.5 ml/m² 10 Days	45.3	1.65 A	91.5	1.96 A
DMSO	6.5 ml/m² 14 Days	49.0	1.70 A	76.0	1.88 A
	Std. Deviation	8.0	0.09	9.0	0.09

[a] Data transformed as the formula log(# of spots + 1).
[b] Treatments followed by the same letter are not significantly different at the P=0.05 level using Tukey's Test.

Table 14. Dollar spot ratings and data analysis of field application of the Tx-1 antibiotic.

		October 6, 1993	
Treatment	Rate	# of Spots	Normalized[a] Data Analysis
18-5-9 Fertilizer	0.85 g N/m² 14 Days	66.0	1.82 A[b]
Triadimefon	0.077 g/m² 21 Days	0.5	0.12 EF
Triadimefon	0.15 g/m² 14 Days	0.0	0.00 F
Chlorothalonil	0.16 g/m² 10 Days	10.5	0.93 CD
Chlorothalonil	0.48 g/m² 10 Days	0.8	0.20 EF
Tx-1 Antibiotic	0.077 g/m² 21 Days	44.5	1.59 AB
Tx-1 Antibiotic	0.15 g/m² 14 Days	18.5	1.16 BCD
Tx-1 Antibiotic	0.16 g/m² 10 Days	19.8	1.29 ABC
Tx-1 Antibiotic	0.48 g/m² 10 Days	3.5	0.64 DE
Solvent	7.5 ml/m² 21 Days	47.8	1.64 AB
Solvent	7.5 ml/m² 14 Days	63.8	1.79 A
Solvent	7.5 ml/m² 10 Days	48.5	1.68 AB
DMSO	6.5 ml/m² 14 Days	54.8	1.74 AB
	Std. Deviation	6.7	0.12

[a] Data transformed as the formula log(# of spots + 1).
[b] Treatments followed by the same letter are not significantly different at the P=0.05 level using Tukey's Test.

SECTION VIII

MANAGEMENT OF SUMMER PATCH WITH THE Tx-1 ANTIBIOTIC

INTRODUCTION

Evaluation of MIC of the Tx-1 antibiotic have identified it as being completely inhibitory to *M. poae* at 10<25 μg/ml. A field evaluation of this antibiotic as a chemical management tool for summer patch was conducted during the summer of 1993. In this study, the Tx-1 antibiotic was compared to the commercial fungicide propiconazole (1-[[2-)2, 4-dichlorophenyl)-4-propyl-1, 3-dioxolan-2yl[methyl]- 1 H-1, 2, 4-triazole) (Banner, Ciba-Geigy, Greensboro, NC) for disease management. Propiconizole is a systemic fungicide and can be applied at lower rates than many contact fungicides. The Tx-1 antibiotic was applied at the same and twice the rate of active ingredient as the recommended rate of propiconizole.

MATERIALS AND METHODS

This study was conducted on an annual bluegrass (*Poa annua* L.) fairway at Dearborn Country Club, Dearborn, MI, with a history of *M. poae* infestation in previous years. The study was set up as a randomized complete block design with 4 replications with plot sizes of 1.8 m x 2.7 m (3′ x 9′) each. All plots were fertilized with 18-5-9 at a capacity to deliver 85 g of N/100 m² per month. The first application was made when the soil temperature reached 18° C

at 5 cm depth. All applications were made on a monthly basis through August 1993.

Propiconizole, Banner, was used as the commercial control agent for summer patch at the recommended rate of 0.12 g of AI/m^2. Tx-1 antibiotic for this study was prepared by fermenting the Tx-1 antibiotic in a 100 L Braun bio-reactor. The resulting cell extract was assayed for antibiotic content after fractionation by vacuum liquid chromatography. Purified cell extract was applied according to the content of active ingredient. The Tx-1 antibiotic was applied at 0.12 g of AI/m^2 and 0.25 g of AI/m^2. The antibiotic was initially applied as a solution in 100% acetone at a rate of 25 ml/m^2. This formulation was changed prior to the third application. The new solvent system was 1:1 hexane:acetone with 2% Tween 20 as used for the dollar spot field study earlier. These applications consisted of 25 ml of solvent mixture and 225 ml of distilled water. Control treatments of the solvent system were applied without the antibiotic to evaluate the solvent effects. All spray applications were applied using a CO_2 hand sprayer at 30 psi. Spray treatments were drenched after application with 2 L of water/m^2 using tap water in a garden water jug.

Plots were rated twice a month as the disease progressed. Ratings were taken as the percentage of annual bluegrass within the plot that had died due to summer patch infection. Data was analyzed with the MSTAT statistical data software, using Tukey's test.

RESULTS AND DISCUSSION

Disease first became evident in late August to early September. Although summer patch was present, disease pressure was not sufficient to identify significant differences among treatments.

CHAPTER II

CONTROL OF DOLLAR SPOT AND SUMMER PATCH WITH *Pseudomonas aureofaciens* Tx-1 and Tx-2

INTRODUCTION

To evaluate Tx-1 and Tx-2 for controlling plant pathogens, it is important to know the mode of antagonism of the bacterial strains. The mode of antagonism by Tx-1 and Tx-2 due to the production of antibiotics, can be determined by isolating mutants of this organism which were unable to produce the antibiotic and assaying them against the pathogens.

This section outlines the procedure used to isolate non-inhibitory strains of Tx-1 and Tx-2 for field evaluation in the biological control study.

Dollar Spot and Summer Patch Control

The focus of recent research into the management of dollar spot disease has been directed toward the application of biological controls. Nelson and Craft (38) have reported the reduction in dollar spot disease with applications of the bacterium *Enterobacter cloacea*.

P. aureofaciens strains Tx-1 and Tx-2 have been identified to produce a novel antibiotic which is completely inhibitory to the summer patch causal agent *M. poae* at concentrations of 10-25 μg/ml and to the dollar spot pathogen, *S. homoeocarpa,* at 25-50 μg/ml. Since *P. aureofaciens* Tx-1 and Tx-2 were isolated from turfgrass root tissue it is assumed that these organisms are capable of colonizing plant roots. If colonization occurs, the bacteria may be able to produce the antibiotic *in situ*. Thomashow et al. (58) have shown that the amount of

antibiotic produced at the microsite by inoculated bacterial biological control agents may produce more active compound per given area than is applied by fungicidal applications. The biologically produced compound is also produced where infection may occur rather than being volatilized or trapped in the soil as occurs with fungicidal spray applications.

In this study, the bacterial strains Tx-1 and Tx-2 were applied as spray applications and as amendments to topdressing applications of Compost Plus. Compost Plus was chosen as the topdressing based on its performance with bacterial amendments in preliminary studies.

MATERIALS AND METHODS

Strain selection

Rifampicin resistant strains of Tx-1 and Tx-2 were isolated by first culturing the bacteria in PDB for 24 h. Rifampicin medium was prepared by delivering 50 mg of rifampicin in 5 ml of 95% ethanol. This was added to 1 L of autoclaved PDA cooled to 50° C. Resistant strains were isolated by plating 0.1 ml aliquots of bacterial culture on the rifampicin medium. Resistant strains were removed following 48 h of growth at 28° C.

Similarity to wild type strains was tested by comparing growth curves. This comparison was initiated by transferring a single colony of each of the test strains and the wild type strains to test tubes of 8.5% saline solution. Serial dilutions were conducted to 10^{-8} from which a 0.1 ml aliquot was transferred to test tubes containing 10 ml of

PDB. PDB cultures were maintained at 22° C on a rotary shaker at 100 rpm. Enumeration of colony forming units (CFU) was conducted every 3 h by performing serial dilutions plated out on PDA medium. This was run for a period of 24 h.

Bacterial strains that were deficient in the production of antibiotic were isolated from seven day cultures of the rifampicin resistant strains of Tx-1 and Tx-2. These cultures were plated on PDA by serial dilution to obtain cell counts of approximately 50 CFUs per plate. Cell colonies that failed to exhibit the characteristic orange pigmentation were isolated and screened against *S. homoeocarpa*. Plate bioassays were conducted by using a sterile toothpick to transfer a portion of a colony to a PDA plate 0.5 cm from the outer perimeter of the plate. Three sample colonies were assayed per plate along with the parent rifampicin strain which was inhibitory. These plates were cultured for 24 h at 28° C. A plug of *S. homoeocarpa* was then transferred to the center of the test plate and incubated for 48 h at which time the zones of inhibition are evaluated. Stability of non-inhibitory strains was assessed by repeated transfers and bioassays of these new generations of isolates. Two isolates of each strain, which exhibited growth characteristics most similar to the wild type strains, were compared by following growth curves as described earlier.

Dollar Spot Control

Dollar Spot control studies using *P. aureofaciens* Tx-1 and Tx-2 were conducted at the Hancock Turfgrass Research Center, East Lansing, MI. The study was initiated August 3, 1992 on a plot of Emerald creeping bentgrass (*Agrostis palustris*). This stand was naturally infested with *S. homoeocarpa* many years prior to this study. *S. homoeocarpa* strains present exhibit resistance to the benzimidazole and dicarboxamide classes of fungicides.

The study was carried out with a randomized complete block design with four replications. Plots were 0.9 m x 8.1 m (3′ x 6′) with 30 cm (1.5′) walkways.

Tx-1 and Tx-2 bacteria for field application, were fermented by first preparing starter inoculum. A single colony of bacteria was transferred to a test tube containing 10 ml of TSB which was cultured for 24 h at 22° C on a rotary shaker at 100 rpm. 1 ml aliquots of these seed cultures were transferred to 2 L erlenmeyer flasks containing 1 L of TSB. Flasks were incubated for 24 h. Following incubation, 250 ml samples were centrifuged in 250 ml polypropylene flasks using a Sorvall centrifuge. Cells were pelleted at 4000 rpm for 10 minutes. Cell pellets were resuspended in 10 ml TSB. If not used immediately, cell suspensions were stored at 4° C. Following refrigeration, cell suspensions were allowed to adjust to room temperature for 6 to 12 h prior to application.

Spray application of bacteria were prepared by adding the 10 ml cell suspension to 240 ml of 8.5% saline solution.

The total volume was applied to a single plot with the use of a nitrogen gas hand sprayer.

Bacterial topdressing treatments were prepared by amending 58 g of the organic carrier Compost Plus (Ringer Corp.) with the 10 ml cell suspensions described previously. Compost Plus is a mixture of animal byproducts (bone meal, feather meal, and other organic components) which contain a 7% nitrogen concentration, and was applied at a rate of 32 g/m² to deliver a rate of 85 g N/100 m² (¼ lb N/1000 ft²). Compost Plus was applied without the addition of bacteria as a control treatment. A chemical control treatment of the fungicide triadimefon was applied in the form of Bayleton 25 at a rate of 0.15 g AI/m².

Treatments were applied on a 14 day schedule. All treatments, except the unfertilized control and Compost Plus treatments, were fertilized with 18-4-10 fertilizer at a rate of 85 g N/100 m² (½ lb N/1000 ft²) every 14 days.

Data was taken as enumeration of dollar spot patches present within a single plot. Ratings were taken at two week intervals and transformed with the equation:

$$Data = Log\ (Number\ of\ Spots + 1)$$

Data transformation was conducted to reduce variation at high incidence of disease. Data was analyzed with Tukey's test.

Treatments exhibiting disease reduction from the first years study were replicated the following year. Treatments were applied on a 14 day schedule beginning July 28, 1993.

Compost Plus was applied to deliver 85 g of N/100 m^2 (0.5 lb of N/1000 ft^2) and 170 g of N/100 m^2 (1.0 lb. of N/1000 ft^2) per month. Fertilizer control treatments consisted of the application of 7-3-1 NPK mix, which is similar to the NPK content of Compost Plus. Fertilizer control treatments were at the same nitrogen levels as Compost Plus. A commercial control agent, triadimefon, was applied at a rate of 0.15 g/m^2 every 14 days. Disease ratings were taken weekly and analyzed as previously described.

Summer Patch Control

Field evaluation of summer patch control during 1992 was conducted at Dearborn Country Club, Dearborn, MI, and at Forest Lake Country Club, Bloomfield Hills, MI and the 1993 trials were carried out at Dearborn Country Club and the Highlands, Grand Rapids MI. The study was designed as a randomized complete block with four replications and plot sizes of 1.8 m x 2.7 m (6′ x 9′). The study was initiated when the soil reached 18° C at 5 cm soil depth. Treatments were conducted on a monthly bases from August.

The bacterial colonies used in this study were those listed in section I of this chapter. Seed cultures were prepared by incubating a single bacterial colony in 10 ml of TSB on a rotary shaker (100 rpm) at 22° C. One ml of the seed culture was transferred to 4 L erlenmeyer flasks with 2 L of TSB. These flasks were incubated for 24 h on a rotary shaker at 100 rpm at 22° C. If not used immediately after incubation the bacteria were stored at 4° C. Application of

the bacteria was conducted by suspending 250 ml of the culture in 750 ml of 0.85% saline solution. The entire volume was spray applied to a single plot using a nitrogen gas hand sprayer at 30 psi.

For application as a topdressing, the 250 ml of bacteria broth culture was centrifuged in a Sorvall centrifuge at 5000 rpm for 10 minutes. The resulting cell pellet was resuspended in 10 ml of YMG broth. The 10 ml volume was mixed with sufficient Compost Plus to provide a rate of 85 g of N/100 m^2 per plot. Compost Plus topdressing applications were applied to plots by hand. A control application of Compost Plus was applied to account for effects due to the organic carrier.

A commercial chemical treatment was included which consisted of propiconizole at a rate of 12.5 g of AI/100 m^2. Propiconizole was applied in the commercial form as Banner.

Data ratings were taken as the percentage of annual bluegrass within a plot which has died due to infection by *M. poae*.

RESULTS AND DISCUSSION

Strain Selection

Comparison of growth curves of rifampicin resistant strains of Tx-1 and Tx-2 with the wild type parents did not show any differences.

Bacterial strains which did not produce antibiotic were selected on the basis of the lack of orange pigmentation. Antibiosis screening showed no signs of inhibition from the

colonies lacking the orange pigmentation while zones of inhibition were evident for the parent Tx-1 and Tx-1. Other than pigmentation, the presumptive antibiotic deficient strains did not exhibit any differences in growth or colony characteristics.

Dollar Spot Control

Only two disease rating dates were included since initial outbreak of dollar spot did not occur until late August 1992 and disease pressure abated in mid September. Data from the rating dates are included in Table 15.

Only Compost Plus and triadimefon treatments showed significant disease reduction in relation to the fertilized control. The Compost Plus treatment which was amended with the antibiotic deficient strain of Tx-1 provided better disease management than those amended with other strains. However, all of the Compost Plus treatments were better than the fertilizer control as of the second rating date. The Compost Plus treatment amended with the antibiotic deficient strain of Tx-1 performed significantly better than the Compost Plus control treatment. The reason of the improved disease management by this strain is may be explained by the lack of phytotoxicity by the Tx-1 antibiotic.

Application of bacteria as a foliar spray failed to provide significant disease reductions. These results suggested that the application of these bacteria provided significant improvements are made. The failure of these bacteria to manage dollar spot may be explained by the

Table 15. Dollar Spot data analysis for the 1992 biological control study.

		August 28, 1992			September 11,1992		
Treatment	Rate[a]	# of Spots	Normalized[b] Data Analysis		# of Spots	Normalized Data Analysis	
No Treatment	---	24.3	1.40	A[c]	155.0	2.17	A
18-4-10 Fertilizer	0.85 g N/m²	12.7	1.11	ABC	73.7	1.86	AB
Compost Plus	32 g/m²	5.3	0.55	ABC	16.0	1.09	CD
Compost Plus/Tx-1	32 g/m² + 250 ml[d]	2.0	0.43	ABC	18.3	1.28	BC
Compost Plus/Tx-1	" "	0.3	0.10	BC	5.3	0.75	CD
Compost Plus/Tx-2	" "	5.3	0.50	ABC	15.0	1.17	BC
Compost Plus/Tx-1	" "	2.3	0.49	ABC	20.3	1.21	BC
Spray Tx-1	250 ml	11.3	0.83	ABC	53.3	1.68	AB
Spray Tx-1	" "	11.7	0.81	ABC	51.3	1.68	AB
Spray Tx-2	" "	17.7	1.26	AB	71.7	1.86	AB
Spray Tx-2	" "	17.7	1.27	AB	89.7	1.95	AB
Triadimefon	0.15 g AI/m²	0.0	0.00	C	0.0	0.00	D
	Std. Deviation	3.5	0.23		11.6	0.16	

[a] Application rates made on plots 0.9 m x 1.2 m.
[b] Data transformation performed as log(# of spots + 1).
[c] Data analyzed with Tukey's test, treatments followed by the same letter are not significantly different at the P=0.05 level.
[d] 250 ml of broth concentrated to 10 ml to apply ≈ 10^{11} CFU/m² or 10^{6} CFU/cm².

inability of these organisms to colonize the phylloplane. These bacteria were isolated from soil samples with significantly different environments than the leaf surface. Blakeman (4) identified bacteria as being the dominant colonizing life forms of the phylloplane from April to mid-June which occurs prior to the initiation of this study and to the fall outbreak of dollar spot. He later identified yeasts as the dominant colonizing organisms beginning in July through to early September at which time filamentous fungi dominate. If yeasts are a dominant colonizer during the period in which dollar spot disease occurs, it may be beneficial to screen yeast populations for antagonistic properties.

The disease management characteristics of Compost Plus were re-evaluated during the 1993 field season. Data for the 1993 field study are provided on Tables 16, 17, and 18. The greening effects caused by Compost Plus seen in the 1992 field study were not evident in 1993 until late September.

Compost Plus applied to deliver 85 g of N/100 m^2 (½ lb. of N/1000 ft^2) per month did not exhibit disease management better than the NPK fertilizer control treatment. However, it provided significantly better disease management than the unfertilized control, with dollar spot reductions of up to 75% of that of the unfertilized control. Compost Plus applied to deliver 170 g of N/100 m^2 (1.0 lb. N/1000 ft^2) per month yielded reductions in dollar spot incidence significantly better than the fertilized control at the same

ratio of NPK. The level of disease management derived from Compost Plus did not reach the level derived from the application of the fungicide triadimefon as in the previous years study.

Dollar spot management by Compost Plus at the 170 g of N/100 m^2 per month provided higher disease reduction as the field season progressed. Dollar spot incidence was reduced by up to 92% of the untreated control and up to 60% of the fertilized control. Significant disease management by Compost Plus in the 1992 field season, which was unseasonably cool and wet, as well as later into the fall of the 1993 season, suggested that certain populations of microorganism are involved in this disease management which are sensitive to harsher conditions of the summer. As stated earlier, it is the filamentous fungi which begin to dominate in colonization of the phyllosphere at the beginning of September. This correlated to the weather conditions in which Compost Plus provides the greatest disease management. The alteration in the microbial populations may also account for the greening effect caused by Compost Plus. Filamentous fungi may also be better suited to utilize the nutrients offered by Compost Plus than organisms better adapted to summer climates. If fungal organisms do play a role in the disease management by Compost Plus, the utilization of fungal biological controls such as *Fusarium heterosporum* (20) as amendments may offer increased management.

Although the disease management offered by Compost Plus is not complete, it provided a tool by which dollar spot disease may be reduced without the use of chemical fungicides.

Summer Patch Control

The 1992 field season was unseasonably cool and wet which resulted in the lack of disease development. Significantly more disease was evident in the 1993 field study, however, due to the lack of severity and consistency, no significant differences were observed.

Table 16. Dollar spot data analysis of Compost Plus treatments for August 17-31, 1993.

		August 17, 1993		August 24, 1993		August 31, 1993	
Treatment	Rate[a]	# of Spots	Normalized Data[b]	# of Spots	Normalized Data	# of Spots	Normalized Data
No Treatment	---	10.0	1.22 A[c]	34.0	1.43 A	46.3	1.60 A
7-3-1 NPK	0.43 g N/m²	10.5	1.04 A	21.8	1.31 A	28.3	1.43 AB
7-3-1 NPK	0.85 g N/m²	9.3	0.94 A	16.5	1.14 A	12.5	1.04 B
Compost Plus	0.43 g N/m²	10.5	0.92 A	19.3	1.15 A	20.8	1.24 AB
Compost Plus	0.85 g N/m²	17.3	1.19 A	20.3	1.27 A	10.3	0.97 B
Triadimefon[d]	0.15 g/m²	0.0	0.00 B	0.0	0.00 B	0.0	0.00 C
	Std. Deviation	3.3	0.12	6.3	0.12	7.5	0.11

[a] Application rates made on plots 0.9 m x 1.2 m.
[b] Data transformation performed as log(# of spots + 1).
[c] Data analyzed with Tukey's test, treatments followed by the same letter are not significantly different at the P=0.05 level.

Table 17. Dollar spot data analysis of Compost Plus treatments for September 13-28, 1993.

		September 13, 1993		September 22, 1993		September 28, 1993	
Treatment	Rate[a]	# of Spots	Normalized Data[b]	# of Spots	Normalized Data	# of Spots	Normalized Data
No Treatment	---	83.8	1.89 A[c]	88.8	1.91 A	99.5	1.99 A
7-3-1 NPK	0.43 g N/m²	44.3	1.65 AB	40.0	1.61 B	60.5	1.85 AB
7-3-1 NPK	0.85 g N/m²	28.0	1.42 BC	22.8	1.37 CD	39.3	1.76 AB
Compost Plus	0.43 g N/m²	37.3	1.53 BC	32.8	1.52 BC	56.3	1.60 AB
Compost Plus	0.85 g N/m²	19.0	1.28 C	14.8	1.20 D	21.8	1.35 AB
Triadimefon	0.15 g/m²	0.0	0.00 D	0.0	0.00 E	0.0	0.00 C
	Std. Deviation	9.5	0.07	10.0	0.04	9.0	0.04

[a] Application rates made on plots 0.9 m x 1.2 m.
[b] Data transformation performed as log(# of spots + 1).
[c] Data analyzed with Tukey's test, treatments followed by the same letter are not significantly different at the P=0.05 level.

Table 18. Dollar spot ratings and data analysis of Compost Plus treatments for October 6, 1993.

		October 6, 1993	
Treatment	Rate[a]	# of Spots	Normalized Data[b]
No Treatment	---	74.1	1.87 A[c]
7-3-1 NPK	0.43 g N/m²	35.5	1.55 AB
7-3-1 NPK	0.85 g N/m²	15.5	1.19 BC
Compost Plus	0.43 g N/m²	20.9	1.32 B
Compost Plus	0.85 g N/m²	6.3	0.80 C
Triadimefon	0.15 g/m²	0.0	0.00 D
	Std. Deviation	6.7	0.09

[a] Application rates made on plots 0.9 m x 1.2 m.
[b] Data transformation performed as log(# of spots + 1).
[c] Data analyzed with Tukey's test, treatments followed by the same letter are not significantly different at the P=0.05 level.

APPENDIX

Average relative growth of fungal turfgrass pathogens on PDA media amended with the Tx-1 antibiotic.

	0.1 µg/ml	1.0 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Bipolaris sorokiniana	84.2[a]	67.1	20.1	0.0	0.0	0.0
Colletotrichum graminicola	94.7	81.2	24.6	0.0	0.0	0.0
Gaeumannomyces graminis	100.0	78.2	0.0	0.0	0.0	0.0
Leptosphaeria korrae	96.6	89.9	16.9	0.0	0.0	0.0
Magnaporthe poae	104.7	97.2	21.5	0.0	0.0	0.0
Microdochium nivale	105.9	97.5	62.6	45.0	26.9	2.5
Rhizoctonia solani	90.0	82.7	67.3	53.6	35.5	24.6
Sclerotinia homoeocarpa (DMI[s])	96.4	92.0	42.0	18.1	0.0	0.0
Sclerotinia homoeocarpa (DMI[r])	92.8	78.3	21.7	11.6	0.0	0.0
Typhula incarnata	84.4	53.1	0.0	0.0	0.0	0.0

[a] Average relative growth is determined by dividing the average radial growth of fungal mycelium on media amended with the Tx-1 antibiotic by the average radial growth of a control plates.

Average relative growth of fungal turfgrass pathogens on PDA media amended with chlorothalonil.

	0.1 µg/ml	1.0 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Bipolaris sorokiniana	70.9[a]	41.5	11.1	0.0	0.0	0.0
Colletotrichum graminicola	93.0	83.8	40.4	21.1	16.2	0.0
Gaeumannomyces graminis	77.5	48.3	39.3	25.8	29.2	0.0
Leptosphaeria korrae	86.1	94.3	53.3	34.4	54.9	15.6
Magnaporthe poae	73.1	38.5	25.4	23.9	3.1	0.0
Microdochium nivale	92.9	78.0	50.0	41.3	36.6	27.2
Rhizoctonia solani	83.5	45.9	19.6	13.5	14.3	10.5
Sclerotinia homoeocarpa (DMI[s])	92.0	52.9	21.0	10.9	10.9	7.6
Sclerotinia homoeocarpa (DMI[r])	91.3	47.1	6.5	3.6	1.5	0.0
Typhula incarnata	97.8	0.0	0.0	0.0	0.0	0.0

[a] Average relative growth is determined by dividing the average radial growth of fungal mycelium on media amended with chlorothalonil by the average radial growth of a control plates.

Average relative growth of fungal turfgrass pathogens on PDA media amended with triadimefon.

	0.1 µg/ml	1.0 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Bipolaris sorokiniana	107.7[a]	104.3	61.5	29.1	25.6	23.5
Colletotrichum graminicola	86.8	73.7	15.8	6.1	0.0	0.0
Gaeumannomyces graminis	101.1	102.3	79.8	47.2	39.3	0.0
Leptosphaeria korrae	96.7	97.5	89.3	89.3	84.4	77.8
Magnaporthe poae	86.2	71.5	15.4	8.5	3.9	0.0
Microdochium nivale	92.9	78.0	50.0	41.3	36.6	27.2
Rhizoctonia solani	83.5	40.6	7.5	0.0	0.0	0.0
Sclerotinia homoeocarpa (DMI[s])	36.2	3.6	0.0	0.0	0.0	0.0
Sclerotinia homoeocarpa (DMI[r])	71.7	34.1	2.9	0.0	0.0	0.0
Typhula incarnata	88.8	33.3	0.0	0.0	0.0	0.0

[a] Average relative growth is determined by dividing the average radial growth of fungal mycelium on media amended with the Tx-1 antibiotic by the average radial growth of a control plates.

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