CHARACTERISTICS OF *RHIZOCTONIA SOLANI* TURF ISOLATES AND EVALUATION OF AN INTEGRATED CONTROL STRATEGY FOR BROWN PATCH AND DOLLAR SPOT ON CREEPING BENTGRASS

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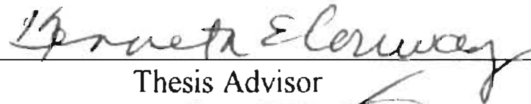

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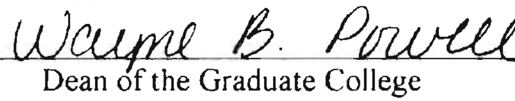

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CHAPTER I

INTRODUCTION

The multi-billion dollar turfgrass industry represents one of the fastest growing, most diversified segments of United States agriculture (34). Turfgrasses are used on home lawns, public parks, playgrounds, air fields, highway rights-of-way, and recreational fields mainly for either aesthetic or functional purposes. Turfgrasses provide value to landscaped areas, and enhance emotional, mental, and physical human welfare. Functionally, turfgrasses: 1) provide soil stability and erosion control, 2) moderate temperatures, especially in urban areas where concrete surfaces can boost ambient temperatures five-to-eight degrees higher than those in rural, more grassy areas; 3) reduce noise and particulate matter; 4) increase real estate value; and 5) provide safe playing surfaces for sports and recreation (14).

Golf courses are one of the most important sectors of the turfgrass industry, contributing billions of dollars to the United States economy annually. Their high financial value makes it imperative to provide healthy, well-managed playing surfaces. The exceptional wear tolerance, high density, low growth, and fine-textured qualities of bentgrass (*Agrostis* spp.) make it highly valuable for use on golf greens, where the majority of golf shots are made and where a golfer's accuracy becomes most critical. Bentgrass can withstand high traffic and rigorous maintenance procedures like frequent mowing to heights as low as 3 mm, and at the same time provide resilient, attractive,

smooth, and uniform playing surfaces. Oklahoma has approximately 25,000 acres of golf course property and 98% of golf course greens are composed of creeping bentgrass (*Agrostis palustris* Huds.) (15).

Bentgrass is a cool-season turfgrass, best adapted to cool, moist environments like those found in the northwestern and northeastern United States. Of the more than 100 bentgrass species, only four are used for turf in the U. S. Creeping bentgrass is the most common (10, 14). The hot, humid conditions of the southern regions of the U. S. (including Oklahoma) restrict the use of bentgrass species to golf course putting greens, where the turf can be intensively managed. The summer climate of the southern U. S. predisposes bentgrass species to several stress conditions. Bentgrass carbohydrate reserves become depleted, making the turfgrass highly susceptible to the pressures of traffic, drought, shade, insects, and disease (14).

Creeping bentgrass is highly susceptible to Rhizoctonia blight (brown patch), incited by *Rhizoctonia solani* Kuhn, and dollar spot, caused by *Sclerotinia homoeocarpa* F. T. Bennett. Brown patch and dollar spot are two of the most important and most destructive turf diseases. It has been estimated that in the United States alone, losses to soilborne fungal pathogens like *R. solani* and *S. homoeocarpa* amount to at least $4 million annually (25). In reference to the brown patch fungus, as of 1994, 12 *R. solani* strains (AG-1 through AG-11 and AG-BI) had been characterized (6) *R. solani* strains are classified according to hyphal anastomosis or mycelial compatibility, a genetic feature which results in the exchange of nuclei and the combining of different genotypes (5, 21). Anastomosis occurs between fungal isolates of the same strain or anastomosis group (AG)

but not between isolates of different AG's. Each AG therefore seems to be genetically independent from all others (27, 28). Four anastomosis groups of *R. solani* isolated from turfgrass have been identified: AG-1 (subgroup IA on cool-season turf in Japan), AG-2 (subgroup 2), AG-4, and AG-5 (1, 4). Subgroup 2 of AG-2 (AG-2-2) has been consistently associated with Rhizoctonia blight of turfgrasses (1, 4, 17, 35). Types AG-2-2 IIIB and IV differ slightly in cultural morphology and in the symptoms they produce. Type IIIB is usually associated with infections on foliar plant portions of family Poaceae, while type IV primarily causes root rots of the Chenopodiaceae (4). The IIIB type causes Rhizoctonia blight (brown patch) on cool-season grasses and type IV causes Rhizoctonia blight (large patch) on warm-season grasses (1, 4, 20, 35). Kataria et al., (21) tested various fungicides against several AGs of *R. solani* and demonstrated that fungicide sensitivity can vary between fungal strains and between isolates of the same strain. A fungicide labeled for control of a particular pathogen may not be equally effective against all strains of the pathogen. Fungicide sensitivity analyses for strains of *R. solani* associated with turfgrass diseases and confirmation of anastomosis grouping are important to ensure that control inputs are not used inappropriately

Reliable disease control for brown patch and dollar spot is commonly achieved through fungicide applications, sometimes several, during a given growing season. In 1996, the nearly 200 Oklahoma golf facilities maintained their courses at an estimated total cost of $220 million (15). A substantial proportion ($865,000) of total maintenance expenses was the result of fungicide costs. The Agricultural Chemical Industry Profile for 1992 estimated that of the total $83.1 million spent by the U S. golf course enterprise on

pesticides, nearly 70% ($56.5 million) was the result of fungicide purchases (11). Goodman and Burpee (16) estimated that typical fungicide application costs for a single golf course can surpass $170/hectare. Although expensive, fungicide applications are cost-effective and essential for satisfactory disease control on highly managed turf where diseases like brown patch and dollar spot can severely reduce turf quality and affect economic value.

The heavy reliance of the golf course industry on fungicides has led to problems other than high prices. Concerns that fungicides may negatively impact the environment by reducing soil and water qualities (23, 33), cause detrimental effects on non-target organisms (12, 23, 30, 33), and promote development of fungicide-resistant fungal pathogen populations (12, 23, 32) have increased public fears and perceptions of fungicide use. Because of these matters, efforts to discover and utilize alternative means of turfgrass pest control are desired and important.

Biological control (biocontrol) is a non-chemical alternative to the use of fungicides for turfgrass disease reduction. Biocontrol can be defined as the manipulation and utilization of antagonistic modes of action of biotic agents other than man for disease reduction. Biocontrol agents can compete with pathogens for essential nutrients, produce toxic metabolites, or parasitize pathogens to provide reductions in pathogen inoculum and disease severity. Biocontrol has several advantages over traditional chemical treatments for disease suppression. Biocontrol organisms reduce environmental toxicity, are selective for particular pathogens and therefore cause fewer nontarget effects, and are less likely to encourage the development of fungicide resistance.

Biocontrol agents have been effective against some turfgrass pathogens. Burpee and Goulty (2) reduced brown patch disease on creeping bentgrass using nonpathogenic binucleate isolates of *Rhizoctonia* spp. Approximately 25 cm^3 of rye grain infested with the nonpathogenic isolates was added to turfgrass plots 24 hours prior to inoculation with *R. solani*. In all three experiments, brown patch disease was significantly suppressed by some of the binucleate isolates. Burpee et al., (3) used a nonpathogenic isolate of *Typhula phacorrhiza* (Reichard:Fr.) Fr. to suppress gray snow mold on creeping bentgrass, incited by *Typhula ishikariensis* Imai and *T. incarnata* Lasch ex. Fr. Wheat-grain infested with *T. phacorrhiza* and applied to turf as a topdressing at rates of 100 and 200 g/m^2 reduced disease severity by 44% and 70%, respectively. Goodman and Burpee (16) found that *Fusarium heterosporum* Nees ex. Fr. applied to creeping bentgrass as a weekly sand-corn meal topdressing at a rate of 400 cm^3/m^2 limited dollar spot disease incidence to as low as 5%, compared to 84% disease incidence on control plots not topdressed and 64% on plots topdressed with noninfested, autoclaved sand-corn meal. Haygood and Mazur (19) compared a strain of *Gliocladium virens* J. H. Miller, J. E. Giddens, and A. A. Foster to the fungicides chlorothalonil, propiconazole, and iprodione for control of dollar spot on bermudagrass. Prill inoculum of *G. virens* formulated by W. R. Grace and Company was applied to the turfgrass at a rate of 32.4 g/m^2 at two week intervals. On the three rating dates, *G. virens* suppressed dollar spot by 70%, 54%, and 46%, compared to 85% disease suppression with the fungicides on all rating dates. Lo et al., (24) tested *Trichoderma harzianum* Rifai strain 1295-22 for control of foliar phases of Pythium root rot, brown patch, and dollar spot diseases. Weekly spray applications of *T. harzianum* at

a rate of 200 ml/m^2 significantly reduced all three diseases on creeping bentgrass and were as effective in control as propiconazole fungicide. Nelson and Craft (26) found that some strains of the bacterium *Enterobacter cloacae* (Jordan) Hormalche and Edwards were able to suppress dollar spot on bentgrass putting greens by 63% in some experiments when applied as monthly sand-corn meal topdressings at a rate of 465 cm^3/m^2 but other strains were only able to provide 3% disease suppression. Although these studies indicated that reductions in turfgrass disease severity can be attained, biocontrols generally do not perform spectacularly and they often do not work as efficiently, or as consistently as fungicides (25).

The use of biological control alone has several disadvantages. Biocontrol agents are effective in reducing disease but they are living organisms and their efficacy and activity depends a great deal more on environmental conditions than fungicides do. For a biological control organism to be successful, it must exist under field situations long enough to protect a plant during the period it is most vulnerable to disease. Secondly, it is often quite difficult to formulate biocontrol agents for proper field testing and application because of the high expenditures of time, energy, space, and money that are frequently associated. Another disadvantage to biological control is that the shelf lives of biocontrol formulations may be limited and may require special preservation and handling to maintain viability. Powell and Faull (29) proposed that biocontrol formulations should have a shelf life of two years at -5°C to +30°C. The mechanisms of action of biocontrol agents are not always known. Knowledge of modes of action is very important for reliably estimating and predicting the persistence and success of disease control under field conditions.

Finally, biological control may not be as fast-acting as traditional fungicides, making assessment of application rates and timing schedules difficult.

In vitro biocontrol tests generally yield positive results for antagonism of one organism by another but field screening results are much more variable since the effectiveness of biocontrol agents depends greatly on the uncontrolled field conditions. As of 1989, it was estimated that approximately only 5% of intentional deliveries of biocontrol agents under field circumstances achieved their objective (29). The problems associated with both chemical and biological controls of plant diseases in general have stirred growing interest in the possibility of combining chemical treatments with biocontrol agents in integrated biological/chemical control systems. The use of integrated disease control strategies has several possibilities and advantages. Combinations of more than one disease management practice can reduce the likelihood of the development of fungicide resistance. The number of pesticide applications and environmental impacts can be reduced. Integrated biological/chemical control tactics may improve the efficacies of biocontrol agents, since fungicides remain effective against a pathogen despite environmental conditions. Fourthly, biological control organisms may be able to provide the long-lasting localization and persistence that fungicides are incapable of providing. Finally, integrated disease control practices could extend effectiveness to several diseases at a given time (31, 36).

Several researchers have investigated the possibilities of integrated disease control strategies. Lifshitz et al., (22) found that integration of the fungicide benodanil with *T. harzianum* resulted in a significant decrease in the incidence of Rhizoctonia pre-emergence

damping off of radish (*Raphanus sativus* L.). Cole and Zvenyika (7) showed that integration of the biological control agent *T. harzianum* with reduced applications of the fungicide triadimenol enhanced disease control of sore shin (*R. solani* and *Fusarium solani* (Mart.) Sacc.) in tobacco (*Nicotiana* spp.) transplants. Conway et al., (8) found that integration of *Laetisaria arvalis* Burdsall with the experimental fungicide CGA 173506 at one-half the recommended rate significantly reduced Rhizoctonia aerial blight and root rot of rosemary (*Rosemarinus officinalis* L.) compared with the fungicide or biocontrol treatments alone. Integration of the fungicide pentachloronitrobenzene (PCNB) and *T. harzianum* resulted in reduced southern blight (*Sclerotium rolfsi* Sacc.) disease severity on apple trees (*Malus domestica* Bork) compared with either treatment alone (9). Harman et al., (18) discovered that applications of *T. harzianum* to control Botrytis bunch rot of grapes in the developmental stage followed by iprodione applications at one-half rate resulted in effective disease control.

Although there have been various research efforts concentrated on integrated control of plant diseases, little is known about integrated control of turfgrass diseases Brown patch and dollar spot diseases can occur on creeping bentgrass greens at approximately the same time of the growing season (11) Although brown patch is suppressed by the biological fungicide azoxystrobin, dollar spot is not (13). It is possible that brown patch can be adequately controlled with biocontrol fungi plus reduced azoxystrobin applications and rates and that dollar spot can be controlled by the same biocontrol fungi. Research on integration of azoxystrobin fungicide and two biological control fungi, *T. harzianum* and *L. arvalis*, for control of brown patch and dollar spot

should help discern whether the selected integrated control strategy in this study can effectively suppress both diseases more so than either the fungicide or the biological agents alone. One of our major objectives was to evaluate whether application schedules and formulations of the biological control organisms are adequate for the integrated control of brown patch and dollar spot on creeping bentgrass greens. This study should also be helpful in ascertaining the efficacy of each biological control fungus and whether one is more effective for control of brown patch and dollar spot than the other. Additional objectives of this study were:

1. To characterize the cultural and pathogenic attributes of fungal isolates obtained from Rhizoctonia blight on cool- and warm-season grasses, to categorize them into their respective anastomosis group, and to determine their sensitivities to azoxystrobin biological fungicide.

2. To evaluate the use of solid vermiculite-corn meal-sand substrate preparations of *T. harzianum* and *L. arvalis* and their applications as topdressings to a creeping bentgrass green surface for potential control of brown patch and dollar spot diseases.

3. To attempt to determine the persistence and localization of *T. harzianum* and *L. arvalis* on a creeping bentgrass green under field conditions.

4. To determine the sensitivities and compatibilities of *T. harzianum* and *L. arvalis* to the biological pesticide azoxystrobin.

5. To ascertain whether the biocontrol rates, topdressing formulations, methods of application, and application schedules of biocontrol fungi were sufficient enough to

provide integrated control of brown patch and dollar spot along with reduced rates and applications of azoxystrobin fungicide.

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CHAPTER II

CHARACTERIZATION OF *RHIZOCTONIA SOLANI* ISOLATES ASSOCIATED WITH PATCH DISEASES ON TURFGRASSES

ABSTRACT

Cultural characteristics and pathogenicity of *Rhizoctonia solani* Kuhn isolates obtained from brown patch on creeping bentgrass (*Agrostis palustris* Huds.) and large patch on zoysiagrass (*Zoysia japonica* Willd) were evaluated and compared with known *R. solani* anastomosis groups AG-2-2 III-B, *R. solani* AG-2-2 IV, *R. solani* AG-1-IA, *R. solani* AG-4, and *R. solani* AG-5. Bentgrass and zoysiagrass isolates were obtained from infected grass leaf sheaths along disease patch margins The bentgrass and zoysiagrass isolates differed culturally from one another. The bentgrass isolate most closely matched *R. solani* AG-2-2 IIIB in both cultural characteristics and pathogenicity on creeping bentgrass cv. "Crenshaw." The bentgrass isolate and the AG-2-2 IIIB tester both showed irregular clusters of mycelia (not sclerotia), concentric zonation, dark brown main hyphae, and sparse aerial hyphae on potato dextrose agar after two weeks of incubation Optimum temperature for growth of both isolates was 25°C. These two isolates caused high levels of disease on creeping bentgrass cv. "Crenshaw" in *in vitro* pathogenicity tests. The zoysiagrass isolate most closely matched *R. solani* AG-2-2 IV in both cultural characteristics and pathogenicity on creeping bentgrass cv. "Crenshaw." The zoysiagrass isolate and the AG-2-2 IV tester both had abundant aerial hyphal growth, dark brown

main hyphae, and no sclerotial formation or zonation on potato dextrose agar after two weeks of incubation. Optimum temperature for growth of both isolates was 25°C but unlike the bentgrass isolate and the AG-2-2 IIIB tester, the zoysiagrass isolate and the AG-2-2 IV tester did not grow at 35°C. The zoysiagrass isolate and the AG-2-2 IV tester caused low levels of disease on creeping bentgrass cv. "Crenshaw" in *in vitro* pathogenicity tests. Results indicated that cultural characteristics and host range of the bentgrass isolate and those of the zoysiagrass isolate are different. Isolates representing *R. solani* AG-2-2 IIIB and AG-2-2 IV were tested for sensitivity to azoxystrobin in *in vitro* tests. Sensitivity to azoxystrobin (effective concentration causing 50% growth inhibition [EC_{50}]) was determined by radial growth on potato dextrose agar amended with 0, 1, 3.2, 10, 31.2, 100, 316, and 1000 mg a.i. azoxystrobin per liter after three days incubation at 22°C. EC_{50} values for AG-2-2 IIIB isolates averaged approximately 193 mg a.i. azoxystrobin/liter while those for AG-2-2 IV isolates averaged approximately <1 mg a.i azoxystrobin/liter. Results suggested there is some variability in fungicide sensitivity between and within *R. solani* anastomosis groups and that *R. solani* AG-2-2 IIIB isolates may be less sensitive to azoxystrobin fungicide than AG-2-2 IV isolates.

Rhizoctonia solani Kuhn is a worldwide, ecologically diverse soilborne fungus belonging to Order Ceratobasidiales of the Basidiomycotina and is the mycelial or imperfect state of *Thanatephorus cucumeris* (A. B. Frank) Donk. Important taxonomic characteristics of the species include: 1) the absence of asexual spores, or conidia; 2) the absence of clamp connections, or structures involved in genetic recombination; 3) the

absence of rhizomorphs; 4) small ($\leq$ 1mm), round, dark brown sclerotia that may or may not be present; 5) multinucleate hyphal cells; 6) pigmented mycelia (shades of brown); 7) right-angled hyphal branching; 8) septum formation in hyphal branches near points of hyphal origin; 9) presence of a dolipore septum; and 10) an optimum growth temperature of 20 to 30°C (2, 4, 6, 23).

The host range of *R. solani* is extensive. The pathogen is capable of causing several diseases symptoms like seedling damping-off, root rot, collar rot, stem canker, crown rot, bud and fruit rots, and foliage blight on a variety of susceptible agriculturally important crops (2, 4) like soybean (*Glycine max* (L.) Merr.) (20), cotton (*Gossypium hirsutum* L.) (5), canola (*Brassica campestris* L.) (26), wheat (*Triticum aestivum* L.) (25), beet (*Beta vulgaris* L.) (8), potato (*Solanum tuberosum* subsp. *tuberosum*) (15), and rosemary (*Rosemarinus officinalis* L.) (11). *R. solani* also infects a number of turfgrass species (12). The fungus was first identified as the causal agent of a disease known as Rhizoctonia blight (brown patch) on creeping bentgrass (*Agrostis palustris* Huds.) in 1913 (6, 12) and has since become one of the most destructive diseases of both warm- and cool-season turfgrasses including zoysiagrasses (*Zoysia* Willd spp.) (1, 6, 12), tall fescue (*Festuca arundinacea* Schreb.) (1, 6, 12), Kentucky bluegrass (*Poa pratensis* L.) (1, 12), centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) (1, 12), and creeping bentgrass (1, 6, 12). Rhizoctonia blight on cool-season grasses is called "brown patch" and on warm-season grasses, the disease is referred to as "large patch" (1, 17) because of slight differences in symptomatology, time of year of disease outbreaks, and *R. solani* isolate cultural morphology (6, 17).

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Variability in disease symptoms, host range, and geographical location of *R. solani* isolates suggests that there are several strains of the species (6). As of 1994, 12 different strains of the fungus (AG-1 through AG-11 and AG-BI) (9) have been recognized based on affinities for hyphal fusion (anastomosis), a genetic feature that results in exchange of nuclei and the combining of different genotypes (6, 18). Anastomosis groups are categorized based upon their mycelial compatibilities for hyphal fusion. Anastomosis occurs between fungal isolates of the same AG but not between isolates of different AGs. Each AG therefore seems to be genetically independent from all others (6, 21, 22).

Anastomosis groups appear to be fairly host plant specific. For instance, AG-3 occurs commonly on Solanaceae and AG-4 is regularly associated with Pinaceae, Chenopodiaceae, Cruciferae, Leguminosae, Malvaceae, and Solanaceae (7). Four anastomosis groups of *R. solani*, AG-1 (specifically, subgroup 1A on cool-season turf in Japan), AG-2, AG-4, and AG-5, have been isolated from turfgrasses (1, 6). Subgroup 2 of AG-2 (AG-2-2) has been consistently associated with Rhizoctonia blight of turfgrasses (1, 6, 16, 30). Reports indicate that brown patch on cool-season turf is typically caused by intraspecific group IIIB of subgroup AG-2-2, while large patch on warm-season turf is incited by intraspecific group IV of subgroup AG-2-2 (1, 6, 17, 30) Type IIIB is usually associated with infections of foliar portions of family Poaceae, while type IV primarily causes root rots of the Chenopodiaceae (6).

Several fungicides have been labeled for control of Rhizoctonia blight including flutolanil (Prostar, AGREVO Corporation, Wilmington, DE), propiconazole (Banner, Ciba-Geigy Corporation (Novartis), Greensboro, NC), fenarimol (Rubigan, Dow-Elanco

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Specialty Products, Indianapolis, IN), iprodione (Chipco 26109, Rhone-Poulenc AG Company, Research Triangle Park, NC), chlorothalonil (Daconil 2787, ISK Biotech Corporation, Mentor, OH), quintozene (Terraclor, Uniroyal Chemical Co., Middlebury, CT), mancozeb (Fore, Rohm and Haas, Co., Philadelphia, PA) (12), and azoxystrobin (Heritage 50WDG, Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire, RG42 6ET, UK) (14). Azoxystrobin is a fairly new beta-methoxyacrylate fungicide that has been used for control of several ascomycete, basidiomycete, and oomycete fungal diseases on such crops as cereals, cucurbits, vegetables, fruits, peanuts, ornamentals, rice, potatoes, and turf. Azoxystrobin is a derivative of the chemically similar strobilurins, a class of naturally occurring fungicides produced by *Strobilurus tenacellus*, a wood-decaying fungus of the mushroom family (Agaricaceae) (14). The fungicide has broad spectrum activity with protectant and acropetal systemic capabilities, meaning the chemical can be taken up by plant xylem and then move upward in the transpiration stream. The chemical is also effective for controlling established infections and can be absorbed by both roots and leaves. Azoxystrobin interferes with cellular respiration in sensitive fungal pathogens by inhibiting transport of mitochondrial electrons (3).

It is not known whether these fungicides are effective against all *R. solani* anastomosis groups or just a select few. Kataria et al., (18) tested different fungicides against various isolates of several anastomosis groups and found variability in fungicide sensitivity between and within anastomosis groups. Knowledge of which anastomosis groupings are involved in a given Rhizoctonia blight outbreak and their sensitivities to

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different fungicides may help to facilitate selection of the most appropriate fungicide for management of the disease in any particular area or situation.

This study was conducted in order to classify *R. solani* isolates from brown patch on creeping bentgrass and large patch on zoysiagrass into appropriate anastomosis groups based on observations of cultural and pathogenicity characteristics and comparisons with those of known anastomosis testers, and to evaluate the anastomosis groups commonly associated with Rhizoctonia blight for variability in sensitivity to azoxystrobin in *in vitro* tests.

MATERIALS AND METHODS

Collection and Isolation. Grass leaf sheaths and blades with symptoms of brown patch and large patch were collected from creeping bentgrass and zoysiagrass, respectively, at the Horticulture Turfgrass Research Center in Stillwater, Oklahoma in 1997. Samples were taken from the extreme margins of patch areas with forceps and transported to the laboratory (Oklahoma State University, Department of Entomology and Plant Pathology, Stillwater, OK) in polyethylene bags. Sections of necrotic, straw-colored tissue were removed from infected plant material and were surface sterilized with 10% Clorox (The Clorox Company, Oakland, CA) for 1 min, plated onto potato dextrose agar (Difco Laboratories, Detroit, MI) amended with streptomycin sulfate (0.3 g/liter medium) (Sigma Chemical Co., St. Louis, MO) contained in petri dishes, and incubated at 22°C (12-h light/12-h dark regime). After 48-h of incubation, hyphal tips from each isolate were transferred to fresh potato dextrose agar (PDA) petri dishes (10 cm X 1.5 cm). Following another 48-h of incubation, hyphal tips were transferred to PDA slants and

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stored in a fungal culture collection at 11°C. The bentgrass isolate was designated as #345 and the one from zoysiagrass as #414 *R. solani* isolates #96, #300, and #309 were obtained from stock slant cultures from a laboratory (Oklahoma State University, Department of Entomology and Plant Pathology, Stillwater, OK) fungal culture collection. These isolates were originally obtained from R. J. Cook (USDA-ARS) and were chosen for the experiment because they are representatives of anastomosis groups AG-4, AG-1-IA, and AG-5, respectively. Also chosen for the experiment were *R. solani* isolates #410 and #411, isolated from zoysiagrass and creeping bentgrass in Kansas and representing anastomosis intraspecific groups AG-2-2 IV and AG-2-2 IIIB, respectively. These two isolates were obtained from N. A. Tisserat (Kansas State Univ., Manhattan, KS). Mycelial fragments were removed from the slant cultures and plated onto PDA then were incubated as above for use in subsequent experiments.

Cultural characteristics. *R. solani* isolates #345 and #414 were identified based on comparisons of cultural characteristics, hyphal anastomosis, and number of nuclei per hyphal cell with those of the known AG testers *R. solani* #96, *R. solani* #300, *R. solani* #309, *R. solani* #410, and *R. solani* #411. Five PDA cultures per isolate were observed for colony color, sclerotial formation, growth zonation, and aerial mycelium after incubation for two weeks at 22°C and the experiment was repeated once. To confirm the multinucleate condition, mycelia from each isolate were removed from PDA cultures and teased apart on a clean glass slide. Hyphae were stained with acridine orange (13) and observed using epifluorescence under an ultraviolet microscope. Following a modified

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procedure described by Aoyagi et al., (1) 15 hyphal cells per isolate were observed for the multinucleate condition in two separate experiments.

Hyphal anastomosis. Hyphal anastomosis reactions were observed by removing mycelial plugs (0.75 cm in diam.) of isolates #345 and #414 from actively growing week-old PDA cultures and pairing them with mycelial plugs of tester isolates of known anastomosis group having the same cultural characteristics as isolates #345 and #414. Tests were conducted on 2% reverse osmosis (RO) water agar in 10 cm X 1.5 cm petri dishes following modified procedures of Parmeter et al., (22), and Zhang and Dernoeden (30). Petri dishes were incubated at 22°C until hyphae from paired isolates began to overlap (two or three days). Overlapping hyphae were then stained with lacto-fuchsin red (10) and were observed for two types of anastomosis reactions using light microscopy at 400X magnification. Perfect anastomosis occurs between hyphae from the same isolate or a genetically identical isolate (clone) and is characterized by complete fusion of cell walls and cytoplasm. Imperfect anastomosis is the result of cell wall fusion but exchanged cytoplasm does not remain viable as with perfect anastomosis (1, 27, 28).

Ten water agar plates per pairing were observed for 25 points of contact each. Pairings of mycelial plugs from the same isolate were designated as control reactions. A pair of separate isolates was considered genetically identical if more than 80% of fusion contacts were perfect anastomoses (1).

Temperature-growth experiment. Mycelial plugs (0.75 cm in diam.) were cut from actively growing two-day-old PDA cultures of isolates #345 (brown patch), #414 (large patch), #411 (AG-2-2 IIIB), and #410 (AG-2-2 IV) and plated onto new PDA petri

dishes. Five replicate petri dishes for each isolate were incubated in the dark at 21, 25, 26, 30, and 35°C. Two perpendicular colony diameters were measured on the bottom of each plate after 24-h incubation. Agar plug diameters were subtracted from every measurement. The two colony diameters for each plate were averaged and a mean growth rate was calculated from the five replicate plates for each temperature. The test was conducted twice with similar results.

Pathogenicity tests using conetainers. All *R. solani* isolates were tested for pathogenicity to creeping bentgrass cv. "Crenshaw." Inoculum was produced by adding one 10 cm X 1.5 cm PDA culture, chopped into approximately 1 cm^2 pieces, to a 250-ml Erlenmeyer flask containing 20 g oat seed and 3 ml RO water that had been autoclaved (121°C, 1.05 kg/cm^2, 20 min) on each of three consecutive days. Flasks containing oat seed were incubated at 22°C (12-h light/12-h dark regime) and after 14 days, the colonized seed was removed and dried overnight under a laminar-flow hood.

Creeping bentgrass seed was obtained from Lofts Great Western Seed Company (Albany, OR). Pathogenicity of *R. solani* isolates on creeping bentgrass was tested in plastic conetainers (3 cm in diam. and 21 cm in depth) (Stuewe & Sons, Inc., Corvallis, OR) following a modified procedure previously described by Wilkinson (24) Conetainers were filled with 100 ml of autoclaved vermiculite (W. R. Grace & Co., Cambridge, MA) and seeded with 0.25 g bentgrass seed that had been surface sterilized with 10% Clorox for 1 min. Seeds were covered with a thin layer of vermiculite, watered every other day with a Peter's 20-20-20 solution (W. R. Grace & Co., Fogelsville, PA), and maintained in

a growth chamber (12-h light/12-h dark, 20 to 22°C). Conetainers were covered with plastic wrap to maintain 100% relative humidity.

Two weeks after planting, the bentgrass was cut to a height of 1 cm with sterile scissors. One infested oat seed was then introduced aseptically to the vermiculite surface of each conetainer except for the controls, which were inoculated with noninfested oat seed. Inoculated bentgrass was then placed in a growth chamber (15-h light/9-h dark, 20 to 25°C) and kept moist (100% relative humidity) with plastic covering. Inoculated bentgrass was watered every other day with Peter's 20-20-20 solution. Disease was rated two weeks after inoculation using the disease index described by Aoyagi et al., (1), where 0 = healthy, 1 = 1 to 25% diseased, 2 = 26 to 50% diseased, 3 = 51 to 75% diseased, and 4 = 76 to 100% diseased. Disease severity was calculated as: disease severity = Σ(disease index X number of inoculated grass samples in each index)/(maximum index X total number of inoculated grass samples) X 100. All treatments consisted of four replicates and tests were conducted two times with similar results.

Fungicide variability tests. Aliquots of a stock solution of azoxystrobin dissolved in molten 3/4 strength PDA were added to subsequent molten 3/4 strength PDA to obtain final concentrations of 1, 3.2, 10, 31.2, 100, 316, and 1000 mg a.i. azoxystrobin per liter medium. These concentrations resulted in equal spacing on a $\log_{10}$ scale (19). Three-quarter strength PDA plates without fungicide were used as controls. Mycelial plugs 0.75 cm in diameter were cut from actively growing cultures of the fungal isolates and placed inverted either onto control or fungicide-amended plates, each containing 20 ml of agar medium. Eight replicate plates were used for each concentration. After three

days of growth at 22°C (12-h light/12-h dark regime), two perpendicular colony diameters were measured on the bottom of each plate. Agar plug diameters were subtracted from every measurement. The two colony diameters for each plate were averaged and a mean diameter was calculated from the eight replicate plates. Percent radial growth inhibition was calculated as:

$$\frac{\text{mean diameter on unamended PDA - mean diameter on fungicide-amended PDA}}{\text{mean diameter on unamended PDA}} \times 100$$

Azoxystrobin dose response curves were constructed for the *R. solani* isolates by plotting probit-transformed (29) percent radial growth inhibition against log-transformed fungicide concentration. The concentration of azoxystrobin causing 50% growth inhibition (EC_{50}) compared to growth on unamended PDA was estimated for each isolate by interpolation from the fitted regression line (second-degree polynomial) using SAS regression. The activity of azoxystrobin fungicide was considered to be strong if the EC_{50} was <10 mg a.i./liter, moderate if the EC_{50} was 11-100 mg a.i./liter, weak if the EC_{50} was 101-1000 mg a.i./liter, and ineffective if the EC_{50} was >1000 mg a.i./liter The experiment was repeated once with similar results.

Statistical analysis. Data from the pathogenicity experiments were subjected to analysis of variance using a general linear model (GLM), and mean separation was performed with Fisher's least significant difference test ($P = 0.05$) (SAS, version 6.10, SAS Institute Inc., Cary, NC). Fungicide variability data were analyzed using SAS regression.

RESULTS AND DISCUSSION

Cultural characteristics. Zhang and Dernoeden (30) suggested that *R. solani* anastomosis classification by the traditional means of microscopic observation of hyphal pairings is tedious and time-consuming and that simple observations of cultural characteristics such as colony color, presence or absence of sclerotia, presence or absence of zonation patterns, and type of mycelial growth are usually reliable enough to tentatively classify isolates into anastomosis groups. In this study, we found that observations of cultural characteristics of our isolates and comparisons of those characteristics with isolates of known anastomosis grouping were quite dependable in classifying our Oklahoma isolates (#345 and #414) into their respective anastomosis groupings. Of the five anastomosis tester turfgrass isolates used (AG-2-2 IIIB, AG-2-2 IV, AG-1-IA, AG-4 and AG-5), isolate #345 from brown patch most closely matched the AG-2-2 IIIB (isolate #411) tester in cultural characteristics while isolate #414 from large patch most closely matched the AG-2-2 IV (isolate #410) tester. *R. solani* isolate #345 obtained from brown patch on creeping bentgrass and AG-2-2 IIIB tester isolate #411 were both buff in color early in the growth development stage but turned to a dark brown color within two weeks. Isolates #345 and #411 also had irregular clusters of mycelia (not sclerotia), zonation or concentric rings, and sparse aerial hyphae on PDA after two weeks of incubation (Table 1). *R. solani* isolate #414 obtained from large patch on zoysiagrass and AG-2-2 IV tester isolate #410 were dark brown early in growth and remained that color after two weeks. These two isolates exhibited abundant aerial mycelia and neither had sclerotial formation or zonation patterns (Table 1).

Hyphal anastomosis. To further confirm our anastomosis groupings, we chose to observe hyphal anastomosis reactions between pairings of the brown patch isolate (#345) and the large patch isolate (#414) with the tester isolates AG-2-2 IIIB (#411) and AG-2-2 IV (#410), respectively, using light microscopy. In this study, imperfect fusion was not observed in positive control pairings between identical isolates and was observed only infrequently between different isolates of the same anastomosis grouping (Fig 1). Following the relationship defined by Aoyagi et al., (1), we considered the relationship between isolates as clonal (i.e., identical anastomosis group) if the frequency of perfect fusion was greater than 80%. In pairings of brown patch isolate #345 with the AG-2-2 IIIB tester (#411) and of large patch isolate #414 with the AG-2-2 IV tester (#410), we observed >80% perfect fusion frequency, lending support to our earlier conclusions of anastomosis grouping of isolates #345 and #414 based on cultural characteristics alone. Results indicated that *R. solani* isolates #345 (from creeping bentgrass) and #414 (from zoysiagrass) were not identical strains (perfect fusion frequency of only 8%) but belonged to separate anastomosis intraspecific groups, AG-2-2 IIIB and AG-2-2 IV, respectively. Perfect fusion was observed among 94.4% of hyphal fusions between isolates #345 and #411 (AG-2-2 IIIB). When #345 was paired with the representative AG-2-2 IV tester #410, a mean perfect anastomosis frequency of 3.33% was obtained. Pairings between isolates #414 and #410 (AG-2-2 IV) resulted in a mean perfect fusion frequency of 84.6%. Pairings between isolate #414 and the representative AG-2-2 IIIB tester #411 resulted in a mean perfect fusion frequency of only 4.4%.

Temperature-growth experiment. *R. solani* isolates #345 and #411 grew at all five temperatures tested (Fig. 2). The optimum temperature of these two isolates was 25°C, with mean colony diameters of 1.52 cm and 1.67 cm, respectively. This evidence lends support to the earlier conclusion made by observations of cultural characteristics and anastomosis reactions that isolate #345, like #411, belonged to AG-2-2 IIIB. Isolates #410 and #414 also had growth rate optima at 25°C, with mean colony diameters of 1.30 cm and 0.94 cm, respectively Neither isolate #414 or #410 grew at 35°C. This evidence further confirmed conclusions from earlier experiments that both isolate #414 and #410 were representatives of AG-2-2 IV.

Pathogenicity tests using conetainers. Butler (7) stated that anastomosis groups appear to be plant host specific. We wanted to examine whether there were any differences in pathogenicity to creeping bentgrass cv. "Crenshaw" between the *R. solani* turfgrass isolates. Our results showed that pathogenicity varies with anastomosis grouping. *R. solani* isolates #345 and #411 caused the highest levels of disease on creeping bentgrass compared to all of the other isolates (Fig. 3). Initial leaf symptoms observed were small, tan lesions that enlarged and became surrounded by reddish brown margins over time. Eventually grass leaves became necrotic and brown in color. These symptoms were similar to symptoms of brown patch on creeping bentgrass under field conditions. Zoysiagrass isolates #410 and #414 were the least aggressive pathogens to bentgrass. Moderate levels of disease were produced by isolates #96, #300, and #309. All uninoculated control bentgrass remained healthy

Fungicide variability tests. Kataria et al., (18) have documented and demonstrated that there is variability in fungicide sensitivity within and between anastomosis groups because of differences in molecular and biochemical characteristics. It has been suggested that knowledge of fungicide sensitivity levels between and within anastomosis groups is useful in selecting appropriate fungicides for reliable and efficient control of *R. solani* diseases (18, 30). Analyses of anastomosis group sensitivity to fungicides allows us to draw firm conclusions about the consistency or variability of performance of a fungicide both within and between anastomosis groups. We wanted to determine if such variations in sensitivity to azoxystrobin, a common fungicide used to control Rhizoctonia blight on cool- and warm-season turfgrasses, were evident between strain AG-2-2 IIIB from brown patch and strain AG-2-2 IV from large patch, and within isolates of strains AG-2-2 IIIB and AG-2-2 IV. The *R. solani* isolates from AG-2-2 IIIB and AG-2-2 IV grew at all seven azoxystrobin concentrations after three days (Fig. 4) However, there was some variability in fungicide sensitivity between strains of *R. solani* and between isolates of AG-2-2 IIIB. We found that fungal isolates #410 and #414 belonging to AG-2-2 IV (large patch) were more sensitive to azoxystrobin fungicide than fungal isolates #345 and #411 belonging to AG-2-2 IIIB (brown patch). At 1 mg a.i azoxystrobin/liter, isolate #410 growth was inhibited by 51% (probit = 5.03) while isolate #414 growth was inhibited by 61% (probit = 5.28). At 1 mg a.i. azoxystrobin/liter, isolate #345 growth was inhibited by only 16% (probit = 4.01) and isolate #411 growth was inhibited by 40% (probit = 4.75). At 1000 mg a.i. azoxystrobin/liter isolates #410 and #414 were inhibited by 82% (probit = 5.92) and 84% (probit=5.99), respectively Isolates

#345 and #411 were inhibited by 66% (probit=5.41) and 70% (probit=5.52) at 1000 mg a.i. azoxystrobin/liter, respectively. The azoxystrobin concentrations that reduced radial growth of isolates by 50% (EC_{50}) were determined to be <1 mg a.i. azoxystrobin/liter for isolates #410 and #414 (both AG-2-2 IV) and approximately 355 mg a.i. azoxystrobin/liter and 31.2 mg a.i. azoxystrobin/liter for isolates #345 and #411 (both AG-2-2 IIIB), respectively.

Our results indicate that Rhizoctonia blight (brown patch) on cool-season turfgrasses and Rhizoctonia blight (large patch) on warm-season turfgrasses are caused by different *R. solani* strains, and that azoxystrobin fungicide may be more effective in controlling large patch infections than in controlling brown patch infections since isolates representing AG-2-2 IV were more sensitive to azoxystrobin than isolates representing AG-2-2 IIIB. However, our results from isolates of two major turfgrass anastomosis groups only approximate azoxystrobin sensitivity levels of representative *R. solani* populations; therefore it may not be safe to draw accurate conclusions about the specificity of these anastomosis groups to azoxystrobin fungicide. We can only speculate that there is variability in azoxystrobin sensitivity within and between entire anastomosis groups. Additional *in vitro* testing with greater numbers of isolates for each anastomosis group, and *in vivo* tests on diseased turfgrass in growth chambers would be necessary to confirm our findings. The fungicide concentration in the recommended label rate is approximately 1500 mg a.i. azoxystrobin/liter Our results indicate that the four *R. solani* isolates are sensitive to fungicide at 1000 mg a.i azoxystrobin/liter, all showing >60%

growth inhibition; therefore, in field situations, the label rate should be effective for inhibiting high proportions of the growth of these four isolates.

ACKNOWLEDGMENTS
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Table 1. Cultural characteristics of *Rhizoctonia solani* isolates commonly associated with turfgrasses

Rhizoctonia isolate	Anastomosis group	Colony Color[1]	Nuclear condition[2]	Aerial mycelium	Sclerotia	Zonation
#96	AG-4	B-DB[3]	>2	Absent	Present	No
#300	AG-1-IA	C	>2	Absent	Present	No
#309	AG-5	B	>2	Absent	Present	No
#345	AG-2-2 IIIB	B-DB	>2	Absent	Absent	Yes
#410	AG-2-2 IV	DB	>2	Present	Absent	No
#411	AG-2-2 IIIB	B-DB	>2	Absent	Absent	Yes
#414	AG-2-2 IV	DB	>2	Present	Absent	No

[1]Cultures grown on PDA at 22°C for two weeks.
[2]Multinucleate condition is a distinguishing characteristic of *R. solani*.
[3]Colony color designations: C=cream, B=buff, DB=dark brown.

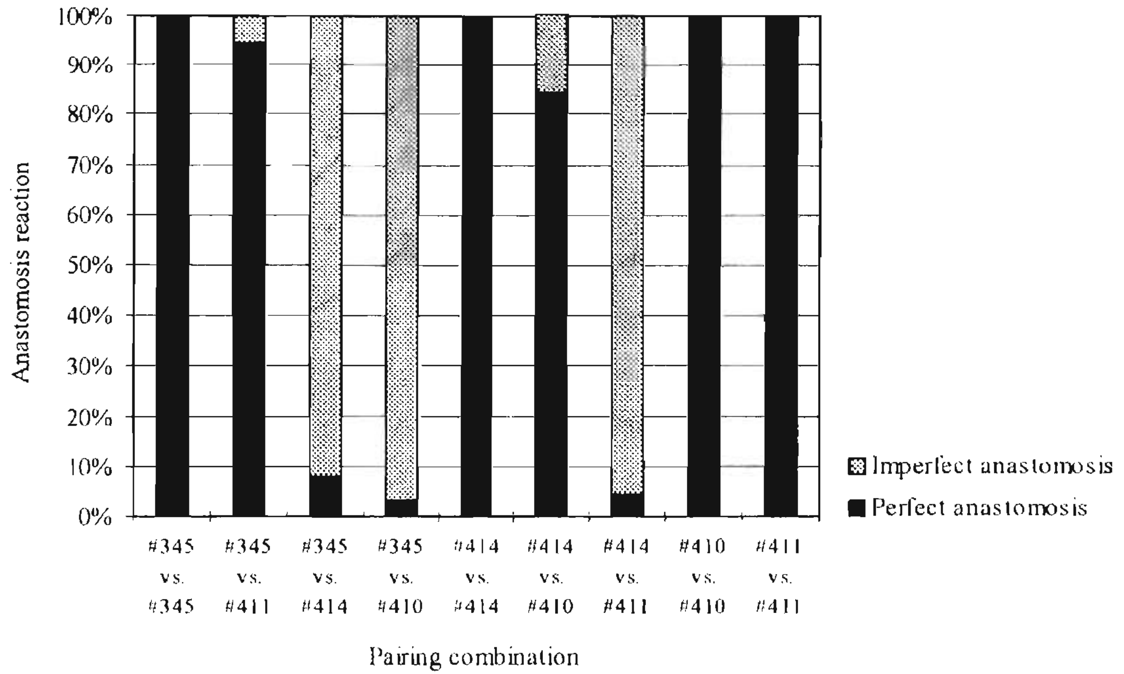

Fig. 1. Anastomosis reactions in various pairings of isolates of *Rhizoctonia solani* commonly associated with turfgrasses. *R. solani* isolates are: #345 = AG-2-2 IIIB; #411 = AG-2-2 IIIB; #414 = AG-2-2 IV; and #410 = AG-2-2 IV.

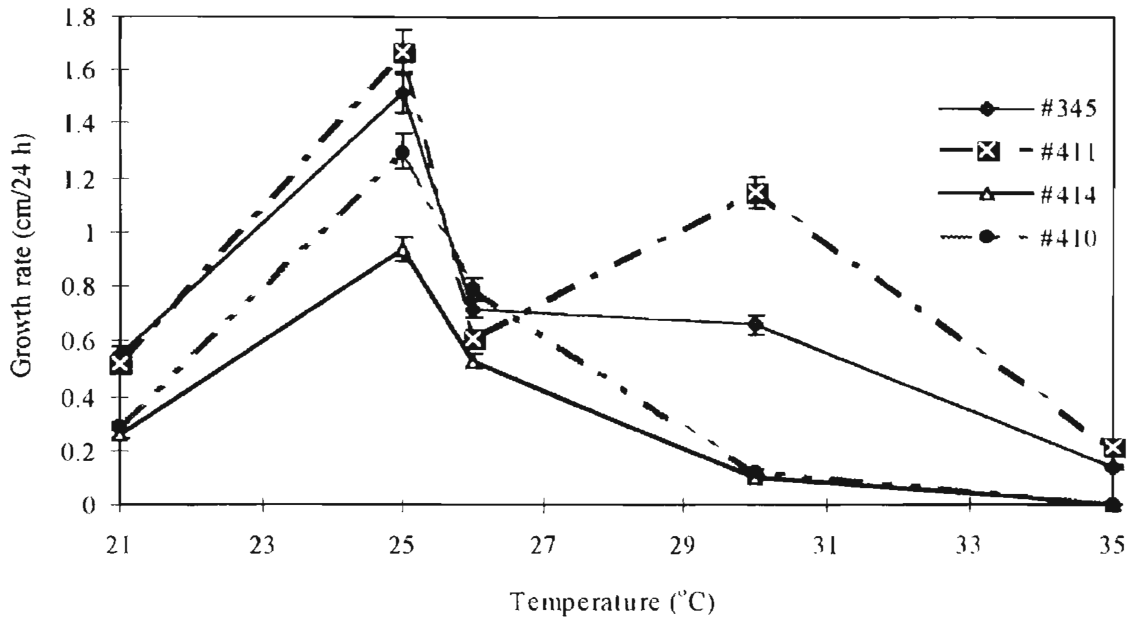

Fig. 2. Growth rates of *Rhizoctonia solani* turfgrass isolates on potato dextrose agar at different temperatures. Growth rates are the averages of five replicate plates. *R. solani* isolates are: #345 = AG-2-2 IIIB; #411 = AG-2-2 IIIB; #414 = AG-2-2 IV; and #410 = AG-2-2 IV. Bars indicate standard errors of means.

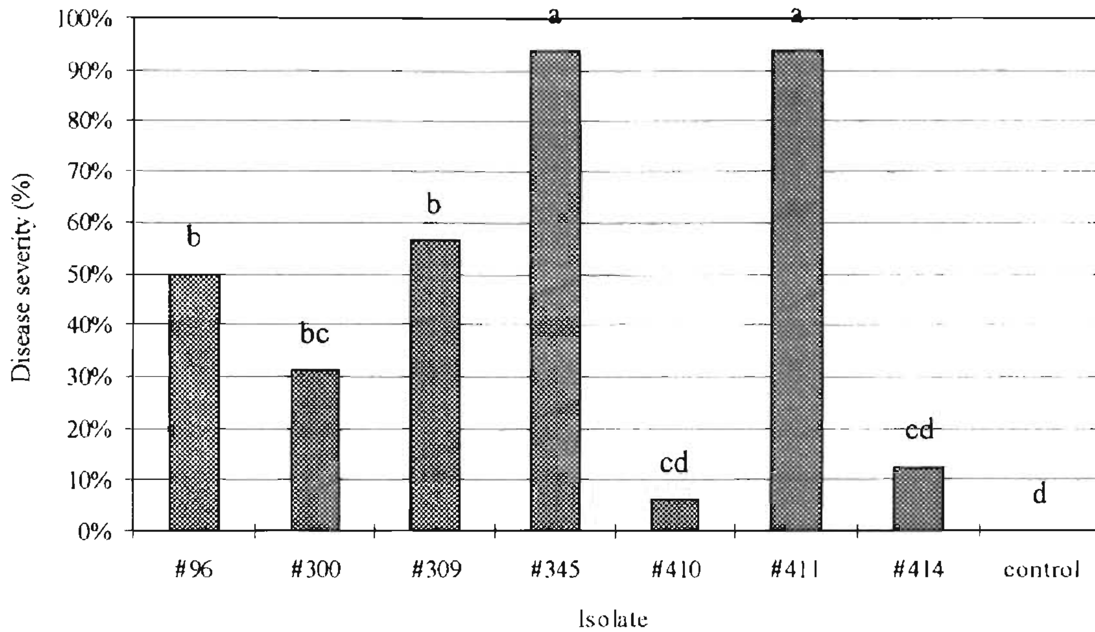

Fig. 3. Pathogenicity of *Rhizoctonia solani* turfgrass isolates on creeping bentgrass cv "Crenshaw" grown in conetainers. Disease severity = Σ (disease index X the number of grass samples in each index)/(maximum index X the total number of grass samples) X 100 (Aoyagi et al., 1998). Disease index of brown patch was rated two weeks after incubation at 20 to 25°C using a scale of 0 to 4, where 0 = no symptoms and 4 = dead grass. Isolate #96 = AG-4, isolate #300 = AG-1-IA, isolate #309 = AG-5, isolate #345 = AG-2-2 IIIB, isolate #410 = AG-2-2 IV, isolate #411 = AG-2-2 IIIB, isolate #414 = AG-2-2 IV. Column values having the same letter(s) do not differ significantly (P = 0.05) according to Fisher's least significant difference test.

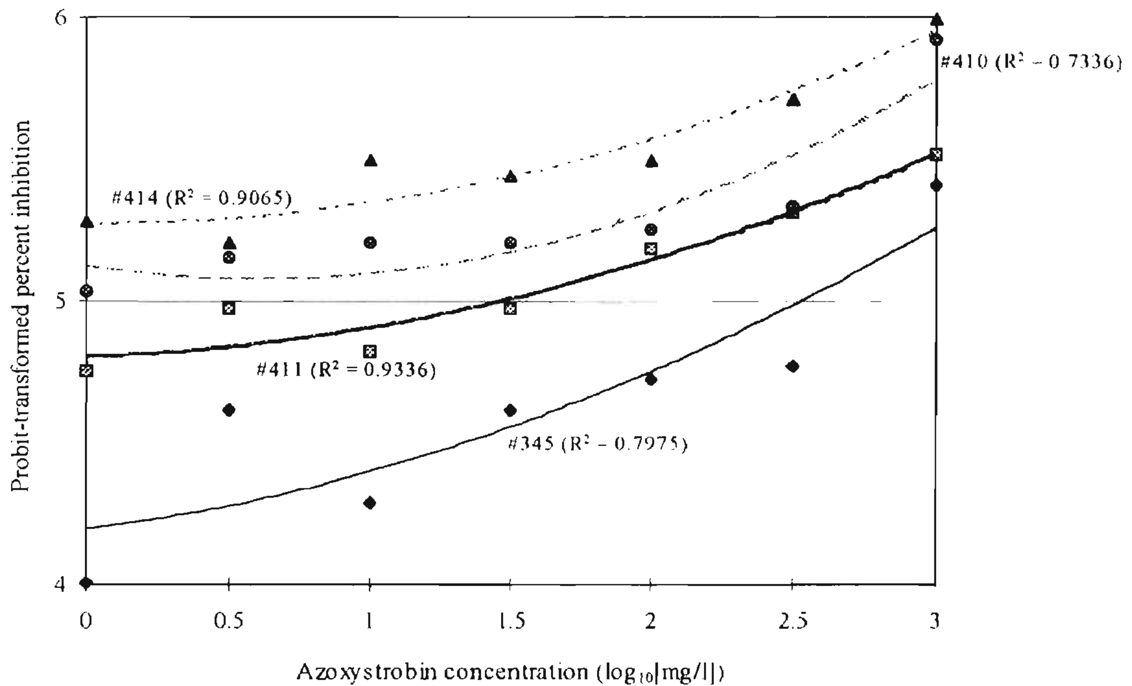

Fig. 4. Dose-response curves for four isolates of *Rhizoctonia solani* to azoxystrobin fungicide. Percent radial growth inhibition = (diameter on unamended medium - diameter on azoxystrobin-amended medium)/(diameter on unamended medium) X 100. *R. solani* isolates were: #345 = AG-2-2 IIIB, #411 = AG-2-2 IIIB; #410 = AG-2-2 IV; and #414 = AG-2-2 IV. The 50% effective concentrations were approximately 355 mg a.i. azoxystrobin/liter and 31.2 mg a.i. azoxystrobin/liter for isolates #345 and #411, respectively, and <1 mg a.i. azoxystrobin/liter for isolates #410 and #414.

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CHAPTER III

EVALUATION OF AN INTEGRATED CONTROL STRATEGY FOR BROWN PATCH AND DOLLAR SPOT ON CREEPING BENTGRASS

ABSTRACT

Brown patch caused by *Rhizoctonia solani* Kuhn AG-2-2 IIIB and dollar spot caused by *Sclerotinia homoeocarpa* F. T. Bennett are two major diseases of creeping bentgrass (*Agrostis palustris* Huds.) golf greens. Isolates of two biological control fungi, *Trichoderma harzianum* Rifai and *Laetisaria arvalis* Burds., were used as topdressing amendments to "Crenshaw" creeping bentgrass. In growth chamber experiments, combined treatment of bentgrass with tolerant isolates of either *T. harzianum* and *L. arvalis* and a foliar spray of the fungicide azoxystrobin at 1/5 label rate suppressed brown patch disease to an equal level as that obtained by fungicide at the full rate In the field study, integration of *T. harzianum* with 1/5 label rate of azoxystrobin provided equivalent control of brown patch as the full rate of fungicide on several rating dates. Integration of azoxystrobin with *L. arvalis* provided significant control compared to the untreated check on some rating dates but was less effective than integration involving *T. harzianum*. *L. arvalis* alone was not significantly different from the control. This integration system was ineffective against dollar spot in both growth chamber experiments and the field study because azoxystrobin had no activity against the disease. Growth chamber tests indicated

that the two biocontrol fungi have potential to control *S. homoeocarpa*. In the future these biocontrols should be tested with a fungicide labeled for dollar spot.

Brown patch caused by *Rhizoctonia solani* Kuhn and dollar spot incited by *Sclerotinia homoeocarpa* F. T. Bennett have been among the most common and destructive turfgrass diseases since their identifications in 1913 and the early 1920s, respectively (10). These two soilborne fungal diseases sometimes occur on turfgrasses at the same time in the growing season. On cool-season turfgrasses like creeping bentgrass (*Agrostis palustris* Huds.), brown patch and dollar spot usually appear from late-spring through early-fall months when average daily temperatures reach between 23 and 29°C and when atmospheric humidity remains at 85% or higher for 48 hours or more (10).

Traditionally, the most effective control of brown patch and dollar spot diseases has been attained by fungicide applications despite enormous costs. An Agricultural Chemical Industry Profile in 1992 estimated that nearly 70% of turfgrass pesticide expenses were attributed to fungicides and that the total annual sales of turfgrass fungicides in the United States surpassed those for other agricultural commodities (11). In the 1960s, reports of development of fungicide resistance by certain plant pathogens and growing concerns over possible harmful effects to the environment through extended pesticide usage warranted the need for a reassessment of turfgrass disease control strategies (11).

Biological control is an attractive non-chemical alternative to fungicides for control of turf diseases since it may pose fewer environmental risks (34). This strategy involves

the manipulation and utilization of antagonistic modes of action of biotic agents that provide beneficial reductions in pathogen inoculum and disease severity. Several reports have indicated that certain individual microbial organisms are capable of reducing the severity of turfgrass diseases. Formulations and delivery systems have included solid substrate amendments (4, 17, 26, 32, 36), alginate prills (19), and liquid formulations (33), and all have demonstrated significant turfgrass disease control under certain field conditions.

A major limitation to the widespread use of biological control of plant diseases has been its inconsistent control. Unlike fungicides, biocontrol organisms are living entities and are extremely responsive to and significantly impacted by alterations in uncontrolled environmental, biological, and physical conditions. As of 1989, it was estimated that approximately only 5% of intentional deliveries of biocontrol organisms in uncontrolled field situations actually provided disease control (39). Problems associated with both chemical and biological controls of plant diseases in general have stirred growing interest in the possibility of combining chemical treatments with biocontrol agents in integrated biological/chemical control strategies. The use of integrated disease control has several possibilites and advantages. The combination of more than one disease management practice could reduce dependence upon a single control practice. Integrated applications may reduce chemical impact on the environment and discourage fungicide resistance. Integrated biological/chemical control tactics should also improve the efficacies of biocontrol agents, because the chemicals remain effective against a pathogen despite environmental conditions. Furthermore, biological control organisms may provide longer

lasting localization and persistence than synthetic chemicals. Finally, where individual chemical or biocontrol treatments may only be effective against a single disease, integrated disease control practices could be effective for control of more than one pathogen (43, 49). Several researchers have successfully integrated chemical and biological control strategies on plants in both greenhouse (8, 30) and field tests (6, 9, 18).

The primary objective of this study was to evaluate the possibility of combining biological and chemical control into an integrated strategy to reduce brown patch and dollar spot on a creeping bentgrass green. Two fungi with biological control activity, *Trichoderma harzianum* Rifai and *Laetisaria arvalis* Burds., and the microbially-derived fungicide azoxystrobin, were chosen for the study based on fungicidal activity.

T. harzianum, a ubiquitous soil-inhabiting deuteromycete, has been studied extensively and has demonstrated control of *R. solani* on various plants (5, 15, 16, 43) including turfgrasses (31, 32, 33), and of dollar spot on turfgrasses (31, 32, 33). *L. arvalis*, a soil-inhabiting basidiomycete fungus of temperate climates, has been investigated mainly as a biological control agent for *R. solani* diseases. Greenhouse (25, 28, 37, 42) and field studies (7, 27, 37) demonstrated that significant suppression of *R. solani* on various plants is possible with *L. arvalis*. This biological agent may also control brown patch on turf. In addition, we investigated the persistence and localization of *T. harzianum* and *L. arvalis* on creeping bentgrass under field conditions prior to and following biocontrol applications. The sensitivities of *T. harzianum* and *L. arvalis* to azoxystrobin, and the efficacy of a topdressing delivery system for a solid substrate

biocontrol formulation consisting of vermiculite-corn meal-sand and its potential incorporation into current turfgrass management practices were also tested.

MATERIALS AND METHODS

Selection of biological control fungi. The biological control fungi *T. harzianum* R110 and *L. arvalis* OK-206 were selected and obtained from a laboratory fungal culture collection (Oklahoma State University, Department of Entomology and Plant Pathology, Stillwater, OK) maintained at 11°C. *T. harzianum* #110, the wild strain of *T. harzianum* R110, was originally isolated in Texas from peanut infected with *Aspergillus niger*, the causal agent of crown rot. *T. harzianum* R110 was selected for resistance to iprodione fungicide at 1000 mg a.i./liter in *in vitro* fungicide tests using fungicide-amended agar petri dishes (K. E. Conway, *personal communication*). *L. arvalis* OK-206, originally isolated in Delaware County, Ohio, was obtained from Forest Products Laboratory (Madison, WI). Cultures of these two fungi were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) for use in all experiments.

Fungicide resistance tests. Dose response curves to azoxystrobin fungicide were constructed for both biological control fungi to determine tolerance. Aliquots of a stock solution of azoxystrobin (Heritage 50WDG, Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire, RG42 6ET, UK) dissolved in molten 3/4 strength PDA were added to subsequent molten 3/4 strength PDA to obtain final concentrations of 1, 3.2, 10, 31.2, 100, 316, and 1000 mg a.i. azoxystrobin per liter medium. These concentrations resulted in equal spacing on a $\log_{10}$ scale (24). Three-quarter strength PDA plates without fungicide were used as controls. Mycelial plugs (0.75 cm in diam.) were

taken from actively growing cultures of the biocontrol isolates and placed inverted onto either control or fungicide-amended plates, containing 20 ml of agar medium. Eight replicate plates were used for each concentration. After three days of growth at 22°C (12-h light/12-h dark regime), two perpendicular colony diameters were measured on the bottom of each plate. Agar plug diameters were subtracted from these measurements. The two colony diameters for each plate were averaged and a mean diameter was calculated from the eight replicate plates. Percent radial growth inhibition was calculated as:

$$\frac{\text{mean diameter on unamended PDA - mean diameter on fungicide-amended PDA}}{\text{mean diameter on unamended PDA}} \times 100$$

Azoxystrobin dose response curves were constructed for *T. harzianum* R110 and *L. arvalis* OK-206 by plotting probit-transformed (48) percent radial growth inhibition against log-transformed fungicide concentration. The concentration of azoxystrobin causing 50% growth inhibition compared to growth on unamended PDA (EC_{50}) was estimated for each isolate from the fitted regression line (second-degree polynomial) using SAS linear regression. The activity of azoxystrobin fungicide was considered to be strong if the EC_{50} was <10 mg a.i. azoxystrobin/liter, moderate if the EC_{50} was 11-100 mg a.i. azoxystrobin/liter, weak if the EC_{50} was 101-1000 mg a.i. azoxystrobin/liter, and ineffective if the EC_{50} was >1000 mg a.i. azoxystrobin/liter. The experiment was repeated once with similar results.

Preparation of biological control formulations. Solid substrate topdressing formulations of *T. harzianum* R110 and *L. arvalis* OK-206 were prepared using vermiculite (W. R. Grace & Co., Cambridge, MA) and white corn meal (Wal-Mart,

Bentonville, AR), following a technique modified by Conway et al., (8). Aluminum pans (48 cm X 30 cm X 8 cm) were filled with 187.48 g vermiculite and 333 g white corn meal. The mixture was autoclaved (121°C, 1.05 kg/cm^2, 20 min) on each of three consecutive days. Small quantities of the topdressing mixture were sprinkled onto PDA plates, incubated at 22°C, and then examined for sterility. If no fungal or bacterial contamination was present, 500 ml of autoclaved reverse osmosis (RO) water was added to the vermiculite-corn meal mixture. Five fresh, actively growing cultures of either *T. harzianum* R110 or *L. arvalis* OK-206 were chopped into approximately 1 cm^2 squares and added aseptically to the topdressing mixture. Biocontrol formulation was then incubated in a growth chamber at 22°C (12-h light/12-h dark regime). After two weeks, formulation was removed and placed onto trays lined with wax paper, covered with cheesecloth, and allowed to dry for two days under a laminar-flow hood. The dried mixture was ground in a blender (Glen Mills Inc., Maywood, NJ) and then stored in polyethylene bags at room temperature until needed one month later.

Estimation of viable biocontrol propagules in topdressing formulations. The number of viable fungal propagules/g mixture (i.e., conidia, chlamydospores, or hyphae in the case of *T. harzianum* R110, and sclerotia or hyphae in the case of *L. arvalis* OK-206) was estimated using a modified procedure described by Henis et al., (20). Biocontrol topdressing formulations were diluted in a series with white silica sand (Unimin Corporation, Guion, AR). Formulation-sand dilutions were mixed in sterile glass petri dishes (10 cm X 1.5 cm) with a sterile metal spatula and then were adjusted to 15% moisture content. A multiple pellet soil-sampler (20) containing 15 metal pistons within

cylinders was calibrated to deliver a total weight of 1.5 g of each formulation-sand dilution (0.1 g per pellet) to each of 10 replicate PDA plates. Dilution plates were incubated at 22°C for two days. The number of pellets exhibiting hyphal growth out of the total 150 (15 pellets per PDA plate X 10 plates per dilution) was counted and recorded. The number of viable propagules per gram of sand for each biocontrol topdressing was then estimated from the mean percentage of pellets colonized by using a multiple infection transformation table (48) to apply the first order of the Poisson distribution ($\log_e X/1\text{-}X$, where X = the proportion of pellets exhibiting at least 10 germinated hyphae) as was done by Henis et al., (20) for *R. solani*. The experiment was done twice with similar results.

Population dynamics of biological control fungi. Both native (prior to any biocontrol applications) and introduced (after biocontrol applications) total populations of *T. harzianum* and *L. arvalis* in soil from field plots of creeping bentgrass were assessed to determine the localization and persistence of the two biocontrol fungi within the turfgrass niche. Three 1 cm X 6 cm soil core samples were obtained monthly at random from each plot using a brass soil core sampler (1 cm in diam. X 33 cm long) and were enumerated for *Trichoderma* and *Laetisaria* populations within one week after sampling. Sample cores contained leaves, roots, stolons, thatch, and soil. Five thatch pieces from each soil core sample from each plot were plated onto each of three petri dishes containing either *Trichoderma* selective medium (TSM) (14) or *Laetisaria* selective medium (LSM) (38). These plates were incubated for two days at 22°C and then observed to obtain counts of thatch exhibiting hyphal growth per plate. Thatch counts for the three replicate plates for

each soil core sample for each plot were pooled and the mean percentage of thatch colonization by each biocontrol organism was calculated.

Each soil sample was divided into upper 3 cm and lower 3 cm sections to determine any differences in biocontrol populations with soil depth. A 1 g subsample from both upper and lower soil core portions was mixed in 10 ml sterile RO water and shaken vigorously by hand. A dilution series was prepared from each soil core portion for each of the three soil core samples per plot. One-milliliter aliquots of the diluted soil core samples were then added to three replicate plates containing either molten TSM or LSM. Remaining soil (2 g) from each soil core portion was placed in an oven (105°C for 24-h) for dry weight determination. After incubation of the dilution plates at 22°C for one week, the colonies were enumerated using a Gallenkamp colony counter (London, UK) and mean population levels for both upper and lower 3 cm soil sections from each soil core sample from each plot were expressed as viable propagules per gram of dry weight of soil.

***In vitro* biological control tests.** Biological control tests were conducted on creeping bentgrass cv "Crenshaw" (Lofts Great Western Seed Company, Albany, OR) using PDA plates and autoclaved vermiculite following modifications to the procedure originally developed by Livneh and described by Inbar et al., (22). Bentgrass seeds were surface sterilized with 10% Clorox (The Clorox Company, Oakland, CA) for 1 min and then rinsed in sterile RO water for 1 min. Mycelial plugs (0.75 cm in diam.) from actively growing cultures of *R. solani* #345 and *S. homoeocarpa* #415 were placed inverted onto PDA petri dishes. Biocontrol formulations + autoclaved sand (11.31 g) in a 1:4 ratio (wt/wt) were mixed with sterile vermiculite (8.75 g) and added to inoculated PDA agar

surfaces. Ten bentgrass seeds were embedded in the vermiculite-formulation-sand mixture. Petri dishes were incubated in a growth chamber (15-h light/9-h dark, 20 to 25°C) and observed after 21 days to obtain counts of germinated seeds and healthy plants to determine pre- and post-emergence disease, respectively. All treatments consisted of four replicates and the experiment was done twice with similar results.

***In vitro* integrated control experiments.** Growth chamber experiments were conducted to evaluate integrated control of brown patch and dollar spot on creeping bentgrass. Inoculum was produced by adding one PDA culture of either *R. solani* #345 or *S. homoeocarpa* #415, chopped into approximately 1 cm^2 pieces, to a 250-ml Erlenmeyer flask containing 20 g oat seed and 3 ml RO water that had been autoclaved (121°C, 1.05 kg/cm^2, 20 min) on each of three consecutive days. After 14 days, the colonized seeds were dried overnight under a laminar-flow hood.

Bentgrass was grown in plastic conetainers (Stuewe & Sons Inc., Corvallis, OR) (3 cm in diam. X 21 cm in depth) following a modified procedure previously described by Wilkinson (47). Conetainers were filled with 100 ml of autoclaved vermiculite and seeded with 0.25 g surface sterilized bentgrass seed. Seeds were covered with a thin layer of vermiculite, watered every other day with a Peter's 20-20-20 solution (W. R. Grace & Co., Fogelsville, PA), and maintained in a growth chamber (12-h light/12-h dark, 20 to 22°C). Conetainers were covered with plastic wrap to maintain 100% relative humidity

Two weeks after planting, the bentgrass was cut to a height of 1 cm with sterile scissors. Two oat seeds infested with either *R. solani* or *S. homoeocarpa* were introduced aseptically to the vermiculite surface of each conetainer except for the controls, which

were inoculated with two noninfested oat seeds. Treatments (Table 2) were replicated three times in each of two experiments. Treated bentgrass was covered with plastic covering to maintain 100% relative humidity, placed in a growth chamber (15-h light/9-h dark, 20 to 25°C), and watered every other day with Peter's 20-20-20 solution. Disease was rated four weeks after inoculation using the Horsfall-Barratt disease rating scale (21), where 1 = 0 to 3% diseased, 2 = 3 to 6% diseased, 3 = 6 to 12% diseased, 4 = 12 to 25% diseased, 5 = 25 to 50% diseased, 6 = 50 to 75% diseased, 7 = 75 to 88% diseased, 8 = 88 to 94% diseased, 9 = 94 to 97% diseased, and 10 = 97 to 100% diseased.

Koch's postulates were tested by plating five diseased grass pieces from conetainers exhibiting disease symptoms onto PDA. Initially, brown patch symptoms included small, tan lesions that enlarged and became surrounded by reddish brown margins over time. Eventually, the grass became necrotic and brown in color. Initial symptoms of dollar spot included yellow-green blotchy leaves, which eventually became blighted and bleached to a straw-colored tan.

Field study. One field study was conducted from late spring to early fall 1999 to evaluate the possibility of integrating azoxystrobin fungicide with *T. harzianum* R110 and *L. arvalis* OK-206 for control of brown patch and dollar spot diseases on a creeping bentgrass green. Field plots (0.5 m X 0.5 m) were established in November, 1998 on a two-year-old stand of creeping bentgrass cv. "Crenshaw" seeded in sand at the Horticulture Turfgrass Research Center in Stillwater, OK. Turfgrass was mowed daily to a height of 0.4 cm and irrigated as needed From August, 1998 to August, 1999, the turf plots received 228.5 kg/ha nitrogen fertilizer.

Eight field study treatments were replicated in triplicate (Table 3). Turfgrass was observed every two to three weeks for brown patch and dollar spot diseases. Brown patch disease severity was estimated visually using the Horsfall-Barratt disease rating scale (21). Dollar spot incidence was determined by counting numbers of spots per plot.

Experimental design and statistical analysis. All experiments were arranged in a completely randomized design. Data from *in vitro* biological control tests, *in vitro* integrated control tests, and from the field study were subjected to analysis of variance using a general linear model (GLM), and mean separation was performed with Fisher's least significant difference test (P = 0.05) (SAS Institute Inc., Cary, NC). Fungicide sensitivity tests were analyzed using SAS regression.

RESULTS

Fungicide resistance tests. The two biological control fungi, *T. harzianum* R110 and *L. arvalis* OK-206, were found to be weakly inhibited and moderately inhibited, respectively, by azoxystrobin fungicide amended to PDA. *T. harzianum* R110 and *L. arvalis* OK-206 both grew at all seven concentrations of azoxystrobin between 1 and 1000 mg a.i./liter (Fig. 5). However, at 1000 mg a.i./liter, radial growth of *L. arvalis* OK-206 was inhibited by 83.8% (probit = 5.99), while *T. harzianum* R110 growth was only inhibited by 61.4% (probit =5.28).

The azoxystrobin concentrations that reduced relative colony diameter of *T. harzianum* R110 and *L. arvalis* OK-206 by 50% (EC_{50}) (probit = 5.00) were estimated to be 661 mg a.i./liter and 81.3 mg a.i./liter, respectively. Our results demonstrated that *T. harzianum* R110 is slightly less sensitive to azoxystrobin than *L. arvalis* OK-206 and that

the full recommended fungicide concentration of 1500 mg a.i./liter (0.62 kg a.i./ha) would inhibit a large proportion of the *L. arvalis* OK-206 population. By interpolation of the dose-response curves for *T. harzianum* R110 and *L. arvalis* OK-206, we estimated that an azoxystrobin concentration of 270 mg a.i./liter inhibited *T. harzianum* R110 and *L. arvalis* OK-206 radial growth by approximately 40% (probit = 4.75) and 61% (probit = 5.28), respectively. This fungicide concentration was considered high enough for sufficient control of the target pest, brown patch, but was low enough to limit detrimental effects on *T. harzianum* R110 and *L. arvalis* OK-206 more so than they would be limited at the full fungicide rate. Therefore, the lower fungicide concentration of 270 mg a.i./liter (0.12 kg/ha) was implemented in further integration experiments.

Estimation of viable biocontrol propagules in topdressing formulations. From the 1:4 dilutions of formulation to sand it was estimated that the *T. harzianum* R110 and *L. arvalis* OK-206 topdressings contained approximately 6.9×10^5 conidia/g sand and 4.7×10^3 sclerotia/g sand, respectively. The 1:4 ratio of either biocontrol formulation to sand was subsequently used in *in vitro* experiments on biological control and integrated control and also in the field study.

Population dynamics of biological control fungi. Introduction of *T. harzianum* R110 to a creeping bentgrass green as a solid-substrate topdressing was effective in establishing high population levels of *Trichoderma* spp. in the thatch. Average thatch colonization remained at a lower level with the untreated control (treatment #1) than with the biocontrol topdressing treatments #6, #7, and #8 (*T. harzianum* R110 alone, *T. harzianum* R110 + azoxystrobin, and azoxystrobin + *T. harzianum* R110, respectively)

(Fig. 6). Average thatch colonization was comparable for treatments #6, #7, and #8. On the first sampling date (day = 0), thatch colonization for these three treatments was comparable to the level in the untreated control (approximately 75% to 80%). However, following biocontrol topdressing application and soil-core sampling 30 days later, thatch colonization was 97%, 96.3%, and 97.8% for treatments #6, #7, and #8, respectively. Thatch colonization for these three treatments remained stable (above 90%) for the remainder of the study. In these experiments, it was not possible to reliably discriminate *T. harzianum* R110 and wild *Trichoderma* spp.

L. arvalis population levels in the thatch were not as high as those of *Trichoderma* spp. Thatch colonization by *Laetisaria* spp. remained at 0% for the untreated control (treatment #1) for all sampling dates. Prior to any biocontrol topdressing applications (day 0), thatch colonization was 0% for treatments #3, #4, and #5 (*L. arvalis* OK-206 alone, *L. arvalis* OK-206 + azoxystrobin, and azoxystrobin + *L. arvalis* OK-206, respectively) (*data not shown*). Even after biocontrol applications, thatch colonization levels only reached approximately 20-30% for treatments #3, #4, #5 (Fig. 7). However, despite the low population levels, monthly biocontrol topdressing applications with treatments #3, #4, and #5 were able to stabilize the *L. arvalis* thatch populations. *L. arvalis* thatch colonization by treatment #4 was slightly lower than in treatments #3 and #5. This finding was most likely the result of moderate sensitivity to the azoxystrobin applied after the biocontrol had been added to the turfgrass. Thatch colonization by *L. arvalis* remained slightly higher with treatment #3 involving no fungicide application than with treatment #5 involving azoxystrobin application prior to topdressing addition.

Thirty days (day 30) after introduction of *T. harzianum* R110 topdressing to creeping bentgrass, population levels of *Trichoderma* spp. in soil core samples containing roots and soil increased by approximately one order of magnitude relative to untreated soil (Fig. 8). *Trichoderma* spp. survived and persisted at high populations (10^5 propagules/g soil) and remained stable or increased slightly during the course of the study. Population levels in the upper 3 cm soil sections were slightly higher than those in the lower soil sections.

L. arvalis OK-206 populations in creeping bentgrass soil did not reach the levels attained by *Trichoderma* spp. Throughout the course of the study, no fungal propagules were present in either upper or lower 3 cm soil core sections of untreated plots. At day 0, prior to biocontrol applications, no *L. arvalis* propagules were detected in any soil core samples from any of the plots for treatments #3, #4, and #5 (*data not shown*). However, at day 30, following topdressing application, population levels in soil core samples containing roots and soil increased 1000-fold (Fig. 9) and persisted at these levels during the course of the study. Population levels in upper 3 cm soil sections were higher than those in lower soil sections.

***In vitro* biological control tests.** After 21 days, 75% of bentgrass seeds germinated in the untreated control and all seedlings were healthy (Fig. 10). In the disease treatments of *S. homoeocarpa* #415 and *R. solani* #345 without biological control, 21.25% and 63.75% of the seeds produced seedlings, respectively. All of these seedlings were infected with the *S. homoeocarpa* #415 treatment while 28.75% of bentgrass seedlings were uninfected in the *R. solani* #345 treatment. These results suggested that *S.*

homoeocarpa is a more aggressive pathogen than *R. solani* on creeping bentgrass cv. "Crenshaw" seedlings. *R. solani* #345 plus *T. harzianum* R110 gave comparable results to the untreated control. With this treatment, 63.75% of seeds produced seedlings and all were healthy at 21 days. Biocontrol of *R. solani* #345 with *L. arvalis* OK-206 resulted in 38.75% bentgrass seed germination with no seedlings infected. Seed germination percentages were slightly lower with treatments involving biocontrol of *S. homoeocarpa* #415 than with treatments involving biocontrol of *R. solani* #345. Biocontrol of *S. homoeocarpa* #415 with *L. arvalis* OK-206 *and T. harzianum* R110 resulted in 30% and 26.25% seed germination, respectively, and all seedlings remained uninfected after 21 days. Results indicated that biological control of both *R. solani* brown patch and *S. homoeocarpa* dollar spot on creeping bentgrass is possible *in vitro* using topdressing applications of either *T. harzianum* R110 or *L. arvalis* OK-206. Both biocontrols were more effective for brown patch than for dollar spot.

***In vitro* integrated control experiments.** Control bentgrass inoculated with *R. solani* #345 gave the highest brown patch disease severity (approximately 95.5% infection) of any of the six treatments (Table 4). All other treatments were significantly different from the control (P = 0.05). Integration treatments (#4 and #6) and biocontrol treatments (#3 and #5) were as effective as the fungicide at the full rate of 0 62 kg a.i./ha (treatment #2) for suppression of brown patch.

In integration experiments on control of dollar spot, treatments #3 and #6 involving *L. arvalis* OK-206 and *T. harzianum* R110 plus azoxystrobin (0.12 kg a.i./ha), respectively, provided significant suppression of dollar spot compared to all other

treatments (P = 0.05) (Table 5). Treatments #1 (control), #2 (azoxystrobin (0.62 kg a.i./ha)), #4 (*L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha)), and #5 (*T. harzianum* R110) did not control dollar spot. These results suggested that *L. arvalis* OK-206 and *T. harzianum* R110 had biocontrol capabilities against dollar spot and that azoxystrobin should have been replaced by a fungicide effective against dollar spot. Azoxystrobin fungicide was ineffective against dollar spot in these tests; reports have suggested that dollar spot incidence may actually increase with azoxystrobin treatment (13). The purpose of these tests was not to evaluate whether azoxystrobin would be effective against dollar spot but to determine whether the selected biocontrol organisms, *L. arvalis* OK-206 and *T. harzianum* R110 could provide suppression of this disease.

Field study. Field plots were examined on 7 June, 14 June, 21 June, 1 July, 12 July, 23 July, 4 August, and 27 August, 1999 for brown patch severity and dollar spot incidence. Average daytime air temperatures for May, June, July, and August were 23.9°C, 28.3°C, 31.7°C, and 31.7°C, respectively, and average nighttime air temperatures were 13.9°C, 19.4°C, 21.7°C, and 22.2°C, respectively. Average precipitation for May, June, July, and August was 0.31", 0.55", 0.02", and 0.14", respectively. On all rating dates, azoxystrobin at the full rate of 0.62 kg a.i./ha (treatment #2) provided significant suppression of brown patch compared with the untreated control (treatment #1) (Table 6). Treatment #3 involving *L. arvalis* OK-206 alone was never significantly different from the control during the four month study. Treatments #4 and #5 involving *L. arvalis* OK-206 applied in conjunction with 1/5 rate (0.12 kg a.i./ha) azoxystrobin never provided significant control of brown patch until 4 August, 1999. Treatments #7 and #8 involving

T. harzianum R110 applied in conjunction with 1/5 rate (0.12 kg a.i./ha) azoxystrobin provided equal control of brown patch as the fungicide at the full rate (treatment #2) on several rating dates and better control than *T. harzianum* R110 alone (treatment #6). There were no significant differences between either treatments #4 and #5 or treatments #7 and #8, suggesting that niche clearing with azoxystrobin fungicide prior to biocontrol topdressing did not improve the efficacy of integrated control of brown patch.

The integrated control strategy was ineffective for control of dollar spot. No significant differences were found for any of the treatments over the course of the study (Table 7). Integration treatments #4, #5, #7, and #8 involving either *L. arvalis* OK-206 or *T. harzianum* R110 applied in conjunction with 1/5 rate (0.12 kg a.i./ha) azoxystrobin provided significant dollar spot control on only one rating date (1 July, 1999). Dollar spot incidence increased over the course of the study. Increases in dollar spot incidence corresponded rather well with temperature and moisture fluxes. Disease increases were noticed especially when average air temperatures reached 23 to 29°C and when grass was continuously wet for 48 hours or more.

Dollar spot incidence on the plot site has been high in past years but brown patch has been scarce. One possible reason for the ineffectiveness of dollar spot control even by the biological control fungi was that the dollar spot inoculum level in the creeping bentgrass soil was too high to be controlled by our chosen treatment rates. The corn meal in our topdressing formulations was intended as a nutritive food base for the two biological control fungi. It is possible that *S. homoeocarpa* outcompeted *T. harzianum*

R110 and *L. arvalis* OK-206 for the food substrate, thereby reducing the efficiency of the biocontrol fungi.

DISCUSSION

Turfgrass diseases like brown patch and dollar spot can cause reductions in turf quality and surface uniformity if control measures are not instituted in a timely and effective manner. Control of these diseases is usually achieved by repeated applications of expensive fungicides, but reports and concerns of soil and water contamination (32, 45), fungicide resistance (12, 32, 44) and other harmful effects (12, 32, 41, 45) have prompted the need for alternative control strategies. Biological control is one alternative to the use of fungicides and the use of biocontrol agents for turfgrass diseases has been well-documented (4, 17, 19, 26, 32, 33, 36). However, reports of unreliability in biological control have limited the widespread application of this disease management strategy on turf. Walsh et al., (46) stated that turfgrass disease biocontrol is an "emerging technology." Biological control as a disease management strategy is still in its infancy basically because many of the uncertainties are still being worked out and explored. There are ongoing efforts to determine the most effective methods of introduction, strategies for maintaining biocontrol population levels when and where they are needed, and the activities of the biocontrol organisms in association with turfgrass. Biological control is unlikely to become the sole management strategy in any disease situation until these problems are resolved and the knowledge base is increased. Currently, there is only one biocontrol organism that has been commercialized and registered with the United States

Environmental Protection Agency for control of turfgrass diseases including brown patch (33) and dollar spot (46). This product is *T. harzianum* strain 1295-22 and is marketed as Bio-Trek (Wilbur-Ellis, Fresno, CA) and has demonstrated a high degree of rhizosphere competence when introduced to turf.

Several researchers have documented successful integration of chemical and biological control for plant disease management (6, 8, 9, 18, 30). Lo et al., (32, 33) have suggested that integration of chemical and biological control for brown patch and dollar spot management is an attractive control strategy. *R. solani* and *S. homoeocarpa* are soilborne pathogens that overwinter in the soil and thatch of turfgrasses. *R. solani* overwinters mainly as sclerotia in the soil or as mycelia in decaying thatch material. *S. homoeocarpa* overwinters either as mycelia in decaying thatch or as ascostroma. Mycelia from both fungi serve as the primary inoculum which infects foliar portions of the turfgrass High density, high-maintenance turfgrass ecosystems provide favorable environments for the rapid, successive, aerial spread of the mycelia of these pathogens from leaf blade to leaf blade and for subsequent disease development. Lo et al., (32), using granular or peat-based topdressing formulations of *T. harzianum strain* 1295-22, were able to suppress the initial infections of brown patch and dollar spot on creeping bentgrass by reducing primary inoculum levels in the soil and thatch but were unable to control subsequent foliar phases of these diseases. They proposed that while soil applications of biocontrol organisms should be used to suppress initial turf infection by reducing pathogen levels in the soil and thatch, compatible fungicides should be incorporated with biocontrol strategies for reductions in subsequent foliar phases of

turfgrass diseases. We explored the possibility of using biocontrol topdressings consisting of vermiculite-corn meal-sand to suppress the initial infection of brown patch and dollar spot in conjunction with a microbially-derived fungicide for control of foliar infections of brown patch.

We chose to use a bulk amendment solid substrate biocontrol topdressing formulation consisting of vermiculite-corn meal-sand because it was inexpensive, relatively easy to prepare and deliver to the turfgrass, and because several researchers have indicated success using a similar type of formulation for control of various soilborne plant diseases (1, 27, 29, 35). Lewis et al., (29) found that use of vermiculite as the inert carrier and wheat bran as a nutritive food base was effective for reduction of the survival and saprophytic growth of *R. solani* in sandy soil. Jeffries and Young (23) further supported the notion that addition of a food base such as wheat bran or corn meal enables a biocontrol organism to proliferate more readily in the rhizosphere and surrounding soil. Topdressing turf greens is a commonly used cultural practice on golf courses to maintain smooth, uniform, and firm surfaces, and topical fertilizers and other materials are routinely applied to golf courses (32). We feel that topdressing applications of *T. harzianum* and *L. arvalis* are compatible with established turf management practices and that these biocontrol topdressings could be coordinated with routine topdressing treatments.

Previous reports have suggested that between 10^5 and 10^7 biocontrol propagules/g soil are needed for effective disease control by *Trichoderma* spp. (35). Lo et al., (32) found that *T. harzianum* 1295-22 population levels of 3 to 5 x 10^5 propagules/g soil sufficiently reduced brown patch and dollar spot in growth chamber and field tests. Based

on the findings of Lo et al., (32), we chose to deliver *T. harzianum* R110 formulation at a rate of 10^5 propagules/g soil. Conway et al., (8) used 5 g dried *L. arvalis* sclerotia/kg potting soil for control of aerial blight and root rot of rosemary. This amount is roughly equivalent to 10^4 sclerotia/g soil. We delivered a slightly lower *L. arvalis* concentration in our formulation (10^3 sclerotia/g soil) but found that control of brown patch and dollar spot in laboratory tests was significantly different from the untreated check.

Our results show that under field conditions, populations of *T. harzianum and L. arvalis* added as monthly vermiculite-corn meal-sand topdressings remained at levels of 10^5 and 10^3 propagules/g soil, respectively, suggesting that both *T. harzianum* R110 and *L. arvalis* OK-206 can survive in association with creeping bentgrass and remain at their initial concentrations following biocontrol topdressing applications. However, future research should investigate other types of delivery systems and application schedules used in conjunction with fungicide applications to determine whether higher biocontrol population levels can be attained.

Data collected during this study indicated that both *T. harzianum* R110 and *L. arvalis* may be capable of some degree of root colonization. Slightly higher fungal populations were found in upper soil portions where a major proportion of bentgrass roots were located, indicating that both biocontrol organisms may be able to survive in the soil by adhering to roots and utilizing root exudates.

The mechanisms by which *T. harzianum* and *L. arvalis* suppress plant pathogens are not totally understood. Production of cell-wall degrading enzymes (43), mycoparasitism (2) and nutrient competition (40) have been proposed mechanisms of

biocontrol activity of *Trichoderma* spp. *T. harzianum* R110 used in this study has been suggested to be a mycoparasite of *R. solani* (8). *L. arvalis* is known to produce a fungicidal compound called laetisaric acid which is inhibitory to *R. solani* and *Pythium* spp. (3).

Laboratory and field tests showed that *S. homoeocarpa* is a more aggressive pathogen on creeping bentgrass cv. "Crenshaw" than *R. solani* We have shown that an integrated control system involving a reduced rate of azoxystrobin fungicide in addition to *T. harzianum* R110 is as reliable as azoxystrobin at the full recommended label rate for control of brown patch on creeping bentgrass, but we found the control strategy to be ineffective against dollar spot. We have also demonstrated that *T. harzianum* R110 can be integrated with foliar fungicidal applications to delay brown patch development with limited detrimental effects on the fungus due to the selected fungicide tolerance level. However, we feel more field trials are necessary to support these findings further. Future research efforts on integrated control of dollar spot should focus on the use of a fungicide effective against that disease. Another worthwhile exploration would be to investigate a tank-mixture of azoxystrobin and a dollar spot-effective fungicide, integrated with biocontrol fungi, for broad spectrum, multicomponent control of more than one disease at the same time. Finally, research efforts should focus on integration of chemical and biological controls for turfgrass diseases since this disease management strategy could be of great benefit to the turfgrass industry. In an agricultural field that utilizes more fungicides than any other crop commodity (32), integrated control could reduce fungicide

expenses, not to mention lower the potential risks for environmental hazards, and decrease the chances for costly liabilities brought about as a result of improper fungicide usage.

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We would like to thank Lofts Great Western Seed Company (Albany, OR) for their generous donation of bentgrass seed used in this study.

Table 2. Treatments evaluated for integrated control of brown patch and dollar spot on creeping bentgrass in growth chamber experiments

Treatment	Rate of biological control	Rate of fungicide
#1 Control[a]	not applicable	not applicable
#2 Azoxystrobin[b]	not applicable	0.62 kg a.i./ ha
#3 *L. arvalis* OK-206[c]	4.7 x 10^3 propagules/g sand	not applicable
#4 *L. arvalis* OK-206 + azoxystrobin[d]	4.7 x 10^3 propagules/g sand	0.12 kg a.i./ha
#5 *T. harzianum* R110[c]	6.9 x 10^5 propagules/g sand	not applicable
#6 *T. harzianum* R110 + azoxystrobin[d]	6.9 x 10^5 propagules/g sand	0.12 kg a.i./ha

[a]Consisted of autoclaved vermiculite-corn meal-sand mixture without biocontrol fungi and was applied immediately following turfgrass inoculation by either pathogen.
[b]Applied immediately following turfgrass inoculation by either pathogen and again two weeks later.
[c]Applied immediately following turfgrass inoculation by either pathogen.
[d]Biological control applied to turfgrass immediately following inoculation and one week prior to azoxystrobin application.

Table 3. Treatments evaluated for integrated control of brown patch and dollar spot on creeping bentgrass under field conditions

Treatment	Rate of biological control	Rate of fungicide
#1 Control[a, f]	not applicable	not applicable
#2 Azoxystrobin[b]	not applicable	0.62 kg a.i./ha
#3 *L. arvalis* OK-206[c, f]	4.7 x 10^3 propagules/g sand	not applicable
#4 *L. arvalis* OK-206 + azoxystrobin[d, f]	4.7 x10^3 propagules/g sand	0.12 kg a.i./ha
#5 Azoxystrobin + *L. arvalis* OK-206[e, f]	4.7 x 10^3 propagules/g sand	0.12 kg a.i./ha
#6 *T. harzianum* R110[c, f]	6.9 x10^5 propagules/g sand	not applicable
#7 *T. harzianum* R110 + azoxystrobin[d, f]	6.9 x10^5 propagules/g sand	0.12 kg a.i./ha
#8 Azoxystrobin + *T. harzianum* R110[e, f]	6.9 x 10^5 propagules/g sand	0.12 kg a.i./ha

[a]Consisted of autoclaved vermiculite-corn meal-sand mixture without biocontrol fungi and was applied on 28 April, 1999 and monthly thereafter.
[b]Applied on 28 April, 1999 and every two weeks thereafter
[c]Applied on 28 April, 1999 and monthly thereafter
[d]Biocontrols applied on 28 April, 1999 and monthly thereafter Azoxystrobin applied on 5 May, 1999 and monthly thereafter.
[e]Azoxystrobin applied on 28 April, 1999 and monthly thereafter. Biocontrols applied on 5 May, 1999 and monthly thereafter. Fungicide was applied one week prior to biocontrol additions to determine if turfgrass niche clearing of potential microbial competitors could increase the control efficacy of the two biocontrol fungi.
[f]Vermiculite-cornmeal formulations were applied in a 1:4 ratio with autoclaved white silica sand (72 g to 288 g) using sterile Mason canning jars. Holes were punched through the lids of the canning jars and topdressing formulations were distributed uniformly over plot areas and lightly rubbed by gloved hand into the turf canopy.

Table 4. *In vitro* integration of *Trichoderma harzianum* R110 and *Laetisaria arvalis* OK-206 with azoxystrobin fungicide for control of brown patch

Treatment[2]	Disease Severity (Horsfall-Barratt ratings)[1]	
#1	9.50	a[3]
#2	1.00	b
#3	1.17	b
#4	1.00	b
#5	1.33	b
#6	1.00	b
LSD (P = 0.05)	0.44	

[1]Horsfall-Barratt disease ratings, where 1 = 0 to 3% diseased, 2 = 3 to 6% diseased, 3 = 6 to 12% diseased, 4 = 12 to 25% diseased, 5 = 25 to 50% diseased, 6 = 50 to 75% diseased, 7 = 75 to 88% diseased, 8 = 88 to 94% diseased, 9 = 94 to 97% diseased, and 10 = 97 to 100% diseased.
[2]Treatments were as follows: #1 = Control; #2 = azoxystrobin (0.62 kg a.i./ha); #3 = *L. arvalis* OK-206; #4 = *L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha); #5 = *T. harzianum* R110; #6 = *T. harzianum* R100 + azoxystrobin (0.12 kg a.i./ha)
[3]Means followed by the same letter within a column are not significantly different according to Fisher's least significant difference test.

Table 5. *In vitro* integration of *Trichoderma harzianum* R110 and *Laetisaria arvalis* OK-206 with azoxystrobin fungicide for control of dollar spot

Treatment[2]	Disease Severity (Horsfall-Barratt ratings)[1]	
#1	10.00	a[3]
#2	9.83	ab
#3	4.83	d
#4	8.33	abc
#5	8.33	abc
#6	7.33	c
LSD (P = 0.05)	2.49	

[1]Horsfall-Barratt disease ratings, where 1 = 0 to 3% diseased, 2 = 3 to 6% diseased, 3 = 6 to 12% diseased, 4 = 12 to 25% diseased, 5 = 25 to 50% diseased, 6 = 50 to 75% diseased, 7 = 75 to 88% diseased, 8 = 88 to 94% diseased, 9 = 94 to 97% diseased, and 10 = 97 to 100% diseased.
[2]Treatments were as follows: #1 = Control; #2 = Azoxystrobin (0.62 kg a.i./ha); #3 = *L. arvalis* OK-206; #4 = *L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha); #5 = *T. harzianum* R110; #6 = *T. harzianum* R100 + azoxystrobin (0.12 kg a.i./ha).
[3]Means followed by the same letter within a column are not significantly different according to Fisher's least significant difference test.

Table 6. Effects of integrated control of brown patch disease on creeping bentgrass cv. "Crenshaw" under field conditions

Treatment[2]	Disease Severity (Horsfall-Barratt ratings)[1]							
	07-Jun	14-Jun	21-Jun	01-Jul	12-Jul	23-Jul	04-Aug	27-Aug
#1	5.33 a[3]	5.67 a	6.33 a	6.67 a	6.67 a	6.33 a	5.33 a	5.33 a
#2	1.00 d	1.00 b	1.00 d	1.67 d	1.33 d	1.00 c	1.00 e	1.00 e
#3	4.00 abc	5.00 a	5.67 a	6.00 ab	5.00 ab	4.33 ab	4.67 ab	4.33 ab
#4	4.33 ab	5.00 a	5.00 ab	5.00 bc	4.00 abcd	4.33 ab	3.67 bc	3.00 cd
#5	2.67 bcd	5.00 a	5.00 ab	5.00 bc	4.67 abc	3.33 bc	3.67 bc	3.33 bc
#6	5.33 a	5.67 a	6.00 a	5.67 ab	3.00 bcd	3.67 b	3.33 cd	3.67 bc
#7	2.00 cd	2.67 b	3.67 bc	4.00 c	3.33 bcd	3.33 bc	3.00 cd	2.67 cd
#8	1.67 d	2.67 b	3.00 c	3.67 c	2.00 cd	2.00 bc	2.00 de	2.00 de
LSD (P = 0.05)	2.09	2 15	1.50	1.54	2.89	2.52	1.58	1.17

[1]Horsfall-Barratt disease ratings (Horsfall and Cowling, 1978).
[2]Treatments were as follows: #1 = Control; #2 = azoxystrobin (0.62 kg a.i./ha); #3 = *L. arvalis* OK-206; #4 = *L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha); #5 = azoxystrobin (0.12 kg a.i./ha) + *L. arvalis* OK-206; #6 = *T. harzianum* R110; #7 = *T. harzianum* R110 + azoxystrobin (0.12 kg a.i./ha); #8 = azoxystrobin (0.12 kg a.i./ha) + *T. harzianum* R110.
[3]Means followed by the same letter(s) within a column are not significantly different according to Fisher's least significant difference test. Percent brown patch was determined by visual estimation.

Table 7. Effects of integrated control of dollar spot disease on creeping bentgrass cv. "Crenshaw" under field conditions

	Disease Incidence[1]							
Treatment[2]	07-Jun	14-Jun	21-Jun	01-Jul	12-Jul	23-Jul	04-Aug	27-Aug
#1	0.67 a[3]	1.00 a	1.00 a	2.33 a	2.67 ab	5.00 ab	14.00 a	8.00 a
#2	1.67 a	2.33 a	2.33 a	9.33 a	12.00 a	14.33 ab	13.67 a	9.67 a
#3	0.00 a	0.33 a	0.33 a	2.33 ab	2.00 b	3.33 b	12.33 a	6.33 a
#4	0.00 a	1.33 a	1.00 a	6.33 bc	6.67 ab	8.33 ab	12.67 a	10.00 a
#5	2.00 a	4.00 a	3.67 a	9.33 bc	10.33 ab	13.00 ab	16.67 a	15.00 a
#6	1.00 a	1 67 a	2.33 a	4.33 ab	4.00 ab	7.67 ab	12.67 a	9.33 a
#7	0.67 a	1.00 a	1.33 a	3.33 c	4.67 ab	9.67 ab	9.67 a	14.67 a
#8	3.33 a	4.33 a	4.00 a	9.33 c	7.33 ab	19.67 a	23.67 a	15.33 a
LSD (P = 0.05)	3.79	5.00	4.65	10.43	9.97	16.32	19.95	15.61

[1] Mean number of spots per plot.

[2] Treatments were as follows: #1 = Control; #2 = azoxystrobin (0.62 kg a.i./ha); #3 = *L. arvalis* OK-206; #4 = *L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha); #5 = azoxystrobin (0.12 kg a.i./ha) + *L. arvalis* OK-206; #6 = *T. harzianum* R110; #7 = *T. harzianum* R110 + azoxystrobin (0.12 kg a.i./ha); #8 = azoxystrobin (0.12 kg a.i./ha) + *T. harzianum* R110.

[3] Means followed by the same letter(s) within a column are not significantly different according to Fisher's least significant difference test.

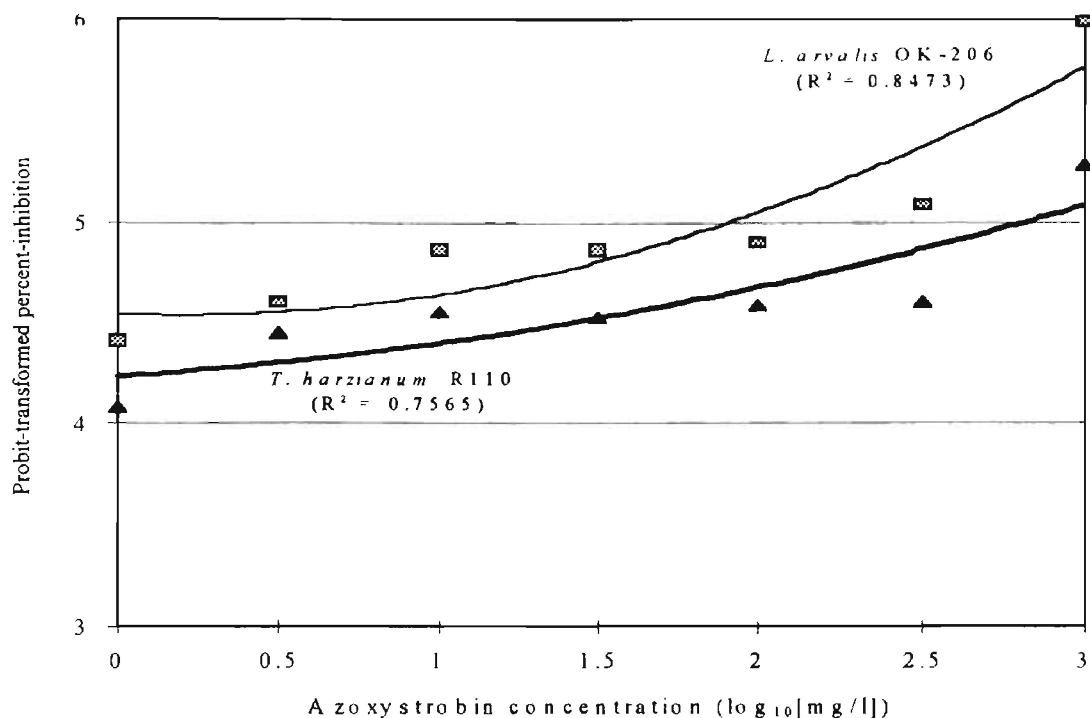

Fig. 5. Dose-response curves for *Trichoderma harziamum* R110 and *Laetisaria arvalis* OK-206 to azoxystrobin fungicide. Percent radial growth inhibition = (diameter on unamended medium - diameter on azoxystrobin-amended medium)/(diameter on unamended medium). The 50% effective concentrations for *T. harziamum* R110 and *L. arvalis* OK-206 were 661 mg a.i. azoxystrobin/liter (log EC_{50} = 2.82) and 81.3 mg a.i. azoxystrobin/liter (log EC_{50} = 1.91), respectively.

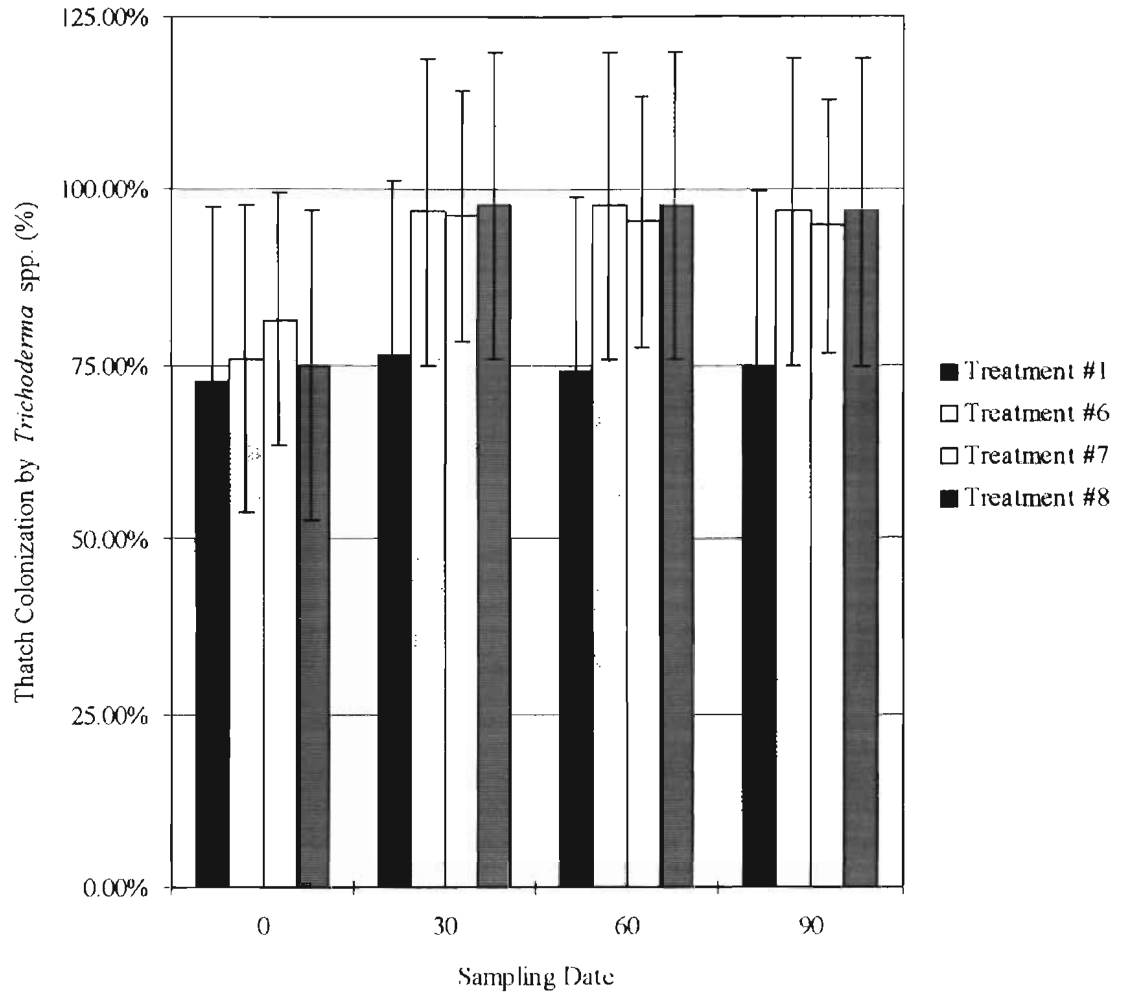

Fig. 6. Percent thatch colonization by *Trichoderma* spp. in creeping bentgrass cv. "Crenshaw" field plots. Treatment #1 = control; treatment #6 = *T. harzianum* R110; treatment #7 = *T. harzianum* R110 + azoxystrobin (0.12 kg a.i./ha); treatment #8 = azoxystrobin (0.12 kg a.i./ha) + *T. harzianum* R110. Columns represent the mean percentages of thatch colonization for three soil core samples from each of three plots per treatment. Bars represent standard errors of means.

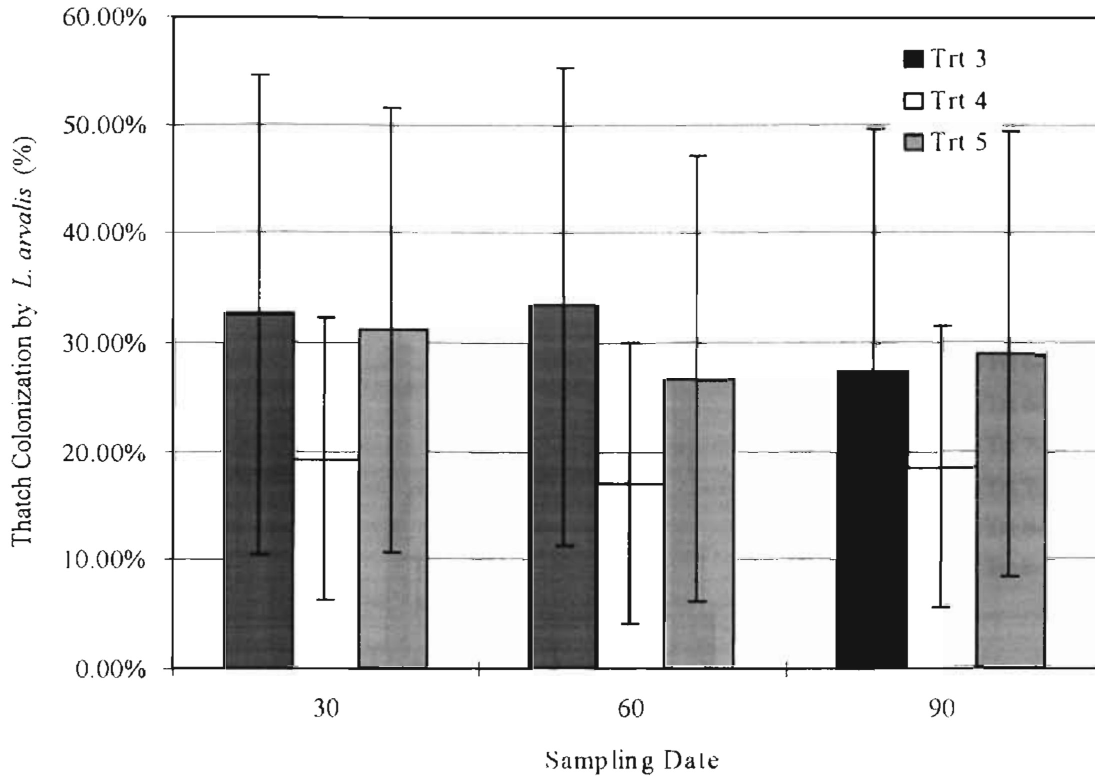

Fig. 7. Percent thatch colonization by *Laetisaria* spp. in creeping bentgrass cv. "Crenshaw" field plots. Treatment #3 = *L. arvalis* OK-206; treatment #4 = *L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha); treatment #5 = azoxystrobin (0.12 kg a.i./ha) + *L. arvalis* OK=206. Columns represent the mean percentages of thatch colonization for three soil core samples from each of three plots per treatment. Bars represent standard errors of means.

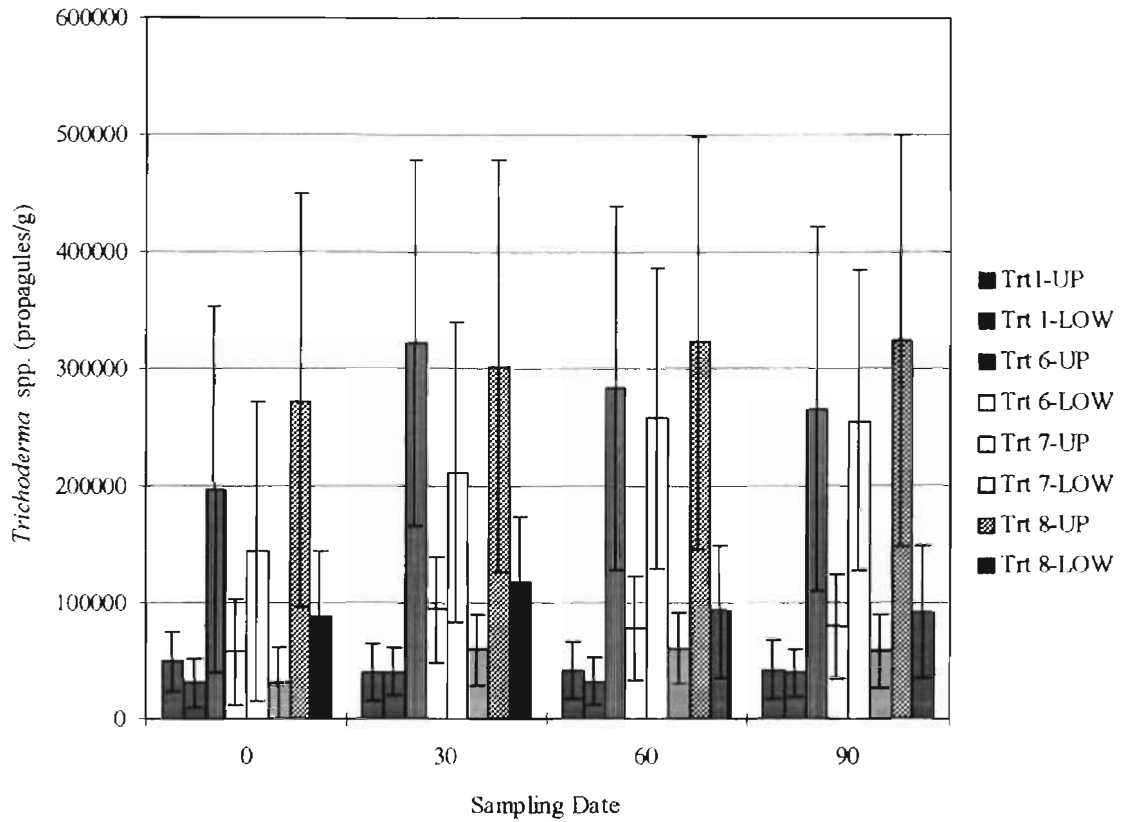

Fig. 8. Population dynamics of *Trichoderma* spp. in upper and lower sections of creeping bentgrass cv. "Crenshaw" field soil. Treatment #1 = control; treatment #6 = *T. harzianum* R110; treatment #7 = *T. harzianum* R110 + azoxystrobin (0.12 kg a.i./ha); treatment #8 = azoxystrobin (0.12 kg a.i./ha) + *T. harzianum* R110. Columns represent mean propagules/g soil for three soil core samples (upper and lower 3 cm sections) from each of three plots per treatment. Bars represent standard errors of means.

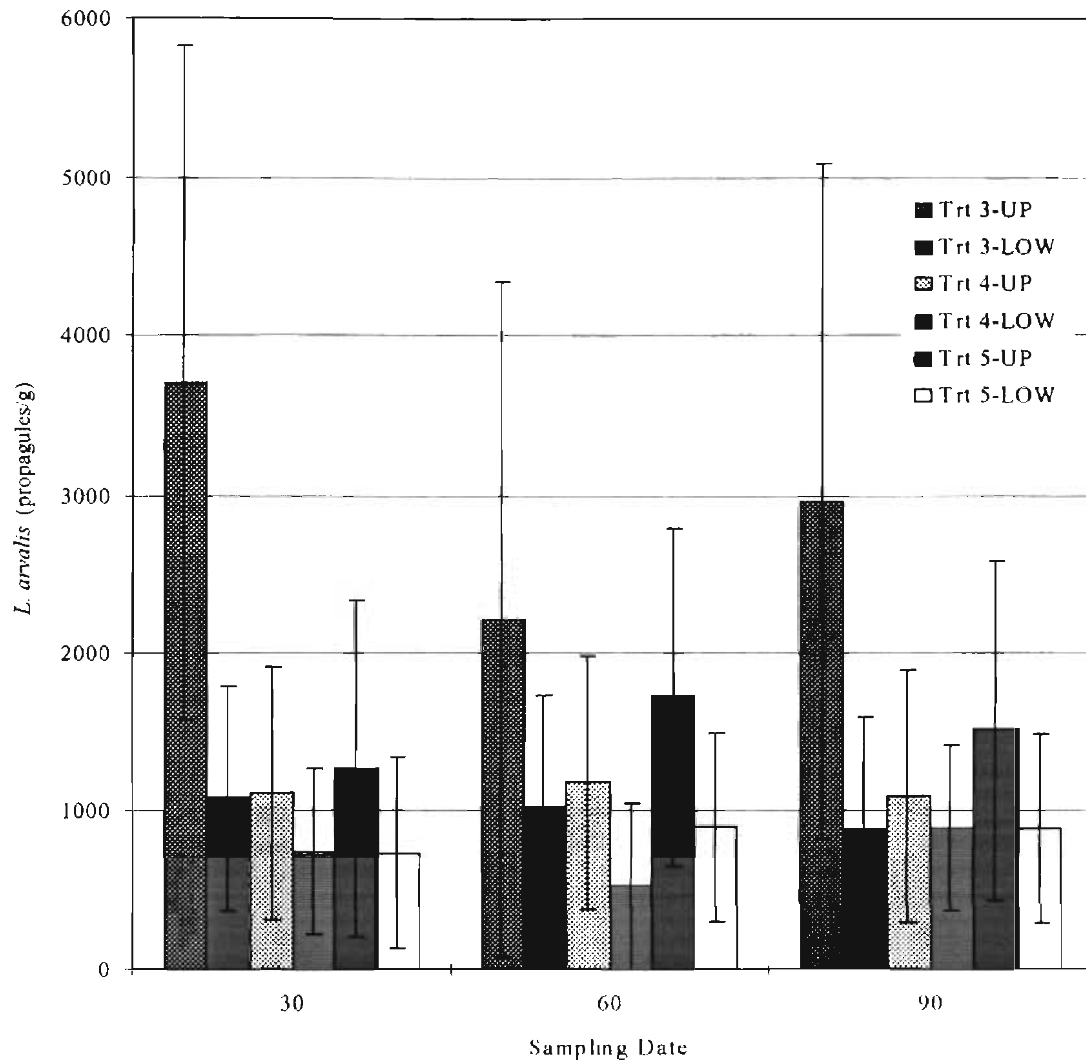

Fig. 9. Population dynamics of *Laetisaria* spp in upper and lower sections of creeping bentgrass cv "Crenshaw" field soil. Treatment #3 = *L. arvalis* OK-206; treatment #4 = *L. arvalis* OK-206 + azoxystrobin (0.12 kg a.i./ha); and treatment #5 = azoxystrobin (0.12 kg a.i./ha) + *L. arvalis* OK-206. Columns represent mean propagules/g soil for three soil core samples (upper and lower 3 cm sections) from each of three plots per treatment. Bars represent standard errors of means.

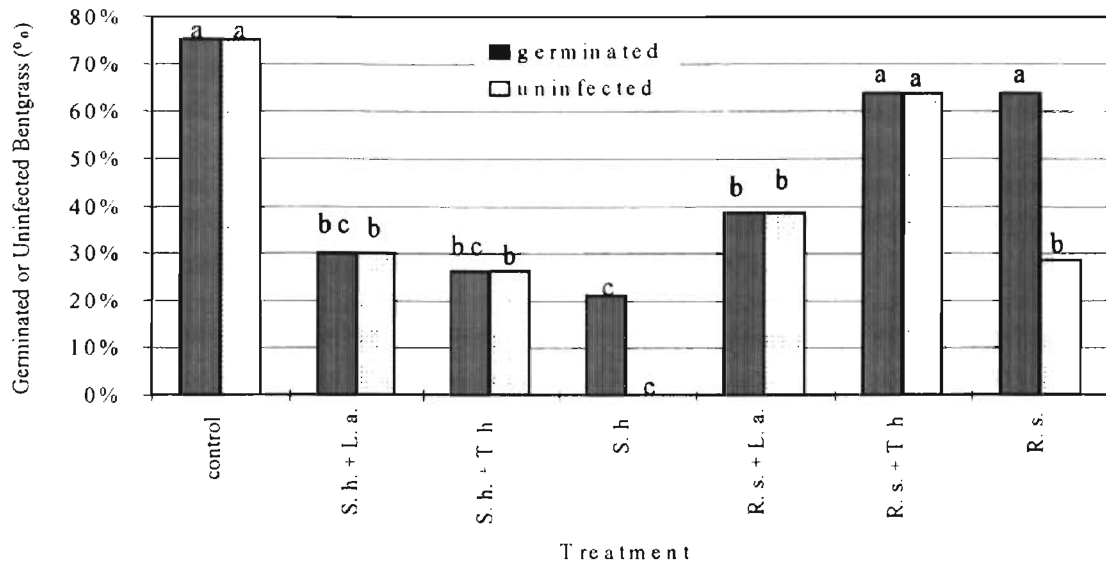

Fig. 10. An *in vitro* system for testing the biocontrol abilities of *Trichoderma harzianum* R110 and *Laetisaria arvalis* OK-206 against brown patch and dollar spot on creeping bentgrass cv. "Crenshaw." Adapted with modifications from Inbar et al., 1996. Control treatment contained no pathogen. Disease treatments consisted of PDA plates inoculated with either *S. homoeocarpa* or *R. solani* with and without biocontrol fungi. Columns headed by the same letter(s) are not significantly different (P = 0.05) according to Fisher's least significant difference test.

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VITA

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