



## ABSTRACT

Title of Dissertation: BIOLOGY OF *OPHIOSPHAERELLA AGROSTIS*,  
EPIDEMIOLOGY OF DEAD SPOT, AND A MOLECULAR  
DESCRIPTION OF THE PATHOGEN

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*Ophiosphaerella agrostis* is a newly described pathogen that incites dead spot disease of creeping bentgrass (*Agrostis stolonifera*) putting greens throughout the eastern United States. The objectives of this research project were to: 1) monitor environmental conditions and develop a predictive model for disease progress and pathogen dispersal under field conditions; 2) determine the effect of various fertilizers on dead spot recovery in bentgrass; and 3) develop molecular methods to diagnose the disease and determine genetic variation among *O. agrostis* isolates. Field studies showed that the appearance of dead spot infection centers was influenced predominantly by air ( $\geq 18^{\circ}\text{C}$ ) and soil ( $\geq 20^{\circ}\text{C}$ ) temperature, but other factors including low relative humidity ( $\leq 80\%$ ), shortened periods of leaf wetness ( $\leq 14$  hours), and high levels of solar radiation ( $\geq 230 \text{ W m}^{-2}$ ) were associated with the development of dead spot epidemics. Patch diameter of *O. agrostis* infection centers and pseudothecia production generally increased at a linear rate

between mid-June and early to mid-August. In a growth chamber study, ascospores were released from pseudothecia following a sharp decrease in relative humidity. Field studies revealed that ascospore release occurred primarily at dawn and dusk or during precipitation events. During precipitation events, ascospores may be forcefully discharged during the entire event, but most ascospores are released within the first 10 hours after precipitation begins. *O. agrostis* successfully over-wintered in bentgrass leaf sheaths, crowns, roots, and stolons, but little or no reactivation of the disease occurred in the second year. Plots treated with ammonium sulfate and isobutylidene diurea were among the fastest and slowest to recover from dead spot, respectively. In the second year (2003), dead spot recurred in plots treated since 2002 with  $\text{KNO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$ , urea, and a complete fertilizer (20-20-20), but disease symptoms did not recur in plots receiving ammonium sulfate. Species-specific primers capable of detecting *O. agrostis* at very low concentrations (5 pg DNA) were developed and can be used to assist in diagnosing dead spot. Amplified fragment length polymorphism (AFLP) DNA fingerprinting resulted in placement of isolates (n=77) into three distinct clades that were  $\geq 69\%$  similar.

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AND A MOLECULAR DESCRIPTION OF THE PATHOGEN

by

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## TABLE OF CONTENTS

LIST OF TABLES .....	VI
LIST OF FIGURES .....	IX
I. LITERATURE REVIEW .....	1
II. INCIDENCE AND SEVERITY OF DEAD SPOT, PSEUDOTHECIA DEVELOPMENT AND OVERWINTERING OF <i>OPHIOSPHAERELLA</i> <i>AGROSTIS</i> IN CREEPING BENTGRASS. ....	9
SYNOPSIS .....	9
INTRODUCTION .....	10
MATERIALS AND METHODS .....	12
RESULTS AND DISCUSSION.....	18
CONCLUSION.....	29
III. ENVIRONMENTAL MONITORING AND DEVELOPMENT OF A PREDICTIVE MODEL FOR THE ONSET AND SEVERITY OF DEAD SPOT SYMPTOMS IN CREEPING BENTGRASS.....	39
SYNOPSIS .....	39
INTRODUCTION .....	41
MATERIALS AND METHODS .....	43
RESULTS AND DISCUSSION.....	52
CONCLUSION.....	61
IV. ENVIRONMENTAL INFLUENCES ON THE RELEASE OF <i>OPHIOSPHAERELLA AGROSTIS</i> ASCOSPORES UNDER CONTROLLED AND FIELD CONDITIONS. ....	75
SYNOPSIS .....	75
INTRODUCTION .....	77
MATERIALS AND METHODS .....	79
RESULTS AND DISCUSSION.....	87
CONCLUSION.....	98
V. NITROGEN SOURCE IMPACT ON RECOVERY OF CREEPING BENTGRASS FROM DEAD SPOT ( <i>OPHIOSPHAERELLA AGROSTIS</i> ).....	112
SYNOPSIS .....	112
INTRODUCTION .....	113
MATERIALS AND METHODS .....	115
RESULTS AND DISCUSSION.....	122
CONCLUSION.....	132
VI. GENETIC VARIATION AMONG <i>OPHIOSPHAERELLA AGROSTIS</i> ISOLATES USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM DNA FINGERPRINTING .....	145
SYNOPSIS .....	145
INTRODUCTION .....	146

MATERIALS AND METHODS .....	149
RESULTS AND DISCUSSION.....	155
CONCLUSION.....	161
VII. A PCR-BASED METHOD FOR THE DETECTION OF <i>OPHIOSPHAERELLA AGROSTIS</i> IN CREEPING BENTGRASS.....	177
SYNOPSIS .....	177
INTRODUCTION .....	178
MATERIALS AND METHODS .....	181
RESULTS AND DISCUSSION.....	186
CONCLUSION.....	190
VIII. EPILOGUE: A ROAD MAP TO FUTURE DEAD SPOT RESEARCH .....	205
APPENDIX A.....	229
APPENDIX B.....	265
APPENDIX C.....	276
LITERATURE CITED .....	278

LIST OF TABLES

CHAPTER II INCIDENCE AND SEVERITY OF DEAD SPOT, PSEUDOTHECIA DEVELOPMENT AND OVERWINTERING OF *OPHIOSPHAERELLA AGROSTIS* IN CREEPING BENTGRASS.

Table 1. Area under the disease progress curve (AUDPC) values for patch diameter and *Ophiosphaerella agrostis* pseudothecia production in creeping bentgrass, 2001-2002.....32

Table 2. Accumulated degree days (DD) based on air (AT) and soil (ST) temperatures from 1 May until the linear growth period of pseudothecia production and linear increase in patch diameter for three years. ....33

Table 3. Predicted regression analyses equations for the percentage of successful *Ophiosphaerella agrostis* isolations from various creeping bentgrass tissues and pseudothecia between 2001 and 2003.....34

CHAPTER III ENVIRONMENTAL MONITORING AND DEVELOPMENT OF A PREDICTIVE MODEL FOR THE ONSET AND SEVERITY OF DEAD SPOT SYMPTOMS IN CREEPING BENTGRASS.

Table 1. Descriptive statistics of environmental and bentgrass dead spot (BDS) data used in correlation analysis.....64

Table 2. Pearson correlation coefficients and significance levels for environmental and disease variables collected for the examination of bentgrass dead spot on a creeping bentgrass in 2000-2002, College Park, MD.....65

Table 3. Variables and point values used to calculate an environmental favorability index (EFI-Epidemic) to provide a warning model for major ( $\geq 20$  infection centers per  $93 \text{ m}^2 \text{ day}^{-1}$ ) dead spot epidemics in creeping bentgrass grown in Maryland. ....66

Table 4. Variables and point values used to calculate an environmental favorability index (EFI-Symptom) to provide a warning model for dead spot symptom expression in creeping bentgrass in Maryland.....67

Table 5. Chi-square ( $\chi^2$ ) analyses of individual and combined environmental variables used to predict the appearance of dead spot symptoms (BDSAny) or major dead spot epidemics (BDSMajor). ....68

	Table 6. Degree days (DD) based on accumulated soil (ST) and air (AT) temperatures from 1 May until the date of primary dead spot symptom expression for three years. ....	69
CHAPTER IV	ENVIRONMENTAL INFLUENCES ON ASCOSPORE RELEASE OF <i>OPHIOSPHAERELLA AGROSTIS</i> UNDER CONTROLLED AND FIELD CONDITIONS.	
	Table 1. Growth chamber conditions during a 12 hour period for eight treatments designed to assess the impact of changing levels of relative humidity and light on <i>Ophiosphaerella agrostis</i> ascospore release.....	101
	Table 2. Repeated measures analysis of variance of ascospore release. ....	102
	Table 3. Influence of precipitation (rain and irrigation) and leaf wetness duration on the release of <i>Ophiosphaerella agrostis</i> ascospores from a creeping bentgrass putting green in 2001 and 2002.....	103
	Table 4. Correlation of percent daily capture of <i>Ophiosphaerella agrostis</i> ascospores in the field with the environmental variables measured. ....	104
CHAPTER V	NITROGEN SOURCE IMPACT ON RECOVERY OF CREEPING BENTGRASS FROM DEAD SPOT ( <i>OPHIOSPHAERELLA AGROSTIS</i> ).	
	Table 1. Number of dead spot ( <i>Ophiosphaerella agrostis</i> ) infection centers and percent change in the original number of infection centers in ‘L-93’ creeping bentgrass as influence by six N-sources, 2000 to 2001. ....	134
	Table 2. ‘L-93’ creeping bentgrass seasonal quality as influenced by six N-sources, 2000 to 2001.....	135
	Table 3. Impact of various nitrogen-sources and manganese on the development of new <i>Ophiosphaerella agrostis</i> infection centers in an ‘L-93’ creeping bentgrass putting green, 2003. ....	136
	Table 4. Impact of various nitrogen-sources and manganese on dead spot recovery in an ‘L-93’ creeping bentgrass putting green, 2003.....	137
	Table 5. Impact of various nitrogen-sources and manganese on seasonal ‘L-93’ creeping bentgrass quality, 2002-2003. ....	138

	Table 6. Impact of various nitrogen-sources and manganese on mat and soil pH in an ‘L-93’ creeping bentgrass putting green, 2002-2003. ....	139
	Table 7. Impact of various nitrogen-sources and manganese on various disorders in a ‘L-93’ creeping bentgrass putting green, 2003.....	140
CHAPTER VI	GENETIC VARIATION AMONG <i>OPHIOSPHAERELLA AGROSTIS</i> ISOLATES USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS.	
	Table 1. Isolate designation; cultivar affected; collection location, date received and collector; and color of 77 <i>Ophiosphaerella agrostis</i> isolates from the United States.....	164
	Table 2. Polymorphic and monomorphic fragments unique to isolates within each of three clades based on unweighted pair-group method with arithmetic mean analyses of amplified fragment length polymorphisms of 77 <i>Ophiosphaerella agrostis</i> isolates.....	167
	Table 3. Analyses of molecular variance of pair-wise distances for <i>Ophiosphaerella agrostis</i> isolates based on classes of color, geographic location (state or region), and pseudothecia production. ....	168
CHAPTER VII	A PCR-BASED METHOD FOR THE DETECTION OF <i>OPHIOSPHAERELLA AGROSTIS</i> IN CREEPING BENTGRASS.	
	Table 1. Isolate designation, cultivar affected, collection location, and date of isolation of <i>Ophiosphaerella agrostis</i> isolates collected from Maryland, USA. ....	191
	Table 2. Isolate designation; cultivar affected; and collection location, date and collector of various <i>Ophiosphaerella agrostis</i> isolates from the USA.....	193
	Table 3. Isolate designation; pathogen; host species and collector of common turfgrass pathogens used to test the specificity of primers OaITS1 and OaITS2. ....	195
	Table 4. Creeping bentgrass cultivar, collection site and location, and seedlot number of seed tested for the presence of <i>Ophiosphaerella agrostis</i> .....	196
	Table 5. DNA concentrations of 80 <i>Ophiosphaerella agrostis</i> isolates.....	197

LIST OF FIGURES

CHAPTER II INCIDENCE AND SEVERITY OF DEAD SPOT, PSEUDOTHECIA DEVELOPMENT AND OVERWINTERING OF *OPHIOSPHAERELLA AGROSTIS* IN CREEPING BENTGRASS.

Figure 1. Day length (A), patch diameter (Diam) and number of pseudothecia (Pseudo) (B) within *Ophiosphaerella agrostis* infection centers in 2000. Each symbol represents the mean patch diameter or pseudothecia count within independently monitored infection centers. New infection centers appeared on 10 June (Diameter and Pseudothecia 1, n=15) and 17 July (Diameter and Pseudothecia 2, n=14).....35

Figure 2. Day length (A), patch diameter (B) and pseudothecia number (C) within *Ophiosphaerella agrostis* infection centers in 2001 and 2002. Each symbol represents the mean patch diameter or pseudothecia count within four independently monitored infection centers. Days listed in the legend indicate dates in which infections centers first appeared.....36

Figure 3. Predicted linear growth phase of *Ophiosphaerella agrostis* patch diameter (A) and pseudothecia production (B) between accumulated day 162 and 238, 2000 to 2002. Regression lines followed by different letters have significantly different slope coefficients ( $P < 0.0001$ ) according to Tukey’s protected least significant difference test.....37

Figure 4. Recovery of *Ophiosphaerella agrostis* isolates from green (A) or bronze (B) leaves; leaf sheaths (C); crowns (D); roots (E); and stolons (F) of infected creeping bentgrass plants and pseudothecia (G). Pathogen isolation was attempted for one year following initial dead spot symptoms (2000-2001= ▲, 2001-2002= ●, and 2002-2003= ■). Open symbols represent outliers that were not included in the regression analyses. Accumulated days began with 1 during the first year of infection and 366 for 1 January during the second year of infection. ....38

CHAPTER III ENVIRONMENTAL MONITORING AND DEVELOPMENT OF A PREDICTIVE MODEL FOR THE ONSET AND SEVERITY OF DEAD SPOT SYMPTOMS IN CREEPING BENTGRASS.

Figure 1. Number of new *Ophiosphaerella agrostis* infection centers per unit area (93 m<sup>2</sup>) per day between 2000 and 2002 on a creeping bentgrass putting green in College Park, MD. Infection centers were counted daily between 30 July and 31 October 2000

(A) and 1 May and 31 October in 2001 (B) and 2002 (C). Arrows indicate the date in which the initial infection centers appeared each year. ....70

Figure 2. The distributions of the environmental variables on days in which *Ophiosphaerella agrostis* infection events occurred (gray box) and days in which no infections were observed (white box). Data represent 10% (left solid line), 25% (left side of box), 50% (center line), 75% (right side of box), and 90% (right solid line) of the observations recorded between May and November of 2000 to 2002. Extreme measurements or outliers are indicated by dots. The distributions for each environmental variable were significantly different at the 5% level where  $P < 0.0025$ . ....71

Figure 3. The distributions of the environmental variables on days in which major ( $\geq 20$  dead spots  $93 \text{ m}^{-2}$ ) *Ophiosphaerella agrostis* infection event occurred (gray box) and days in which no infections were observed (white box). Data represent 10% (left solid line), 25% (left side of box), 50% (center line), 75% (right side of box), and 90% (right solid line) of the observations recorded between May and November of 2000 to 2002. Extreme measurements or outliers are indicated by dots. The distributions for each environmental variable were significantly different at the 5% level where  $P < 0.0025$ . ....72

Figure 4. Relationship between daily mean soil temperature and the number of new dead spot infection centers appearing daily, 2000 to 2002. Significance for cubic regression lines are  $P = 0.0159, 0.0001, \text{ and } 0.0001$  for 2001, 2000, and 2002, respectively. ....73

Figure 5. Relationship of mean daily relative humidity (%) and maximum daily air temperature ( $^{\circ}\text{C}$ ) to the environmental favorability index for major dead spot outbreaks (EFI-Epidemic). Five-variable indexes (A) were calculated from field observations. Two variable indices (B) were predicted from the regression equation. Each color represents incremental changes in the EFI values. ....74

CHAPTER IV ENVIRONMENTAL INFLUENCES ON ASCOSPORE RELEASE OF *OPHIOSPHAERELLA AGROSTIS* UNDER CONTROLLED AND FIELD CONDITIONS.

Figure 1. Total number of *Ophiosphaerella agrostis* ascospores ( $\sqrt{(20 \times \text{ascospores})}$ ) released after 12 hours of incubation under changing relative humidity or light treatments. Treatments were as follows: (A) ~50% RH, light to dark; (B) ~50% RH, dark to light;

(C) 100% RH, light to dark; (D) 100% RH, dark to light; (E) ~50% to 100% RH, light; (F) 100% to ~50% RH, light; (G) ~50% to 100% RH, dark; and 100% to ~50 RH, dark. Treatment means followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's least significant difference test.....105

Figure 2. Interaction between hours of incubation and *Ophiosphaerella agrostis* ascospore release under varying controlled conditions (A to H) in which relative humidity (solid line) or light (white background) or dark (gray background) treatments were imposed at hour 6 of the 12 hour incubation period. ....106

Figure 3. Release of *Ophiosphaerella agrostis* ascospores as influenced by changing levels of relative humidity (RH) or light (white background) and dark (shaded) during 12 hours of incubation at 25°C. Ascospore data were square root transformed ( $\sqrt{(20 \times \text{ascospores})}$ ). Due to background levels of ascospores in all treatments, only treatments in which mean ascospore release ( $n$ ) was  $\geq 50$  12 hours<sup>-1</sup> were included. Treatments were as follows: (C) 100% RH, light to dark; (E) ~50% to 100% RH, light; (F) 100% to ~50% RH, light; and 100% to ~50 RH, dark. For individual treatments, bars labeled with the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's least significant difference test.....107

Figure 4. Seasonal relationship between the daily release of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] (A) and the appearance of new *Ophiosphaerella agrostis* infection centers (B), 2001. Days in which a major (2.0%) or minor (1.0%) percent of the total number of ascospores (4758 ascospores) released (A) are indicated by the solid and dashed lines, respectively. New infection centers (B) were counted daily between 1 May and 31 October and ascospores (A) were collected using a Burkard volumetric spore trap between 10 June and 31 October 2001. Estimated curves (C) were fitted using a non-parametric, locally weighted regression analyses (Proc Loess).....108

Figure 5. Seasonal relationship between the daily release of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] (A) and the appearance of new *Ophiosphaerella agrostis* infection centers (B), 2002. Days in which a major (2.0%) or minor (1.0%) percent of the total number of ascospores (9488 ascospores) released (A) are indicated by the solid and dashed lines, respectively. New infection centers (B) were counted daily between 1 May and 31 October and ascospores (A) were collected using a Burkard volumetric spore trap between

14 May and 31 October 2002. Estimated curves (C) were fitted using a non-parametric, locally weighted regression analyses (Proc Loess).....109

Figure 6. Percent of the total number [ $\sqrt{(20 \times \text{ascospores})}$ ] of *Ophiosphaerella agrostis* ascospores collected in 2001 (A) and 2002 (B) during each hour of the day. Ascospores were collected hourly between 10 June and 31 October 2001 and 1 May and 31 October in 2002 on a creeping bentgrass putting green.....110

Figure 7. Relationship between the number and percentage of *Ophiosphaerella agrostis* ascospores released each hour since the beginning of a precipitation event. Data are mean values of 227 precipitation (rain and irrigation) events on a creeping bentgrass putting green from 14 June to 31 October in 2001 and 14 May to 31 October 2002. A= observed frequency of the number of hours for each rain event; B= mean number of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] collected each hour since the beginning of the precipitation event; and C= percent of the total ascospores collected from 227 precipitation events during each hour of the event. Error bars indicate standard error of the means between 2001 and 2002.....111

CHAPTER V NITROGEN SOURCE IMPACT ON RECOVERY OF CREEPING BENTGRASS FROM DEAD SPOT (*OPHIOSPHAERELLA AGROSTIS*).

Figure 1. Impact of various nitrogen (N)-sources and manganese on dead spot incidence and bentgrass recovery in an ‘L-93’ creeping bentgrass putting green, 2002 (A) and 2003 (B). Significant differences ( $P \leq 0.05$ ), according to Tukey’s protected least significant difference test, among treatments first appeared on 4 September 2002 and are indicated by different letters. There were no differences among N-sources on any rating date between 17 September 2002 (\*) and the final rating date (23 September 2003). During the aforementioned period, all N-sources reduced dead spot severity, when compared to plots not receiving N (unfertilized and manganese).....141

Figure 2. Impact of various nitrogen (N)-sources and manganese on percent of plot area damaged by dead spot in an ‘L-93’ creeping bentgrass putting green, 2002. After 23 August, all plots in which N was applied had significantly less dead spot ( $P \leq 0.0001$ ), when compared to the unfertilized control or manganese alone. There were no differences in percent dead spot among plots receiving N on any rating date.....142

Figure 3. Plots treated with ammonium sulfate incurred little damage from yellow patch (*Rhizoctonia cerealis*). Photo taken 31 March 2003. ....143

Figure 4. Algae was not observed on any rating date in plots treated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Photo taken 13 September 2003. ....144

CHAPTER VI GENETIC VARIATION AMONG *OPHIOSPHAERELLA AGROSTIS* ISOLATES USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS.

Figure 1. Colony color and cultural morphology of *Ophiosphaerella agrostis* (n=16) isolates grouped into clade A based on amplified fragment length polymorphism fingerprinting analysis using primers *EcoRI* + AG and *MseI* + C. ....169

Figure 2. Colony color and cultural morphology of *Ophiosphaerella agrostis* (n=48) isolates grouped into clade B based on AFLP analysis using primers *EcoRI* + AG and *MseI* + C. ....170

Figure 3. Colony color and cultural morphology of *Ophiosphaerella agrostis* (n=13) isolates grouped into clade C based on AFLP analysis using primers *EcoRI* + AG and *MseI* + C. ....171

Figure 4. DNA fragment banding pattern of 77 *Ophiosphaerella agrostis* isolates after fluorescent amplified fragment length polymorphism analysis with automated capillary electrophoresis. ....172

Figure 5. Unweighted pair-group method with arithmetic mean dendrogram of 77 isolates of *Ophiosphaerella agrostis* collected from 21 different locations throughout the USA. Genetic distances were calculated by the SAHN similarity coefficient. ....173

Figure 6. Unweighted pair-group method with arithmetic mean dendrogram of 77 isolates of *Ophiosphaerella agrostis* collected from 21 different locations throughout the USA. Genetic distances were calculated by the Neighbor joining similarity coefficient. ....174

Figure 7. Typical variation in colony color and morphology of *Ophiosphaerella agrostis* isolates separating into each of three clades determined by amplified fragment length polymorphism (AFLP) DNA fingerprinting. Isolates shown include OpNC-1, OpMD-14 and OpPA-8 and represent clades A, B, and C, respectively. ....175

Figure 8. Pseudothecia development from various isolates of *Ophiosphaerella agrostis* after incubation at 25°C in constant darkness for 30 days. Error bars represent standard errors of the

differences among isolates on individual rating dates ( $P<0.05$ ).....176

CHAPTER VII A PCR-BASED METHOD FOR THE DETECTION OF *OPHIOSPHAERELLA AGROSTIS* IN CREEPING BENTGRASS.

Figure 1. Oligonucleotide primers specific to *Ophiосphaerella agrostis* (OaITS1 and OaITS2) were developed based on the ITS regions of isolates OpOH-1, OpMD-6 and OpVA-1, previously sequenced by Câmara et al., 2000.....198

Figure 2. Internal transcribed spacer region depicting location of primers OaITS1 and OaITS2 specific for *Ophiосphaerella agrostis*. The primer pair amplified a 445 to 446 bp region of all 80 *O. agrostis* isolates tested. ....199

Figure 3. Polymerase chain reaction (PCR) product amplified from the ITS region of DNA from (n=53) *Ophiосphaerella agrostis* isolates collected in Maryland, USA. A negative control (NC) and a 100 bp ladder are shown. ....200

Figure 4. Polymerase chain reaction (PCR) product amplified from the ITS region of DNA from (n=27) *Ophiосphaerella agrostis* isolates from 10 states. A negative control (NC) and a 100 bp ladder are shown. ....201

Figure 5. Polymerase chain reaction (PCR) amplification of fungal DNA from various turfgrass pathogens. From left to right: 100 bp ladder, negative control (NC); positive control (PC; OpVA-4); *Ophiосphaerella korrae* (#162, Lk-5); *O. herpotricha* (#189,#217); *O. narmari* (#370); *Bipolaris cynodontis* (BpMD-1); *Colletotrichum graminicola* (CgMD-6); *Gauemannomyces graminis* var. *avenae* (GgaFR-1, GgaMD-9, GgaPA-1); *Gloeocercospora sorghi* (GsMD-1), *Magnaporthe poae* (MpMD-3), *Rhizoctonia cerealis* (RcCT-1); *R. solani* (RsMD-4); *R. zeae* (RzMD-3); *Sclerotinia homoeocarpa* (ShVA-1); and a 100 bp ladder.....202

Figure 6. Polymerase chain reaction (PCR) amplification of fungal DNA from field-infected creeping bentgrass. From left to right: 100 bp ladder; negative control (NC); positive control (PC; OpVA-4); *Ophiосphaerella agrostis*-infected bentgrass plants (G1-G8), asymptomatic creeping bentgrass (Bent1-Bent4); hybrid bermudagrass (Berm1-Berm4) plants; and a 100 bp ladder. ....203

Figure 7. Amplification of *Ophiосphaerella agrostis* at various DNA dilutions. From left to right: ladder (L); OpPA-6 (1-5); and

OpVA-3 (6-10) at 50 ng, 5 ng, 0.5 ng, 50 pg, and 5 pg DNA. ....204

## I. LITERATURE REVIEW

Creeping bentgrass (*Agrostis stolonifera* L.) is a commonly used turfgrass species on golf course putting greens throughout the United States because of its ability to withstand low mowing heights and intense cultural practices, and because it provides a high quality putting surface. In 1998, Dernoeden et al. (1999) discovered a new disease of creeping bentgrass incited by an unidentified species of *Ophiosphaerella*. Through morphological and molecular study, it was shown that the pathogen constituted a new species, *Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum et M.E. Palm (Câmara et al., 2000). The pathogen and disease were documented in eleven states between 1998 and 2004. In addition to creeping bentgrass, the pathogen also has been isolated from diseased hybrid bermudagrass (*Cynodon dactylon* [L.] Pers. x *C. transvaalensis* Burt-Davy) in Texas and Florida (Kaminski and Dernoeden, 2002; Krausz et al., 2001).

Spegazzini (1909) described *Ophiosphaerella graminicola* Speg., the type species of the genus, which he found to be a pathogen of sprangletop (*Leptochloa virgata* (L.) P. Beauv.) in Argentina. The type species is characterized as having pleosporaceous (many spored) ascocarps with bitunicate asci containing brown scolecospores (filiform spores). The scolecospores lay parallel to one another and generally are slightly twisted near their middle and have no swollen cells, gelatinous sheaths, or appendages (Walker, 1980). Other species of *Ophiosphaerella* occur on *Gramineae* or *Cyperaceae* and have thin-walled pseudothecia (20-40 µm) made up of radially flattened cells (Walker, 1980). Species of *Ophiosphaerella* produce long, bitunicate asci containing eight, pale-brown, filiform, multiseptate ascospores. Ascospores are produced in pseudothecia and range in

length from 100-200 x 1.5-3  $\mu\text{m}$  (Walker, 1980).

Three other turfgrass pathogens in the genus *Ophiosphaerella* have been described. *Ophiosphaerella herpotricha* J.C. Walker, *O. korrae* (J.C. Walker and A.M. Smith) Shoemaker and C.E. Babcock (formerly *Leptosphaeria korrae*), and *O. narmari* (J.C. Walker and A.M. Smith) Wetzal, Hulbert and Tisserat (formerly *Leptosphaeria narmari*) were determined to cause spring dead spot of bermudagrass (*Cynodon dactylon* (L.) Pers) (Crahay et al., 1988; Endo et al., 1985; Smith, 1965; Tisserat et al., 1989; Walker and Smith, 1972; and Wetzal et al., 1999). *O. herpotricha* also causes spring dead spot in buffalograss (*Buchloë dactyloides* (Nutt.) Engelm) (Tisserat et al. 1999). Necrotic ring spot of creeping red fescue (*Festuca rubra* L. var. *rubra* Smith), and Kentucky (*Poa pratensis* L.) and annual (*Poa annua* L.) bluegrasses is incited by *O. korrae* (Dernoeden et al., 1995; Landschoot, 1996; and Worf et al., 1986). All of the aforementioned *Ophiosphaerella* species, except *O. graminicola*, are turfgrass root pathogens. These three root pathogens are characterized as producing darkly pigmented hyphae on roots, but none have been reported to infect creeping bentgrass.

When grown on potato dextrose agar (PDA), the optimum temperature for growth of *O. agrostis* is between 25 and 30°C (Kaminski et al., 2002). When incubated at 25°C, *O. agrostis* generally can be differentiated from other *Ophiosphaerella* spp. by its rose-quartz colony color. A few isolates, however, may produce an olive-green or gray-colored mycelium (Kaminski, 2001). *O. herpotricha* grown on PDA produces a white, cottony mycelium that turns tan or brown within 3 to 7 days (Tisserat et al., 1989). Wetzal et al. (1996) reported a brownish-black liquid exuding from the center of *O. herpotricha* isolates after 2 weeks of incubation on half-strength PDA. In addition,

optimum growth of *O. herpotricha* occurred at 20 to 25°C and maximum colony growth was between 3.5-4.1 mm 24 hour<sup>-1</sup> on PDA (Tisserat et al., 1989). Maximum colony growth for *O. korrae* and *O. narmari* is 4 to 5 mm 24 hour<sup>-1</sup> at 25°C on PDA (Walker and Smith, 1972). When incubated at 25°C on PDA, aerial mycelium of *O. korrae* and *O. narmari* initially is white to buff and darkens as colonies age (Walker and Smith, 1972; Wetzal et al., 1996). *O. korrae* colonies also have a distinctive raised or “domelike” growth habit (Wetzal et al., 1996). *O. herpotricha* and *O. korrae* exhibit little or no growth when incubated at 30°C on PDA (Crahay et al., 1988; Tisserat et al., 1989). Growth of *O. narmari* at 30°C has not been reported.

On creeping bentgrass grown on golf course putting greens, dead spot initially appears as small, reddish-brown or copper-colored spots approximately 1.0 cm in diameter, which may increase to about 8.0 cm in diameter (Dernoeden et al., 1999). During early stages of disease development, the spots often are confused with other turfgrass diseases such as dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett), copper spot (*Gloeocercospora sorghi* Bain & Edgerton ex Deighton), and Microdochium patch (*Microdochium nivale* [Fr.] Samuels & I.C. Hallett). Spots also can mimic damage from black cutworms (*Agrotis ipsilon* Hufnagel) or ball-marks, which commonly are found on putting greens. As the disease progresses, grass in the center of the spots becomes tan, while leaves in the periphery of the spot appear reddish-brown. *O. agrostis* was isolated from bentgrass and bermudagrass leaves, roots, crowns and stolons, demonstrating that *O. agrostis* attacks all plant tissues. Dark-brown to black hyphal masses can be found near or on the nodes of creeping bentgrass stolons, but generally are lacking in the internode region of stolons or on roots. On bermudagrass, hyphae generally are found

within roots and stolons and only occasionally are found growing ectotrophically (Krausz et al., 2001). Patches may be distributed throughout the putting green or localized, but the spots and patches generally do not coalesce. Active dead spot infection centers generally appear in areas with full sun and good air circulation. In particular, *O. agrostis* infection centers first may appear along ridges and on mounds and south-facing slopes of greens. These areas are particularly prone to higher soil temperatures and often are the first to exhibit drought symptoms. Dead spot severity may vary from a few spots to several hundred per green and depressions or pits may form in the putting surface.

Turf recovers very slowly, as stolon growth into dead patches appears restrained or inhibited. A frog-eye symptom (i.e., a dead ring area with living plants in the center) occasionally may occur if one or more plants in the center of the original patch survives or escapes infection from the previous year. Active disease symptoms generally are not observed during the winter months; however, spots that have not fully recovered often remain visible until the subsequent summer. Laboratory studies revealed that reactivation of dead spot in winter-dormant, infected creeping bentgrass may occur after 12 to 20 days incubation at constant temperatures (25 and 30°C), which are the same temperatures that are optimum for the growth of *O. agrostis* on PDA (Kaminski et al., 2002). Foliar mycelium is not observed in the field, but when diseased plants are incubated under high humidity for 3 to 5 days a white to pale-pink foliar mycelium may develop. Numerous pseudothecia often can be found on necrotic leaf, sheath, and stolon tissues.

Pseudothecia range from 150 to 350  $\mu\text{m}$  wide ( $\bar{X}$  = 223  $\mu\text{m}$ ), and have necks ranging from 30 to 160  $\mu\text{m}$  long ( $\bar{X}$  = 86  $\mu\text{m}$ ) (Câmara et al., 2000; Kaminski, 2001). Field observations suggest that dead spot is a polycyclic disease and that *O. agrostis* exists

solely in its sexual state (Câmara et al., 2000; Kaminski and Dernoeden, 2002).

Although the anamorph of *O. agrostis* has not been observed, pseudothecia and ascospores are abundant and serve as the secondary source of inoculum throughout the summer months. Ascospore length can range between 68 and 188  $\mu\text{m}$  ( $\bar{X}$  = 118  $\mu\text{m}$ ). Asci length and width range from 63 to 233  $\mu\text{m}$  ( $\bar{X}$  = 128  $\mu\text{m}$ ) and 8 to 15  $\mu\text{m}$  ( $\bar{X}$  = 11  $\mu\text{m}$ ), respectively. On average, ascospores have 13 septations (range = 6 to 15). Ascospores of *O. agrostis* can be forcefully ejected through ostioles of pseudothecia or exuded *en masse* in the presence of water (Kaminski et al., 2002). In the mid-Atlantic region, dew and leaf surface exudates develop in a bentgrass canopy at about 2000 hours in the summer and leaves often remain wet until 1000 hours when daytime temperatures reach  $\geq 25^{\circ}\text{C}$ . Ascospore germination can occur in as little as 2 hours (Kaminski et al., 2002). During the early hours of incubation, ascospores generally germinate in larger numbers in the presence of light and bentgrass leaves or roots. Ascospores incubated with bentgrass leaves exhibited similar or greater levels of germination, when compared to ascospores incubated with roots. Ascospores incubated with either tissue type for 2 to 12 h, however, generally resulted in a greater percent germination when compared to ascospores incubated in water alone. Germination occurs from the terminal ends of ascospores and occasionally from the interior cells. Up to four germ tubes may develop from an individual ascospore, and each is capable of directly penetrating leaves and roots or entering open stomates on leaves. Leaf surface exudates appear to be an important factor in rapid germination and subsequent infection of leaves by *O. agrostis* during the morning hours. The timing of ascospore release under field conditions, however, is unknown.

Dead spot may be active between May and December, but generally is most active during the summer months. Although dead spot typically develops between 1 and 2 years following bentgrass establishment, outbreaks of dead spot have been observed on creeping bentgrass that was less than 1 year old and as old as 6 years of age (Kaminski and Dernoeden, 2002). Dead spot generally is most severe during the first or second year of symptom expression and declines as greens age (Kaminski and Dernoeden, 2002). The decline phase may last from 1 to 3 years after the first year of disease expression, with the number of infection centers per green normally decreasing in subsequent years. Although dead spot is observed primarily on putting surfaces, occasionally it has been found on creeping bentgrass maintained at higher (i.e., > 0.2 mm) mowing heights. Bentgrass dead spot appears restricted to sand-based greens, collars, and tees, and has not been found in bentgrass or bermudagrass grown on native soil. The disease also was shown to be introduced by sod (Kaminski and Dernoeden, 2002). These observations support the premise that the pathogen may be most damaging where soil microbial populations are low; however, the inoculum potential on older greens may be too low to incite an epidemic in subsequent years.

Little information is available with regards to cultural and chemical management of dead spot in creeping bentgrass. Kaminski and Dernoeden (2002), reported that *O. agrostis* was capable of infecting the common *Agrostis* species and cultivars grown on golf courses. The velvet bentgrass (*Agrostis canina* L.) cultivars ‘SR7200’ and ‘Bavaria’ generally were the most and least susceptible cultivars, respectively. ‘Bardot’ colonial bentgrass (*Agrostis capillaris* L.) was highly susceptible to dead spot, but exhibited the greatest amount of recovery prior to winter. Among the creeping bentgrass cultivars

(n=17), 'L-93' generally had the greatest number of infection centers, but the number of infection centers was not significantly different from most of the other creeping bentgrass cultivars evaluated.

Field fungicide evaluation trials reported by Towers et al. (2000) and Wetzel and Butler (2001) showed that propiconazole, chlorothalonil, thiophanate methyl, fludioxonil, pyraclostrobin and iprodione effectively controlled dead spot, but little or no control was provided by triadimefon, trifloxystrobin, mefenoxam, or a formulation of *Bacillus subtilis*. Curative chemical management with the aforementioned fungicides is less efficacious (Wetzel and Butler, 2000). *In vitro* studies by Kaminski (2001) revealed that triadimefon, trifloxystrobin, chlorothalonil, and azoxystrobin reduced the total growth of three *O. agrostis* isolates (OpVA-1, OpTX-1, and OpOH-1), but the magnitude of the reduction was small. Except for one isolate (OpOH-1) grown on iprodione, thiophanate methyl and iprodione generally reduced the total growth of the *O. agrostis* isolates at all rates  $\geq 10 \mu\text{g a.i. ml}^{-1}$ . Although the growth of OpOH-1 was reduced at iprodione rates  $\geq 50 \mu\text{g a.i. ml}^{-1}$ , total growth of the isolate was significantly greater than both OpVA-1 and OpTX-1 at all rates  $> 0.5 \mu\text{g a.i. ml}^{-1}$  of iprodione. According to Wetzel and Butler (2000), weekly applications of urea in conjunction with fungicides reduced the number and diameter of dead spot infection centers. When applied weekly, however, urea alone did not significantly reduce dead spot severity, when compared to the untreated control (Wetzel and Butler, 2000).

This research project was designed to further investigate the biology of *O. agrostis* and the epidemiology of bentgrass dead spot. A goal of these studies was to elucidate environmental conditions surrounding the incidence and severity of dead spot

and the release of *O. agrostis* ascospores and subsequent appearance of infection centers. Additionally, little is known about the impact of different types of nitrogen sources on dead spot. Therefore, the effect of applications of water-soluble and water-insoluble nitrogen fertilizers was evaluated in the field to determine their impact on dead spot incidence, severity and recovery of the turf from the disease. Furthermore, molecular methods were explored to describe genetic variation and improve detection of the pathogen. Genetic variation among *O. agrostis* isolates from various geographic regions and the use of species-specific oligonucleotide primers as a means of identifying the pathogen were evaluated. The objectives of this research project therefore were to: 1) monitor disease severity and pseudothecia development and determine pathogen survival during winter under field conditions; 2) develop a predictive model for determining dead spot incidence and severity; 3) determine conditions that are necessary for ascospore release; 4) determine the effect of various fertilizers on the incidence, severity and recovery of bentgrass affected by dead spot; 5) determine genetic variation among *O. agrostis* isolates using amplified fragment length polymorphism DNA fingerprinting; and 6) develop species-specific oligonucleotide primers capable of identifying *O. agrostis*.

## II. INCIDENCE AND SEVERITY OF DEAD SPOT, PSEUDOTHECIA DEVELOPMENT AND OVERWINTERING OF *OPHIOSPHAERELLA AGROSTIS* IN CREEPING BENTGRASS.

### SYNOPSIS

Dead spot (*Ophiophaerella agrostis*) attacks relatively young creeping bentgrass (*Agrostis stolonifera*) golf greens throughout the eastern United States. Little information is available regarding the development of dead spot and pseudothecia as well as the ability of *O. agrostis* to overwinter. The objectives of this research were to determine the peak period of dead spot activity based on increasing patch diameter and pseudothecia development, and to determine the ability of *O. agrostis* to overwinter within or on various bentgrass tissues. Over the course of this three year field study, patch diameter of *O. agrostis* infection centers generally increased at a linear rate between mid-June and early-August. Pseudothecia production closely followed increasing patch diameter. Pseudothecia could be found within necrotic or dead tissue as early as the first day of symptom expression and as many as 478 pseudothecia were found in a single dead spot. Periods of rapid dead spot development coincided with air and soil temperatures in the range of 22 to 26°C. Although new infection centers appeared between late-August and early-October, increases in patch diameter and pseudothecia development were negligible during this period. Dead spot severity varied each year with the most severe dead spot occurring the year after the initial green construction or year after fumigation with methyl bromide. *O. agrostis* was capable of overwintering as pseudothecia or as hyphae within or on bentgrass leaf sheaths, roots, stolons and crowns, especially the nodes of stolons. Despite this survival and the apparent movement of the pathogen from stolons to crowns and leaf sheaths in late-April or early-May, little or no reactivation of the disease occurred in the second year at two juxtaposed study sites.

## INTRODUCTION

Dead spot of creeping bentgrass (*Agrostis stolonifera* L.) and hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burt-Davy) is incited by *Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum, et M.E. Palm (Câmara et al., 2000; Dernoeden, 2000; Dernoeden et al., 1999; Krausz et al., 2001). A relatively new disease, dead spot affects golf course putting greens throughout the eastern United States (Dernoeden et al., 1999; Kaminski and Dernoeden, 2002). Dead spot primarily is a problem on newly constructed, sand-based putting greens and generally appears within 1 to 2 years following establishment. In addition, the disease may occur on older bentgrass putting greens following fumigation with methyl bromide. Initially, the disease appears as small, copper or reddish-brown spots approximately 1 to 2 cm in diameter, which slowly increase to approximately 8 to 10 cm in diameter throughout the summer months.

Once infection occurs, turfgrass in the center of dead spots dies and often forms pits or depressions, which adversely affect the putting surface. Recovery of bentgrass into infected spots is slow and dead spots often remain present throughout the winter until bentgrass growth resumes in the spring. On creeping bentgrass grown in the mid-Atlantic region of the United States, dead spot incidence may occur as early as May and active disease symptoms may be present as late as December (Kaminski and Dernoeden, 2002).

Little information is available regarding the seasonal development of individual infection centers and the progression of *O. agrostis* to other plant parts. Based on field observations, the disease is most severe in sunny locations and often first appears in areas prone to heat stress such as ridges, mounds, and slopes of putting greens (Kaminski and

Dernoeden, 2002). *O. agrostis* only has been found in its sexual state and no anamorph has been reported. Pseudothecia often can be found embedded in necrotic leaf, sheath and stolon tissues of infected or dead bentgrass (Câmara et al., 2000; Dernoeden et al., 1999; Kaminski and Dernoeden, 2002). Although other *Ophiosphaerella* spp. cause several turfgrass diseases, *O. agrostis* is the only species in which pseudothecia commonly are found in nature. Knowing peak periods of disease severity and development of pseudothecia as well as the winter survival of *O. agrostis* are important in understanding the biology and spread of the pathogen.

This study was designed to further investigate the biology of *O. agrostis* and the epidemiology of dead spot. The primary goal was to increase the understanding of some basic biological functions of the pathogen. The objectives of this field research therefore were to: 1) monitor and determine the timing of pseudothecia development; 2) measure the growth rate of diseased spots over time; and 3) determine the ability of *O. agrostis* to overwinter within or on various bentgrass tissues.

## MATERIALS AND METHODS

### **Field Study**

Environmental and disease monitoring were conducted at the University of Maryland Paint Branch Turfgrass Research Facility in College Park, MD between 2000 and 2002. In August of 1999, a research putting green was constructed to United States Golf Association (USGA) specifications (USGA Green Section Staff, 1993). Soil was a modified sand mix (97% sand, 1% silt, and 2% clay) with a pH of 4.9 and 10 mg organic matter g<sup>-1</sup> of soil. Soil P (42 to 79 kg ha<sup>-1</sup>) and K (4 to 16 kg ha<sup>-1</sup>) varied throughout the three years and generally were low to moderate. A total of 1075 kg ha<sup>-1</sup> ground agricultural limestone was applied between November 1999 and April 2000 to raise soil pH. The soil pH in the top 5 cm was raised to 6.9 by 25 September 2000. Each year, field evaluations were initiated on separate sections of the aforementioned research green. All cultural and chemical practices were the same as those described in Chapter III. Air and soil temperatures were monitored between 1 May and 31 October in 2000, 2001 and 2002. Air temperature was measured with a thermistor placed 30 cm above the plant canopy (model HMP35C, Campbell Scientific, Logan, Utah). Soil temperature was measured by averaging the data from two or three thermistor sensors (model 107, Campbell Scientific, Logan, Utah) placed 2.5 cm below the turfgrass canopy near the thatch and soil interface. All temperature recording instruments were connected to a CR-10 datalogger (Campbell Scientific, Logan, Utah) and programmed to measure temperature at 2 min intervals and mean measurements were recorded every 60 min. Day length data collected for College Park were provided by the U.S. Naval Observatory located in Washington, D.C.

## Disease Severity and Pseudothecia Development

The research areas were monitored daily for the presence of dead spot infection centers between 2000 and 2002. Each year, a mix of tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran (1:1 v/v) inoculum, infested with *O. agrostis*, was prepared using the method described by Kaminski et al. (2002). Approximately 0.5 g of inoculum was placed at the soil surface in a grid pattern spaced on 0.9 (2000) or 1.5 (2001 and 2002) m centers over a 465 m<sup>2</sup> area. All isolates used to inoculate each area in this study were described previously (Câmara et al., 2000; Kaminski et al., 2002; Kaminski, Chapter VI). For the 2000 study year, the 'L-93' creeping bentgrass area (seeded 20 September 1999) was inoculated with isolate OpVA-1 on 8 October 1999. When disease symptoms initially appeared, fifteen infection centers were chosen at random and measurements made every 2 to 5 days between 10 June and 14 July 2000. Disease severity was assessed by measuring patch diameter of individual infection centers in two directions with a ruler. Mean patch diameter of each infection center was used for the statistical analyses. Pseudothecia development also was monitored within each of the fifteen spots on a weekly basis. Pseudothecia were counted within each spot using a portable microscope with a magnification of 25x (Macroscope 25, Ben Meadows Company, Janesville, WI). On 17 July, new infection centers (n=14) were selected and patch diameter was measured and pseudothecia counted until 1 September 2000.

In 2001, the 'Providence' creeping bentgrass area (seeded 20 September 1999) was inoculated with isolates OpMD-9 + OpVA-1 on 12 March. To monitor the

development of pseudothecia and increases in patch diameter over the course of the entire season, new infection centers (n=4) were selected periodically throughout year. In 2001, four new infection centers were marked for data collection on 1 June, 20 July, 20 August, and 3 and 21 September. Patch diameter and the development of pseudothecia within each infection center were monitored in each spot until 2 November.

Due to a decline in disease activity in 2001, the site was fumigated with methyl bromide (98% methyl bromide + 2% chloropicrin) on 23 August 2001. Prior to fumigation the area was vertically mowed in three directions and core aerated to a depth of 7.6 cm with 12.5 mm diameter hollow tines. On 30 August 2001, the area was seeded to 'L-93' creeping bentgrass. On 21 March 2002, the area was inoculated with two isolates (OpOH-1 and OpVA-1) of *O. agrostis* as described previously. In 2002, four new infection centers appearing on 13 and 27 May, 18 June, 8 July, 12 August and 9 September were selected randomly and data were recorded for all spots until 31 October.

### **Statistical Analyses**

A square root transformation of pseudothecia count data was completed prior to analyses. Regression analyses were performed to determine the rate of increasing patch diameter and numbers of pseudothecia, and slope coefficients were compared using the method described by Neter and Wasserman (1974). Additionally, the area under the disease progress curve (AUDPC) was determined for the aforementioned patch diameter and pseudothecia data over the course of each year. Except for 2000, dates used to calculate AUDPC values were 1 June to 2 November 2001 and 13 May to 31 October 2002. In 2000, patch diameter and pseudothecia counts were collected for shorter

periods, and therefore these data were not included in the AUDPC analyses. All AUDPC values were subjected to analysis of variance using the MIXED procedure in SAS (SAS Institute Inc., 2000). The AUDPC values were calculated using the formula:  $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$ , where  $i = 1, 2, 3 \dots n-1$ , where  $y_i$  is the diameter (mm) of *O. agrostis* infection centers or number of pseudothecia spot<sup>-1</sup> and  $t_i$  is the time of the  $i$ th rating (Campbell and Madden, 1990).

### ***Development of a degree day model***

To determine the impact of accumulating heat leading up to the period of peak disease activity, cumulative degree-days (DD) were computed (Ritchie and NeSmith, 1991). Accumulation of DD began on 1 May of each year and values were calculated by the following equation:  $DD = \sum^n [T_{(s \text{ or } a)} - T_b]$ , where  $T_{(s \text{ or } a)}$  is the mean daily soil ( $s$ ) or air ( $a$ ) temperature;  $T_b$  is the base temperature, and  $n$  is the number of days elapsed since 1 May (Ritchie and NeSmith, 1991). A  $T_b$  of 15°C was utilized for reasons described below.

### ***In planta Distribution and Overwintering of Ophiosphaerella agrostis***

The survival of *O. agrostis* within or on plants was determined by attempting to isolate the pathogen from the roots, crowns, leaf sheaths, stolons, and bronze/tan and green creeping bentgrass tissue collected along the periphery of infection centers, as well as from pseudothecia. Attempts to isolate *O. agrostis* from 'L-93' creeping bentgrass (seeded 1999) previously infected by the pathogen were made between 31 January and 26 June 2001. Three to four infection centers were removed from the field and immediately

prepared for study. Symptomatic bentgrass plants from the edge of diseased spots were removed and short, young roots exhibiting discoloration near the stem were selected. The crowns of plants that appeared to have been infected by *O. agrostis* the previous year were cleaned of all leaves and roots. Crown tissue generally appeared healthy once excess tissue was removed. Tan or green leaf tissue was detached above the collar region and leaf sheaths of discolored tissue were detached below the collar prior to surface disinfecting. Stolons generally exhibited severe discoloration at the nodes, but not the internodal regions. Finally, pseudothecia were removed from dead or decaying tissue.

In the second year, isolation of *O. agrostis* from the aforementioned plant tissues was attempted from infected 'Providence' creeping bentgrass (seeded 1999) between initial symptom development on 31 May 2001 and 1 March 2002. Unlike the first year, pathogen isolation from healthy-appearing crown tissue was not attempted. Only discolored crowns with visibly symptomatic tissue were selected for pathogen isolation. During the final year (2002-2003), attempts to isolate the pathogen from 'L-93' creeping bentgrass (seeded 2001) generally were performed monthly between 20 June 2002 and 26 June 2003.

In all years, infected leaf, leaf sheath, root, and stolon tissue were sectioned into 3 to 6 mm long pieces. For crowns and pseudothecia, plant tissue was removed from individual samples prior to surface disinfecting. All creeping bentgrass tissue and pseudothecia were disinfested in 0.53% sodium hypochlorite for 60 sec and triple washed for 30 sec in sterilized, distilled water. Tissue then was blotted dry on sterilized filter paper and placed on antibiotic water agar (AWA). The AWA was prepared by mixing 20 g agar L<sup>-1</sup> deionized water, then adding to cooled media (50°C) 0.5 g of benzylpenicillin

(Penicillin G, Sigma-Aldrich Co., St. Louis, MO) and/or 0.5 g streptomycin sulfate (Sigma-Aldrich Co., St. Louis, MO). The Petri dishes were incubated at 25°C, and after 3 to 4 days actively growing mycelia were transferred to PDA. Positive identification of *O. agrostis* was based on the growth rate and the rose-quartz colony color and morphology described by Kaminski et al. (2002). A total of 10 tissue sections or pseudothecia were chosen for each isolation attempt and data were reported as a percentage of successful *O. agrostis* isolations.

To determine the ability of *O. agrostis* to overwinter and to track the progression of the pathogen from its overwintering site to other plant parts, regression analyses were conducted on percent successful isolation data using SAS (SAS Institute Inc., 2000). To perform regression analyses, dates were expressed as numeric values representing accumulated days beginning with the first day of the year for each study. For example, 1 = 1 January for the initial year of each study and 366 = 1 January for the subsequent year. For the 2000-2001 year, however, 1 January 2001 = 367 due to the leap year in 2000. Regression analyses were performed on numeric dates ranging from 151 (31 May 2001, study year 2) and 542 (26 June 2003, study year 3). To prevent misinterpretation of regression lines for the data from all three years, stepwise regression analyses were performed separately on data collected from 2001-2002 and 2002-2003 to determine the best fit line for each year. Since isolation attempts during the first year only were collected between January and June 2001, these data were not included in the initial analysis. Significant polynomial transformations of accumulated numeric days for each year were selected and regression analyses again were performed on all isolation data collected from the three years.

## RESULTS AND DISCUSSION

### **Dead Spot and Pseudothecia Observations**

*Study year 1.* Dead spot symptoms first appeared on 8 June 2000 in spots inoculated the previous October and infection centers first appearing on 10 June were monitored further. Patch diameter measured on 10 June (n=15) increased from an average initial diameter of 39 mm to 63 mm by the final rating date (14 July) (Figure 1). Although pseudothecia initially were not observed within these infection centers, an average of 24 pseudothecia infection center<sup>-1</sup> were observed on 16 June (6 days after initial symptoms). Pseudothecia continued to develop in necrotic tissue within patches and an average of 127 pseudothecia were observed on 14 July.

A second set of infection centers (n=14) appearing on 17 July were marked and patch diameter and pseudothecia development were monitored until 1 September. On 17 July, patches averaged 22 mm in diameter (Figure 1). Patch diameter of the second set of infection centers increased more slowly than the initial infection centers (i.e., 10 June) and by the final rating date (1 September), patches only averaged 38 mm in diameter. A small number of pseudothecia already were present within several infection centers when symptoms first became visible, and an average of 139 pseudothecia spot<sup>-1</sup> were present on 21 August. Throughout the year, as many as 248 pseudothecia were found inside a single patch. In 2000, no new infection centers appeared after 11 October. Immature ascospores, however, could be found inside pseudothecia throughout winter.

*Study year 2.* The area was inoculated on 12 March 2001 and although no disease symptoms were present, the pathogen was isolated from young roots and crown tissue of plants adjacent to the inoculum on 12 April. By 18 April, mature pseudothecia were

found embedded on the inoculum mix. No pseudothecia, however, were found on bentgrass tissue at this time. Visual symptoms in the form of *O. agrostis* infection centers first were observed on 31 May. Four new infection centers appearing on 1 June were marked and patch diameter and pseudothecia development were monitored until 2 November (Figure 2). Average patch diameter increased from 13 mm on 1 June to 25 mm on 18 June. Mean pseudothecia production within these spots, however, was low ( $\leq 8 \text{ spot}^{-1}$ ) until the end of June. Between 29 June and 16 July, the average number of pseudothecia within patches increased to 101. After 16 July, the average number of pseudothecia per patch rapidly declined and an average of only 5 pseudothecia were observed by the final rating date (2 November). It is unclear if the decomposition of pseudothecia resulted in a reduced ability to observe fruiting bodies in each patch. It was unlikely, however, that pseudothecia forming in the depressed patches were physically removed by mowing.

Few new infection centers developed in the 1999 seeded 'Providence' bentgrass throughout 2001 and disease severity generally was low. Four additional sets of infection centers were selected between 20 July and 21 September. Although disease symptoms were observed, there was little or no increase in patch diameter from these infection centers (Figure 2). In addition, very few pseudothecia ( $\leq 3$ ) were observed within monitored infection centers appearing in July or August. Pseudothecia were observed, however, within new infection centers appearing on 3 and 21 September. Observations during the second year of the study were similar to previous reports in which dead spot incidence and severity generally declines within one to three years following initial infection (Kaminski and Dernoeden, 2002). Similar to 2000, no new infection centers

occurred after early October.

**Study year 3.** To stimulate declining dead spot activity, the site used to monitor dead spot in 2002 was fumigated and reseeded in August 2001 and the area was inoculated on 21 March 2002. Unlike 2001, dead spot incidence was severe and results were similar to those observed in 2000. In 2002, new infection centers were apparent within inoculated spots on 12 May. Despite the continued appearance of new infection centers between early-May and early-June, patch diameter (n=8) did not begin to increase until mid-June (Figure 2). Patch diameter continued to increase in all monitored spots (n=16) until early to mid-August. Little or no increase in patch diameter was observed in any spots after mid-August, and no new infection centers developed after 9 October.

Pseudothecia began to develop within necrotic bentgrass tissue on 9 June and their production generally coincided with the increase in patch diameter. Pseudothecia were produced in very high numbers within spots (n=12) appearing between 13 May and 18 June. Maximum pseudothecia production within the aforementioned patches occurred by mid-August and as many as 478 fruiting bodies were observed within a single spot. New *O. agrostis* infection centers appearing on 8 July, however, only had an average of 106 pseudothecia spot<sup>-1</sup> by mid-August. After this peak, the number of pseudothecia decreased and by 31 October there was an average range of 46 to 136 pseudothecia per patch in each set of monitored spots. Few pseudothecia (mean  $\leq 11$  spot<sup>-1</sup>) developed within new infection centers appearing on 12 August or 9 September.

Each study site was located adjacent to the previous year's study. Despite the development of new infection centers within each study site, disease symptoms generally did not recur in areas affected during the previous year. Also, despite the 2001

fumigation of the study site and an increase in dead spot severity in 2002, very few infection centers were observed within this site in 2003. These observations again demonstrate that a decline in dead spot incidence and severity can occur in the year following initial infection (Kaminski and Dernoeden, 2002; Kaminski et al., 2002). Kaminski and Dernoeden (2002) suggested that dead spot activity may be promoted in sand-based root zones with initially low soil microbial activity, but mentioned that low inoculum potential may impact disease incidence in the second year. Based on the results of this study, it appears that secondary inoculum (pseudothecia and ascospores) is drastically reduced following the first year of infection. Inoculum in the form of ascospores, however, was present each year as discussed in an unrelated study conducted on the same research green (Kaminski, Chapter IV). The reduction in disease severity, therefore, likely is the result of factors other than inoculum density such as turf age and possibly the buildup of microbial antagonists.

### **Regression Analyses and Area Under The Disease Progress Curve**

Regression analysis was used to determine the rate of increase for the number of pseudothecia and patch diameter during each of the three study years. Additionally, AUDPC values for 2001 and 2002 were determined for patch diameters as well as for pseudothecia development within infection centers. Due to the truncated measurements collected in 2000, these data were excluded from the AUDPC analyses.

In 2000, patch diameter and pseudothecia counts were measured between 10 June and 14 July (n=15) and 17 July and 14 August (n=14). Although variation in the initial diameter of patches was great (range=20 to 62 mm), the initial infection centers appearing on 10 June increased linearly throughout the period ( $R^2=0.27$ ;  $P<0.0001$ )

(Figure 3). A similar linear increase in patch diameter ( $R^2=0.46$ ;  $P<0.0001$ ) initially was observed from infection centers appearing on 17 July. The rate of increase in patch diameter of the aforementioned infection centers, however, slowed by early to mid-August. The patch diameter of all infection centers appearing prior to 17 July increased at a similar rate during the period in which linear growth occurred (i.e., 10 June to 14 August). As was the case with patch diameter, pseudothecia also developed linearly in both sets of infection centers ( $R^2=0.65$  and  $0.70$ ,  $P<0.0001$ ).

As previously mentioned, dead spot was less severe in 2001 and few infection centers were observed throughout the season. The patch diameter of new infection centers ( $n=4$ ) occurring on 1 June, however, was monitored until 2 November. After an initial increase in patch diameter, infection centers appearing on 1 June expanded at a slow rate and only reached a mean patch diameter of approximately 30 mm during the season. Regression analysis of these infection center diameters was weakly linear ( $R^2=0.14$ ;  $P=0.0013$ ) between June and August (Figure 3). Unlike 2000, the patch diameter of infection centers appearing in late July 2001 did not increase in size. Pseudothecia developed within the initial infection centers, but unlike 2000 a linear increase in the number of pseudothecia only occurred for a short period (29 June to 20 July).

Although *O. agrostis* infection centers first appeared on 12 May 2002, the linear growth phase of these infection centers did not occur until approximately mid-June. Regardless of when infection centers ( $n=16$ ) first appeared, all dead spots increased at a similar linear rate ( $R^2=0.63$  to  $0.92$ ;  $P<0.0001$ ) between 14 June and 12 August. Patch diameter of infection centers appearing on 12 August and 9 September remained small

and patches did not appreciably increase in size throughout the remainder of the season. In 2002, the linear stage of pseudothecia production ( $R^2=0.49$  to  $0.90$ ,  $P<0.0001$ ) began in late June, approximately 7 to 10 days following the sharp increase in patch diameter (Figure 3). Additionally, the number of pseudothecia per spot continued to increase until late August, approximately 2 weeks after increases in patch diameter had ceased.

Although the  $R^2$  values for new infection centers appearing each year were low due to variation in the initial patch diameters, there were no differences in the slope coefficients among infection centers during the linear growth period. Additionally, the rate of increasing patch diameter (slope coefficient) of the combined data for each year was similar in 2000 and 2002 (i.e., initial years). The rate of increasing patch diameter from infection centers appearing in 2001 (second year), however, was significantly different ( $P=0.0001$ ) from the other years. These results indicate that although the disease may be active between May to December in the mid-Atlantic region, the dead spots only increased in size and most pseudothecia only were produced during a relatively short period between June and August. Additionally, data again revealed that dead spot may be less severe in the second year following seeding as shown by a reduction in the overall diameter of infection centers as well as the number of pseudothecia produced.

Pseudothecia developed at a similar rate within each infection center over the course of the study. In the second year of the study (2001), however, this linear growth period was shortened and only lasted approximately 3 weeks. In 2000 and 2002 (first year after construction and first year after fumigation, respectively), pseudothecia production coincided with increasing patch diameter. In growth chamber studies,

pseudothecia developed when incubated in constant light and under a range of temperatures (13 to 28°C), but did not develop in the dark (Kaminski et al., 2002). In all three years, pseudothecia development generally began as day length approached the longest day of the year (14.9 hours sunlight day<sup>-1</sup>). Although the development of the sexual fruiting bodies was initiated when day length approached  $\geq 14.8$  hours sunlight day<sup>-1</sup>, the linear phase of pseudothecia production continued until day length decreased to  $\leq 13.5$  hours sunlight day<sup>-1</sup>. As day length increased to  $\geq 13.5$  hours sunlight day<sup>-1</sup> into May, mean soil and air temperatures for May were  $\leq 20^\circ\text{C}$ . It is unclear, however, if a minimum period of light or day length or an interaction between temperature and day length trigger the initiation or are necessary for the production of *O. agrostis* pseudothecia. In bermudagrass, pseudothecia can be found within necrotic tissues as early as March suggesting that temperature may be more important in their development.

Differences in total disease (AUDPC) were observed among starting dates of new infection centers in 2001 and 2002 ( $P < 0.0001$ ). Based on patch diameter data collected from infection centers in 2001, AUDPC (AUDPC=784) was greatest when infection centers developed on 1 June (Table 1). There were no differences in the AUDPC values (AUDPC=139 to 239) from infection centers appearing between 20 July and 21 September. Unlike 2001, dead spot was very severe in 2002 and total disease varied among infection center sets (May to September) occurring throughout the season. Total disease (AUDPC=1032 to 1049) was greatest from new infection centers (n=8) appearing in May 2002. The AUDPC values became significantly less from infection centers appearing on 18 June, 8 July and 12 August, when compared to those appearing in May 2002. Total disease (AUDPC=183 and 186) was low from infection centers appearing in

August and September, which were not significantly different.

The AUDPC values for the development of pseudothecia within each infection center generally were similar to the values for patch diameter (Table 1). Total pseudothecia development was greatest (AUDPC=879) within infection centers first appearing on 1 June 2001. Pseudothecia were produced in very low numbers within infection centers appearing between 20 July and 21 September. Although not statistically significant, pseudothecia production within infection centers appearing later in the 2001 season (3 and 21 September) was greater than in spots appearing in July or August of the same year. In 2002, prodigious numbers of pseudothecia developed within infection centers appearing between 13 May and 18 June (AUDPC=1548 to 1700). Moderate pseudothecia numbers (AUDPC=806) were found over the data collection period within infection centers appearing on 8 July 2002. Unlike 2001 (second year after seeding), pseudothecia development within infection centers appearing on 8 July and later continued to decrease and few differences existed among the dates.

Not surprisingly, data indicated that infection centers appearing early in the season likely will result in the greatest total disease throughout the year. Patch diameter and the number of pseudothecia within the dead spots, however, appeared only to increase substantially between mid-June and mid- to late-August. Therefore, chemical dead spot management prior to and during this period likely would be most effective. Due to the limited recovery of bentgrass within diseased patches, it is important to reduce or delay the development of new infection centers with fungicides as soon as the first infection centers appear in the spring. Hence, May and June fungicide application timings would appear to most critical for preventing new infections. Despite the

occurrence of new infection centers between late-August and early-October, increases in patch diameter and pseudothecia development were negligible during this period.

Although dead spot may be active as late as December in the mid-Atlantic region (Kaminski and Dernoeden, 2002), new infection centers rarely appeared after early-October. Based on the results of this and an unrelated study involving the application of a fungicide and several nitrogen-sources, it appears that there may be little or no benefit obtained from dead-spot targeted fungicide applications beginning in September in the mid-Atlantic region (Kaminski, Chapter V).

#### *Development of a degree day model*

The development of a degree day (DD) model was attempted based on the delay in the point at which initial symptoms occurred and when new infection centers began to increase at a linear rate. Kaminski et al. (2002) found that dead spot symptoms re-developed in winter dormant, infected plugs incubated at temperatures  $\geq 20^{\circ}\text{C}$ . Although no reactivation was visible when infected creeping bentgrass was incubated at  $15^{\circ}\text{C}$ , isolation of the pathogen from these plugs was possible. In this study, disease symptoms generally did not appear at temperatures  $\leq 15^{\circ}\text{C}$ , therefore a base temperature of  $15^{\circ}\text{C}$  was selected for the accumulated DD model.

Degree day totals of 218 (2000), 285 (2002), and 390 (2001) were associated with the period in which pseudothecia began to develop (Table 2). Due to the reduced disease severity and limited increase in patch diameter, degree days were not calculated for increasing patch diameter in 2001. In 2000 and 2002, however, the accumulated DD associated with an increase in patch diameter was 218 and 213, respectively. Although

the range of accumulated DD between 1 May and the period in which pseudothecia and patch diameter began to increase was small, further study in multiple geographic regions is warranted with respect to the use of degree days for predicting the peak period of dead spot activity.

### ***In planta* Distribution and Overwintering of *Ophiosphaerella agrostis***

The distribution of *O. agrostis* throughout various creeping bentgrass plant tissues was assessed between 2001 and 2003. Isolation of *O. agrostis* primarily was attempted for one year following the development of initial symptoms within each site. Data collected from all three years were combined and percent isolation of *O. agrostis* from bentgrass tissue and pseudothecia are shown in Figure 4. In addition, the predicted linear equations for percent isolation within each tissue type resulting from regression analysis are shown in Table 3.

Isolation of *O. agrostis* from green tissue only was successful during the initial stages of symptom expression, and isolation mainly coincided with periods of highest disease activity (Figure 4). Percent isolation of the pathogen from bronze or tan leaves or from discolored leaf sheaths generally was highest after the appearance of initial symptoms and declined during the autumn months. During the winter months, isolation of *O. agrostis* from the aforementioned tissues generally was unsuccessful ( $\leq 20\%$ ). Although pathogen isolation from leaf sheath tissue declined during the winter months, the percentage of successful isolations from leaf sheaths increased during the spring and early summer.

Isolation of *O. agrostis* from root tissue varied throughout the year, but percent

isolation generally declined from the time of initial symptom expression (100 to 50%) until the final isolation attempts in the second year (0 to 60%) (Figure 4). Attempts to isolate the pathogen directly from mature pseudothecia also were made throughout the study. Pseudothecia, however, were not always present in the collected samples. Isolation of *O. agrostis* from sexual fruiting bodies was greatest during late summer and autumn following initial symptom expression. Isolation of *O. agrostis*, however, declined throughout the winter and spring and pseudothecia generally were not observed or isolation was unsuccessful by June or July in the year following initial symptom expression. Previous laboratory studies showed that pseudothecia within inactive winter patches could germinate directly to produce mycelium (Kaminski et al., 2002). Based on results of this study, however, viable cells within pseudothecia appear to decline rapidly overtime.

*O. agrostis* generally was isolated in greatest percentages from stolon tissue. Isolation of the pathogen from bentgrass stolons commonly was  $\geq 80\%$  throughout the autumn (Figure 4). A decline in percent isolation from stolon tissue occurred during the winter months; however, the pathogen was isolated from at least 3 out of 10 stolons on all rating dates. Isolation of *O. agrostis* from crown tissue followed a pattern similar to that of the stolons, although the percentages isolated from crown tissue generally were lower. Similar to that observed for leaf sheaths, pathogen isolation from bentgrass stolons and crowns again increased during late spring and early summer of the second year. Despite this increase in successful isolations in late April and early May in the second year, dead spot symptoms rarely were visible in the field.

These data show that *O. agrostis* can be found throughout the entire plant during

periods of peak disease activity, but that it overwinters best in bentgrass tissue residing near or below the soil surface. During the winter months, *O. agrostis* generally was not isolated from previously infected leaf or sheath tissue or from green leaf tissue. *O. agrostis*, however, routinely was recovered from bentgrass stolons, roots, and crowns during winter and spring. The survival of the pathogen in or on underground tissue likely occurs in response to the temperature buffering capacity of thatch and soil and probably other factors. In the year following initial symptom expression, regression analysis revealed an increase in the percent of successful *O. agrostis* isolations only from leaf sheath (31 March), crown (19 April) and stolon (20 April) tissue (Figure 4). Hence, although the recurrence of dead spot symptoms generally was negligible in the year following initial symptom expression, the pathogen does spread from overwintering tissues to other plant parts without necessarily causing symptoms in the second year.

#### CONCLUSION

Similar to previous observations, dead spot development in this study was most severe during the year following construction and seeding of the research putting green or following fumigation and reseeded (Kaminski and Dernoeden, 2002; Dernoeden et al., 1999; Dernoeden, 2000). Disease severity was very low in 2001 following seeding. Although 'Providence' creeping bentgrass was used in the second year (2002), cultivar evaluations revealed that both cultivars used in this study were equally susceptible to dead spot. Therefore, the rapid decline in dead spot incidence and severity likely was due to factors other than differences in cultivar susceptibility. A slower decline occurs with take-all (*Gaeumannomyces graminis* (Sacc.) Arx and D. Olivier var. *avenae* (E. M.

Turner) Dennis) in *Agrostis* turf in response to a build-up of bacterial antagonists (Smiley et al., 1992; Smith et al., 1989).

New *O. agrostis* infection centers appeared as early as May, but dead spot was most severe between late-June and mid-August. Monthly mean air and soil temperatures for June, July and August ranged from 22 to 26°C. During this period, regression analyses showed that patch diameter and pseudothecia production increased at a linear rate. For this reason, fungicide application at the onset of *O. agrostis* infection centers is an important strategy for reducing total disease potential. A reduction in the inoculum potential and total disease severity also may be possible by physically removing infection centers. Pseudothecia generally began to appear within infected bentgrass tissue in June and maximum numbers of pseudothecia were produced by mid-August during conditions of long day lengths (13.5 to 14.9 hours sunlight day<sup>-1</sup>) and elevated air (22 to 26°C) and soil (24 to 26°C) temperatures. Daily increases in patch diameter and pseudothecia production, however, generally were not correlated with temperature or day length. This likely was influenced by the inability to precisely measure relatively small (a few mm) changes in patch diameter under field conditions. Greatest pseudothecia production was achieved soon after (1 to 2 weeks) patch size reached its maximum. Although new infection centers continued to appear into early October of all three years, dead spots appearing after late-August or early-September remained small and few pseudothecia developed within these patches. Based on AUDPC analyses, total disease was greatest in spots developing earlier in each season.

Immature ascospores could be found within pseudothecia during the winter months. *O. agrostis* was capable of overwintering as hyphae within or on bentgrass

roots, stolons, crowns and leaf sheaths as well as pseudothecia. Despite this survival and the apparent movement of the pathogen on stolons, crowns and leaf sheaths between late-March and into June in the second year, little or no reactivation of disease symptoms occurred in the second year of each study. It should be noted that dead spot has been observed on greens as old as six years and that new *O. agrostis* infections may be promoted by certain water-soluble nitrogen sources during the characteristic decline phase of the disease (Kaminski and Dernoeden, 2002; Kaminski, Chapter V). While the disease characteristically declines in the second year, there are other known and unknown factors that may promote dead spot for several years following initial symptom expression.

Table 1. Area under the disease progress curve (AUDPC) values for patch diameter and *Ophiosphaerella agrostis* pseudothecia production in creeping bentgrass, 2001-2002.

Date of initial infection <sup>x</sup>	AUDPC	
	Patch diameter	Pseudothecia
	mm	no.
2001		
1 June	784 a <sup>y</sup>	879 a
20 July	231 b	8 b
20 August	168 b	12 b
3 September	239 b	178 b
21 September	139 b	90 b
2002 (Fumigated site)		
13 May	1049 a	1548 a
27 May	1032 a	1565 a
18 June	849 b	1700 a
8 July	638 c	806 b
12 August	186 d	145 bc
9 September	183 d	3 c

<sup>x</sup> New infection centers were periodically marked throughout the study and patch diameter and the number of pseudothecia within each spot were recorded.

<sup>y</sup> For each year, means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Table 2. Accumulated degree days (DD) based on air (AT) and soil (ST) temperatures from 1 May until the linear growth period of pseudothecia production and linear increase in patch diameter for three years.

Year	Pseudothecia		Diameter	
	DD <sub>ST</sub>	DD <sub>AT</sub>	DD <sub>ST</sub>	DD <sub>AT</sub>
2000	218	178	218	178
2001	390	295	- <sup>x</sup>	-
2002	285	248	213	177

<sup>x</sup> Data in 2001 were not included in determining accumulated DD due to the reduced disease severity and limited increase in patch diameter during the year.

Table 3. Predicted regression analyses equations for the percentage of successful *Ophiophaearella agrostis* isolations from various creeping bentgrass tissues and pseudothecia between 2001 and 2003.

Tissue	Equation <sup>x</sup>	MS	MSE	P > F	R <sup>2</sup>
Green	$BDS = 0.56(D) - 22.8(\sqrt{D}) + 228.3$	1833	25	<0.0001	0.82
Bronze/Tan	$BDS = 0.00079(D)^2 - 0.77(D) + 186.7$	14461	216	<0.0001	0.81
Sheath	$BDS = 8.59E-10(D)^4 - 13.8(\sqrt{D}) + 264.8$	13561	55	<0.0001	0.78
Crown	$BDS = 1.39E-8(D)^4 - 9.69E-6(D)^3 + 26.4(\sqrt{D}) - 227.3$	8347	611	<0.0001	0.57
Root	$BDS = -0.13(D) + 99.2$	9190	542	0.0002	0.34
Stolon	$BDS = 1.05E-8(D)^4 - 7.31E-0.6(D)^3 + 20.4(\sqrt{D}) - 144.6$	4100	226	<0.0001	0.64
Pseudothecia	$BDS = -0.28(D) + 159.0$	15781	219	<0.0001	0.74

<sup>x</sup> Regression equation represents the percentage of successful *O. agrostis* isolations from various bentgrass tissues and pseudothecia based on cumulative days (D) beginning in the initial year (i.e., 1 = 1 January for study year 1 and 366 = 1 January for the subsequent year following initial infection).

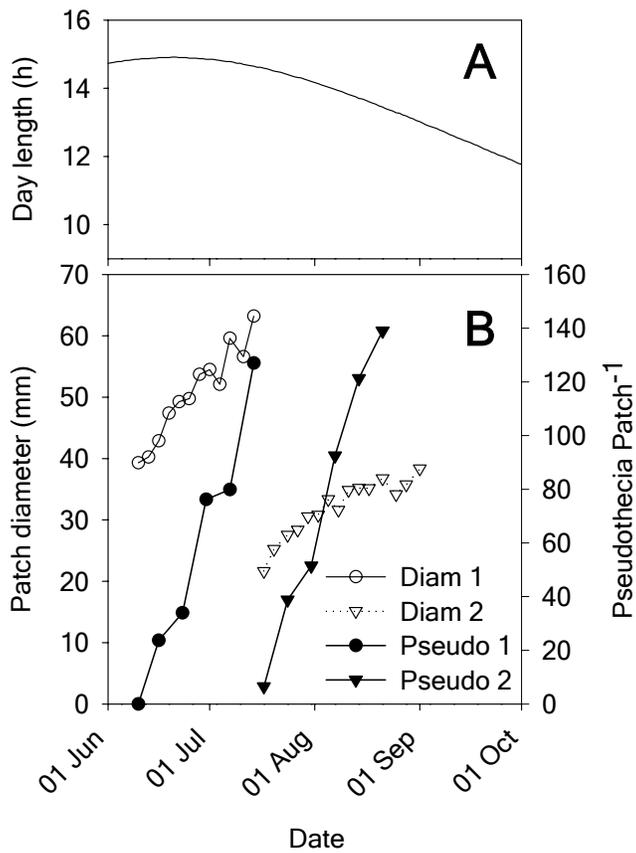


Figure 1. Day length (A), patch diameter (Diam) and number of pseudothecia (Pseudo) (B) within *Ophiosphaerella agrostis* infection centers in 2000. Each symbol represents the mean patch diameter or pseudothecia count within independently monitored infection centers. New infection centers appeared on 10 June (Diameter and Pseudothecia 1, n=15) and 17 July (Diameter and Pseudothecia 2, n=14).

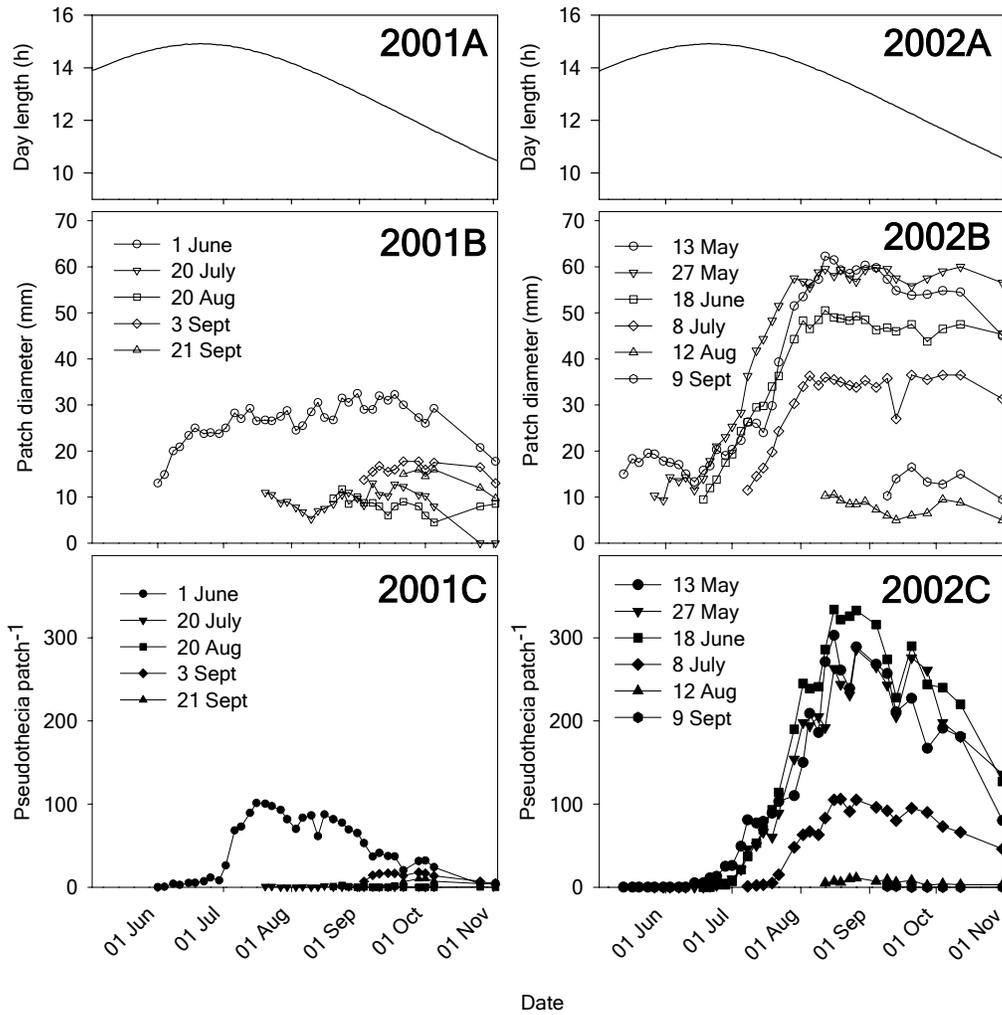


Figure 2. Day length (A), patch diameter (B) and pseudothecia number (C) within *Ophiosphaerella agrostis* infection centers in 2001 and 2002. Each symbol represents the mean patch diameter or pseudothecia count within four independently monitored infection centers. Days listed in the legend indicate dates in which infections centers first appeared.

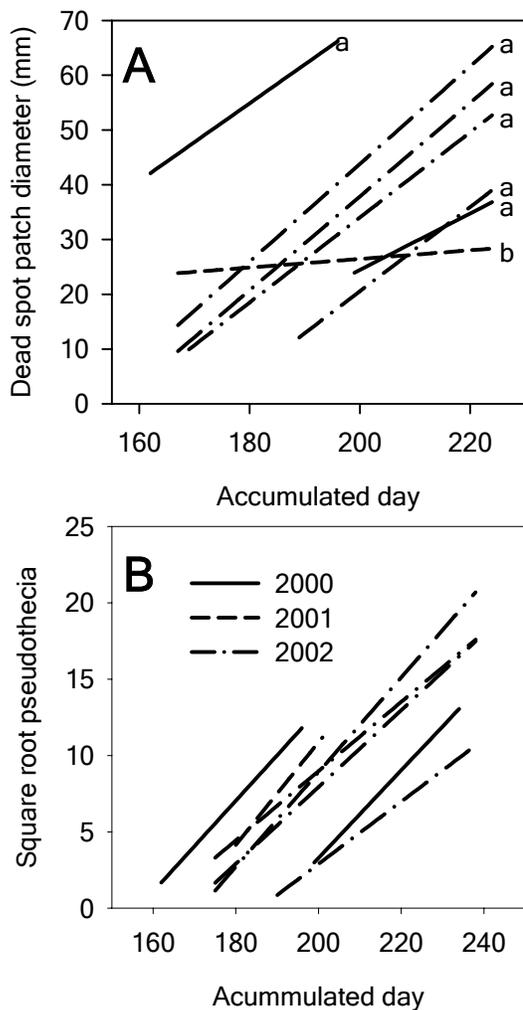


Figure 3. Predicted linear growth phase of *Ophiosphaerella agrostis* patch diameter (A) and pseudothecia production (B) between accumulated day 162 and 238, 2000 to 2002. Regression lines followed by different letters have significantly different slope coefficients ( $P < 0.0001$ ) according to Tukey's protected least significant difference test.

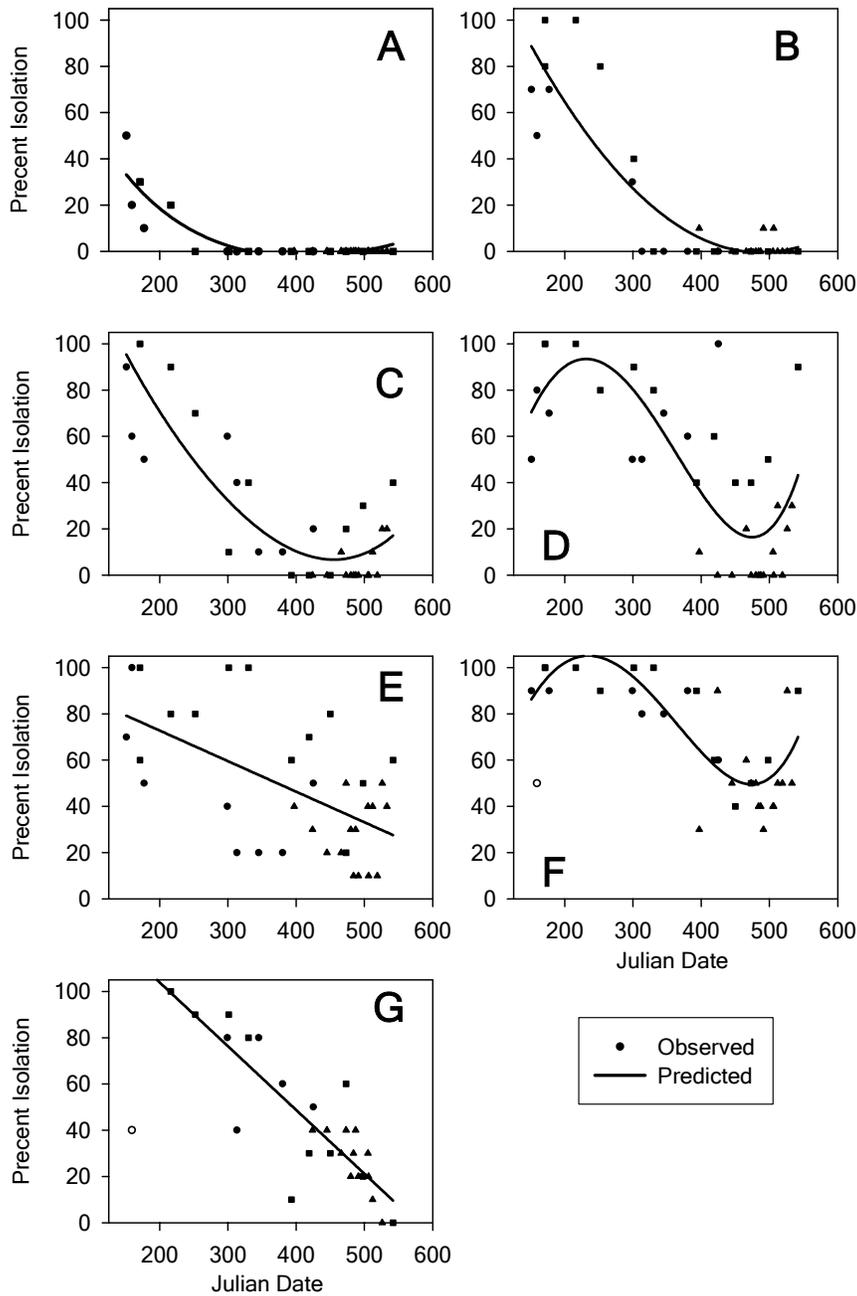


Figure 4. Recovery of *Ophiosphaerella agrostis* isolates from green (A) or bronze (B) leaves; leaf sheaths (C); crowns (D); roots (E); and stolons (F) of infected creeping bentgrass plants and pseudothecia (G). Pathogen isolation was attempted for one year following initial dead spot symptoms (2000-2001= ▲, 2001-2002= ●, and 2002-2003= ■). Open symbols represent outliers that were not included in the regression analyses. Accumulated days began with 1 during the first year of infection and 366 for 1 January during the second year of infection.

### III. ENVIRONMENTAL MONITORING AND DEVELOPMENT OF A PREDICTIVE MODEL FOR THE ONSET AND SEVERITY OF DEAD SPOT SYMPTOMS IN CREEPING BENTGRASS.

#### SYNOPSIS

Dead spot of creeping bentgrass is incited by *Ophiosphaerella agrostis*. The disease may be active between May and December, but little is known about the environmental conditions that influence symptom development. The objectives of this three year field study were to: 1) elucidate environmental conditions surrounding expression of dead spot symptoms, and 2) develop a simplified statistical model to assist in predicting the appearance of dead spot symptoms and epidemics in creeping bentgrass. Environmental parameters measured included relative humidity (RH), air (AT) and soil (ST) temperature, solar irradiance (SOL), precipitation and irrigation (RAIN), and leaf wetness duration (LWD). Dead spot symptoms generally did not occur at temperatures below 15°C. Three models were developed that accurately predicted the appearance or severity of dead spot. Two environmental favorability indexes were developed, which predicted dead spot appearance (79%) and severity (74%). In years with severe levels of dead spot (2000 and 2002), the appearance of major infection events ( $\geq 20$  infection centers per unit area (ICUA) (i.e.,  $93 \text{ m}^{-2} \text{ day}^{-1}$ ) was predicted on 37 of 40 days. A combination of elevated air ( $AT_{\text{Max}} \geq 27^{\circ}\text{C}$ ) and soil ( $ST_{\text{Mean}} \geq 18^{\circ}\text{C}$ ) temperatures, low relative humidity ( $RH_{\text{Mean}} \leq 80\%$ ), shortened periods of leaf wetness ( $LWD \leq 14$  hours), and high levels of solar radiation ( $SOL_{\text{Mean}} \geq 230 \text{ W m}^{-2}$ ) were associated with the development of major dead spot epidemics. Between 1 May and 31 October (2000-2002), the appearance of new dead spot infection centers was most accurately predicted (79%) by the single parameter of  $ST_{\text{Mean}} \geq 20^{\circ}\text{C}$ . False predictions based on elevated soil temperatures generally occurred during late spring. A cumulative degree-day model,

however, did not accurately predict the development of initial infection centers. Data indicated that preventive fungicide applications may be most efficacious when initiated prior to the time when soil temperatures consistently reach  $\geq 15^{\circ}\text{C}$  in late spring.

## INTRODUCTION

Dead spot (*Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum, et M.E. Palm) of creeping bentgrass (*Agrostis stolonifera* L.) and hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burt-Davy) is a relatively new disease of golf putting greens throughout the eastern half of the United States (Câmara et al., 2000; Dernoeden et al., 1999; Kaminski and Dernoeden, 2002). *O. agrostis* is unusual with respect to most turfgrass pathogens in that it appears to exist solely in its sexual state in nature and no anamorph has been found. Additionally, pseudothecia and ascospores commonly develop within infected bentgrass tissues and their ascospores serve as a secondary source of inoculum (Câmara et al., 2000; Kaminski and Dernoeden, 2002; Kaminski et al., 2002). The disease may be active between May and December, but the disease is most severe between mid-June and early-August (Kaminski, Chapter II).

Predictive models have been developed for various plant disease systems. In turfgrass, weather-based prediction models have been developed for several diseases including anthracnose (*Colletotrichum graminicola* [Ces.] Wils.), dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett), Pythium blight (*Pythium aphanidermatum* [Edson] Fitzp.), brown patch (*Rhizoctonia solani* Kühn), and others (Danneberger et al., 1984; Hall, 1984; Nutter et al., 1983; Fidanza et al., 1996). Predictive models can improve the timing of fungicide applications for turfgrass disease management, and may be effective in reducing overall pesticide use.

Based on field observations, dead spot is most severe in sunny locations and often first appears in areas prone to heat stress such as ridges, mounds, and slopes of individual

putting greens (Kaminski and Dernoeden, 2002). Little information, however, exists regarding the environmental conditions that trigger the development of dead spot. This study was designed to define the environmental conditions surrounding dead spot symptom expression and epidemics. The objectives of this research therefore were to: 1) elucidate environmental conditions surrounding expression of dead spot symptoms, and 2) develop a simplified statistical model to assist in predicting dead spot outbreaks.

## MATERIALS AND METHODS

### **Epidemiological Field Study**

Environmental and disease monitoring were conducted at the University of Maryland Paint Branch Turfgrass Research Facility in College Park between 2000 and 2002. In August of 1999, a research putting green was constructed to United States Golf Association (USGA) specifications (USGA Green Section Staff, 1993). Soil was a modified sand mix (97% sand, 1% silt, and 2% clay) with a pH of 4.9 and 10 mg organic matter g<sup>-1</sup> of soil. Soil P (42 to 79 kg ha<sup>-1</sup>) and K (4 to 16 kg ha<sup>-1</sup>) levels varied throughout the three years and generally were low to moderate. A total of 1075 kg ha<sup>-1</sup> ground agricultural limestone was applied between November 1999 and April 2000 to raise soil pH to 6.9 by 25 September 2000. Each year, field evaluations were initiated on separate sections of the same research green.

***Study Year 1.*** On 20 September 1999, a 465 m<sup>2</sup> area was seeded (50 kg seed ha<sup>-1</sup>) with 'L-93' creeping bentgrass. At the same time, an equivalent area adjacent to the 'L-93' section also was seeded at the same rate with 'Providence' creeping bentgrass. Following seeding, the sites were lightly raked, rolled and covered with 100% spunbonded polypropylene (Reemay Inc., Old Hickory, TN) to prevent seeds from dislodging. Both areas were irrigated to maintain adequate moisture. Covers were removed after bentgrass seedling emergence, which occurred approximately 5 to 8 days after seeding. Both 'L-93' and 'Providence' areas were maintained using the same conditions throughout the autumn of 1999. During this period, the area received N, P, and K at 196, 260, and 54 kg ha<sup>-1</sup>, respectively from a starter fertilizer (N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O at 19, 25, and 5%, respectively, The Scotts Company, Marysville, Ohio). The starter

fertilizer contained 4.3% ammoniacal N, 7.4% urea N, 6.3% other water-soluble N, 1.0% water insoluble N, and 1.8% S. Superphosphate (0-45P<sub>2</sub>O<sub>5</sub>-0) and K<sub>2</sub>SO<sub>4</sub> (0-0-50 K<sub>2</sub>O) were the P and K sources, respectively. Plots initially were mowed on 21 October 1999 to a height of 12.5 mm using a walk-behind greens mower and the mowing height gradually was lowered to 4.3 mm by the following May. The area was routinely irrigated, vertical mowed, and topdressed throughout the study. A detailed description of all cultural practices can be found in Appendix B. Inoculum consisted of a mix of tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran (50/50% v/v), which was prepared as described by Kaminski and Dernoeden (2002). On 8 October 1999, the 'L-93' area was inoculated with *O. agrostis* isolate OpVA-1. All isolates used in this study were previously described (Kaminski et al., 2002). *O. agrostis* infested tall fescue seeds were placed below the thatch and at the soil surface on 0.9 m centers.

**Study Year 2.** The area seeded to 'Providence' creeping bentgrass in 1999 was used to monitor dead spot incidence and severity in 2001. Cultivar evaluations previously reported by Kaminski and Dernoeden (2002) revealed similar levels of dead spot severity on both cultivars used in this study. In 2001, the study site was inoculated with *O. agrostis* isolate OpMD-9 + OpVA-1 using the previously described inoculation technique with the following modifications. In 2001, the area was inoculated on 12 March and the infested tall fescue seed/wheat bran mixture was placed at the soil surface in a grid pattern spaced approximately every 1.5 m.

Triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone) (2.4 kg a.i. ha<sup>-1</sup>), which does not impact dead spot, was applied preventively

three times to control dollar spot (*Sclerotinia homoeocarpa* F. T. Bennett) (Towers et al., 2000). Sod webworm (*Crambus* spp.) and black cutworm (*Agrotis ipsilon* Hufnagel) were controlled with pyrethroid insecticides. Wetting agents were applied as needed to control localized dry spot beginning in June 2001.

**Study Year 3.** Due to a general decrease in disease activity in 2001, the study site for 2002 was fumigated. Prior to fumigation, the area was vertically mowed in three directions and core aerated to a depth of 7.6 cm with 12.5 mm hollow tines. On 23 August 2001, a 465 m<sup>2</sup> area was covered and fumigated with methyl bromide (98% methyl bromide + 2% chloropicrin). On 25 August, covers were removed and the area was seeded to 'L-93' creeping bentgrass on 30 August as previously described. The aforementioned starter fertilizer was applied at 196 kg N ha<sup>-1</sup> between September and November 2001. The study area initially was mowed to a height of 5.1 mm three times week<sup>-1</sup> and the height was gradually lowered to 4.3 mm.

On 21 March 2002, the area was inoculated with isolate OpVA-1. On 4 April 2002, the area received 37 kg N ha<sup>-1</sup> from the aforementioned starter fertilizer. A complete fertilizer (20N-3.87% ammoniacal, 5.87% nitrate, and 10.26% urea, 20P<sub>2</sub>O<sub>5</sub>, and 20K<sub>2</sub>O; Plant Marvel Laboratories, Inc., Chicago, IL) was applied at 6 kg N ha<sup>-1</sup> on 26 April and 10 May. Between 11 May and 11 October, a total of 123 kg N ha<sup>-1</sup> from urea was applied to the study area.

### **Environmental Monitoring**

Environmental conditions were monitored between 1 May and 31 October in 2000, 2001 and 2002. Air temperature (AT) and relative humidity (RH) were measured with a thermistor and a Vaisala® capacitive sensor, respectively, which were placed 30

cm above the plant canopy (model HMP35C, Campbell Scientific, Logan, Utah). Sensors were housed in a 12-plate, louvered radiation shield to protect them from sunlight and rain. Soil temperature (ST) was measured by averaging the data from two thermistors (model 107B, Campbell Scientific, Logan, Utah) placed 2.5 cm below the turfgrass canopy at the thatch and soil interface. All temperature (AT and ST) and RH instruments were programmed to measure environmental conditions at 2 min intervals and the mean, maximum and minimum measurements were recorded every 60 min.

Leaf wetness duration (LWD) was estimated by placing two (2001 and 2002) or three (2000) electrical impedance sensors (model 237, Campbell Scientific, Logan, Utah) horizontally on the turf canopy. The sensors were coated with flat-white, latex paint to improve their accuracy in detecting LWD (Gillespie and Kidd, 1978). The electrical resistance for the sensors at the transition between wet and dry was 150 kohms. Sensors for LWD were programmed to record readings every 15 min and resistance values for each sensor were recorded as either 0 for dry or 0.25 for wet during each quarter hour.

In 2000, daily rainfall was determined using a rain gauge placed adjacent to the study site. Additionally, total precipitation from irrigation events was estimated based on the water output from each irrigation head  $\text{min}^{-1}$ . For the 2001 and 2002 season, hourly precipitation was determined using a tipping rain bucket (Texas Electronics Inc., Dallas, TX) situated 30 cm above the turf canopy. Solar irradiance (SOL) and wind speed (WS) data were collected using a United States Department of Agriculture weather station located approximately 0.3 km from the study site. The SOL sensor (LI200X, LI-COR, Lincoln, NE) consisted of a silicon photocell pyranometer mounted 3 m above the ground on a tripod. Wind speed measurements were recorded at the same location using a Model

5103 Wind Monitor (R.M. Young Company, Traverse City, MI).

With the exception of the SOL and WS equipment, all monitoring instruments were connected to a CR-10 datalogger (Campbell Scientific, Logan, Utah) encased in a weatherproof aluminum box and powered by a 12-V rechargeable lead battery connected to a solar panel. All instruments were programmed to measure environmental conditions on either 2 min (AT, ST, and RH) or 15 min (LWD and SOL) intervals. All data were downloaded weekly using a NEC Ready 330T laptop computer (NEC Computers International, Wijchen, The Netherlands).

The conditions evaluated provided 20 environmental variables that were tested for use in a dead spot predictive model. All variables were determined from hourly data collected in a 24 hour period beginning at 0800 hours. Daily averages collected prior to 0800 hours were considered to have occurred the previous day. This interval was chosen to coincide with disease incidence data collection described below. The 20 environmental variables were: mean percent RH for the 24 hour (RHMean), 48 hour (RHMean48), and 72 hour (RHMean72) period prior to 0800 hours; minimum daily RH (RHMin); hours of RH  $\geq$  90% (RH90),  $\geq$  75% (RH75), or  $\geq$  60% (RH60); minimum, mean and max air temperature ( $^{\circ}$ C) (ATMin, ATMean or ATMax); minimum, mean and max soil temperature ( $^{\circ}$ C) (STMin, STMean or STMax); hours of LWD; total precipitation (mm) during the 24 hour (RAIN24) or 48 hour (RAIN48) period prior to 800 hours; mean and maximum solar radiation ( $W\ m^{-2}$ ) (SOLMean and SOLMax); and mean and max wind speed ( $m\ sec^{-1}$ ) (WSMean and WSMax).

### **Disease Variables Measured**

*Daily Infection Centers.* The presence of new *O. agrostis* infection centers was

monitored daily between 700 and 900 hours throughout the study. Infection data were recorded during this period because new infection centers generally were easier to visually observe during the morning hours. To confirm the presence of *O. agrostis*, the pathogen was isolated from tissue of initial infection centers and colony color and morphological characteristics were compared to previously published descriptions (Câmara et al., 2000; Kaminski and Dernoeden, 2002). In 2000, the number of new spots within the study area was recorded between 30 July and 31 October 2000. In 2001 and 2002, the number of new dead spot infection centers was recorded daily between 1 May and 31 October. To ensure that individual infection centers were not counted twice, a small dot (~10 mm in diameter) was painted next to each dead spot. Daily infection center data were adjusted to represent the number of new *O. agrostis* infection centers  $93 \text{ m}^{-2} \text{ day}^{-1}$  (ICUA).

### **Development of a Dead Spot Descriptive Model**

Descriptive information regarding the appearance of dead spot infection centers and the development of a predictive model were conducted using previously described methods (Fidanza et al., 1996; Nutter et al., 1983). Disease incidence data, or days in which new *O. agrostis* infection centers were observed (BDSAny), were converted to a binary value in which 0 = no new infection centers and 1 = new infection center(s) observed. Due to variation in the number of *O. agrostis* infection centers on individual rating dates, a secondary disease variable (BDSMajor) was created. The variable BDSMajor also consisted of binary values in which 1 = days in which major infection events of  $\geq 20$  ICUA occurred and 0 = days in which  $< 20$  ICUA appeared.

Data for the 20 environmental variables were subjected to correlation analyses to

identify any variable(s) significantly associated with BDSAny, BDSMajor, and ICUA as well as those highly correlated with each other. The strength of association among the variables was assessed based on the computed correlation coefficients. Multicollinearity (i.e., intercorrelated variables) may occur when many variables measure a similar event (such as with environmental data) and are included in the model (Freund and Littell, 1991). Simple correlation coefficients also were used to determine variables with multicollinearity, which may be of concern when selecting parameters for use in model development (Draper and Smith, 1981).

Comparisons were made between the binary values of each disease variable (BDSAny and BDSMajor) and each of the 20 environmental variables. These comparisons were made in an attempt to discern the specific environmental conditions that occurred on days in which BDSAny and BDSMajor events occurred. Data were subjected to univariate analysis, a statistical procedure which combines frequency or binary data and descriptive statistics (Cody and Smith, 1991). The statistical procedure used was similar to that employed by Fidanza et al. (1996) to determine the impact of environmental variables on outbreaks of brown patch. The original procedure was described by Scherm and van Bruggen (1994). A Bonferroni correction factor (i.e., Type I error adjustment) was used to compare the 20 variables at the appropriate  $P < 0.05$  level. The corrected probability value was calculated taking into account the 20 variables as follows:  $(0.05/20) = 0.0025$ .

To develop an explanatory model for dead spot incidence and severity, an environmental favorability index (EFI) was developed using those variables that were significantly correlated with and exhibited the strongest relationship to dead spot. Due to

the variation in the number of daily *O. agrostis* infection centers ( $n = 418$ ,  $\bar{X} = 7.1$ , and  $\sigma = 16.5$ ), a dead spot severity ranking system was developed. A 0 to 5 dead spot severity ranking was developed in which 0 = 0; 1 = 0.1 to 5.0 ICUA; 2 = 5.1 to 10.0 ICUA; 3 = 10.1 to 20.0 ICUA; 4 = 20.1 to 30.0 ICUA; and 5  $\geq$  30.0 ICUA.

The validity of the EFI was determined using chi-square ( $\chi^2$ ) analyses, which compared observed infection events with the predicted or expected EFI values. The  $\chi^2$  analysis and EFI procedure were used to test relationships and to develop forecasting models for other turfgrass diseases including Pythium blight and brown patch (Nutter et al., 1983; and Fidanza et al., 1996). Chi-square analyses were performed using the FREQ procedure in SAS (SAS Institute Inc., 2000). The value of  $\chi^2$  was calculated by the equation  $\chi^2 = \sum [(f_o - f_e) / (f_e)]$ , where  $f_o$  equals the number or frequency of observed outcomes and  $f_e$  represents the expected outcomes, as expressed by a “yes” or “no” event (Witte, 1980). Additionally, stepwise regression analyses were performed on the environmental variables to determine if a simplified model could be developed that accurately predicted the presence or absence of dead spot infection centers. The aforementioned regression analyses were performed using the LOGISTIC procedure in SAS (SAS Institute Inc., 2000).

To determine if the accumulation of heat was a factor in the appearance of dead spot symptoms, cumulative degree-days (DD) were computed (Ritchie and NeSmith, 1991). Accumulation of DD began on 1 May of each year and were calculated by the following equation:  $DD = \sum^n [STMean] - [T_b]$ , where STMean is the mean daily soil temperature;  $T_b$  is the base soil temperature, and  $n$  is the number of days elapsed since 1 May (Ritchie and NeSmith, 1991). A  $T_b$  of 15°C was utilized for reasons described

below.

## RESULTS AND DISCUSSION

### **Field Observations**

***Study year 1 (2000).*** Bentgrass dead spot symptoms first appeared on 7 June in spots inoculated the previous October. Mature pseudothecia were observed embedded in inoculum within diseased spots, however, pseudothecia were not observed within diseased bentgrass tissue until 15 June. An increase in the number of infection centers was noted on 19 June and again on 4 July, and new spots generally appeared in the areas immediately surrounding the initial dead spots. *O. agrostis* infection centers continued to appear throughout the summer. Disease severity, based on the number of infection centers, was considered severe in 2000. The number of new infection centers occurring daily, however, only was recorded between 30 July and 31 October. During this period, 332 new *O. agrostis* ICUA were observed (Figure 1). The maximum number of new infection centers counted on a single day (9 August) was 25 ICUA. No new infection centers appeared after 10 October.

***Study year 2 (2001).*** The area used to monitor dead spot in 2001 was inoculated on 12 March 2001. Although no disease symptoms were present, the pathogen was isolated from roots and crown tissue of plants adjacent to the inoculum on 12 April. Initial disease symptoms appeared on 30 May 2001 (Figure 1). Pseudothecia first were observed within infected creeping bentgrass tissue on 4 June. Not including initial symptoms developing within inoculated spots on 30 and 31 May (14 ICUA), the maximum number of new infections occurring in a single day (2 September) only was 11 ICUA. Disease pressure was very low in 2001 and a total of only 161 ICUA were observed. Observations in 2001 were similar to previous field observations, which

indicated that disease severity is greatly reduced in the second year following green construction (Kaminski and Dernoeden, 2002). Despite this reduction in disease severity, small numbers ( $\bar{X} \leq 4.3$  ICUA) of new infection centers continued to appear until 4 October.

***Study year 3 (2002).*** The study area used to monitor dead spot in 2002 was fumigated the previous August. The study area was inoculated on 21 March 2002 and dead spot symptoms first were observed on 11 May 2002. Relatively few new infection centers appeared between 11 May and 15 June (87 ICUA); however, a dramatic increase in the number of infection centers occurred between mid-June and mid-August (Figure 1). In 2002, disease incidence was extremely severe and total dead spot infections for May, June, July, August, September and October averaged 32, 528, 1349, 342, 197, and 14 ICUA, respectively. The maximum number of infection centers observed on a single day was 142 ICUA and occurred on 15 July. Similar to the previous two years, no new *O. agrostis* infection centers appeared after early-October.

### **Development of a Dead Spot Descriptive Model**

Development of a descriptive model was attempted from environmental and disease data collected between 2000 and 2002. Descriptive statistics for all environmental and disease data used in the analyses are shown in Table 1. Correlation analyses of the 20 environmental variables among the parameters measured revealed several variables that were highly intercorrelated (i.e., condition of multicollinearity) (Table 2). Multicollinearity may occur in models containing too many variables that explain the same phenomena (Freund and Littell, 1991). High correlation coefficients

were observed between RAIN and RAIN48 ( $r=0.75$ ); SOLMean and SOLMax ( $r=0.93$ ); and WSMean and WSMMax ( $r=0.70$ ). Correlations among all relative humidity factors (RH90, RH75, RH60, RHMean, RHmin, RH48Mean, and RH72Mean) also were high and correlation coefficients ranged from  $r = 0.65$  to  $0.94$ . Similarly, mean, maximum, and minimum AT and ST measurements were highly correlated ( $r \geq 0.73$ ). Lower correlation coefficients were observed among all RH parameters versus LWD ( $r = 0.43$  to  $0.63$ ) and both SOL variables ( $r = -0.41$  to  $-0.65$ ).

Correlation analyses also were performed between each of the environmental variables and infection events (BDSAny and BDSMajor) and ICUA (Table 2). The aforementioned disease variables were most correlated ( $P<0.001$ ) with all temperature measurements including minimum, mean, and maximum AT and ST. Correlation coefficients for these environmental variables and BDSAny ranged from  $r=0.49$  to  $0.60$ . When compared to ICUA and BDSMajor, correlation coefficients were considerably weaker and ranged from  $r = 0.25$  to  $0.36$  and  $0.23$  to  $0.33$ , respectively. Additionally, weak correlations were observed between all disease variables and SOLMean ( $r = 0.15$  to  $0.22$ ), SOLMax ( $r = 0.13$  to  $0.23$ ), LWD ( $r = -0.11$  to  $-0.19$ ) and RH90 ( $r = -0.11$  to  $-0.19$ ).

The correlation data provided a moderately strong association between temperature (air and soil) and the presence of dead spot symptoms, and the distributions of the environmental variables were plotted for days with and days without the appearance of infection centers (BDSAny) (Figure 2.). Additionally, similar distributions were determined using only those days in which major infection events were observed (BDSMajor) (Figure 3). Using the Bonferroni correction factor, the statistical separations

of the aforementioned variables were significant at  $P \leq 0.0025$ . Distribution data for the 20 variables and BDSAny revealed several statistical separations including minimum, mean, and maximum AT and ST, SOLMean, and SOLmax. On average, ATMean ( $\bar{X} = 23.7^\circ\text{C}$ , standard deviation ( $\sigma$ ) = 4.3) and STMean ( $\bar{X} = 24.5^\circ\text{C}$ ,  $\sigma = 3.2$ ) were higher on days in which dead spot symptoms were observed, when compared to days with no infection centers present (ATMean:  $\bar{X} = 16.1^\circ\text{C}$ ,  $\sigma = 5.4$ ; STMean:  $\bar{X} = 17.5^\circ\text{C}$ ,  $\sigma = 4.7$ ) (Figure 2). Similarly, average SOLMean and SOLmax were 34 and 97  $\text{W m}^{-2}$  greater on days when dead spot infections were observed versus those days with none. Except for RHMin, all relative humidity variables (RH90, RH75, RH60, RHMean, RH48 and RH72) were significantly different for BDSMajor and indicated that there was an increase in major dead spot infection events on days with relatively low humidity. The average daily RHMean was 9% lower for days with BDSMajor ( $\bar{X} = 69\%$ ,  $\sigma = 10$ ) than days with  $< 20$  ICUA ( $\bar{X} = 78\%$ ,  $\sigma = 12$ ) (Figure 3). Additionally,  $\geq 20$  infection centers  $93\text{m}^{-2} \text{ day}^{-1}$  only were observed on 3 out of 40 days when RHMean was  $> 80\%$ . Furthermore, all temperature and RH variables, LWD and SOLMean also exhibited distinct statistical separations, when compared to BDSMajor. On average, days in which  $\geq 20$  infections centers  $93\text{m}^{-2}$  appeared had approximately 2 hours fewer LWD and 40  $\text{W m}^{-2}$  greater SOLMean, when compared to days in which  $< 20$  ICUA were observed. Environmental variables with significant data distributions for BDSAny and/or BDSMajor may signify their potential use to predict periods when dead spot symptoms appear.

Based on the correlation coefficients and examination of statistical distributions and the environmental data (data not shown), it was apparent that the development of dead spot infection centers was influenced by a combination of environmental variables.

While AT and ST were most related to dead spot incidence, a combination of low RH, shortened periods of LWD, and high levels of solar radiation (SOLMean and SOLMax), likely contribute to the appearance of dead spot symptoms. An EFI was developed to summarize the influence of the aforementioned environmental variables on the appearance and severity of dead spots.

The EFI was developed to elucidate critical periods for dead spot epidemics. To develop the EFI, important environmental variables identified previously were selected and point values assigned to each variable based on their importance in predicting the appearance and severity of dead spot symptoms. Dead spot severity was based on the 0 to 5 ranking system previously described. The environmental variables selected for the development of each EFI included minimum, mean, and maximum AT and ST, RH48, RH72, RH90, RH75, RH60, LWD, SOLMean and SOLMax. The use of point values for developing plant disease prediction models was reviewed previously (Krause and Massie, 1975; Fry and Fohner, 1985). Point values for each environmental variable included those that provided the largest  $\chi^2$  value for the EFI model.

Dead spot severity was low and no major infection events occurred in 2001. Therefore, environmental data from 2000 and 2002 were used to develop the severity EFI (EFI-Epidemic). The EFI-Epidemic model resulted in a  $\chi^2$  value of 49 ( $P < 0.0001$ ) and included the variables STMean, ATMax, SOLMean, RHMean and LWD (Table 3). An EFI-Epidemic  $\geq 4$  was the best indicator for the appearance of  $\geq 20$  infection centers  $93\text{m}^{-2} \text{ day}^{-1}$ . When EFI-Epidemic was limited to higher (e.g., EFI-Epidemic  $\geq 5$ ) or that included lower (e.g., EFI-Epidemic  $\geq 3$ ) accumulated point values, it was more likely to give a false prediction due to under or over estimation of days in which  $\geq 20$  infection

centers  $93 \text{ m}^{-2}$  appeared, respectively. The aforementioned model accurately predicted BDSMajor (i.e., “yes” or “no” events) on 175 of 238 days (74%) in 2000 and 2002 (Table 5). The EFI-Epidemic accurately predicted the appearance of major dead spot infection epidemics on 37 out of 40 days (93%). On three days (10, 13 and 25 July), the EFI-Epidemic did not predict major disease severity events.

Although five variables were used to determine the initial model (EFI-Epidemic), a simplified EFI (EFI-Epidemic<sub>2</sub>) was developed using stepwise regression. Stepwise regression analysis was performed on the environmental data used to develop the predicted values of EFI-Epidemic. The environmental parameters that provided the simplest and best-fit model included RHMean and ATMax (Figure 4) and resulted in the equation:  $\text{EFI-Epidemic}_2 = 9.68 \times 10^{-3} T^2 - 0.31 T - 3.79 \times 10^{-6} RH^3 - 1.11 \times 10^{-3} TRH - 1.83 \times 10^{-4} T^2 RH - 4.46 \times 10^{-6} T^3 RH$ , where EFI-Epidemic<sub>2</sub> is the two parameter environmental favorability index,  $T$  is the maximum daily air temperature ( $^{\circ}\text{C}$ ), and  $RH$  is the mean daily relative humidity (%). The model had an adjusted  $R^2 = 0.80$  and all estimated coefficients were significant ( $P < 0.001$ ). Comparison between the 2 parameter regression model and the 5 parameter EFI, however, revealed a reduction in the number of correct predictions of dead spot epidemics. The model EFI-Epidemic<sub>2</sub> only predicted 26 out of 40 days in which  $\geq 20$  ICUA appeared; a reduction from 93% to 65% accuracy. The five parameter EFI model provided relatively high accuracy with regards to predicting major dead spot infection events. Comparable disease warnings, however, were not achieved with fewer environmental inputs.

An EFI that could determine the appearance of a single infection also was developed. The most accurate EFI model for predicting the appearance of dead spot

symptoms (EFI-Symptom) included the variables STMean, ATMean, ATMin, SOLMax and LWD and the point values assigned to each variable are shown in Table 4. Similar to EFI-Epidemic, an  $\text{EFI-Symptom} \geq 4$  was the best indicator for the appearance of dead spot symptoms for reasons previously discussed. An  $\text{EFI-Symptom} \geq 4$  provided a  $\chi^2$  value of 121 ( $P < 0.0001$ ) and accurately predicted dead spot on 304 of 387 days (79%) between 2000 and 2002 (Table 5).

Due to the limited ability of golf course superintendents to measure numerous environmental parameters, single factor and logistic regression models also were developed using easily obtainable environmental parameters. Stepwise logistic regression analyses of BDSAny data from the final and most severe year (2002) resulted in a four factor model:  $\text{BDSLog} = 1.40 \times 10^{-5}(\text{STMean}^3 \times \text{RHMean}) + 8.83 \times 10^{-3}(\text{SOLMax}) - 0.01(\text{LWD}^2) - 0.70(\sqrt{\text{STMax}}) - 3.70$ , where BDSLog is the logistic regression prediction of disease incidence. Environmental variables resulting in a  $\text{BDSLog} > 0$  were indicative of the appearance of dead spot symptoms, while  $\text{BDSLog} \leq 0$  indicated no symptoms would appear. Chi-squared analyses to test predicted (BDSLog) versus actual dead spot symptom development (BDSAny) was accurate on 136 of 143 days (95%;  $P < 0.0001$ ) in 2002. When data from 2000 and 2001 were included in the regression equation, the model was 77% accurate ( $P < 0.0001$ ). The single factor models using either  $\text{STMean} \geq 20$  or  $\text{ATMean} \geq 18^\circ\text{C}$  resulted in  $\chi^2$  values of 135 and 113 ( $P < 0.0001$ ), respectively (Table 5). Temperatures of  $\text{STMean} \geq 20^\circ\text{C}$  and  $\text{ATMean} \geq 18^\circ\text{C}$  predicted dead spot infection centers with 80 and 77% accuracy, respectively. In the regression and single factor models, most inaccurate disease predictions occurred prior to the appearance of initial disease symptoms in late spring or in October when few new dead spots

appeared.

***Degree Days.*** False predictions based on elevated ST early in the season may indicate there is a cumulative heat effect for dead spot symptom expression, which may be more accurately explained by a degree day model. Kaminski et al. (2002) found that visual dead spot symptoms developed in winter-dormant, infected creeping bentgrass plugs incubated at temperatures  $\geq 20^{\circ}\text{C}$ . Despite the lack of symptoms when plugs were incubated at  $15^{\circ}\text{C}$ , the pathogen was successfully isolated from individual plants (Kaminski et al., 2002). In this study, disease symptoms generally did not appear at temperatures  $\leq 15^{\circ}\text{C}$ , therefore a base temperature of  $15^{\circ}\text{C}$  was selected for the accumulated degree day (DD) model (Figure 4).

Based on STMean, initial dead spot symptoms developed after 31, 125 and 199 DD in 2002, 2001 and 2000, respectively (Table 6). The lack of accuracy of the DD model indicated that further study is warranted with respect to the use of DD for predicting the appearance of initial dead spot symptoms. It is unclear if inoculation timings influenced the appearance of initial dead spot symptoms. For 2000, the study area was inoculated in October 1999 and disease symptoms did not appear until 7 June 2000 (DD=199). For the remaining two years, the areas were inoculated on 12 March 2001 and 21 March 2002. In the aforementioned years, disease symptoms developed in mid- to late-May (DD=31 and 125). Kaminski (Chapter II) found that although the pathogen was capable of overwintering in various bentgrass tissues, percent recovery of the pathogen generally declined throughout the autumn and winter months. Early disease development in 2001 and 2002 may have been due to the spring inoculation and therefore greater levels of viable inoculum earlier in the year. Due to the early development of

dead spot symptoms in 2002, future investigations should begin DD data collection earlier in the season (i.e., ~ 1 April in Maryland). Plots should be inoculated in the autumn rather than spring so that the pathogen can more naturally colonize plants and overwinter.

## CONCLUSION

Except for the DD model, the environmental models (EFI-Symptom, single parameter, and BDSLog) were able to accurately predict (73 to 80%) the appearance of new dead spot infection centers. The EFI-Epidemic was accurate (74%) in predicting dead spot epidemics. Environmental factors that were associated with dead spot incidence and/or days with major infection events included AT, ST, SOL, LWD, and RH. Based on the results of these field studies, dead spot symptom development appears to be most associated with temperature. Daily  $ST_{Mean} \geq 20^{\circ}C$  or  $AT_{Mean} \geq 18^{\circ}C$  between late spring and late summer were the best single parameter indicators for the appearance of dead spot infection centers. Previous research, however, indicated that reactivation of dead spot symptoms in winter-dormant bentgrass only occurred after incubation for 12 to 28 days at temperatures between 20 and 30°C (Kaminski and Dernoeden, 2002). Therefore, although the aforementioned temperatures (i.e.,  $ST_{Mean} \geq 20^{\circ}C$  and  $AT_{Mean} \geq 18^{\circ}C$ ) accurately predicted the appearance of dead spot symptoms with between 77 and 80% accuracy, it is likely that an accumulation of heat in the spring is required prior to the development of initial disease symptoms. Based on the severity model (EFI-Epidemic), it appears that in addition to elevated temperatures ( $AT_{Max} > 27^{\circ}C$  and  $ST_{Mean} \geq 18^{\circ}C$ ), several environmental parameters contributed to major dead spot epidemics. Days with  $\geq 20$  ICUA generally were predicted by a combination of lower  $RH_{Mean} (\leq 80\%)$ , fewer hours of leaf wetness ( $\leq 14$  hours), and an increase in  $SOL_{Mean} (\geq 230 W m^{-2})$ . These conditions support previous reports in which dead spot was found to be most severe on putting greens exposed to full sun (increased SOL), adequate wind movement (lowered RH), and on mounds or ridges and south facing

slopes that would be more prone to heat and drought stress and reduced leaf wetness periods (Kaminski and Dernoeden, 2002). Regardless, dead spot is a serious problem for newly established creeping bentgrass putting greens. Due to the ability of the pathogen to rapidly produce prodigious numbers of pseudothecia, relatively few infection centers may provide sufficient inoculum to severely impact the playability of infected greens. Therefore, the disease threshold for dead spot is extremely low. Based on results from this and other studies (Kaminski, Chapter V), preventive fungicide applications may be most efficacious when initiated prior to soil temperatures consistently reaching  $\geq 15^{\circ}\text{C}$  in late spring and possibly continued until soil temperatures drop consistently below  $20^{\circ}\text{C}$ . It is important to note, however, that new infection centers appearing in September and October generally remain small, and complete recovery from these infection centers likely would be achievable with autumn applied nitrogen-fertilizer applications (Kaminski, Chapter V).

The environmental conditions surrounding *O. agrostis* infection of creeping bentgrass were not studied. During periods of peak disease activity, new pseudothecia may be found within necrotic tissue at the time initial symptoms appear (Kaminski, Chapter II). In growth chamber studies, pseudothecia developed in as few as 4 days when incubated at  $25^{\circ}\text{C}$  (Kaminski et al., 2002). Following infection, it appears that *O. agrostis* enters a latent phase in which dead spot symptoms go unnoticed for a period of days. In the field, small dead spots (approximately 1 to 2 cm in diameter) generally appeared between 4 and 10 days following a large release of ascospores (Kaminski, Chapter IV). The length of this latent period likely is dependent upon environmental conditions on days following infection. While extended periods of leaf wetness duration

likely are necessary for infection to occur, dead spot symptoms appear to be influenced by environmental conditions that promote plant stress. While the conditions that influence dead spot symptom expression include elevated temperatures and solar radiation and decreased hours of leaf wetness and relative humidity, the environmental conditions associated with plant infection are imperfectly understood.

Ascospores released during the summer months play an important role in the infection and development of secondary dead spot symptoms. Kaminski et al. (2002) reported that mature ascospores were capable of germinating in as little as 2 hours when incubated at 25°C and that ascospores were capable of directly penetrating bentgrass leaves and roots. On golf putting greens, irrigation commonly is applied and leaf wetness duration routinely averages 16 hours day<sup>-1</sup>. For this reason, conditions necessary for germination of *O. agrostis* ascospores and possibly infection may be artificially promoted by common cultural practices. Although ascospore release was monitored in 2001 and 2002, spore release only was weakly associated with the appearance of infection centers (data not shown). Future studies should address the development and release of ascospores and the conditions that influence the length of time between *O. agrostis* infection and the expression of dead spot symptoms.

Table 1. Descriptive statistics of environmental and bentgrass dead spot (BDS) data used in correlation analysis.

Variable <sup>x</sup>	N	Mean	Std Dev	Sum	Minimum	Maximum
Relative humidity (RH) $\geq$ 90 (hours)	532	9.5	6.0	5033	0.0	24.0
RH $\geq$ 75 (hours)	532	14.0	5.6	7452	0.0	24.0
RH $\geq$ 60 (hours)	532	18.1	5.1	9629	0.0	24.0
Mean RH (%)	531	77.8	11.4	41300	32.1	100.0
Mean RH for 48 hours (%)	523	77.8	10.2	40697	37.0	99.5
Mean RH for 72 hours (%)	515	77.9	9.3	40095	43.4	97.5
Minimum RH (%)	531	48.4	16.4	25687	13.2	99.6
Mean soil temperature (ST) (°C)	520	21.4	4.6	11141	7.2	29.0
Minimum ST (°C)	520	17.2	4.7	8918	3.9	24.9
Maximum ST (°C)	520	27.2	5.4	14124	8.7	37.0
Mean air temperature (AT) (°C)	518	20.2	5.5	10473	3.9	31.4
Minimum AT (°C)	518	13.9	6.0	7223	-3.1	25.5
Maximum AT (°C)	518	26.8	5.8	13887	4.7	40.3
Leaf wetness duration (hours)	519	17.1	3.1	8863	6.3	24.0
Rain and irrigation (mm)	520	4.6	8.2	2399	0.0	51.1
Rain and irrigation for 48 hours (mm)	511	9.1	12.0	4673	0.0	63.8
Mean solar irradiance (W m <sup>-2</sup> )	547	193.3	75.8	105724	16.1	338.8
Maximum solar irradiance (W m <sup>-2</sup> )	547	653.1	202.2	357244	68.9	1152.0
Mean wind speed (m s <sup>-1</sup> )	552	1.2	0.6	686	0.3	4.0
Maximum wind speed (m s <sup>-1</sup> )	552	6.2	2.0	3426	2.1	16.6
Dead spot infection centers 93 m <sup>-2</sup>	418	7.1	16.5	2955	0.0	142.0
Dead spot infection center > 1	418	0.6	0.5	243	0.0	1.0
Dead spot infection center $\geq$ 20	418	0.1	0.3	40	0.0	1.0

<sup>x</sup> All variables represent the data collected for the 24 hour period prior to 0800 hours unless otherwise stated.

Table 2. Pearson correlation coefficients and significance levels for environmental and disease variables collected for the examination of bentgrass dead spot on a creeping bentgrass in 2000-2002, College Park, MD.

Variables <sup>x</sup>	RH90	RH75	RH60	RH	RH48	RH72	RH	ST	ST	ST	AT	AT	AT	LWD	Rain	Rain	48	SOL	WS	WS	SOL	Max	Max	SOL	Max
				Mean	Mean	Mean	Min	Mean	Min	Max	Mean	Min	Max					Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
RH90	-	***y	***	***	***	***	***	NS	NS	***	***	NS	***	***	***	***	***	***	***	***	***	***	***	***	***
RH75	.849	-	***	***	***	***	***	NS	**	***	*	*	***	***	***	***	***	***	***	***	***	***	***	***	***
RH60	.639	.850	-	***	***	***	***	NS	***	*	NS	***	**	***	***	***	***	***	***	***	***	***	***	***	***
RH Mean	.861	.932	.874	-	***	***	***	NS	***	***	NS	**	***	***	***	***	***	***	***	***	***	***	***	***	***
RH48 Mean	.735	.824	.808	.897	-	***	***	NS	***	*	NS	*	**	***	**	***	***	***	***	***	***	***	***	***	***
RH72 Mean	.653	.736	.731	.797	.941	-	***	NS	***	NS	NS	NS	**	***	NS	NS	***	***	***	***	***	***	***	***	***
RH Min	.685	.805	.789	.852	.757	.650	-	NS	***	***	NS	***	***	***	***	***	***	***	NS	***	NS	***	***	***	***
ST Mean	-1.29	-0.56	.059	-0.04	.024	.029	.020	-	***	***	***	***	***	***	***	**	***	***	***	***	***	***	***	***	***
ST Min	.025	.144	.253	.184	.187	.161	.261	.948	-	***	***	***	***	*	***	***	***	***	***	***	***	***	***	***	***
ST Max	-.237	-.213	-.100	-.151	-.100	-.071	-.212	.940	.795	-	***	***	***	***	NS	NS	*	***	***	***	***	***	***	***	***
AT Mean	-.202	-.112	.018	-.072	-.059	-.067	.007	.923	.859	.870	-	***	***	***	*	*	*	***	***	***	***	***	***	***	***
AT Min	-.015	.101	.215	.139	.101	.067	.253	.857	.868	.734	.933	-	***	**	***	***	***	***	***	***	***	***	***	***	***
AT Max	-.258	-.223	-.114	-.175	-.136	-.121	-.228	.883	.754	.920	.929	.772	-	***	NS	NS	NS	***	***	***	***	***	***	***	***
LWD	.599	.630	.514	.609	.494	.427	.603	-.262	-.095	-.383	-.301	-.134	-.401	-	***	***	***	***	*	***	***	***	***	***	***
Rain	.256	.240	.209	.244	.129	.082	.273	.123	.186	.067	.095	.162	.036	.315	-	***	***	***	NS	***	***	***	***	***	***
Rain48	.282	.274	.244	.277	.235	.160	.297	.158	.223	.092	.097	.161	.032	.273	.750	-	***	***	NS	NS	NS	NS	NS	NS	NS
SOL Mean	-.518	-.595	-.540	-.580	-.576	-.507	-.652	.414	.178	.585	.404	.210	.526	-.603	-.149	-.131	-	***	NS	NS	NS	NS	NS	NS	NS
WS Mean	-.303	-.218	-.175	-.261	-.269	-.227	-.048	-.209	-.168	-.250	-.213	-.198	-.266	-.101	.017	-.010	.031	-	***	NS	NS	NS	NS	NS	NS
WS Max	-.230	-.222	-.217	-.257	-.241	-.205	-.201	-.143	-.149	-.123	-.175	-.236	-.114	-.167	.100	.070	.126	.696	-	***	***	***	***	***	***
SOL Max	-.495	-.549	-.451	-.519	-.515	-.405	-.623	.433	.212	.609	.407	.215	.547	-.565	-.115	-.100	.933	.014	.183	-	***	***	***	***	***
ICUA	-.181	-.147	-.145	-.200	-.228	-.226	-.113	.317	.319	.250	.364	.339	.304	-.186	-.024	.025	.192	-.046	-.090	.160	***	***	***	***	***
Significance	***	**	**	***	***	***	*	***	***	***	***	***	***	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BDSAny	-.111	-.040	.009	-.046	-.075	-.107	.021	.603	.594	.537	.563	.560	.491	-.112	.077	.124	.215	-.093	-.101	.232	***	***	***	***	***
Significance	*	NS	NS	NS	NS	*	NS	***	***	***	***	***	***	*	NS	**	***	NS	NS	*	***	***	***	***	***
BDSMajor	-.193	-.177	-.175	-.220	-.238	-.244	-.143	.268	.256	.225	.326	.295	.277	-.179	.019	.020	.152	-.062	-.098	.132	***	***	***	***	***
Significance	***	***	***	***	***	***	**	***	***	***	***	***	***	***	NS	NS	NS	NS	NS	*	***	***	***	***	***

<sup>x</sup> RH = relative humidity; ST = soil temperature; AT = air temperature; LWD = leaf wetness duration; SOL = solar irradiance; WS = wind speed; ICUA = infection centers 93 m<sup>-2</sup>; BDSAny = presence or absence of infection centers; and BDSMajor = ≥20 ICUA.

<sup>y</sup> Significance levels are as follows: NS = not significant, \* = ≤0.05, \*\* = ≤0.01, and \*\*\* = ≤0.001.

Table 3. Variables and point values used to calculate an environmental favorability index (EFI-Epidemic) to provide a warning model for major ( $\geq 20$  infection centers per  $93 \text{ m}^2 \text{ day}^{-1}$ ) dead spot epidemics in creeping bentgrass grown in Maryland.

Environmental variable <sup>x</sup>	Condition	Point value <sup>y</sup>
STMean	< 18°C	0
	18-24°C	1
	> 24°C	2
ATMax	< 27°C	0
	$\geq 27^\circ\text{C}$	1
RHMean	> 80%	0
	$\leq 80\%$	1
SOLMean	< $230 \text{ W m}^{-2}$	0
	$\geq 230 \text{ W m}^{-2}$	1
LWD	> 14 hours	0
	$\leq 14$ hours	1

<sup>x</sup> Variables were measured during a 24 hour period prior to 0800 hours for all days where: STMean = mean soil temperature ( $^\circ\text{C}$ ); ATMean = mean air temperature ( $^\circ\text{C}$ ); RHMean = mean relative humidity (%); SOLMean = mean solar irradiance ( $\text{W m}^{-2}$ ); and LWD = hours leaf wetness duration.

<sup>y</sup> Point values were added to calculate the environmental favorability index.

Table 4. Variables and point values used to calculate an environmental favorability index (EFI-Symptom) to provide a warning model for dead spot symptom expression in creeping bentgrass in Maryland.

Environmental variable <sup>x</sup>	Condition	Point value <sup>y</sup>
STMean	< 15°C	0
	15-20°C	1
	> 20°C	2
ATMean	< 18°C	0
	≥ 18°C	1
ATMin	< 10°C	0
	≥ 10°C	1
SOLMax	< 580 W m <sup>-2</sup>	0
	≥ 580 W m <sup>-2</sup>	1
LWD	> 18 hours	0
	≤ 18 hours	1

<sup>x</sup> Variables were measured during a 24 hour period prior to 0800 hours for all days where: STMean = mean soil temperature (°C); ATMean = mean air temperature (°C); ATMin = minimum air temperature (°C); SOLMax = maximum solar irradiance (W m<sup>-2</sup>); and LWD = hours leaf wetness duration.

<sup>y</sup> Point values were added to calculate the environmental favorability index.

Table 5. Chi-square ( $\chi^2$ ) analyses of individual and combined environmental variables used to predict the appearance of dead spot symptoms (BDSAny) or major dead spot epidemics (BDSMajor).

Disease Parameter(s) <sup>x</sup>	BDSAny		$\chi^2$ <sup>w</sup> (Accuracy) <sup>z</sup>
	Yes	No	
STMean $\geq$ 20°C	204	50	135
STMean < 20°C	30	115	(80%)
ATMean $\geq$ 18°C	204	61	113
ATMean < 18°C	27	102	(77%)
ATMin $\geq$ 10°C	214	81	92
ATMin < 10°C	18	82	(75%)
ATMax $\geq$ 23°C	208	84	72
ATMax < 23°C	24	79	(73%)
<hr/>			
EFI-Symptom $\geq$ 4 <sup>y</sup>	206	63	120
EFI-Symptom < 4	20	98	(79%)
<hr/>			
	BDSMajor		
	Yes	No	
EFI-Epidemic $\geq$ 4 <sup>y</sup>	37	59	53
EFI-Epidemic < 4	3	139	(74%)

<sup>w</sup> All values are significant ( $P < 0.0001$ ) according to the Pearson chi-square test.

<sup>x</sup> Variables were measured during a 24 hour period prior to 0800 hours for all days where: STMean = mean soil temperature (°C); ATMean = mean air temperature (°C); ATMin = minimum air temperature (°C); and ATMax = maximum air temperature (°C).

<sup>y</sup> Environmental parameters and their corresponding point values included in the environmental favorability index models EFI-Symptom and EFI-Epidemic are shown in Tables 3 and 4, respectively.

<sup>z</sup> Accuracy (%) represents the number of correct predictions for days in which BDSAny = “Yes” or “No”, averaged over all dates.

Table 6. Degree days (DD) based on accumulated soil (ST) and air (AT) temperatures from 1 May until the date of primary dead spot symptom expression for three years.

Year	Primary symptoms	
	DD <sub>ST</sub>	DD <sub>AT</sub>
2000	199	157
2001	125	66
2002	31	24

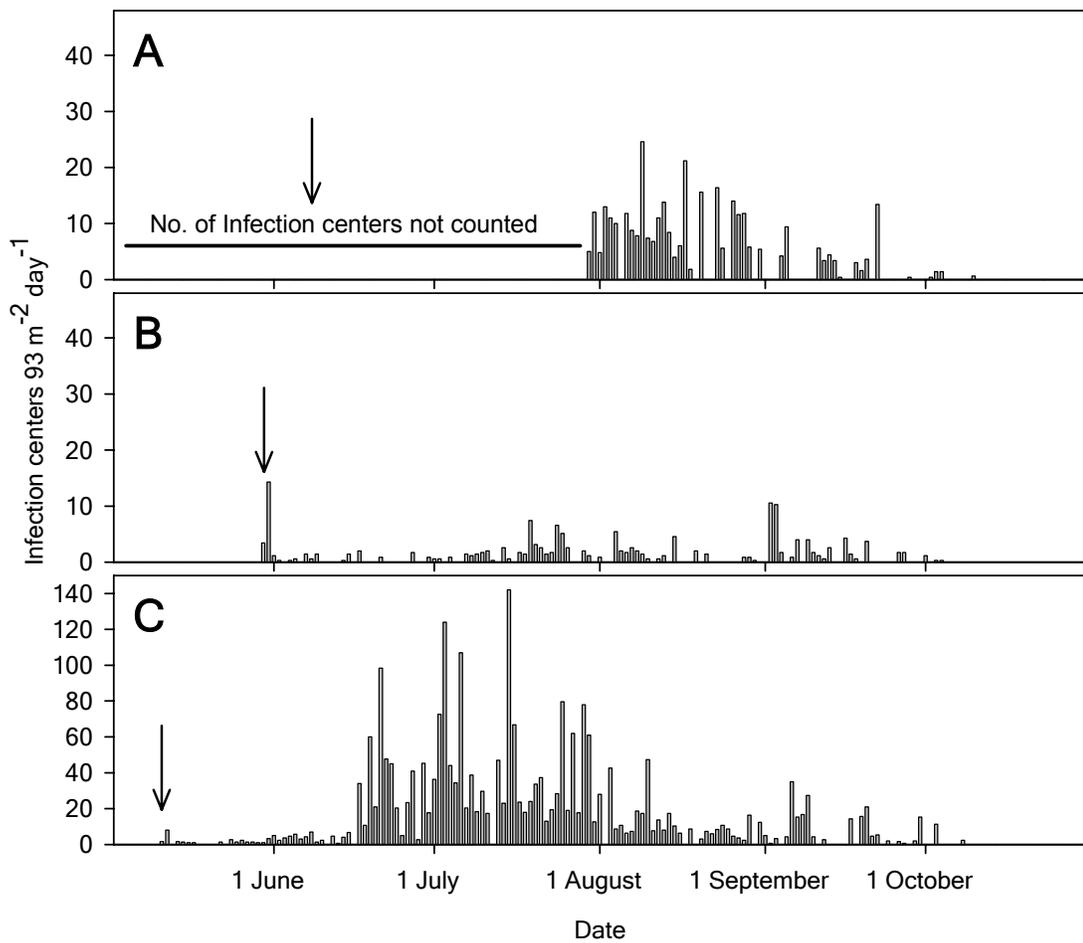


Figure 1. Number of new *Ophiosphaerella agrostis* infection centers per unit area ( $93 \text{ m}^2$ ) per day between 2000 and 2002 on a creeping bentgrass putting green in College Park, MD. Infection centers were counted daily between 30 July and 31 October 2000 (A) and 1 May and 31 October in 2001 (B) and 2002 (C). Arrows indicate the date in which the initial infection centers appeared each year.

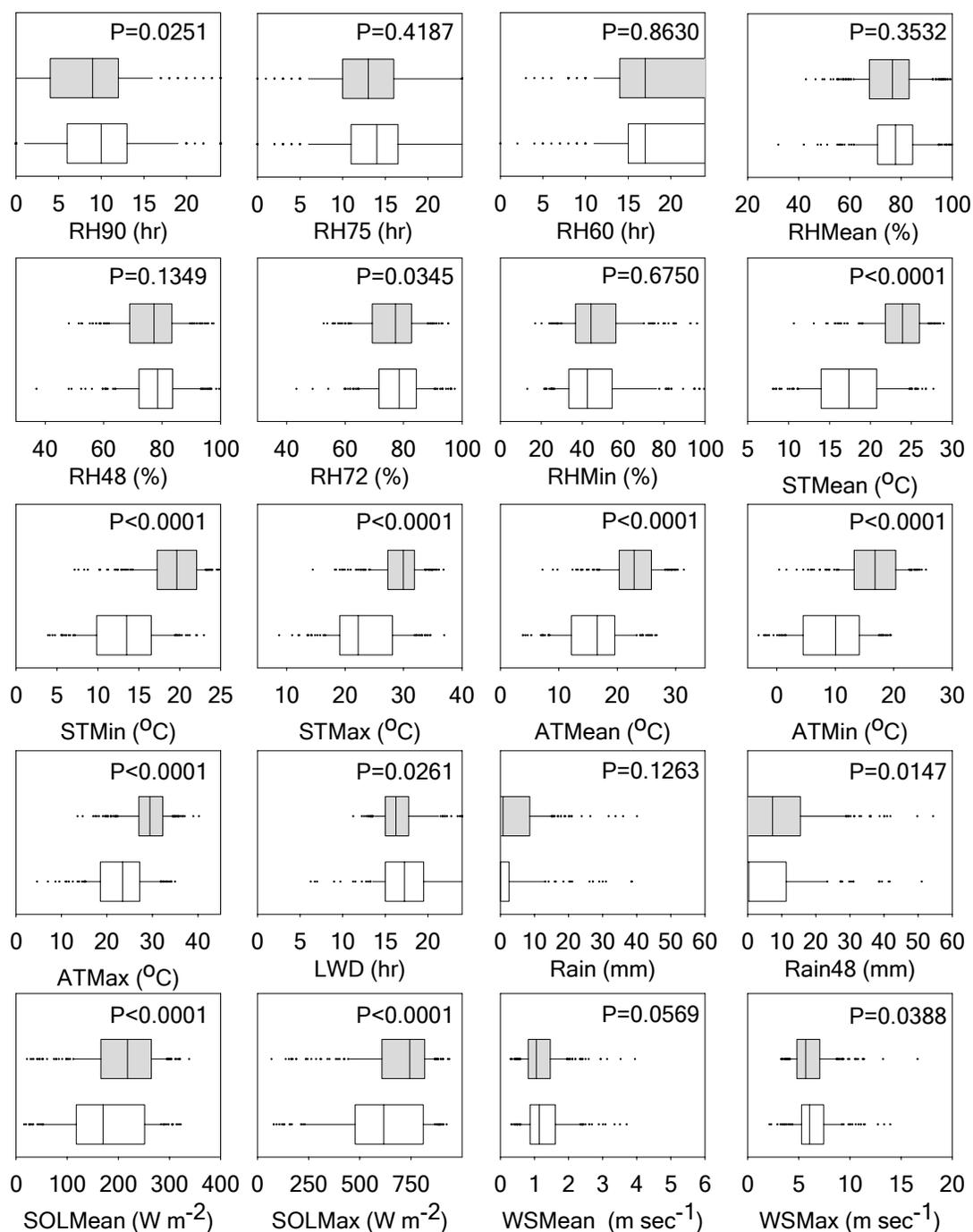


Figure 2. The distributions of the environmental variables on days in which *Ophiosphaerella agrostis* infection events occurred (gray box) and days in which no infections were observed (white box). Data represent 10% (left solid line), 25% (left side of box), 50% (center line), 75% (right side of box), and 90% (right solid line) of the observations recorded between May and November of 2000 to 2002. Extreme measurements or outliers are indicated by dots. The distributions for each environmental variable were significantly different at the 5% level where  $P < 0.0025$ .

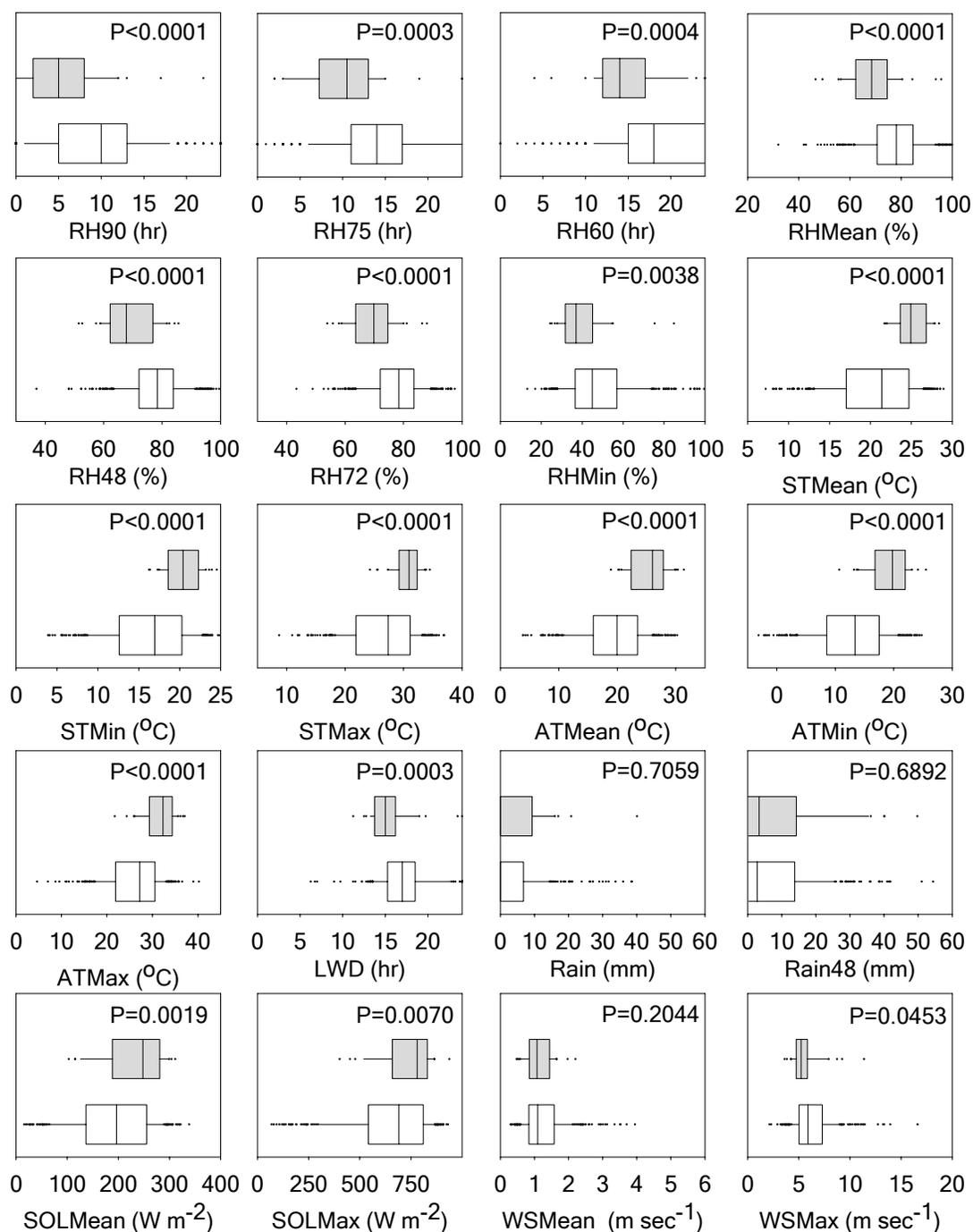


Figure 3. The distributions of the environmental variables on days in which major ( $\geq 20$  dead spots  $93 \text{ m}^2$ ) *Ophiostroma agrostis* infection event occurred (gray box) and days in which no infections were observed (white box). Data represent 10% (left solid line), 25% (left side of box), 50% (center line), 75% (right side of box), and 90% (right solid line) of the observations recorded between May and November of 2000 to 2002. Extreme measurements or outliers are indicated by dots. The distributions for each environmental variable were significantly different at the 5% level where  $P < 0.0025$ .

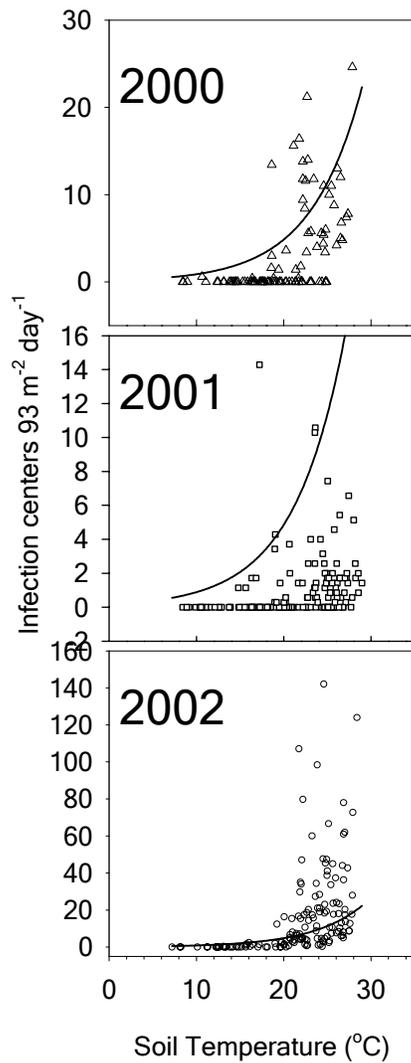


Figure 4. Relationship between daily mean soil temperature and the number of new dead spot infection centers appearing daily, 2000 to 2002. Significance for cubic regression lines are  $P = 0.0159, 0.0001, \text{ and } 0.0001$  for 2001, 2000, and 2002, respectively.

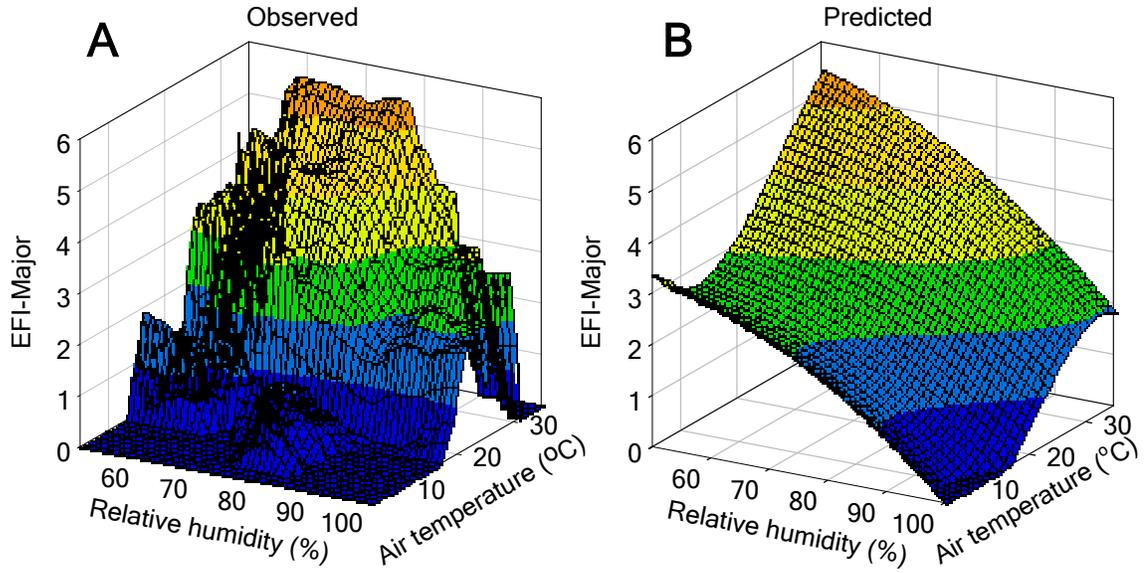


Figure 5. Relationship of mean daily relative humidity (%) and maximum daily air temperature ( $^{\circ}\text{C}$ ) to the environmental favorability index for major dead spot outbreaks (EFI-Epidemic). Five-variable indexes (A) were calculated from field observations. Two variable indices (B) were predicted from the regression equation. Each color represents incremental changes in the EFI values.

#### IV. ENVIRONMENTAL INFLUENCES ON THE RELEASE OF *OPHIOSPHAERELLA AGROSTIS* ASCOSPORES UNDER CONTROLLED AND FIELD CONDITIONS.

##### SYNOPSIS

*Ophiosphaerella agrostis*, the causal agent of dead spot of creeping bentgrass (*Agrostis stolonifera*), routinely produces prodigious numbers of pseudothecia and ascospores throughout the summer months. The environmental conditions associated with the release, as well as the daily and seasonal release timings of *O. agrostis* ascospores, are unknown. The objectives of this research therefore were to: 1) determine the influence of changes in light and relative humidity on ascospore release in a controlled environment; 2) document the seasonal and daily discharge patterns of ascospores in the field; and 3) elucidate those environmental conditions that promote ascospore release under field conditions. In a growth chamber study, a sharp decrease (100% to ~50%) in relative humidity resulted in a rapid (1 to 3 hours) discharge of ascospores, regardless of whether pseudothecia were incubated in constant light or dark. In field studies, ascospores were collected between May and October 2001 and 2002. Daily ascospore release increased between 1900 and 2300 hours and again between 700 and 1000. The release of ascospores mostly occurred during the early morning hours when relative humidity was decreasing and the canopy began to dry or during the evening hours when relative humidity was low and dew began to form. Few ascospores were released between 1100 and 1800 when the bentgrass canopy was dry. The release of ascospores also was triggered by precipitation. A large percentage of the total number of ascospores (87%) collected during precipitation events was released within 10 hours of the beginning of each event. Although few precipitation events lasted longer than 10 hours (n=27), ascospores generally were collected throughout the duration of these

events. Ascospore discharge and the appearance of new infection centers occurred in a cyclic pattern that peaked about every 12 days. New infection centers appeared approximately 3 to 10 days after a large release of ascospores. Conditions favoring ascospore germination (i.e., leaf wetness and suitable temperatures) and probably bentgrass infection routinely occur during the critical periods when large numbers of ascospores are released.

## INTRODUCTION

*Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum, et M.E. Palm is an ascomycete capable of producing prodigious numbers of pseudothecia and viable ascospores (Câmara et al., 2000, Kaminski et al., 2002). This pathogen causes dead spot of both creeping bentgrass (*Agrostis stolonifera* L.) and hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burt-Davy) golf putting greens throughout the eastern and southern regions of the United States (Dernoeden et al., 1999; Kaminski and Dernoeden, 2002; Krausz et al., 2001).

Unlike the other *Ophiosphaerella* spp. associated with turfgrass disease, *O. agrostis* commonly produces pseudothecia in nature (Kaminski and Dernoeden, 2002). In the mid-Atlantic region of the United States, pseudothecia generally develop within necrotic bentgrass tissues between late-June and mid-August. Ascospores serve as a source of secondary propagules throughout the growing season and play an integral role in the dead spot disease cycle (Kaminski et al., 2002; Kaminski, Chapter III). In nature, the pathogen is believed to exist solely as a teleomorph and attempts to detect an anamorph have been unsuccessful (Câmara et al., 2000; Kaminski and Dernoeden, 2002; Kaminski et al., 2002). An anamorph only has been reported for *O. herpotricha* J. Walker, but its conidial state has been questioned and it was suggested that the fungus belonged to either the genus *Urohendersoniella* Petrak or *Scolescosporiella* Petrak (Webster and Hudson, 1957; Walker, 1980).

Although ascospores rarely are found in nature for other *Ophiosphaerella* spp., ascospore production and the influence of environmental factors on ascospore release has been examined for other plant pathogens (Ingold, 1971). Rain, moisture and relative

humidity have been shown to play a major role in the release of ascospores by other ascomycetes (Hong and Michailides, 1998; Gadoury et al., 1984; MacHardy and Gadoury, 1986; McGee and Petrie, 1979; Mengistu et al., 1993; and Spotts and Cervantes, 1994). Temperature also can impact ascospore release (Hong and Michailides, 1998).

Previous studies of *O. agrostis* revealed that pseudothecia were produced *in vitro* on a tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran mix (Kaminski et al., 2002). Under constant light, pseudothecia developed in as few as 4 days. Pseudothecia were not produced in the dark. Ascospores incubated at 25°C germinated in as little as 2 h and germination during the first 4 h of incubation was enhanced by both light and the presence of bentgrass leaves or roots (Kaminski et al., 2002). The environmental conditions associated with the release, as well as the daily and seasonal release timings of *O. agrostis* ascospores, are unknown. Elucidation of these conditions will lead to a better understanding of the epidemiology of dead spot and therefore improve the successful development of management strategies for the disease.

This research project was designed to investigate the timing of and environmental conditions that influence the release of *O. agrostis* ascospores. The objectives of this research therefore were to: 1) determine the influence of changes in light and relative humidity on ascospore release in a controlled environment; 2) document the seasonal and daily discharge patterns of ascospores in the field; and 3) elucidate the impact of environmental conditions that promote ascospore release under field conditions.

## MATERIALS AND METHODS

### **Ascospore Collection and Quantification**

Ascospore release was monitored using a seven day recording volumetric spore sampler (Burkard Manufacturing Co. Ltd., Rickmansworth Hertfordshire, United Kingdom). The Burkard trap samples  $0.6 \text{ m}^3 \text{ air hour}^{-1}$  through an orifice (14 x 2 mm) placed 45 cm above the turfgrass canopy. Ascospores in the sampled air were trapped on melinax tape, which had been coated with a silicone/hexane mixture (5:1 w/v). Estimations of hourly ascospore release were determined as described by the manufacturer. Briefly, the coated melinax tape rotates at a rate of  $2 \text{ mm hour}^{-1}$ ; therefore, each 2 mm x 14 mm section of tape represents 1 h of spore collection. Because the orifice is 2 mm wide, total ascospores captured within any given hour may have been deposited during a 2 hour period (4 mm section). Spores deposited along a narrow (100  $\mu\text{m}$ ) linear traverse across the 14 mm wide opening will have been deposited within a single hour. Ascospores quantified along the narrow linear traverse, therefore, may be regarded as an estimate of the total number of spores collected for one hour. In this study, ascospore release was determined by counting all spores deposited within a 100  $\mu\text{m}$  x 14 mm traverse. Spores counted along the traverse represent  $1/20^{\text{th}}$  of the total spores estimated for the hour, therefore data were transformed (ascospores x 20) according to the manufacturers recommended estimation process.

### ***In Vitro* Ascospore Release**

The effect of relative humidity (RH) and light on ascospore release were monitored in a walk-in growth chamber (Percival Scientific, Perry, Iowa). Due to

extreme fluctuations in RH within the growth chamber, a boxed-in enclosure (~1.5 m<sup>3</sup>) was constructed using 4 mil clear plastic. Relative humidity was maintained using a Model M3DH30B2A dehumidifier (Maytag, Newton, Iowa). Air temperature within the enclosure was maintained at 25 ± 3°C.

Microclimatic conditions including air temperature (°C) and relative humidity (%) monitored within the plastic enclosure were recorded throughout the study. Air temperature (AT) and relative humidity (RH) were measured with a thermistor and a Vaisala<sup>®</sup> capacitive sensor (model HMP35C, Campbell Scientific, Logan, Utah), respectively. To ensure that light and dark treatments were properly functioning, a solar irradiance device (LI200X, LI-COR, Lincoln, NE) also was placed in the enclosure. All monitoring instruments were connected to a CR-10 datalogger (Campbell Scientific, Logan, Utah) encased in a weatherproof aluminum box and powered by a standard wall outlet. Environmental instruments were programmed to measure parameters at 1 min intervals and data were averaged each hour by the CR-10. All data were downloaded daily using a NEC Ready 330T laptop computer (NEC Computers International, Wijchen, The Netherlands).

Eight treatments were evaluated as follows: A) 6 hours of light followed by 6 hours of dark at ~50% relative humidity (RH); B) 6 hours of dark followed by 6 hours of light at ~50% RH; C) 6 hours of light followed by 6 hours of dark at 100% RH; D) 6 hours of dark followed by 6 hours of light at 100% RH; E) 6 hours of ~50% RH followed by 6 hours of 100% RH under constant light; F) 6 hours of 100% RH followed by 6 hours of ~50% RH under constant light; G) 6 hours of ~50% RH followed by 6 hours of 100% RH under constant darkness; and H) 6 hours of 100% RH followed by 6 hours of ~50%

RH under constant darkness. All treatments are listed in Table 1.

Pseudothecia were produced using the method previously described with the following modifications (Kaminski et al., 2002). Briefly, a mix of tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran (1/1 v/v) was prepared by soaking tall fescue seeds in tap water overnight. Seeds then were rinsed three times and mixed with wheat bran (v/v), placed in 1L flasks, and autoclaved for 1 h on two consecutive days. Mycelia from the edge of three actively growing colonies (OpMD-4, OpMD-9 and OpOH-1) were removed and separately placed on the surface of the mixture. Isolates were described previously by Kaminski (Chapter VI). Flasks were incubated in a dark growth chamber (I30BLL, Percival Scientific, Inc., Perry, IA) at 25°C for at least 16 d. Inoculum was mixed every 2 to 3 days to promote aeration and to allow mycelia to become evenly distributed throughout the medium. Approximately 0.2 g of each infested mix then was placed on sterile, moist filter paper (Qualitative 415, VWR Scientific, West Chester, PA) in a 90 by 15 mm Petri dish (VWR Scientific, West Chester, PA). Petri dishes containing infested media were placed in a growth chamber at 25°C in constant light ( $88 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ) from four fluorescent, cool-white, 20 watt bulb (F20T12/CW, Philips Lighting, Somerset, NJ). The inoculum and filter paper initially were moistened with 1 mL sterile water and periodically re-moistened after the filter paper dried. After 7 days, the tall fescue seed/wheat bran mixture contained pseudothecia with mature ascospores. Prior to use of the pseudothecia-infested mixture, ascospore release was confirmed visually under a stereo microscope. Although the tall fescue/bran mixture contained varying numbers of pseudothecia, only mixtures in which ascospores were forcibly ejected from ostioles were used. The Petri dishes containing the mixture

(12 to 34 d old) were placed in the growth chamber under the initial starting conditions six hours prior to treatment initiation. The mixture containing mature pseudothecia was placed directly in front of the spore trap collection orifice and was replaced with a fresh mixture of pseudothecia prior to each treatment. After the mixture was removed from the growth chamber, pseudothecia were microscopically examined for the presence of ascospores as described previously.

Only one volumetric spore sampler was available; therefore, *in vitro* treatments were conducted in the same growth chamber. The experiment was repeated three times. Prior to analyses, all data were square root transformed. Data from each treatment were summed across the three replications and the total numbers of ascospores collected during the 12 hour period were subjected to analyses of variance using the MIXED procedure in SAS (SAS Institute Inc., 2000). Data also were subjected to repeated measures analyses using the REPEATED statement within the MIXED procedure in SAS (SAS Institute Inc., 2000). The covariance structure of the repeated measures procedure was selected based on Akaike's Information Criterion (AIC) (SAS Institute Inc., 2000).

### **Field Ascospore Release and Dead Spot Symptoms**

The release of ascospores and the subsequent development of dead spot infection centers under field conditions were monitored at the University of Maryland Paint Branch Turfgrass Research Facility in College Park, MD in 2001 and 2002. In August of 1999, a research putting green was constructed to United States Golf Association (USGA) specifications (USGA Green Section Committee, 1993). Soil was a modified sand mix (97% sand, 1% silt, and 2% clay) with a pH of 4.9 and 10 mg organic matter gram<sup>-1</sup> of

soil. A total of 1075 kg ha<sup>-1</sup> ground agricultural limestone was applied between November 1999 and April 2000 and raised soil pH in the top 5 cm to 6.9 by 25 September 2000. During the study period, soil P (79 kg ha<sup>-1</sup>) and K (16 kg ha<sup>-1</sup>) levels were in the low to moderate range.

A 465 m<sup>2</sup> area was seeded with 'Providence' creeping bentgrass (50 kg seed ha<sup>-1</sup>), and irrigated to maintain adequate moisture on 10 September 1999. Bentgrass seedling emergence occurred on 15 September and the turf was maintained under putting green conditions thereafter. Inoculum (OpVA-1 + OpMD-9) was prepared as described previously. The site was inoculated by placing 0.5 g inoculum at the soil surface in a grid pattern spaced approximately every 1.5 meters on center on 12 March 2001. Due to relatively low disease activity in 2001, an area adjacent to the aforementioned study site was fumigated for the 2002 study year. On 23 August 2001, a 465 m<sup>2</sup> area was fumigated with methyl bromide (98% methyl bromide + 2% chloropicrin). The area was seeded to 'L-93' creeping bentgrass on 30 August 2001 and after seedling emergence the turf was maintained under putting green conditions. On 21 March, 2002, the area was inoculated with a mixture of isolates (OpOH-1 + OpVA-1) as previously described.

In 2001 and 2002, the number of new dead spot infection centers was recorded daily between 1 May and 31 October. To ensure that individual infection centers were not counted twice, a small dot (~10 mm in diameter) was painted next to each dead spot. Daily infection center data were transformed and expressed as the number of new *O. agrostis* ICUA. Field release of ascospores was monitored hourly between 10 June and 31 October 2001 and 14 May and 31 October 2002 using the Burkard volumetric spore trap and ascospores were quantified as previously described. Development of

pseudothecia within the tall fescue/bran inoculum and the bentgrass tissue also was monitored.

**Environmental Monitoring.** Environmental conditions were monitored between 1 May and 31 October in 2001 and 2002. Air temperature (AT) and relative humidity (RH) were measured with a previously described sensor placed 30 cm above the plant canopy (model HMP35C, Campbell Scientific, Logan, Utah) near the center of the each study area. Sensors were housed in a 12-plate, louvered radiation shield to protect them from sunlight and rain. Soil temperature (ST) was measured by averaging the data from two or three thermistors (model 107, Campbell Scientific, Logan, Utah) placed 2.5 cm below the turfgrass canopy at the thatch and soil interface and spaced approximately 10 m apart. All temperature (AT and ST) and RH instruments were programmed to measure environmental conditions at 2 min intervals and the mean, maximum and minimum measurements were recorded every 60 min.

Leaf wetness duration (LWD) was estimated by placing two or three electrical impedance grids (model 237, Campbell Scientific, Logan, Utah) horizontally on the turf canopy. The sensors were coated with flat-white, latex paint to improve their accuracy in detecting LWD (Gillespie and Kidd, 1978). The electrical resistance for the sensors at the transition between wet and dry was 150 kohms. Sensors for LWD were programmed to record readings every 15 min and resistance values for each sensor were recorded as either 0 for dry or 0.25 for wet.

Hourly precipitation was determined using a tipping rain bucket (Texas Electronics Inc., Dallas, TX) situated 30 cm above the turf canopy. Precipitation

included both natural rainfall events and irrigation applied using an automatic, overhead irrigation system. Solar irradiance (SOL) and wind speed (WS) data were collected at the United States Department of Agriculture, Beltsville Agricultural Research Station located approximately 0.3 km from the study site. The SOL sensor (LI200X, LI-COR, Lincoln, NE) consisted of a silicon photocell pyranometer mounted 3 m above the ground on a tripod. Wind speed measurements were recorded at the same location using a Model 5103 Wind Monitor (R.M. Young Company, Traverse City, MI).

With the exception of the solar radiation and wind speed equipment, all monitoring instruments were connected to a CR-10 datalogger (Campbell Scientific, Logan, Utah), which was encased in a weatherproof aluminum box and powered by a 12-volt rechargeable lead battery connected to a solar panel. All instruments were programmed to measure environmental conditions on either 2 min (AT, ST, and RH) or 15 min (LWD, SOL, WS) intervals. All data were downloaded weekly using a NEC Ready 330T laptop computer (NEC Computers International, Wijchen, The Netherlands).

**Data Analyses.** The percent of the total number of ascospores collected during each hour in the day (000 to 2300) were determined for both years to reveal any daily patterns of spore release. Daily means for the environmental variables described previously were obtained by averaging hourly data. Additionally, the number of hours with precipitation (natural rainfall and irrigation) and the mean and maximum intensity of precipitation ( $\text{mm hour}^{-1}$ ) were tabulated for each day. Correlation analyses were performed between daily ascospore release ( $\sqrt{(20 \times \text{ascospores})}$ ) and measured

environmental variables and a predictive model was developed using stepwise regression (SAS Institute Inc., 2000).

To examine the effect of the duration of a precipitation event on ascospore capture, the number of spores deposited during each hour since the beginning of each precipitation event was determined. To account for intermittent breaks in precipitation, a precipitation event was determined as described by Pinkerton et al. (1998). A precipitation event was defined by Pinkerton et al. (1998) as a continuous series of hours during which the individual hours in the event met one of the following criteria: rain occurred, the turfgrass canopy was wet ( $LWD > 0$ ) and rain occurred during the following hour; or the turfgrass canopy was wet and rain occurred during at least one of the two preceding hours. Hourly means of the number of ascospores captured and the percent of the total ascospores collected during all rain events were regressed on hours since the beginning of a precipitation event by using polynomial functions.

To determine seasonal patterns or cadences in ascospore release for each year, the numbers of ascospores released and infection centers appearing each day were analyzed utilizing a nonparametric method (LOESS) for estimating regression surfaces (Cleveland et al., 1988; Cleveland and Grosse, 1991). The LOESS procedure in SAS makes statistical inferences, but does not make assumptions about the parametric form of the regression surface (SAS Institute Inc., 2000). The fraction of the data (i.e., smoothing parameter) in each local neighborhood controls the smoothness of the estimated surface (SAS Institute Inc., 2000). Based on plots of the residuals, a smoothing parameter of 0.06 was selected for daily ascospore release and the number of new infection centers appearing in 2001 and 2002.

## RESULTS AND DISCUSSION

### **Growth Chamber Ascospore Release**

Due to large variation in the numbers of ascospores released within each treatment, ascospore data were square root transformed prior to analyses. Therefore, mean values presented in the results and labeled ‘ascospores’ represent the square root of ascospores  $\times 20$  ( $\sqrt{(20 \times \text{ascospores})}$ ). Treatment regimes (A to H) are shown in Table 1. The total number of ascospores collected throughout the 12 hour period were significantly different ( $P=0.0037$ ) among treatments. The greatest release of spores (213 and 215 ascospores  $12 \text{ h}^{-1}$ ) occurred from those treatments (F=100% to ~50%, light; H=100% to ~50%, dark) in which RH was lowered from 100% to ~50%, regardless of light treatment (Figure 1). Similar numbers of ascospores also were released in treatment C (157 ascospores  $12 \text{ h}^{-1}$ ; 100% RH; light to dark). When compared to treatments F and H, fewer ascospores were released (84 ascospores  $12 \text{ h}^{-1}$ ) from treatment E in which RH was increased from ~50% to 100% in constant light. Very few ascospores were collected within treatments D (27 ascospores  $12 \text{ h}^{-1}$ ; 100% RH; dark to light) and G (28 ascospores  $12 \text{ h}^{-1}$ ; ~50% to 100% RH; dark). Ascospores were not released or were released in extremely low numbers ( $\leq 3$  ascospores  $12 \text{ h}^{-1}$ ) in treatments A (~50% RH; light to dark) and B (~50% RH; dark to light) in which the RH remained ~50% for the entire 12 hour period. During periods of low RH (~50%), the tall fescue seed/wheat bran mixture generally appeared dry.

To assess the impact of changing environmental conditions (light and RH), treatment means and their standard errors were plotted for the twelve hour period (Figure 2). Repeated measures analysis revealed a significant treatment by time interaction

( $P < 0.0001$ ) (Table 2). Due to this interaction, the release of ascospores during the 12 hour incubation period was assessed individually for each treatment. Based on the total release of ascospores throughout the 12 hour period (Figure 1) and the distribution of the spore release (Figure 2), only treatments in which the mean was  $\geq 50$  ascospores  $12 \text{ h}^{-1}$  (treatments C, E, F and G) were subjected to repeated measures analyses. Based on AIC values, the best covariance structure was determined to be first order autoregressive. Although fairly high levels of ascospores (84 to 157 ascospores  $12 \text{ hours}^{-1}$ ) were released throughout the 12 hour period in treatments C (100% RH; light to dark) and E (~50% to 100% RH; light), there were no differences in the number of ascospores released each hour (Figure 3). Ascospores collected over the course of the study ranged from an average of 7 to 26 and 0 to 11 ascospores  $\text{hour}^{-1}$  for treatments C and E, respectively. The impact of decreasing relative humidity, however, was apparent in treatments F and H. Regardless of light (dark or light), both treatments in which RH was lowered from 100% to ~50% resulted in a significant increase in the release of ascospores. The release of ascospores in both aforementioned treatments began immediately following the drop in RH and a significant increase in the number of spores released continued only for a short period (1 to 2 hours). In both treatment F and H, peak ascospore release occurred at hour 7.

In summary, ascospores generally were not released in treatments where the RH was low (~50%) throughout the 12 hour period. The release of ascospores from other fungi also occurs only after hydration by free moisture or RH (Alderman, 1993; Aylor and Anagnostakis, 1991; Bertrand and English, 1976). Varying numbers of ascospores were released when the RH was adjusted to or maintained at 100%. Low to moderate

numbers of ascospores were released when pseudothecia were exposed to 100% RH and light for the first 6 hours (i.e., Treatment C), however few ascospores were collected when pseudothecia were maintained at 100% RH and in darkness for the first 6 hours (i.e., treatment D). While ascospores were released under varying levels of light and RH, only a sharp drop in RH stimulated the greatest and most rapid release of ascospores in constant light or darkness. Hong and Michailides (1998) also noted a release of *Monilinia fructicola* (G. Wint.) Honey ascospores in response to an abrupt decrease in relative humidity. Results and observations from this experiment showed that a sharp drop in RH resulted in the release of a large number of spores. Free moisture, however, was required for ascospore release and ascospores generally were not collected when pseudothecia were dry.

### **Field Ascospore Release**

**2001.** In 2001, the area was inoculated on 12 March and dead spot symptoms first appeared on 1 June. Pseudothecia first were observed within necrotic bentgrass tissue on 4 June. Ascospore collection began immediately after the volumetric spore trap was placed in the field on 10 June. The total number of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] counted between 10 June and the final rating date (31 October) was 4758. Major and minor ascospore release events were based on the percent of total ascospores collected each year and counted in a single day. Throughout the year, a total of 11 major ( $>95$  spores day<sup>-1</sup>, 2.0% of seasonal release) and 22 minor ( $\geq 48$  to 95 spores day<sup>-1</sup>, 1%) ascospore release events were recorded (Figure 4A). The first major discharge of ascospores occurred on 16 June (226 ascospores day<sup>-1</sup>) and accounted for 4.8% of the

total number of ascospores counted. Nine major events occurred between June and July with the final two events occurring on 30 August and 20 September. Minor ascospore release events ( $n=22$ ) generally occurred until late August, with low to moderate numbers of ascospores released in September and October. Although a low number of ascospores ( $9 \text{ spores day}^{-1}$ ) were collected as late as 26 October, no new infection centers appeared after 5 October (Figure 4B).

**2002.** Due to the declining nature of dead spot in the field, the site was fumigated and reseeded with 'L-93' creeping bentgrass in August 2001 to encourage the disease in 2002. On 21 March 2002, the area was inoculated and infection centers first appeared within inoculated spots on 12 May. Ascospore counts began on 14 May. Pseudothecia first were observed within the tall fescue/bran inoculum on 18 April 2002 and a total of 2439 (26% of seasonal total) ascospores were collected prior to the development of pseudothecia within necrotic bentgrass tissue on 10 June 2002. Hence, fruiting bodies developing within the infested inoculum likely were responsible for the major discharge events ( $\geq 190 \text{ ascospores day}^{-1}$ , 2.0%) occurring on 18, 19 and 27 May and 6 June. In 2002, dead spot was severe and a total of 9488 ascospores were collected. Throughout the season, a total of 8 major and 24 minor ( $\geq 95$  and  $< 190 \text{ ascospores day}^{-1}$ , 1%) ascospore release events were observed (Figure 5A). Similar to 2001, low levels of ascospores were collected until early autumn (21 October); however, no new infection centers developed after 9 October (Figure 5B).

**Daily and Hourly Ascospore Release.** The release of ascospores was monitored over 7120 hours between mid-May (2001) or mid-June (2002) and 31 October of each year. To determine any patterns in ascospore release during the day, the percent of the

total number of ascospores collected during each hour was regressed on the hour of the day (0000 to 2300) by using polynomial functions. The percent of ascospores collected during each hour (i.e., 0000 to 2300) occurred in a cyclic fashion and ranged from 1.9 to 7.8% and 1.4 to 7.0% in 2001 and 2002, respectively (Figure 6). Regression analyses ( $R^2 \geq .78$ ,  $P < 0.0001$ ) indicated an increase in the release of ascospores during the late afternoon and into the evening hours (1900 to 2300). In 2001, the percent of ascospores collected decreased after the aforementioned increase between 2100 and 2200 hours and then peaked again during the morning hours (0800 hours). In 2002, large numbers of ascospores were collected throughout the night, but ascospore release again peaked during the morning hours (0700 hours). In 2001 and 2002, only 15% and 23% of the total ascospores released, respectively, were collected between 1100 and 1800 hours. Averaged across both study years, the reduced release period (i.e., 1100 to 1800 hours) was associated with lower air (25 versus 19°C) and soil (26 versus 20°C) temperatures, fewer percent of hours with a wet bentgrass canopy (28% versus 94% of hours with leaf wetness), and lower relative humidity (59% versus 84%), when compared to the environmental conditions occurring between 1900 and 2300 and 0000 and 1000 hours (i.e., dawn to dusk). Similar to the results of the growth chamber study, it was apparent that the release of ascospores occurred during periods in which the bentgrass canopy was wet and that discharge was stimulated by a decrease in RH. Unlike the growth chamber study, however, ascospore release also occurred as the canopy became wet and when moisture levels within pseudothecia would have increased. In the field, the bentgrass canopy became wet in the evening hours while RH was still low (mean RH = 60% at 1900 hours). It is likely that moisture changes within pseudothecia stimulated the

discharge of ascospores. Ascospore discharge again was stimulated as RH decreased and when pseudothecia began to dry during the early morning hours (700 to 1000). Few ascospores (10 to 12%), however, were collected during periods when RH was low and the canopy was dry (Table 3).

To determine the environmental conditions that influence ascospore release, correlation analyses were performed between the number of ascospores collected and daily environmental variables. Because fewer ascospores were released in 2001 (4758 ascospores) when compared to 2002 (9488 ascospores), the percent of total ascospores released on each day were used in the analyses. Correlation analyses of all data revealed only weak associations between a select set of environmental variables and percent daily ascospore collection (Table 4). Significant positive correlations occurred between ascospores counted and precipitation duration (hours day<sup>-1</sup>;  $r = 0.31$ ); air and soil temperatures (°C;  $r = 0.14$  to  $0.32$ ); and total precipitation (mm day<sup>-1</sup>) ( $r = 0.25$ ). Percent of ascospores collected daily also was negatively correlated to the maximum hourly decrease in relative humidity day<sup>-1</sup> ( $r = -0.26$ ), indicating a relationship between abrupt decreases in RH and increasing release of ascospores.

Stepwise regression analyses performed on the percent of ascospores collected day<sup>-1</sup> and mean daily environmental variables resulted in a weak ( $R^2 = 0.25$ ,  $P < 0.0001$ ) predictive model. The five parameter model contained six environmental variables including total hours precipitation day<sup>-1</sup>; minimum air and soil temperature; maximum decrease in relative humidity hour<sup>-1</sup>; total precipitation day<sup>-1</sup> (mm); and minimum relative humidity. The regression equation is  $A = 2.4E^{-8}(H_m)^2(P_t) - 1.1(P_h)(H_d)^2 - 8.8E^{-7}(T_a)^3 + 1.4E^{-6}T_s^3 + 2.5E^{-5}(P_h)^2 + 4.0E^{-4}$  where  $A$  is the percent of ascospores released day<sup>-1</sup>;  $H_m$  is

the minimum relative humidity  $\text{day}^{-1}$ ;  $P_t$  is the total precipitation  $\text{day}^{-1}$  (mm);  $P_h$  is the number of hours of precipitation  $\text{day}^{-1}$ ;  $H_d$  is the maximum decrease in relative humidity during a 1 hour period  $\text{day}^{-1}$ ; and  $T$  is the minimum air ( $a$ ) or soil ( $s$ ) temperature. Hourly correlations between the percent of ascospores collected and all environmental variables also were weak ( $r \leq 0.23$ ) and did not contribute to the development of a simplified model for predicting ascospore release.

**Impact of Precipitation on Ascospore Release.** Examination of the data and correlation analyses suggested that precipitation (rain and irrigation) most influenced the release of ascospores in the field. Precipitation accounted for 16% (2001) and 20% (2002) of the total number of hours monitored in this study. The percent of ascospores collected each year during precipitation events was 33 and 42% in 2001 and 2002, respectively (Table 3). Additionally, the maximum number of ascospores collected each hour generally was higher during precipitation events, when compared to hours in which no rain event occurred (Table 3). Ascospore release patterns were visible when data were compared to hours since the beginning of each precipitation event (Figure 7). The number of ascospores collected increased during the first five hours of precipitation and ascospores continued to be collected throughout much of each event (Figure 7B). Because the average precipitation event lasted  $\leq 7$  hours, the greatest percentages of ascospores were collected during this period. A total of  $53 \pm 4\%$  and  $87 \pm 5\%$  of the total number of ascospores collected during all precipitation events were captured during the first 4 and 10 hours of an event, respectively. Additionally, examination of ascospore data collected during precipitation events indicated that spore release declined as precipitation rate increased and vice versa (data not shown). This was similar to a

previous report in which ascospores were observed to exude out of pseudothecia in the presence of free water (Kaminski et al., 2002). These data indicate that although ascospore release may continue for the duration of a rain event, pseudothecia begin ejecting large quantities of ascospores immediately after the initiation of a precipitation event.

**Seasonal Ascospore Release and Dead Spot Symptom Development.** Based on a visual examination of the daily release of ascospore data over the course of the season, it was apparent that *O. agrostis* ascospores were released in a cyclical fashion. To investigate this pattern, data were subjected to non-parametric statistical smoothing using the LOESS procedure in SAS (SAS Institute Inc., 2000). Field data collected in 2001 and 2002 were analyzed separately. Days in which major and minor spore release events occurred for each year are shown in Figure 4A and Figure 5A.

In 2001, the thirteen estimated peak periods associated with the release of ascospores occurred on 16 and 30 June, 12, 17 and 27 July, 14, 22, and 30 August, 13, 21 and 30 September, and 16 and 26 October 2001 (Figure 4C). The 11 major release events observed in 2001 all grouped into 7 of the 13 peaks in ascospore release as estimated by nonparametric statistical analyses. In other words, major ascospore release events that occurred on consecutive days were grouped within a single peak in the estimation procedure. The remaining 6 estimated peaks were the result of moderate or small increases in spore release throughout the season. The average number of days between each estimated peak in ascospore release was  $11 \pm 1.1$  days (standard error) in 2001. The number of infection centers appearing in 2001 was low and the magnitude of each daily count was small (Figure 4). Regardless, 10 estimated peaks in the number of new dead

spot infection centers that appeared each day occurred every  $12 \pm 1$  days between 31 May and 18 September. Peaks in new infection centers occurring on 31 May and 8 June appeared prior to the placement of the volumetric spore trap in the field. Out of the 6 estimated major ascospore release events in 2001, four were followed by an increase in the number of new infection centers observed in the plot area. Ascospores released around 30 June and 12, 18, and 26 July were followed by a sharp increase in new infection centers on 12 (12 days), 19 (7 days) and 24 (6 days) July, and 4 (9 days) August, respectively. Finally, the increase in the number of infection centers occurring in early September was preceded by three minor spore release events on 15, 24 and 30 August.

A total of 8 major and 24 minor daily ascospore release events were observed in 2002, and they resided within a total of 13 estimated release cycles (Figure 5C). Only one minor release event occurring on 31 May resided between two estimated peaks in spore release. The first 8 observed release events (major and minor) were the result of ascospores originating from pseudothecia that had developed within or on the tall fescue/bran inoculum and all fit into three estimated release periods. The remaining 24 observed ascospore release events were located within 11 estimated periods of ascospore release, which peaked on 15 and 29 June, 11, 17 and 23 July, 6, and 27 August, 12 and 24 September, and 10 October 2002. The average number of days between each estimated peak in ascospore release was  $12 \pm 1.3$  days in 2002. Unlike 2001, dead spot infection was severe in 2002. Estimated periods associated with the daily appearance of infection centers peaked on 21 June, 4, 15 and 28 July, 10 and 28 August, and 7, 19, and 30 September 2002. On average, each peak in the appearance of new infection centers

occurred every  $13 \pm 0.9$  days. Throughout 2002, most infection centers (82%) appeared between 17 June and 10 August. During this period, each peak in the estimated number of infection centers was preceded (3 to 6 days) by a peak in ascospore release. The remaining number of new infection centers peaked 3 to 10 days after spore release. Despite a large number of ascospores collected in late-August, new infection centers appearing thereafter were relatively few in number.

In summary, ascospore discharge and the appearance of new infection centers generally occurred every 11 to 14 days. Additionally, new infection centers appeared 3 to 10 days after peak periods of spore release. The influence of temperature likely plays an important role in the infection process by ascospores. Although ascospores were released throughout May 2002, mean air and soil temperature during the period in which ascospores were released was 10 and 13°C, respectively. An increase in the number of dead spot infection centers, however, did not occur until mid-June when both air and soil temperatures averaged 24°C. Temperatures during the early release events likely were too low for infection to occur. Pseudothecia can develop in growth chambers within four days when exposed to constant light and fruiting bodies may be present within necrotic tissue when dead spot symptoms first appear in the field (Kaminski et al., 2002; Kaminski, Chapter II). Results from this study indicate that symptom development can occur within a short period (3-10 days) following ascospore release. Infection and subsequent symptom expression (i.e., infection centers) likely is influenced most by temperature at the time ascospores alight on moist bentgrass tissues and in the days following infection. Kaminski et al. (2002) reported that dead spot reactivation and

symptom expression of winter-dormant field samples occurred in as little as 12 days after incubation at 25°C. Undoubtedly, other factors also are involved in the infection process.

## CONCLUSION

Ascospores of *O. agrostis* are discharged from pseudothecia in large numbers throughout the year and serve as an important source of inoculum. Although no conidial state has been found, the pathogen is capable of producing multiple cycles of fruiting bodies and viable ascospores within a short time period (Kaminski et al., 2002; Kaminski Chapter II; Kaminski Chapter III). This is in contrast to many other plant pathogenic fungi that produce ascocarps as an overwintering mechanism and whose spores generally serve as a primary source of inoculum the following spring (Pinkerton et al., 1998; Hong and Michailides, 1998; Cox and Scherm, 2001; Mondal et al., 2003).

In the growth chamber study, sharp decreases in relative humidity resulted in a rapid (1 to 3 hours) discharge of ascospores, regardless of whether pseudothecia were incubated in constant light or dark. Once relative humidity dropped and remained low (~50%) ascospore release was minimal. Similarly, few or no ascospores were collected when pseudothecia were incubated at ~50% for the entire 12 hour period. Although ascospore release was not stimulated by rapid increases in RH, low to moderate numbers of ascospores were collected throughout periods in which RH remained elevated. In field studies, daily ascospore release was greatest in the morning (600 to 900) and again in the early evening (1900 to 2300 hours). Ascospores also were released at various levels throughout the night when the bentgrass canopy was wet. In both years, very few ascospores were released between 1100 and 1800 hours, when the bentgrass canopy generally was dry. The release of ascospores was associated with several environmental factors. In particular, precipitation (rain and irrigation), relative humidity and leaf wetness appeared to most influence the release of ascospores. During each day,

ascospore release occurred cyclically and was greatest during the morning and evening hours when the bentgrass canopy was wet and RH was low or decreasing. This likely occurs from the swelling and subsequent contraction of the bitunicate asci. In other words, as the moisture available within the pseudothecium decreases, the ectoascus layer will rupture allowing the elastic endoascus to extend upwards, rupture, and forcefully release its ascospores through the ostiole. This phenomenon was further supported in growth chamber studies where a sharp decrease (100% to ~50%) in relative humidity resulted in the immediate discharge of ascospores. Additionally, large percentages (33 and 42%) of all ascospores were discharged during precipitation (rain or irrigation) events in both years. Precipitation events, however, only occurred during 16 and 20% of the hours in which ascospores were monitored in 2001 and 2002, respectively. During these precipitation event periods, ascospore discharge began immediately and most of the spores (87%) were collected within the first 10 hours after precipitation had begun. Ascospores probably ooze from ostioles when precipitation rates and/or relative humidity levels are high enough to saturate pseudothecia. This phenomenon likely would explain the lack of forcefully discharged ascospores following the rapid increase in relative humidity and subsequent saturation of pseudothecia in the growth chamber experiment.

Ascospore release began as early as May and continued until late October. During this period, the release of ascospores occurred in cycles, which peaked about every 12 days. A similar length cycle also was observed with the appearance of dead spot infection centers, and was delayed approximately 3 to 10 days following ascospore release. In a related study, it was observed that the development of pseudothecia within infection centers increased until late July or late August in 2001 and 2002, respectively

(Kaminski, Chapter II). The aforementioned timing for maximum pseudothecia development occurred approximately 6 to 10 days prior to the final major release event in 2001 (26 July) and 2002 (1 September). Although large numbers of ascospores were released as early as May and as late as October, these spore release events were not followed by proportionally large numbers of new infection centers. Most new infection centers appeared between June and August, which coincided with periods of high ST (~25°C) and AT (~24°C). Hence, relatively high temperatures appear to be required for ascospore germination and/or subsequent infection. Elevated soil temperatures ( $\geq 20$  °C) in the days following infection also appear to be necessary for dead spot symptoms to appear (Kaminski, Chapter III).

Kaminski et al. (2002) reported that mature ascospores were capable of germinating in water in as little as 2 hours when incubated at 25°C and that ascospore germ tubes and appressoria directly penetrated bentgrass leaves and roots. On golf putting greens, irrigation commonly is applied and leaf wetness duration routinely exceeds 16 hours day<sup>-1</sup>. For this reason, conditions favoring the germination of *O. agrostis* ascospores and possibly infection may be artificially maintained by routine irrigation practices. Although conditions necessary for ascospore release occur routinely, the complete discharge of mature ascospores during a major release event may preclude subsequent releases until new pseudothecia are produced. Hence, the potential cyclical development of pseudothecia and subsequent release of mature ascospores may add to the complexity of developing a predictive model.

Table 1. Growth chamber conditions during a 12 hour period for eight treatments designed to assess the impact of changing levels of relative humidity and light on *Ophiosphaerella agrostis* ascospore release.

Treatment	Relative humidity (%) <sup>x</sup>		Light <sup>y</sup>	
	0-6 h	6-12 h	0-6 h	6-12 h
A	~50%	~50%	Light	Dark
B	~50%	~50%	Dark	Light
C	100%	100%	Light	Dark
D	100%	100%	Dark	Light
E	~50%	100%	Light	Light
F	100%	~50%	Light	Light
G	~50%	100%	Dark	Dark
H	100%	~50%	Dark	Dark

<sup>x</sup> Percent relative humidity was maintained at either 100% or ~50%.

<sup>y</sup> Light treatments were as follows: light (120 W m<sup>-2</sup>) or dark.

Table 2. Repeated measures analysis of variance of ascospore release.

Source <sup>w</sup>	df <sup>x</sup>	MS <sup>y</sup>	F	<i>P</i> > F <sup>z</sup>
Time	11	325.5	1.66	0.25
Treatment	3	2816.1	14.36	<0.0001
Time x Treatment	11	1157.0	5.90	<0.0001

<sup>w</sup> Time = 12 hour period and treatment = 8 relative humidity (100% or ~50%) and light (light or dark) interactions as shown in Table 1.

<sup>x</sup> df = Degrees of freedom

<sup>y</sup> MS = Mean square

<sup>z</sup> Probability associated with the F test.

Table 3. Influence of precipitation (rain and irrigation) and leaf wetness duration on the release of *Ophiosphaerella agrostis* ascospores from a creeping bentgrass putting green in 2001 and 2002.

Environmental factor	2001 <sup>x</sup>	2002 <sup>x</sup>
Total hours of precipitation	508	789
Total hours of leaf wetness	2390	2560
Percentage <sup>y</sup> of spores collected		
with leaf wetness	87	86
without leaf wetness	12	10
during a rain event	33	42
with leaf wetness and no rain	54	42
Maximum number of spores collected		
1 hour with rain	38	91
1 hour without rain	25	59
5 hour with rain	113	161
5 hour without rain	94	128
10 hour with rain	151	264
10 hour without rain	174	194
Number of hours when spore release		
was moderate (>10 to 20 ascospores hour <sup>-1</sup> ) with rain	44	137
was moderate without rain	47	103
was major (>20 ascospores hour <sup>-1</sup> ) with rain	6	50
was major without rain	3	11
Total number of rain events	109	118
Total number of rain events $\geq$ 6 hours	29	49
Total number of rain events $\leq$ 5 hours	80	69
Average duration of rain events (hours)	5	7

<sup>x</sup> Ascospores were collected were between 14 June and 31 October 2001 and 14 May and 31 October 2002.

<sup>y</sup> Percentages not totaling 100% indicate missing environmental or ascospore data.

Table 4. Correlation of percent daily capture of *Ophiosphaerella agrostis* ascospores in the field with the environmental variables measured.

Environmental variable	Correlation coefficient <sup>x</sup> %
Mean relative humidity (%)	0.19***
Mean air temperature (°C)	0.21***
Mean soil temperature (°C)	0.27***
Mean solar radiation (W m <sup>-2</sup> )	0.10
Mean wind speed (m sec <sup>-1</sup> )	0.09
Total rain (mm day <sup>-1</sup> )	0.25***
Leaf wetness duration (hours day <sup>-1</sup> )	0.06
Total daily hours of precipitation	0.31***
Minimum relative humidity (%)	0.18**
Minimum air temperature (°C)	0.30***
Minimum soil temperature (°C)	0.32***
Maximum relative humidity (%)	0.09
Maximum air temperature (°C)	0.14*
Maximum soil temperature (°C)	0.22***
Maximum decrease in relative humidity hour <sup>-1</sup>	-0.26***
Maximum increase in relative humidity hour <sup>-1</sup>	0.02
Maximum rain intensity (mm hour <sup>-1</sup> )	0.15**

<sup>x</sup> \*, \*\*, and \*\*\* indicate significance at  $P < 0.05$ ,  $<0.01$ , and  $<0.001$ , respectively.

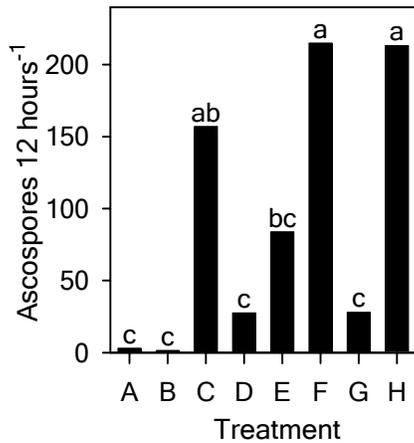


Figure 1. Total number of *Ophiosphaerella agrostis* ascospores ( $\sqrt{(20 \times \text{ascospores})}$ ) released after 12 hours of incubation under changing relative humidity or light treatments. Treatments were as follows: (A) ~50% RH, light to dark; (B) ~50% RH, dark to light; (C) 100% RH, light to dark; (D) 100% RH, dark to light; (E) ~50% to 100% RH, light; (F) 100% to ~50% RH, light; (G) ~50% to 100% RH, dark; and 100% to ~50 RH, dark. Treatment means followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's least significant difference test.

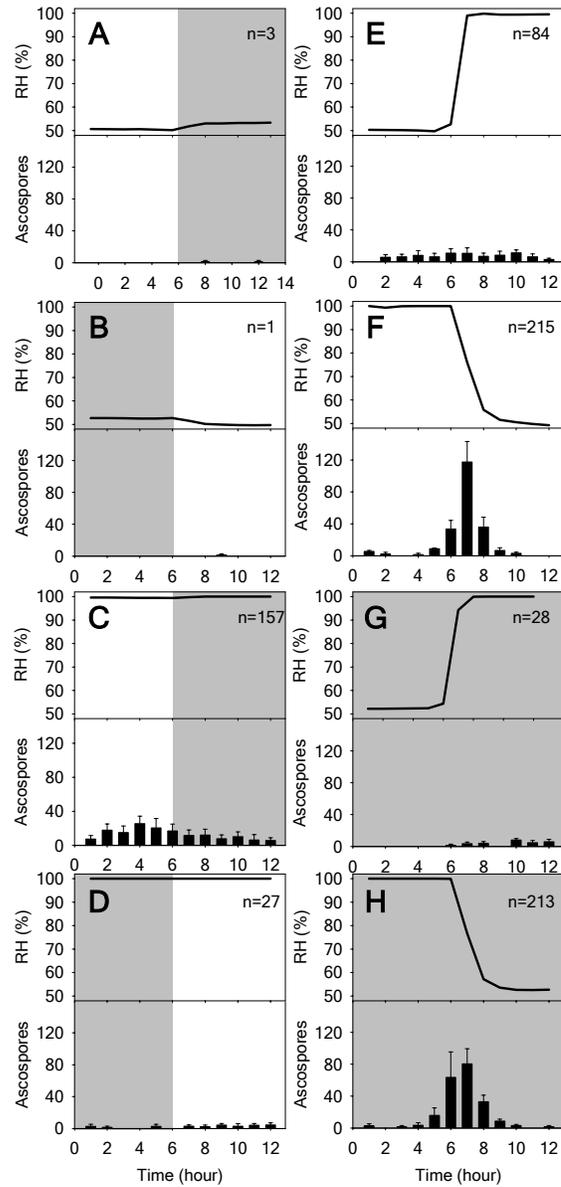


Figure 2. Interaction between hours of incubation and *Ophiosphaerella agrostis* ascospore release under varying controlled conditions (A to H) in which relative humidity (solid line) or light (white background) or dark (gray background) treatments were imposed at hour 6 of the 12 hour incubation period. Treatments were as follows: (A) ~50% RH, light to dark; (B) ~50% RH, dark to light; (C) 100% RH, light to dark; (D) 100% RH, dark to light; (E) ~50% to 100% RH, light; (F) 100% to ~50% RH, light; (G) ~50% to 100% RH, dark; and (H) 100% to ~50% RH, dark. Error bars represent the standard error of the mean and n equals the mean number of ascospores ( $\sqrt{(20 \times \text{ascospores})}$ ) released throughout the 12 hour period of 3 replications of the experiment.

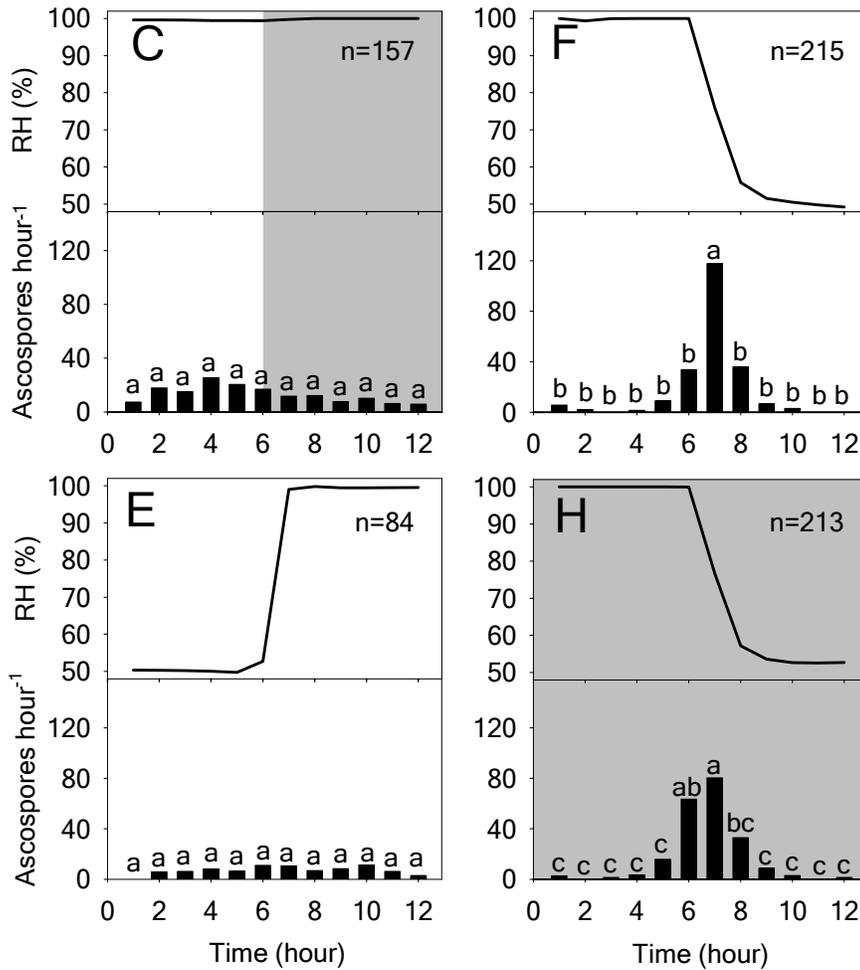


Figure 3. Release of *Ophiosphaerella agrostis* ascospores as influenced by changing levels of relative humidity (RH) or light (white background) and dark (shaded) during 12 hours of incubation at 25°C. Ascospore data were square root transformed ( $\sqrt{(20 \times \text{ascospores})}$ ). Due to background levels of ascospores in all treatments, only treatments in which mean ascospore release (n) was  $\geq 50 \text{ 12 hours}^{-1}$  were included. Treatments were as follows: (C) 100% RH, light to dark; (E) ~50% to 100% RH, light; (F) 100% to ~50% RH, light; and 100% to ~50 RH, dark. For individual treatments, bars labeled with the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's least significant difference test.

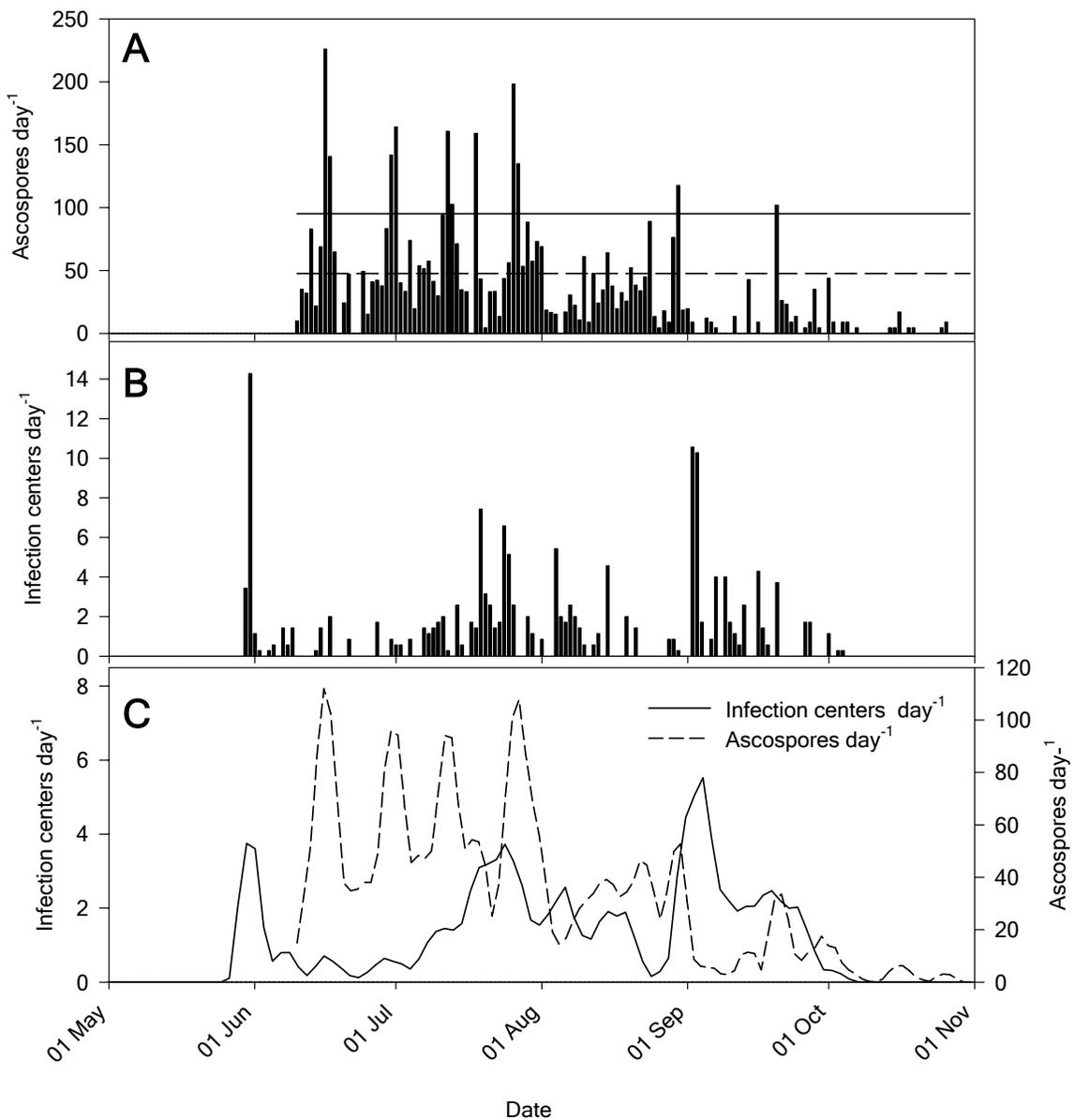


Figure 4. Seasonal relationship between the daily release of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] (A) and the appearance of new *Ophiosphaerella agrostis* infection centers (B), 2001. Days in which a major (2.0%) or minor (1.0%) percent of the total number of ascospores (4758 ascospores) released (A) are indicated by the solid and dashed lines, respectively. New infection centers (B) were counted daily between 1 May and 31 October and ascospores (A) were collected using a Burkard volumetric spore trap between 10 June and 31 October 2001. Estimated curves (C) were fitted using a non-parametric, locally weighted regression analyses (Proc Loess).

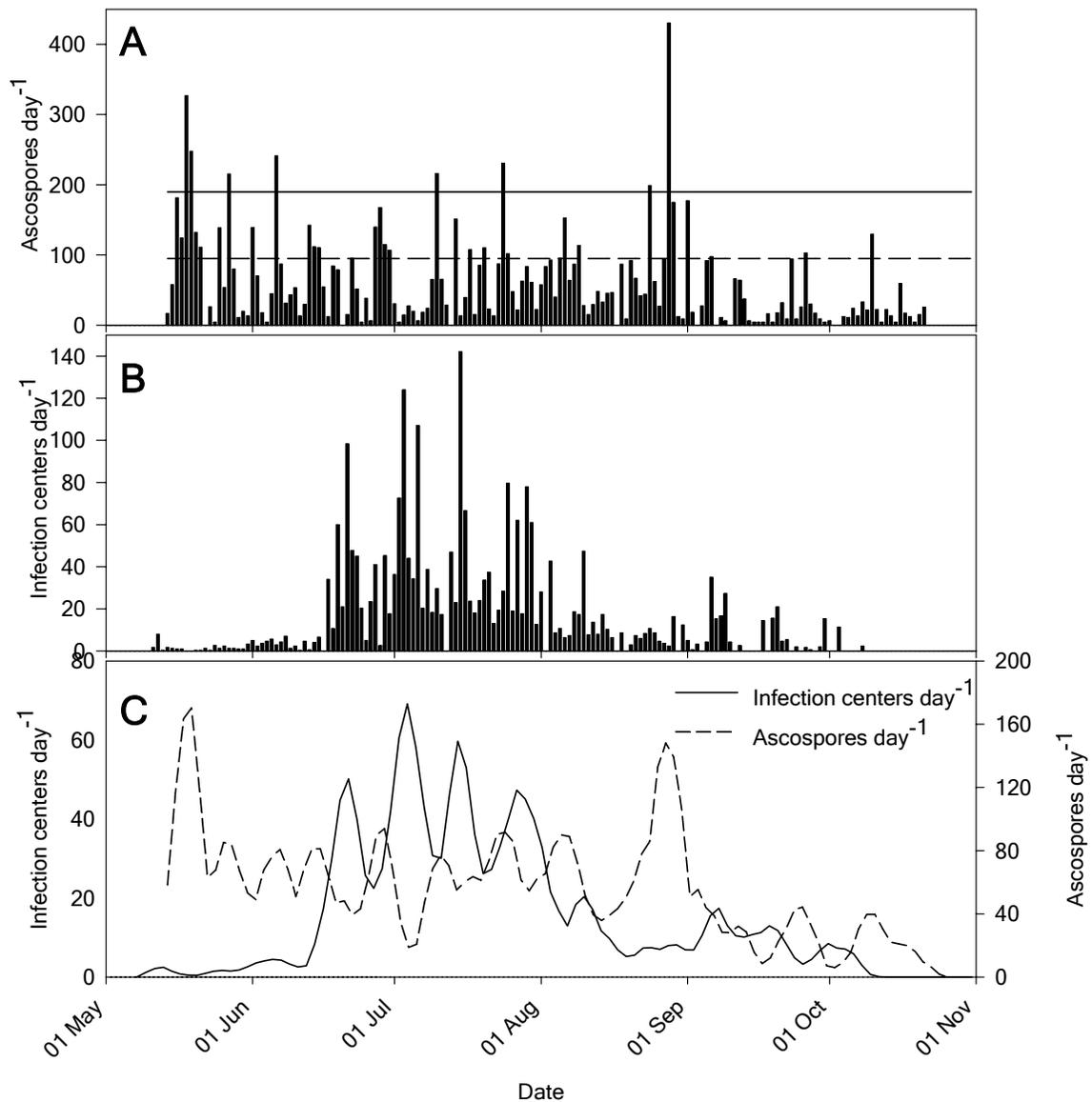


Figure 5. Seasonal relationship between the daily release of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] (A) and the appearance of new *Ophiosphaerella agrostis* infection centers (B), 2002. Days in which a major (2.0%) or minor (1.0%) percent of the total number of ascospores (9488 ascospores) released (A) are indicated by the solid and dashed lines, respectively. New infection centers (B) were counted daily between 1 May and 31 October and ascospores (A) were collected using a Burkard volumetric spore trap between 14 May and 31 October 2002. Estimated curves (C) were fitted using a non-parametric, locally weighted regression analyses (Proc Loess).

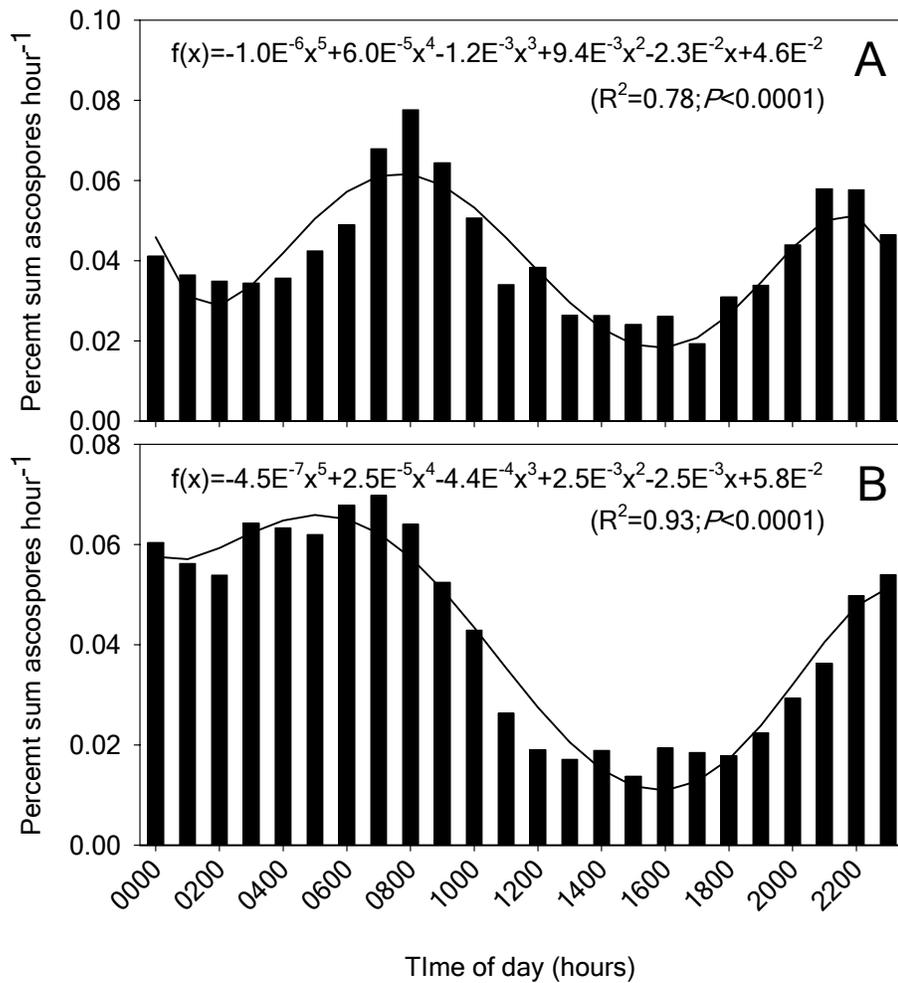


Figure 6. Percent of the total number  $[\sqrt{(20 \times \text{ascospores})}]$  of *Ophiosphaerella agrostis* ascospores collected in 2001 (A) and 2002 (B) during each hour of the day. Ascospores were collected hourly between 10 June and 31 October 2001 and 1 May and 31 October in 2002 on a creeping bentgrass putting green.

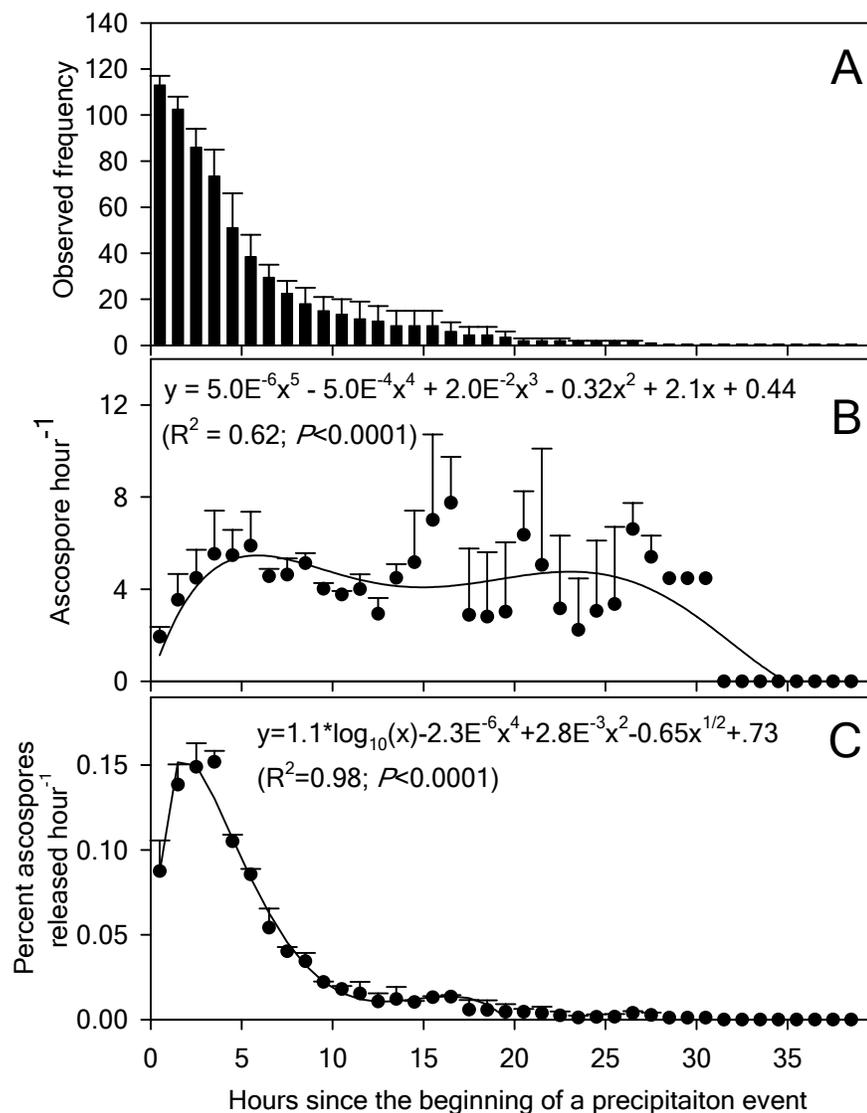


Figure 7. Relationship between the number and percentage of *Ophiosphaerella agrostis* ascospores released each hour since the beginning of a precipitation event. Data are mean values of 227 precipitation (rain and irrigation) events on a creeping bentgrass putting green from 14 June to 31 October in 2001 and 14 May to 31 October 2002. A= observed frequency of the number of hours for each rain event; B= mean number of ascospores [ $\sqrt{(20 \times \text{ascospores})}$ ] collected each hour since the beginning of the precipitation event; and C= percent of the total ascospores collected from 227 precipitation events during each hour of the event. Error bars indicate standard error of the means between 2001 and 2002.

V. NITROGEN SOURCE IMPACT ON RECOVERY OF CREEPING BENTGRASS FROM DEAD SPOT (*OPHIOSPHAERELLA AGROSTIS*).

SYNOPSIS

Dead spot is a relatively new disease of creeping bentgrass incited by *Ophiophaerella agrostis*. Limited information is available on chemical and cultural management strategies for dead spot. Two field studies were designed to evaluate the influence of various nitrogen (N) fertilizers and the fungicide iprodione on the recovery of dead spot when applied after or during peak dead spot activity. When applied after peak activity, all N-sources (ammonium sulfate, IBDU, SCU, urea, Ringer Greens Super, and methylene urea) aided in the recovery and healing of dead spot, but none provided for complete recovery before winter. Plots treated with ammonium sulfate and IBDU were among the fastest and slowest to recover, respectively. Iprodione failed to improve dead spot recovery, when applications were initiated in late summer after most injury was evident. When applied during the early stages of symptom expression, none of the five N-sources ( $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , urea, and 20-20-20) examined in a second study prevented new infection centers from occurring. The percent of plot area diseased, however, continued to increase in plots not receiving N and significantly less dead spot was observed in all N-treated plots by early to mid-September of the first year (2002). In the second year (2003), dead spot recurred in plots treated with  $\text{KNO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$ , 20-20-20, and urea. Conversely, dead spot did not recur in plots receiving  $(\text{NH}_4)_2\text{SO}_4$  or the unfertilized plots. In plots receiving N, the number of dead spot infection centers  $\text{plot}^{-1}$  in 2003 was positively correlated with pH and disease incidence appeared to be favored at a pH greater than 6.0 and 6.6 in the mat and soil, respectively.

## INTRODUCTION

Dead spot (*Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum, et M.E. Palm) primarily is a disease of newly constructed creeping bentgrass (*Agrostis stolonifera* L.) golf greens grown on sand-based root zones (Câmara et al., 2000; Dernoeden et al., 1999). The disease first was discovered on a Maryland golf course in 1998, and since has been found on creeping bentgrass putting greens in at least 11 states (Dernoeden et al., 1999; Kaminski and Dernoeden, 2002). The pathogen also attacks hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burt-Davy) putting greens in Florida and Texas (Kaminski and Dernoeden, 2002; Krausz et al., 2001).

On golf putting greens, dead spot may be active between May and December, but the pathogen is most severe during the summer months (June to August) in the mid-Atlantic region of the USA. As grass in the center of infection centers dies, depressions or pits often form which disrupt both the playability and aesthetic quality of the putting surface. Although *O. agrostis* infection centers remain relatively small (5 to 8 cm diameter), creeping bentgrass regrowth into diseased spots is slow. Hence, spots that have not fully recovered prior to winter remain visible until bentgrass growth resumes the following spring.

Limited information is available with regard to chemical and cultural management strategies for dead spot. Several fungicides are effective in reducing dead spot severity, when applied prior to the onset of disease symptoms (Towers et al., 2000; Wetzel and Butler, 2000; Wetzel and Butler, 2001). Chemical management of dead spot, however, is more difficult once symptoms are present and fungicides often must be applied every 7 to

10 days. Various nitrogen (N) sources have been effective for managing several turfgrass diseases. Ammonium-based N fertilizers help to lower soil pH, and have been shown to reduce the severity of several turfgrass diseases including spring dead spot (*Ophiosphaerella korrae* Walker and Smith), summer patch (*Magnaporthe poae* Landschoot and Jackson), and take-all (*Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *avenae* (E.M. Turner) Dennis) (Dernoeden, 1987; Dernoeden et al., 1991; Davidson and Goss, 1972; Smiley et al., 1992; Smith, 1956). Additionally, applications of manganese (Mn) with  $(\text{NH}_4)_2\text{SO}_4$  reduce the severity of take-all in creeping bentgrass (Hill et al., 1999). Conversely, some alkaline-reacting  $\text{NO}_3$ -based N-sources are thought to increase the severity of several turfgrass patch diseases (Smiley et al., 1992). In Kentucky bluegrass (*Poa pratensis* L.), applications of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{NaNO}_3$  generally were associated with increased summer patch severity (Hill et al., 2001; Thompson et al., 1995). The severity of Microdochium patch (*Microdochium nivale* (Fr.) Samuels and Hallett) also was shown to increase with increasing soil pH (Smith, 1958).

The influence of various N-sources, Mn and pH on dead spot incidence, severity and recovery is unknown. Therefore, the primary objectives of this study were to: 1) determine the influence of several N-sources on creeping bentgrass recovery from dead spot; 2) determine curative and preventive effects of N-sources and Mn on dead spot incidence and severity; and 3) elucidate the role of pH on dead spot severity. Secondary objectives were to evaluate the influence of the N-sources on turfgrass quality and other potential pest problems.

## MATERIALS AND METHODS

Two field studies were conducted at the University of Maryland Paint Branch Turfgrass Research Facility located in College Park, MD. In August 1999, a research putting green was constructed to United States Golf Association (USGA) specifications (USGA Green Section Staff, 1993). The soil was a modified sand mix (97% sand, 1% silt, and 2% clay) with a pH of 6.9 and 10 mg organic matter g<sup>-1</sup> of soil. Soil P (42 to 79 kg ha<sup>-1</sup>) and K (4 to 16 kg ha<sup>-1</sup>) levels varied throughout the three years and generally were low to moderate. Turf was 'L-93' creeping bentgrass and was maintained as a golf course putting green. Turf was mowed with a walk-behind mower to a height of 4.0 to 5.0 mm at least 3 times week<sup>-1</sup>. The area routinely was vertical mowed and topdressed in 2000 and 2001. A detailed description of all chemical and cultural practices can be found in Appendix A. The study areas were irrigated as needed to prevent drought stress.

### *Autumn Recovery-Study I*

In study I, the effect of various N-sources and iprodione (3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide) on the autumn recovery of dead spot was assessed. Dead spot was monitored between September 2000 and July 2001 following seeding of 'L-93' creeping bentgrass on 20 September 1999. Six N-sources were evaluated including isobutylidene diurea (31-0-0); sulfur coated urea (29-0-0; Lesco Elite, Strongsville, OH); urea (46-0-0); (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (21-0-0); methylene urea (40-0-0); and Ringer Greens Super (10-2-6) (Ringer Corporation, Minneapolis, MN). Isobutylidene diurea (IBDU), sulfur-coated urea (SCU) and methylene urea are synthetic slow release organic N-sources that contain 4.6%, 7.3% and 25.5% water-soluble

nitrogen (WSN), respectively. Urea is a quickly available synthetic organic N-source. Ammonium sulfate is a quickly available inorganic source of N. Ringer Lawn Restore is a slowly available natural organic N-source that contains 1.5% WSN.

In 2000, treatments were applied about every two weeks beginning 6 September 2000 at a rate of 24 kg N ha<sup>-1</sup> and a total of 120 kg N ha<sup>-1</sup> was applied by 3 November 2000. Prior to initiation of fertility and fungicide treatments, the area was fertilized with 123 kg N ha<sup>-1</sup> from urea. Each main plot (i.e., fertilizer treatment) was split and received either iprodione (6.1 kg a.i. ha<sup>-1</sup>) or no fungicide treatment, except for those mentioned below. Iprodione was included to determine if a fungicide effective in managing dead spot would assist in speeding turf recovery (Wetzel and Butler, 2001). Iprodione was applied every two weeks between 11 September and 3 November 2000. In spring 2001, dead spot activity within previously infected spots was minimal and thereafter iprodione treatments were deleted from the study. Fertilizer treatments initially were applied at 49 kg N ha<sup>-1</sup> on 1 May 2001. Thereafter, treatments were applied at 24 kg N ha<sup>-1</sup> on 22 May, 8 June and 13 July for a total of 120 kg N ha<sup>-1</sup> in 2001. Due to the declining turfgrass quality in plots receiving no fertilizer, untreated plots received N applications (24 kg N ha<sup>-1</sup>) from a starter (19N-25P<sub>2</sub>O<sub>5</sub>-5K<sub>2</sub>O) fertilizer (Contec<sup>®</sup>, Scott's Co., Marysville, OH) on 22 May and 8 June and from Jack's Classic Interiorscape Design (21-7-16: J.R. Peters Inc., Allentown, PA) on 13 July. The starter fertilizer contained 4.3% ammoniacal N, 7.4% urea N, 6.3% other WSN, 1.0% water-insoluble N and 1.8% combined S and Jack's Classic contained 4.8% ammoniacal N, 10.1% urea N, and 6.1% nitrate N, respectively. Additionally, 24 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> from 0-46-0 were applied to the entire study area on 29 May 2001. All soluble fertilizers (i.e., urea, ammonium sulfate,

and Jack's Classic) and fungicide treatments were applied with a CO<sub>2</sub> pressurized (262 kPa) sprayer calibrated to deliver 1016 L ha<sup>-1</sup> water. All other fertilizers were applied manually using a shaker bottle. Plots were irrigated immediately after each fertilizer application.

Fenarimol ( $\alpha$ -(2-chlorophenyl)- $\alpha$ -(4-chlorophenyl)-5-pyrimidinemethanol) and triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone) were used to control dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett) based on studies by Wetzel and Butler (2000 and 2001) and Towers et al. (2000) showing that they had little or no activity on dead spot. On 15 September 2000, non-fungicide-treated plots received an application of fenarimol (0.37 kg a.i. ha<sup>-1</sup>). In addition, the entire area was treated with 1.5 kg a.i. ha<sup>-1</sup> triadimefon and 0.37 kg a.i. ha<sup>-1</sup> fenarimol + 6.1 kg a.i. ha<sup>-1</sup> chlorothalonil (tetrachloroisophthalonitrile) on 20 October 2000 and 10 November 2000, respectively.

Main plots measured 1.5 by 3.0 m, and were arranged as a randomized complete split-block design with four replications. A mix of tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran (1:1 v/v) inoculum, consisting of a single isolate (OpVA-1), was prepared using the method described by Kaminski et al. (2002). Approximately 0.5 g of inoculum was placed at the soil surface in a grid pattern spaced on 0.9 meter centers on 8 October 1999. The number of dead spot infection centers plot<sup>-1</sup> were counted. Due to variation in initial disease incidence among plots, data from each rating date were transformed as a percent of infection centers plot<sup>-1</sup> based on the initial rating date (13 September 2000) and these data represented dead spot recovery. Percent dead spot recovery and actual infection center data were analyzed

using PROC MIXED procedure (SAS Institute Inc., 2000). Additionally, infection center data from each year were used to determine the area under the disease progress curve (AUDPC). The AUDPC values were calculated using the formula:  $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$ , where  $i = 1, 2, 3 \dots n-1$ , where  $y_i$  is the number dead spot infection centers and  $t_i$  is the time of the  $i$ th rating (Campbell and Madden, 1990). The AUDPC data then were standardized by dividing the AUDPC value by the total time duration ( $t_n - t_1$ ) of the experiment (Fry, 1978). The dates used to calculate the AUDPC were collected between 13 September and 29 November 2000 and from 15 May to 24 July 2001.

In 2000 and 2001, turfgrass quality was rated every two weeks on a 0 to 10 visual scale where 0 = brown or dead turf, 8.0 = minimal acceptable quality for a creeping bentgrass putting green, and 10 = optimum uniformity, green color and texture. The putting green threshold for unacceptable levels of dead spot was  $\geq 1$  spot plot<sup>-1</sup>; hence, quality data disregarded the presence of *O. agrostis* infection centers. Therefore, quality ratings were designed primarily to determine the impact of repeated applications of an N-source on turf color and texture. Quality data were seasonally combined as follows: winter = January-March; spring = April-June; summer = July-September; and autumn = October-December.

### ***Curative Management and Recovery-Study II***

Study II was conducted on a separate area seeded in 1999 on the aforementioned USGA research green. In study I, mostly slow release N-sources were evaluated in what was to be a long term study. The decline of dead spot in the second year precluded further data collection. In study II, only water soluble N-sources were evaluated to

determine if acid or alkaline reacting fertilizer varied in their ability to promote bentgrass recovery from dead spot. In study II, the fertilizers were applied at low rates generally used by superintendents in the summer. In the autumn, higher rates of N were applied. Due to a dramatic decrease in disease activity in 2001, the site was fumigated. Prior to fumigation the area was vertically mowed in three directions and core aerated to a depth of 7.6 cm with 12.5 mm diameter hollow tines. On 23 August 2001, a 0.06 ha area was fumigated with methyl bromide (98% methyl bromide + 2% chloropicrin) and seeded to 'L-93' creeping bentgrass on 30 August. The aforementioned starter fertilizer (19-25-5) was applied at 196 kg N ha<sup>-1</sup> between September and November. In 2002, the area received 110 kg N ha<sup>-1</sup> from urea prior to treatment initiation. On 21 March 2002, the area was inoculated with two isolates (OpOH-1 and OpVA-1) of *O. agrostis* on 1.5 m centers as previously described above.

The effect of N-sources and Mn on dead spot incidence and severity was assessed between June 2002 and November 2003. Five water-soluble N-sources were assessed including Ca(NO<sub>3</sub>)<sub>2</sub> (15.5-0-0); KNO<sub>3</sub> (13-0-44); (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (21-0-0); urea (46-0-0); and a complete fertilizer (20-20-20, Nutriculture; Plant Marvel Laboratories, Inc., Chicago Heights, IL). The complete fertilizer contained 3.87% ammoniacal, 5.87% nitrate, and 10.26% urea N-sources. The N-sources were applied beginning 28 June 2002. In addition, Mn (MnSO<sub>4</sub>; VWR scientific, West Chester, PA) was applied alone or in combination with urea. Dead spot was active prior to initiation of the study.

In 2002, the N-sources were applied weekly at a rate of 4.9 kg N ha<sup>-1</sup> between 28 June and 17 August. On 26 August, 4, 13, and 30 September and 21 October 2002, N was applied at 12 kg N ha<sup>-1</sup>. In 2003, N was applied about every two weeks at 12 kg ha<sup>-1</sup>

between 29 April and 11 July. On 18 and 28 July 2003, fertilizer treatments were applied at a rate of 4.9 kg N ha<sup>-1</sup>. On 13 and 27 September 2003, N-sources were applied at 12 kg N ha<sup>-1</sup>. The final fertilizer application was 24 kg N ha<sup>-1</sup> and was applied on 19 November 2003. Total amounts of N applied were 99 and 132 kg N ha<sup>-1</sup> in 2002 and 2003, respectively. On all application dates, Mn was applied at a rate of 2.0 kg ha<sup>-1</sup>. All fertilizer and Mn treatments were applied as described previously. Only N treatments in which  $\geq 12$  kg N ha<sup>-1</sup> were applied were immediately watered-in to prevent burning.

Due to periodic epidemics of dollar spot, triadimefon was applied as needed throughout the study. On three dates (13 September 2002 and 14 May and 9 June 2003), however, fungicide applications (chlorothalonil and propiconazole [1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1H-1,2,4-triazole]) that impact dead spot severity were mistakenly applied to the site. All materials were applied as described previously.

Dead spot incidence was rated by counting the number of infection centers plot<sup>-1</sup>. Between 2 August and 31 October 2002, dead spot recovery was assessed on a 0 to 100% scale, where 0 = plots free of dead spot infection centers and 100 = entire plot area covered with dead spot infections. In second year following initial infection (30 May to 29 July 2003), dead spot recovery was rated on a 0 to 5 scale in which 0.0 = infection centers completely healed, 3.0 = bare spots with visible bentgrass encroachment and 5.0 = bare spots with no visible bentgrass encroachment. In the second year, recurring dead spot infection centers also were counted between 21 August and 7 October 2003. The AUDPC was determined as previously described. Data used to calculate the AUDPC were collected between 27 July and 31 October 2002 and between 26 March and 23

September 2003. For new infection centers appearing in late summer of 2003, AUDPC was calculated from data collected between 21 August and 7 October.

In 2002 and 2003, turfgrass quality (dead spot disregarded) was rated approximately every two weeks as previously described. Ratings also were made on several other pests and stress problems in 2003. Yellow patch (*Rhizoctonia cerealis* Van der Hoeven) and algae (blue-green, filamentous species unknown) were rated visually on a linear 0 to 100% scale as previously described. Sod webworm (*Crambus* spp.) damage was rated by counting the number of damaged spots plot<sup>-1</sup>.

Soil and mat pH were determined one month after treatment initiation (26 July 2002) and also after the recurrence of dead spot in the second year (29 August 2003). To measure pH, four soil cores (19 mm diameter) were removed from each plot and immediately processed. All green leaf tissue was removed and the 0 to 2.5 cm organic matter zone (organic matter plus roots and sand or mat layer) from each core was separated into small pieces by hand. Similarly, the 2.6 to 5.0 cm soil zone was separated. For each replicate, the four cores at each depth were combined and a total of 10 g were removed from the total sample and placed into a small plastic cup. When all samples were weighed, 20 ml ddH<sub>2</sub>O were added to each cup and allowed to set for 30 minutes. After 30 minutes, cups were briefly agitated and allowed to set for an additional 30 minutes prior to measuring pH. Following calibration, pH was measured using a Model 8000 pH meter (VWR, West Chester, PA). All data were subjected to analysis of variance using the PROC MIXED function of SAS (SAS Institute Inc., 2000) and means were separated using Tukey's protected least significant difference t-test ( $P \leq 0.05$ ).

## RESULTS AND DISCUSSION

### *Autumn Recovery-Study I*

**Bentgrass Recovery From Dead Spot.** In 2000, dead spot symptoms first appeared on 8 June and the disease was allowed to progress throughout the area prior to treatment initiation on 6 September. Although few new infection centers occurred after treatment initiation, *O. agrostis* infection centers remained active into November 2000. Dead spot incidence in 2001 was minimal and only a few active infection centers were present throughout the study site. Inactive infection centers from 2000, however, continued to recover throughout late spring and early summer of 2001. There were no fertilizer by fungicide interactions and the main effect of fungicide was not significant on any rating date. The main effect of fertilizer on the number of infection centers plot<sup>-1</sup> and percent change in the number of infection centers (percent dead spot recovery), however, were significant on several rating dates.

In 2000, the number of infection centers plot<sup>-1</sup> (AUDPC = 12 to 30 infection centers plot<sup>-1</sup> day<sup>-1</sup>) was similar among the fertilizer treatments on all rating dates (data not shown). There was an increase in the percent dead spot from the initial rating (13 September) in the untreated plots and in plots treated with either IBDU or SCU on 6 October (Table 1). On 6 October (1 month after the initial fertilizer application), plots treated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> had the greatest reduction (30%) in dead spot. Dead spot reduction in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-treated plots, however, was not significantly different from the reductions observed in plots treated with methylene urea, Ringer Greens Super, urea or SCU on 6 October. By 29 November, dead spot recovery was observed in all plots treated with N; however, the percent change in plots treated with IBDU was not

significantly different from the untreated control. There were no differences in AUDPC values among any treatments between 13 September and 29 November 2000 (data not shown).

There was no reactivation of dead spot in the study site in 2001. Bentgrass recovery from the infection centers that developed in 2000, however, was minimal until temperatures increased in early June 2001. On 15 May, there were no differences in dead spot recovery among plots receiving N (12 to 48% recovery plot<sup>-1</sup>), when compared to the unfertilized control (5% recovery plot<sup>-1</sup>). Despite the application of N to untreated control plots on 22 May 2001, little recovery (18%) was observed in these plots by 18 June (Table 1). All plots treated with N, however, exhibited a large reduction in dead spots with the number of infection centers ranging from 3 to 8 plot<sup>-1</sup> and percent recovery  $\geq 60\%$ . Although not statistically significant, ammonium sulfate-treated plots continued to exhibit the greatest recovery and by the final rating date (24 July) there were no visible dead spots in those plots. By 24 July, the application of N to turf in the untreated control plots appeared to result in moderate levels of disease recovery (42%), and those plots had 16 dead spots plot<sup>-1</sup> by the end of the study. Similar to 2000, the AUDPC values from data collected between 15 May and 24 July 2001 revealed no differences in total disease among fertilizer treatments. All treatments receiving N between September 2000 and 13 July 2001, however, resulted in a reduction in total number of dead spots, when compared to the untreated control.

Although dead spot remained active until November 2000, iprodione applications did not have an impact on dead spot recovery. Iprodione has been used with varying levels of success with respect to dead spot management. Wetzels and Butler (2001)

reported excellent preventive dead spot control with iprodione ( $3.1 \text{ kg a.i ha}^{-1}$ ) applied on a 14 day schedule. Towers et al. (2000) found that iprodione provided only fair disease control when applied at a similar rate and spray interval as used by Wetzel and Butler (2001). Curative dead spot control with iprodione, however, was less effective, even when applied at  $6.1 \text{ kg a.i. ha}^{-1}$  on a 7 day interval (Wetzel and Butler, 2000). In this study, iprodione may have had little or no curative activity or it was too late to use the fungicide after numerous infection centers were allowed to develop during the summer. As previously observed, dead spots were very slow to recover as stolon growth into the center of infected spots appears to be inhibited (Kaminski and Dernoeden, 2002). The use of fungicides may be unnecessary once the development of new infection centers slows. While  $(\text{NH}_4)_2\text{SO}_4$  was somewhat more effective in promoting recovery, plots treated with IBDU exhibited low levels of recovery on several rating dates that were similar to unfertilized plots.

**Quality.** Creeping bentgrass quality (dead spot injury not included in ratings) was assessed between October 2000 and August 2001. In the autumn (i.e., October to December), excellent quality was observed in plots treated with  $(\text{NH}_4)_2\text{SO}_4$  and urea (Table 2). Additionally, applications of methylene urea or SCU resulted in acceptable bentgrass quality ( $\geq 8.0$ ). Plots treated with IBDU and Ringer Greens Super generally had good quality ( $\geq 7.0$ ) in the autumn of 2000. Quality ratings in unfertilized plots quickly declined between October and December and on average were extremely poor. In 2001, winter quality of all plots was poor and no treatments reached acceptable quality until summer (July to September). During the summer, overall quality of creeping bentgrass treated with  $(\text{NH}_4)_2\text{SO}_4$  was excellent, while plots treated with urea generally

were good. Plots treated with all other fertilizers exhibited poor quality for the remainder of the study. Among fertilizer treatments, IBDU and Ringer Lawn Restore provided unacceptable quality in all seasons.

### ***Curative Management and Recovery-Study II***

Slow and rapid release N-sources were applied in study I after the majority of new infection centers had appeared and increases in patch diameter had slowed. In study II, water soluble N-sources were evaluated in frequent and low rate applications in a foliar feeding program to determine if this would be a more efficient method of suppressing dead spot severity and promoting disease recovery.

**Dead Spot Incidence.** This site was fumigated on 23 August 2001 and seeded with 'L-93' creeping bentgrass one week later (i.e., 30 August). *O. agrostis* infection was first noticed on 12 May 2002 and treatments were initiated on 28 June. On 27 June, there was an average of 21 *O. agrostis* infection centers plot<sup>-1</sup>. The number of infection centers continued to increase throughout the summer, with most dead spots appearing by early to mid-August. By late August, the bentgrass in all plots had begun to recover, and there were no differences in dead spot frequency among treatments. On 4 September, plots treated with KNO<sub>3</sub> had the fewest number of infection centers (42 plot<sup>-1</sup>), but disease levels were similar among all plots in which N was applied (Figure 1). Disease levels continued to decrease in all fertilized plots (except Mn alone) until mid-September. Between 17 and 30 September, the number of infection centers plot<sup>-1</sup> again increased in all fertilized plots. During this period, plots treated with KNO<sub>3</sub>, urea, or urea + Mn had an increase of 14, 11 and 8 infection centers plot<sup>-1</sup>, respectively. Conversely, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-

treated plots only had an increase of one infection plot<sup>-1</sup>. Thereafter, the most rapid recovery occurred in plots receiving N, regardless of source. On the final 2002 rating date (31 October), plots treated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> had the fewest number of infection centers (15 plot<sup>-1</sup>) (Figure 1). Between 27 July and 31 October 2002, standardized AUDPC values were greatest ( $P=0.0083$ ) in unfertilized plots (86 plot<sup>-1</sup>) and least (47 to 52 plot<sup>-1</sup>) in plots treated either with KNO<sub>3</sub> or ammonium sulfate (data not shown). Total disease in all other plots receiving N averaged between 57 and 62 dead spots plot<sup>-1</sup>, and there were no significant dead spot differences among these N-source treatments.

In 2003, College Park, MD experienced excessively wet weather conditions and moderate temperatures throughout the spring and early summer. As a result, there was no reactivation of dead spot at this time. Recovery of dead spots from the previous year was slow and few differences among N-sources existed until mid-June (Figure 1; all data not shown). Between 12 June and 12 July, the number of infection centers in 20-20-20-treated plots declined greatly from 23 to 5 dead spots plot<sup>-1</sup>. After 12 July, diseased spots continued to heal in all plots receiving N and no differences were observed among fertilizer treatments. Standardized AUDPC values (26 March to 23 September) were highly significant ( $P=0.0001$ ) and ranged from 13 to 55 spots plot<sup>-1</sup> (data not shown). Total disease was greatest in unfertilized and Mn alone (50 to 55 plot<sup>-1</sup>) plots, but again no differences existed among plots (13 to 24 plot<sup>-1</sup>) in which N was applied.

Although recovery of inactive diseased spots was occurring, new infection centers appeared in mid-August 2003. Between 21 August and 27 September, new infection centers developed in plots treated with Ca(NO<sub>3</sub>)<sub>2</sub>, KNO<sub>3</sub>, urea, urea + Mn, and 20-20-20 (Table 3). No new or reactivated dead spots were observed in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-treated plots on

any rating date. Although quality was extremely poor and turf density declined, no new infection centers were observed in unfertilized plots or those plots receiving only Mn. Except on 21 August, plots treated with  $\text{KNO}_3$  had the greatest number of new *O. agrostis* infection centers. On 21 August, plots treated with  $\text{Ca}(\text{NO}_3)_2$  had the greatest number of *O. agrostis* infection centers, but the number of infection centers was similar to those plots treated with  $\text{KNO}_3$ , urea and urea + Mn. Dead spot incidence in 20-20-20-treated plots was delayed and no new infection centers were present until 23 September. After 23 September, *O. agrostis* infection centers in the 20-20-20-treated plots increased dramatically and by 27 September the number of centers was similar to all treatments in which dead spot recurred. After 27 September, the number of infection centers began to decrease. Total disease (AUDPC) during the period of reactivation was greatest in plots treated with either  $\text{Ca}(\text{NO}_3)_2$  or  $\text{KNO}_3$ . Moderate reactivation occurred in plots receiving urea or urea + Mn and disease severity in these plots was similar to the aforementioned  $\text{NO}_3$ -N-treated plots. Manganese did not reduce dead spot incidence when applied with urea.

**Percent Dead Spot.** Dead spot severity and recovery were visually rated on a 0 to 100 percent scale in 2002 and on a 0 to 5 recovery scale in 2003. There were no differences in the percent of plot area diseased (1.5 to 5.1%) among treatments between 2 and 16 August 2002 (data not shown). The impact of N-applications on dead spot, however, was apparent between August and October. During this period, percent area diseased decreased or remained relatively stable within plots receiving N, while the percent plot area diseased continued to increase in all plots not receiving N (Figure 2). Creeping bentgrass treated with  $(\text{NH}_4)_2\text{SO}_4$  had the least amount of disease (1%) prior to

winter.

Despite the application of N throughout the spring and summer in 2003, slow recovery was observed in most plots throughout the year. Between 19 May and 7 August, plots treated with  $(\text{NH}_4)_2\text{SO}_4$  generally exhibited the greatest bentgrass recovery (Table 4; all data not shown). On five out of eight rating dates (19 and 30 May, 6 and 12 June, and 12 July),  $\text{Ca}(\text{NO}_3)_2$ -treated plots had significantly less bentgrass regrowth, when compared to dead spots in plots treated with  $(\text{NH}_4)_2\text{SO}_4$ . Based on a playability threshold for a creeping bentgrass green, acceptable dead spot levels ( $\leq 1.0$  spot per plot) were observed in  $(\text{NH}_4)_2\text{SO}_4$ -treated plots by 30 May;  $\text{KNO}_3$  and 20-20-20 plots by 26 June; urea + Mn-treated plots by 12 July; and  $\text{Ca}(\text{NO}_3)_2$  and urea plots by 29 July 2003. Little or no recovery was observed in unfertilized (unfertilized control and Mn alone) plots on any rating date.

**Bentgrass Quality.** Quality (dead spot disregarded) during summer and autumn 2002 generally was acceptable ( $\geq 8.0$ ) for all plots in which N was applied and few differences existed among the N-sources (Table 5). In spring 2003, bentgrass in all treatments had unacceptable quality. Plots treated with  $\text{KNO}_3$  had the highest spring quality (quality=7.9), whereas quality of plots treated with  $(\text{NH}_4)_2\text{SO}_4$  were poor (quality=6.7) due to a widening of the leaf blades and a reduction in color. Quality of creeping bentgrass treated with  $(\text{NH}_4)_2\text{SO}_4$ , however, was good throughout the summer. Although bentgrass quality varied by season and N-source, plots treated with  $\text{KNO}_3$  generally had the best quality throughout the study. Unfertilized plots (unfertilized control and Mn alone) had the poorest quality in all seasons.

**Soil and Mat pH.** Approximately one month after treatment initiation (26 July

2002), significant pH differences ( $P < 0.0001$ ) were observed in the 0 to 2.5 cm organic matter zone (i.e., mat) among the various fertilizer treatments. Plots receiving  $\text{Ca}(\text{NO}_3)_2$  and  $\text{KNO}_3$  generally exhibited the highest mat pH (pH=6.58 to 6.59) (Table 6). The mat pH in plots receiving urea+Mn (pH=6.49), however, were not significantly ( $P \leq 0.05$ ) different than the pH in the aforementioned plots. The lowest mat pH (6.24) was observed in plots receiving  $(\text{NH}_4)_2\text{SO}_4$ . Mat pH for the remaining fertilizer-treated plots was similar, and ranged from an average pH of 6.41 to 6.46.

On 29 August 2003, mat pH again was lowest in plots receiving ammonium sulfate (pH=5.57) and was greatest in plots receiving  $\text{NO}_3\text{-N}$  (pH=6.61 to 6.76) (Table 6). Except for ammonium sulfate-treated plots, all treatments had mat pH levels similar to the untreated control. Soil sampled at the 2.6 to 5.0 cm depth had pH levels higher than in the mat. In 2003, new dead spot infection centers began to develop on 21 August. Based on pH measurements on 29 August 2003 and dead spot infection centers at the time of peak disease activity in 2003 (27 September), there was a significant correlation between the number of new infection centers and mat ( $r=0.71$ ,  $P < 0.0001$ ) and soil ( $r=0.78$ ,  $P=0.0001$ ) pH. Among the N-fertilized plots, dead spot did not recur in plots with a pH below 5.91 and 6.54 in the mat and soil layer, respectively. Plots in which dead spot recurred, however, had pH values greater than 6.17 and 6.62 in the mat and soil layer, respectively. Smiley and Cook (1973) attributed the suppression of wheat take-all to acidification of the root zone through active plant uptake of  $\text{NH}_4$  and subsequent release of  $[\text{H}^+]$  from plant roots. Similarly, Hill et al. (2001) found a linear positive relationship between soil pH (0 to 2.5 and 2.6 to 5.0 cm depth) and summer patch severity of Kentucky bluegrass. It is likely that nitrification of  $\text{NH}_4\text{-N}$  by soil microbes

as well as release of  $[H^+]$  due to the plant uptake of  $NH_4$  played a significant role in the acidification of mat and soil in this study.

**Yellow patch, sod webworm and algae.** Various disorders were monitored in 2003. In March, a severe outbreak of yellow patch was observed in the study site. In plots treated with 20-20-20 and  $KNO_3$ , 75% and 44% of the bentgrass was blighted by the pathogen, respectively (Table 7). Moderate disease levels were observed in plots treated with  $Ca(NO_3)_2$ , urea, urea + Mn and in the untreated control. Very low levels of yellow patch were observed in plots treated with  $(NH_4)_2SO_4$  (Figure 3).

All fertilizer-treated plots had severe sod webworm damage, while little damage was observed in plots receiving no fertilizer on 2 May 2003 (Table 7). The greatest insect damage (45 to 46 spots  $plot^{-1}$ ) occurred in plots treated with  $KNO_3$  or  $Ca(NO_3)_2$ . A similar level of insect damage occurred in plots treated with 20-20-20. Among fertilized plots,  $(NH_4)_2SO_4$ -treated plots sustained the least webworm damage (17 spots  $plot^{-1}$ ), however, there were no significant differences among plots treated with  $(NH_4)_2SO_4$ , urea, urea + Mn and the unfertilized plots.

A blue-green, filamentous algae (unidentified) began to develop in the study site in early to mid-May 2003 following extended periods of overcast and rainy weather. Between 19 May and 13 September, the percentage of the plot area blackened by algae generally was greatest in plots treated with 20-20-20 (Table 7). By late August and into September, the level of algae blackening was similar in plots treated with  $MnSO_4$  alone, 20-20-20 and the unfertilized control. On all rating dates, no blackening was observed in  $(NH_4)_2SO_4$ -treated plots (Figure 4). A low level of algae was present in plots treated with

$\text{KNO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$ , urea, and urea + Mn. There were, however, no algal blackening differences among the aforementioned N-sources and plots treated with  $(\text{NH}_4)_2\text{SO}_4$ .

## CONCLUSION

**Study I.** Data revealed few differences in the ability of water soluble and water insoluble N-sources to enhance recovery of bentgrass in the autumn following heavy summer disease pressure. All N-sources aided in the recovery and healing of dead spot, but none provided for complete recovery before winter. Plots treated with  $(\text{NH}_4)_2\text{SO}_4$  and IBDU, however, were among the fastest and slowest to recover, respectively. Additionally, plots treated with  $(\text{NH}_4)_2\text{SO}_4$  generally were superior in quality to those plots received IBDU. The quality of bentgrass treated with predominantly water soluble nitrogen sources generally was superior to slow release N-sources. Iprodione failed to improve dead spot recovery when applications were initiated in late summer after most injury was evident. In this study, dead spot severity had peaked prior to the application of iprodione and few new infection centers appeared after early to mid-September. While fungicides may prevent infection, they likely have little impact on the recovery of existing dead spot patches. Therefore, this study indicated that fungicides may only be effective if applied preventively or early curative. In the mid-Atlantic region, this period would correspond with periods of increasing patch diameter and pseudothecia development (i.e., June through August) (Kaminski, Chapter II). For this reason, it appears more important to apply water-soluble N-sources rather than fungicides during the autumn months following peak dead spot activity.

**Study II.** Study II was initiated to assess the impact of five water soluble N-sources and Mn on preventing new *O. agrostis* infection centers and reducing dead spot severity. When applied during the early stages of symptom development, none of the water soluble N-sources prevented new infection centers from occurring and the number

of *O. agrostis* infection centers increased in all plots until mid-August 2002. Although new infection centers were minimal in late August and September, the percent of plot area diseased continued to increase in plots not receiving N. When compared to unfertilized plots, significantly less dead spot (percent and no. infection centers plot<sup>-1</sup>) was observed in all plots receiving N by early to mid September 2002. In the second year (2003), reactivation of previously infected dead spot patches was minimal. In August 2003, however, plots treated with KNO<sub>3</sub>, Ca(NO<sub>3</sub>)<sub>2</sub>, and urea exhibited a recurrence of new *O. agrostis* infection centers. The application of 20-20-20 delayed, but did not prevent new dead spot symptoms. Conversely, dead spot did not recur in unfertilized plots or plots receiving (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 2003. The number of dead spot infection centers plot<sup>-1</sup> in 2003 was positively correlated with pH and disease incidence appeared to be favored at a pH greater than 6.0 and 6.6 in the mat and soil, respectively. Furthermore, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> generally was most effective in reducing yellow patch and sod webworm damage as well as algal growth.

Applications of N-alone were not very effective in reducing the severity of active dead spot. In study I, plots treated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were fastest to completely recover. Ammonium sulfate also was the only N-source to prevent dead spot recurrence in the second year of study II. Because both studies were initiated after dead spot developed, it is unknown if any N-source would have reduced or enhanced the damage when applied preventively. Regardless, it would appear prudent to utilize (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the primary N-source during establishment. Due to the potential of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to lower pH to detrimental levels, soils tests should be conducted routinely and appropriate measures taken to correct pH extremes.

Table 1. Number of dead spot (*Ophiostoma agrostis*) infection centers and percent change in the original number of infection centers in 'L-93' creeping bentgrass as influence by six N-sources, 2000 to 2001.

Fertilizer	Percent change in no. dead spots plot <sup>ix</sup>						Dead spot infection centers plot <sup>i</sup>					
	2000			2001			2001			2001		
	6 Oct	3 Nov	29 Nov	15 May	18 June	24 July	15 May	18 June	24 July	AUDPC <sup>y</sup>		
Ammonium sulfate	-30 c <sup>z</sup>	-55 c	-59 b	-45 a	-90 c	-100 c	14 a	3 b	0 b	4 b		
IBDU	+24 ab	-14 a	-14 a	-12 a	-60 b	-83 b	19 a	8 b	3 b	7 b		
Methylene urea	-20 c	-44 bc	-45 b	-27 a	-73 bc	-95 bc	15 a	7 b	1 b	6 b		
Ringer Greens Super	-21 c	-22 ab	-43 b	-46 a	-72 bc	-89 bc	12 a	7 b	3 b	6 b		
Sulfur coated urea	+2 bc	-53 c	-51 b	-32 a	-78 bc	-89 bc	11 a	3 b	2 b	4 b		
Urea	-15 c	-48 bc	-43 b	-48 a	-82 c	-94 bc	12 a	4 b	2 b	4 b		
Untreated	+41 a	-3 a	+4 a	-5 a	-18 a	-42 a	26 a	25 a	16 a	20 a		

<sup>x</sup> The percent change of infection centers within each plot was based on the initial rating of the number of infection centers on 13 September 2000.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 15 May to 24 July 2001.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's protected least significant difference test.

Table 2. 'L-93' creeping bentgrass seasonal quality as influenced by six N-sources, 2000 to 2001.

Fertilizer	Quality <sup>x</sup>			
	2000	2001		
	Autumn	Winter	Spring	Summer
	0-10 scale <sup>y</sup>			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	9.4 a <sup>z</sup>	4.8 a	7.4 a	8.3 a
IBDU	7.6 d	4.4 ab	5.1 d	4.6 d
Methylene urea	8.7 b	4.1 bc	6.4 c	6.2 c
Ringer Greens Super	7.3 d	2.7 d	3.7 e	4.8 d
SCU	8.2 c	3.8 c	6.0 c	6.2 c
Urea	9.1 a	4.3 b	6.9 b	7.6 b
Untreated	5.2 e	0.2 e	1.3 f	5.2 d

<sup>x</sup> Quality ratings were averaged across seasons as follows: winter = January-March; spring = April-June; summer = July-September; and autumn = October-December.

<sup>y</sup> Creeping bentgrass quality was assessed visually on a 0 to 10 scale, where 0 = bentgrass brown or dead, 8 = minimum acceptable quality for a bentgrass putting green, and 10 = optimum density, green color and texture.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Table 3. Impact of various nitrogen-sources and manganese on the development of new *Ophiosphaerella agrostis* infection centers in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>x</sup>	New <i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>					AUDPC <sup>y</sup>
	21 Aug	13 Sept	23 Sept	27 Sept	7 Oct	
				no.		
Calcium nitrate	2.8 a <sup>z</sup>	2.3 ab	6.5 ab	12.5 a	9.0 ab	5.3 a
Potassium nitrate	1.8 a	3.8 a	9.8 a	15.3 a	13.3 a	6.9 a
Ammonium sulfate	0.0 b	0.0 b	0.0 c	0.0 b	0.0 c	0.0 c
Urea	1.8 a	1.5 ab	4.5 abc	9.3 ab	5.8 bc	3.6 ab
Urea + manganese	1.5 ab	2.3 ab	4.3 bc	6.8 ab	5.8 bc	3.4 abc
Manganese	0.0 b	0.0 b	0.0 c	0.0 b	0.0 c	0.0 c
20-20-20	0.0 b	0.0 b	1.0 c	6.3 ab	5.8 bc	1.7 bc
Unfertilized	0.0 b	0.0 b	0.0 c	0.0 b	0.0 c	0.0 c

<sup>x</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 21 August to 7 October 2003.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's protected least significant difference test.

Table 4. Impact of various nitrogen-sources and manganese on dead spot recovery in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>x</sup>	Dead spot recovery			
	30 May	26 June	12 July	29 July
	0-5 scale <sup>y</sup>			
Calcium nitrate	2.3 bz	1.1 b	1.4 b	0.9 b
Potassium nitrate	1.5 bc	0.9 b	0.8 c	0.4 bc
Ammonium sulfate	1.0 c	0.5 b	0.5 c	0.3 bc
Urea	2.0 b	1.3 b	1.0 bc	0.5 bc
Urea + manganese	2.0 b	1.0 b	0.6 c	0.3 bc
Manganese 20-20-20	4.1 a	4.4 a	4.3 a	3.9 a
Unfertilized	1.5 bc	0.9 b	0.5 c	0.1 c
	4.4 a	4.1 a	4.4 a	3.9 a

<sup>x</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dead spot severity was rated visually on a 0 to 5 scale; where 0.0 = infection centers completely healed, 3.0 = bare spots with visible bentgrass encroachment and 5.0 = bare spots with no visible bentgrass encroachment.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's protected least significant difference test.

Table 5. Impact of various nitrogen-sources and manganese on seasonal 'L-93' creeping bentgrass quality, 2002-2003.

Treatment	Quality <sup>x</sup>			
	2002		2003	
	Summer	Autumn	Spring	Summer
	0-10 scale <sup>y</sup>			
Calcium nitrate	8.2 ab <sup>z</sup>	8.1 b	7.4 ab	7.7 ab
Potassium nitrate	8.6 a	8.1 b	7.9 a	8.1 a
Ammonium sulfate	7.9 b	9.3 a	6.7 c	8.3 a
Urea	8.5 ab	9.0 ab	7.2 bc	7.7 ab
Urea + manganese	8.6 a	8.8 ab	7.2 bc	7.9 ab
Manganese	6.4 c	4.9 c	2.2 d	2.8 c
20-20-20	8.5 ab	8.3 ab	7.3 bc	7.1 b
Unfertilized	6.3 c	4.8 c	1.9 d	2.1 c

<sup>x</sup> Quality ratings were combined as follows: spring = April-June; summer = July-September; and autumn = October-December.

<sup>y</sup> Creeping bentgrass quality was assessed visually on a 0 to 10 scale, where 0 = bentgrass brown or dead, 8 = minimum acceptable quality for a bentgrass putting green, and 10 = optimum density, green color and texture.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's protected least significant difference test.

Table 6. Impact of various nitrogen-sources and manganese on mat and soil pH in an 'L-93' creeping bentgrass putting green, 2002-2003.

Treatment <sup>x</sup>	pH		
	26 July 2002	29 Aug 2003	
	Mat <sup>y</sup>	Mat	Soil
Calcium nitrate	6.58 a <sup>z</sup>	6.61 ab	6.89 ab
Potassium nitrate	6.59 a	6.76 a	7.02 a
Ammonium sulfate	6.24 c	5.57 d	6.36 c
Urea	6.42 b	6.44 bc	6.78 ab
Urea + manganese	6.49 ab	6.29 c	6.71 b
Manganese	6.44 b	6.50 abc	6.84 ab
20-20-20	6.46 b	6.32 c	6.72 b
Unfertilized	6.41 b	6.51 abc	6.81 ab

<sup>x</sup> Nitrogen treatments were applied as follows: 5 kg N ha<sup>-1</sup> on 28 June; 5, 12, 19 and 27 July; and 2 10 and 17 August and 12 kg N ha<sup>-1</sup> on 26 August; 4, 13 and 30 September; and 21 October 2002 and 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Measurements of pH were made at the 0-2.5 cm depth (mat) and 2.6-5.0 cm depth (soil).

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's protected least significant difference test ( $P < 0.0001$ ).

Table 7. Impact of various nitrogen-sources and manganese on various disorders in a 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>z</sup>	Yellow patch		Sod webworm		Algae		
	26 Mar	%	2 May	No. spots plot <sup>-1</sup>	29 July	7 Aug	29 Aug
Calcium nitrate	21 c <sup>y</sup>		46 a	0.2 b	0.0 b	1.4 b	5.6 b
Potassium nitrate	44 b		45 ab	0.0 b	0.0 b	0.8 b	1.1 b
Ammonium sulfate	4 d		17 cd	0.0 b	0.0 b	0.0 b	0.0 b
Urea	25 b		25 bcd	0.2 b	1.0 b	1.4 b	2.5 b
Urea + manganese	25 b		21 cd	0.5 b	0.1 b	2.6 b	3.1 b
Manganese	18 cd		4 d	0.9 b	2.0 b	13.0 a	17.5 a
20-20-20	75 a		31 abc	7.3 a	7.3 a	21.0 a	17.5 a
Unfertilized	22 b		4 d	0.3 b	0.3 b	14.0 a	21.3 a

<sup>z</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

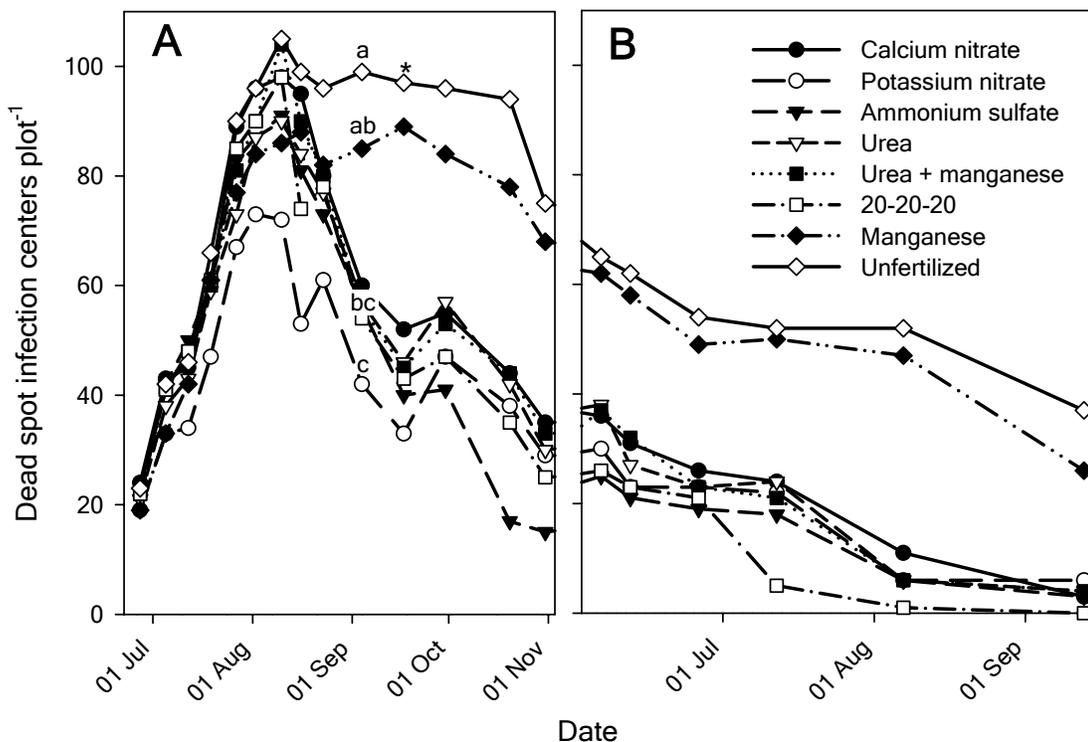


Figure 1. Impact of various nitrogen (N)-sources and manganese on dead spot incidence and bentgrass recovery in an 'L-93' creeping bentgrass putting green, 2002 (A) and 2003 (B). Significant differences ( $P \leq 0.05$ ), according to Tukey's protected least significant difference test, among treatments first appeared on 4 September 2002 and are indicated by different letters. There were no differences among N-sources on any rating date between 17 September 2002 (\*) and the final rating date (23 September 2003). During the aforementioned period, all N-sources reduced dead spot severity, when compared to plots not receiving N (unfertilized and manganese).

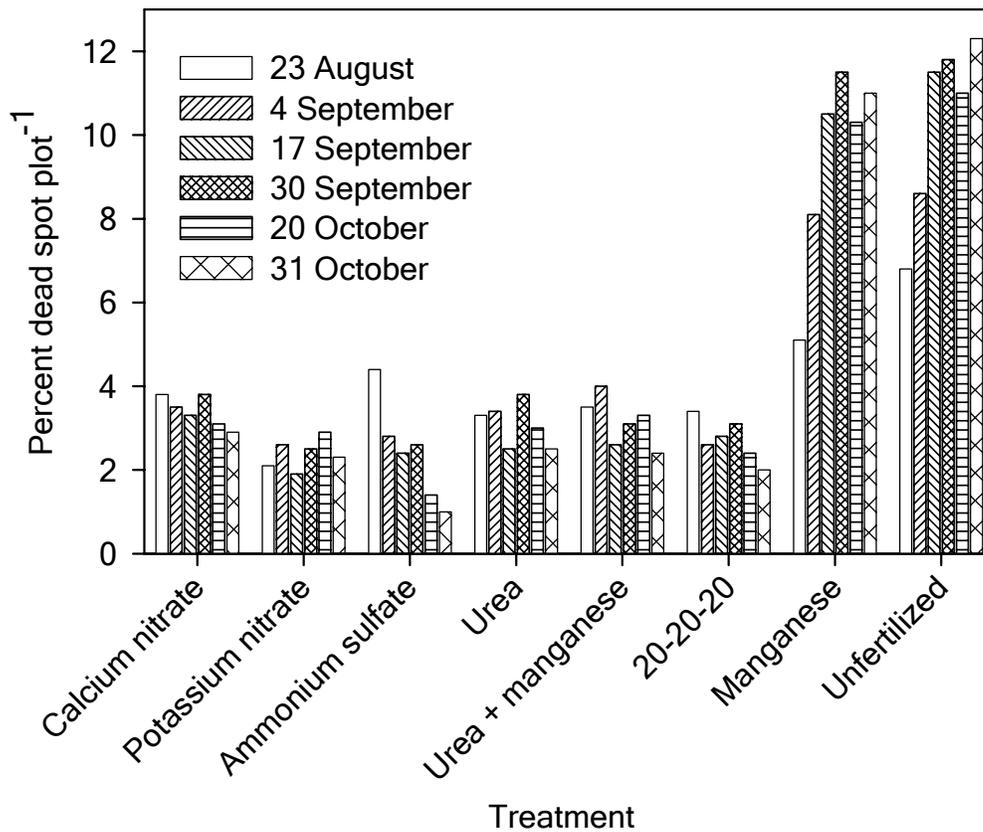


Figure 2. Impact of various nitrogen (N)-sources and manganese on percent of plot area damaged by dead spot in an 'L-93' creeping bentgrass putting green, 2002. After 23 August, all plots in which N was applied had significantly less dead spot ( $P \leq 0.0001$ ), when compared to the unfertilized control or manganese alone. There were no differences in percent dead spot among plots receiving N on any rating date.



Figure 3. Plots treated with ammonium sulfate incurred little damage from yellow patch (*Rhizoctonia cerealis*). Photo taken 31 March 2003.



Figure 4. Algae was not observed on any rating date in plots treated with  $(\text{NH}_4)_2\text{SO}_4$ . Photo taken 13 September 2003.

## VI. GENETIC VARIATION AMONG *OPHIOSPHAERELLA AGROSTIS* ISOLATES USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM DNA FINGERPRINTING

### SYNOPSIS

Dead spot (*Ophiосphaerella agrostis*) is a relatively new disease of young creeping bentgrass and hybrid bermudagrass putting greens in the USA. *O. agrostis* is unusual in that it produces prodigious numbers of pseudothecia in the field throughout the summer months and has no known asexual stage. The objectives of this study were to: 1) identify genetic diversity among *O. agrostis* isolates using fluorescent AFLP DNA fingerprinting; 2) re-examine colony color and other characteristics that may distinguish isolates; and 3) determine individual isolates' capacity for and rate of pseudothecia production. A total of 77 *O. agrostis* isolates were collected from twenty-one different creeping bentgrass or hybrid bermudagrass putting greens in eleven states. From all isolates evaluated, 78 out of 97 markers were polymorphic (80.4%) and provided 57 unique profiles. Genetic variation of *O. agrostis* was diverse and separated into three distinct clades with  $\geq 69\%$  similarity in an unweighted pair-group with arithmetic mean analyses. Analysis of molecular variance indicated that *O. agrostis* isolates collected from each state and the ability to produce pseudothecia were the best indicators for genetic similarity. Colony color varied among the isolates, but generally was similar for isolates residing within clade B or clade C. Colony color of isolates within clade A appeared to be a mixture of the colony colors exhibited by clades B and C.

## INTRODUCTION

Dead spot is a new disease of young creeping bentgrass (*Agrostis stolonifera* L.) putting greens in the USA. The disease is incited by *Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum, et M.E. Palm, a previously undescribed pathogen that first was isolated in 1998 from a golf course in Maryland (Câmara et al., 2000; Dernoeden et al., 1999). Since its description, *O. agrostis* has been found in at least 13 states and has been isolated from several additional turfgrass species including hybrid bermudagrass (*Cynodon dactylon* [L.] Pers. x *C. transvaalensis* Burt-Davy) and colonial (*A. capillaris* L.) and velvet (*A. canina* L.) bentgrasses (Kaminski and Dernoeden, 2002; Kaminski et al., 2002; Krausz et al., 2001).

Dead spot generally appears within 1 to 2 years following establishment of creeping bentgrass putting greens. In addition, the disease may occur on older putting surfaces following fumigation with methyl bromide. In the mid-Atlantic region of the USA, disease symptoms may appear as early as May and disease activity often peaks between July and August. Initially, the disease appears as small, copper or reddish-brown spots approximately 1 to 2 cm in diameter that may increase to approximately 6 to 8 cm throughout the summer months. Once infection occurs, turfgrass in the center of dead spots dies and often forms pits or depressions, which adversely affect the putting surface. Regrowth of bentgrass in infected spots is slow and dead spots often remain present throughout the winter until bentgrass growth resumes in the spring.

Other species within the genus *Ophiosphaerella* [*O. herpotricha* J. Walker; *O. korrae* Walker and Smith (synonym *Leptosphaeria korrae*); and *O. narmari* Walker and Smith (synonym *L. narmari*)] cause various turfgrass diseases (Crahay et al., 1988;

Dernoeden et al., 1995; Endo et al., 1985; Landschoot, 1996; Smith, 1965; Tisserat et al., 1989; Wetzel et al., 1999; Worf et al., 1986). The aforementioned *Ophiosphaerella* spp., however, have not been reported to infect creeping bentgrass.

Unlike other *Ophiosphaerella* spp. found in association with turfgrasses, the production of pseudothecia by *O. agrostis* is common. Field observations revealed that *O. agrostis* is capable of producing prodigious numbers of pseudothecia in necrotic leaf, sheath, and stolon tissues (Dernoeden et al., 1999; Kaminski and Dernoeden, 2002). Growth chamber studies confirmed that pseudothecia development can occur within four days and that mature ascospores may be produced in as little as one week when subjected to a range of temperatures (13 to 28°C) and constant light (Kaminski et al., 2002). In a Maryland field study, pseudothecia developed in the greatest numbers during the summer months (June to August), but occasionally developed as late as September (Kaminski, Chapter III). Additionally, immature ascospores were found in small numbers within pseudothecia throughout the winter (Kaminski, Chapter II). The pathogens' proclivity to proliferate sexually during the summer months increases the chance for genetic recombination. The capability of *O. agrostis* to undergo numerous meiotic cycles in a single season ultimately may lead to increased pathogen fitness.

Genetic analyses, based on amplified fragment length polymorphism (AFLP) fingerprinting of *O. agrostis* isolates collected in 1998 revealed an 87% or greater similarity among isolates from 5 different states (Câmara et al., 2000). Previous studies revealed that a single isolate (OpOH-1) exhibited differences in colony color, sequence within the internal transcribed spacer (ITS) 2 region of ribosomal DNA, and fungicide sensitivity, when compared to the type species (Câmara et al., 2000; Kaminski, 2001).

Unlike the rose-quartz or pink colony color of most isolates collected in 1998, *O. agrostis* isolate OpOH-1 exhibited white to olive-gray mycelia when grown on potato dextrose agar (PDA) at 25°C (Câmara et al., 2000; Kaminski et al., 2002). Although within the range (75 to 150 µm) reported by Câmara et al. (2000) in the original description of *O. agrostis*, ascospores of isolate OpOH-1 produced on PDA were shorter (68 to 98 µm) and had fewer septations (6 to 8) than most other isolates (Kaminski, 2001). Additionally, rDNA sequencing of three *O. agrostis* isolates revealed a single base pair (bp) insertion of thymine nested between a cytosine and a five thymine repeat in two different locations of the ITS2 region in OpOH-1 (Câmara et al., 2000). Finally, *in vitro* experiments indicated reduced sensitivity of the isolate OpOH-1 to the fungicide iprodione (3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide) (Kaminski, 2001).

Additional AFLP fingerprinting from a larger population is needed to elucidate genetic diversity within *O. agrostis*. Since the initial description of the pathogen, additional isolates exhibiting varying colony characteristics have been collected from a broader geographic distribution and host range. The goal of this study was to identify variation within *O. agrostis* based on morphological, biological and molecular characters. The objectives of this study were to: 1) identify genetic diversity among *O. agrostis* isolates using fluorescent AFLP DNA fingerprinting; 2) re-examine colony color and other characteristics that may distinguish isolates; and 3) determine individual isolates' capacity and rate of pseudothecia production.

## MATERIALS AND METHODS

**Fungal Isolates.** Between 1998 and 2003, 77 *O. agrostis* isolates were collected from twenty-one different creeping bentgrass or hybrid bermudagrass putting greens in eleven states. Isolates from creeping bentgrass were collected from Maryland (MD) (n=52), Virginia (VA) (n=3), Ohio (OH) (n=1), New Jersey (NJ) (n=4), Pennsylvania (PA) (n=4), Massachusetts (MA) (n=3), New York (NY) (n=1), Illinois (IL) (n=5), North Carolina (NC) (n=1), and Michigan (MI) (n=2). Additionally, a single isolate from hybrid bermudagrass was collected from a research putting green at Texas A&M University in College Station, Texas (TX). The isolates were grown on PDA in a dark incubator maintained at 25°C for 10 days. Colony morphology of all isolates was described and photographed (Table 1). Prior to DNA extraction, isolates were stored in potato dextrose broth (PDB) or on PDA slants at 4°C or at -20°C in a 20% glycerol solution.

**DNA Isolation.** DNA was extracted from fungal cultures grown at room temperature for 7 to 10 days in 100 ml of PDB (24 g L<sup>-1</sup>) on a shaker table (LabLine Orbit Shaker, Lab-Line Instruments Inc., Melrose, IL) set to 90 rpm's. Cultures were filtered through Whatman #1 filter paper, rinsed in distilled, deionized H<sub>2</sub>O (ddH<sub>2</sub>O) and lyophilized in a Freezemobile 6 (The VirTis Company, Gardiner, NY) for 24 to 48 h. Freeze-dried mycelial mats were stored at -20°C or immediately prepared for DNA extraction. Approximately 20 to 30 mg of freeze-dried mycelia were placed in a microcentrifuge tube (VWR, West Chester, PA) and ground into a fine powder using a micro-pestle (VWR, West Chester, PA). Liquid nitrogen occasionally was used to aid in the grinding process.

Total genomic DNA was extracted using Qiagen Mini-Prep Kits (Qiagen Inc., Valencia, CA). A total of 400  $\mu$ l of Buffer AP1 and 4  $\mu$ l of RNase A stock solution was added to pulverized mycelia and vortexed. To lyse cells, the mixture then was incubated for 10 minutes at 65°C, mixing 2 to 3 times during incubation. To precipitate proteins and polysaccharides, 130  $\mu$ l of Buffer AP2 was added to the lysate and incubated on ice for 5 minutes. The tubes then were centrifuged for 5 minutes at 16000 rpm and the lysate was added to the supplied QIAshredder column. The material then was centrifuged for 2 minutes at 16000 rpm. The eluate material was placed in a 1.5 ml microcentrifuge tube and 675  $\mu$ l Buffer AP3/E was added. DNA was separated from the mixture by centrifugation in a DNeasy mini-spin column. Buffer AW (500  $\mu$ l) was added to the mini-spin column and centrifuged for 1 minute at 8000 rpm. An additional 500  $\mu$ l Buffer AW was added and the spin column membrane was dried by centrifuging for 2 minutes at 16000 rpm. For the final step, 50  $\mu$ l of the preheated (65°C) elution buffer was added, the column centrifuged for 1 minute at 8000 rpm, and the step repeated.

**Amplified Fragment Length Polymorphism Analyses.** Fluorescent AFLP with automated capillary electrophoresis (fAFLP-CE) was used to examine 77 *O. agrostis* isolates. DNA fragments were amplified by a multi-step procedure, originally proposed by Vos et al. (1995), and modified as follows. *O. agrostis* template DNA was digested with *Eco*RI and *Mse*I (New England BioLabs, Beverly, MA) and ligated to commercial *Eco*RI and *Mse*I oligonucleotide adapters (Applied Biosystems, Foster City, CA) in a single step with incubation overnight at room temperature. The procedure described previously was followed (Saunders et al., 2001; Saunders et al., 2002; Viji et al., 2004), with modifications as noted below. Commercial *Eco*RI and *Mse*I AFLP preselective

primers and AFLP core mix (Applied Biosystems) were used for the preselective amplification of the restricted and ligated fragments. The mixture was subjected to amplification at 94°C for 3 minutes followed by 20 cycles of the following profile: 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 minutes with a final hold of 60°C for 30 minutes (GeneAmp® 9700 PCR system, Applied Biosystems). For selective amplification, custom primers with a fluorescent WellRED™ D4 dye-labeled phosphoramidite (D4) added to the 5' end of the *EcoRI*+AG primer (Invitrogen™ Life Technologies, Carlsbad, CA) were used. No modifications were made to the *MseI*+C primer (Qiagen, Valencia, CA). The products from the preselective amplification were diluted as described previously and used as template DNA for selective amplification (Saunders et al., 2001). Labeled *EcoRI* primer (0.05 μM) and the unlabeled *MseI* primer (0.10 μM) were added to the template and the mixture was subjected to a thermocycling profile of 94°C for 2 minutes, followed by 10 cycles of 94°C for 20 sec, 1°C per cycle stepdown of annealing temperature from 66°C held for 30 sec and 72°C for 2 minutes. This was followed by 25 cycles of 94°C for 20 sec; 56°C for 30 sec and 72°C for 2 minutes; and a final hold of 60°C for 30 minutes.

Samples were prepared for capillary electrophoresis by diluting the final amplified product 1:30 (v/v) in commercial Sample Loading Solution (Beckman-Coulter, Fullerton, CA), which included 1% (v/v) Beckman-Coulter 420 bp DNA size standards. Fragment separation and detection were performed by capillary electrophoresis on each sample with a genetic analysis system (CEQ 8000™, Beckman-Coulter, Fullerton, CA), beginning with a 30 second electrophoretic injection at 2.0 kV, followed by a 35 minute separation at 50°C and 6.0 kV. Data were analyzed using CEQ 8000 fragment analysis

software with parameters that recognized peaks exceeding a height threshold of 10% of the height of the second largest peak and a slope threshold of 10. The latter parameter was determined by the minimum rate of signal increase relative to the baseline noise for the sample. A 95% confidence level was imposed upon size estimation. The size standard fit coefficient was 0.38 and used a cubic model. For AFLP analysis, maximum bin (fragment) width was 1.0 nucleotide. Peaks that clearly were present, but not recognized by the automated scoring, were added manually. Minor peaks that appeared in most or all samples, but were recognized sporadically by the software, were manually removed from the analyses. Samples were scored as “1” if a fragment was present, and “0” if absent. In rare cases where two fragments were present in one bin, the bin was scored as “1” for that sample.

All data, including non-polymorphic bins, were imported into the multivariate data analysis program, NTSYSpc, version 2.1 (Exeter Software, Setauket, NY). The module SimQual was used to generate a similarity matrix based on simple matching coefficients, and the module SAHN was used to perform hierarchical clustering using an unweighted pair-group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973). The tree matrix generated by SAHN was converted into a dendrogram using the module Tree Plot. Grouping of isolates into distinct clades was confirmed using the neighbor joining (NJ) method (Saitou and Nei, 1987).

Analysis of molecular variance (AMOVA) in GenALEX was calculated to determine genetic similarity among isolates based on certain characteristics, including isolate color, geographic state and region, and pseudothecia production (Peakall and Smouse, 2001). The AMOVA can be used to determine if a specific observable

characteristic would predict genetic similarity. Within each categorical class, the AMOVA compares the genetic distances among individuals sharing similar characteristics to the genetic distance among individual isolates outside of their type. Pair-wise genetic distances for AMOVA were calculated as described by Huff et al. (1993) as  $D = n (1 - (2n_{xy}/2n))$ , where  $n_{xy}$  = the number of shared bands and  $n$  = total banding positions. The AMOVA partitions genetic distance variance among and within characteristic classes, and tests of significance were calculated using a 999 replication bootstrap. Greater genetic variation of isolates among classes when compared to genetic variation within a class, indicates genetic similarity. Only categorical classes containing 2 or more isolates were included in the AMOVA analyses.

**Pseudothecia Production.** The ability of an *O. agrostis* isolate to produce pseudothecia and its rate of pseudothecia development were used as a determinant of each isolate's ability to reproduce sexually. Pseudothecia were produced using the method developed by Kaminski et al. (2002). Briefly, a mix of tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran (1:1 v/v) was prepared by soaking tall fescue seeds in tap water overnight. Seeds then were rinsed three times and mixed with wheat bran (v/v), placed in 250 ml flasks, and autoclaved for 1 h on two consecutive days. Mycelia from the edge of an actively growing colony were removed and placed on the surface of the cooled seed/bran mix. Flasks then were incubated in a growth chamber under constant light ( $88 \mu\text{mol m}^{-2} \text{sec}^{-1}$  from four F20T12/CW, 20 watt bulbs (Philips Lighting, Somerset, NJ) at 25°C for 21 days. Inoculum was mixed every 1 to 3 days to promote aeration and to allow mycelium to become evenly distributed throughout the medium. After incubating for 21 days, approximately 0.20 g of the

infested mix was placed on sterile, moist filter paper (Qualitative 415, VWR Scientific, West Chester, PA) in a 60 by 15 mm Petri dish (VWR Scientific, West Chester, PA). Petri dishes containing infested media were placed in the aforementioned growth chamber. The mixture and filter paper were kept moist throughout the study. Every other day, seeds were rated visually for abundance of pseudothecia on a 0 to 5 linear scale where 0 = none, 3 = 11 to 15 and  $5 \geq 21$  pseudothecia with necks partially embedded in tall fescue seed. The experiment was arranged in a randomized complete block with 3 replications (growth chambers). Within each growth chamber, each isolate was replicated twice and mean pseudothecia rating data were combined prior to statistical analyses. Data from each rating date were analyzed using the MIXED procedure (SAS Institute Inc., 2000). Additionally, regression analyses were performed to determine the rate of pseudothecia production for each isolate.

## RESULTS AND DISCUSSION

**Fungal Isolates.** All isolates characterized in this study (n=77) were determined to be *O. agrostis* by the use of species-specific oligonucleotide primers developed previously (Kaminski, Chapter VII). Colony color varied when grown on PDA and incubated in the dark at 25°C for 10 days. Most isolates (n=52) exhibited the typical rose-quartz or pink colony color previously described (Dermeiden et al., 1999; Câmara et al., 2000; Kaminski, 2001; Kaminski et al., 2002) (Table 1, Figures 1 to 3). The Ohio isolate (OpOH-1) typified the olive-gray or buff colony color observed from several isolates (n=21). Three isolates (OpMD-12, OpNC-1 and OpNJ-6) separated into a third mycelial color class, which appeared predominantly gray. *O. agrostis* isolate OpMD-10, which appeared as rose-quartz after the initial isolation (26 October 1998), grew more appressed to the agar surface and was beige or buff in color when removed from storage for DNA isolation (Figure 2).

**Amplified Fragment Length Polymorphism Analyses.** Amplification of *O. agrostis* DNA using the primers *EcoRI*+AG and *MseI*+C resulted in a total of 97 bins within the range of 64 to 411 bp (Figure 4). The resulting matrix had 7469 data points. Data were used to produce a similarity tree using both the SAHN and NJ method. The similarity tree produced using the SAHN method resulted in a similarity of 69% or higher (Figure 5). The 77 isolates analyzed in this study separated into three distinct clades. Isolates belonging to clade A (n=16) and clade B (n=48) were highly variable and had similarities of 77% and 84%, respectively. Clade A and clade B each had 10 and 4 unique fragments, respectively, but all were polymorphic among isolates within each

clade (Table 2). Less variation existed among isolates within clade C (n=13), which were 99% similar. Clade C had 4 unique fragments, and all were monomorphic among the isolates (Table 2). Molecular groupings of the 77 isolates were confirmed by analysis using the NJ method (Figure 6).

Isolates separating into clade A included isolates collected from creeping bentgrass in NJ, MD, PA, and NC and hybrid bermudagrass from TX. Colony color of isolates in this group was the most diverse of the three clades (Figure 3). Colony color ranged from rose-quartz to gray, and all three colony colors described previously were represented by isolates of clade A. The majority of isolates analyzed fit into clade B, and included all of the isolates collected from VA, IL, and MI and a subset of isolates from NJ and MD. Additionally, isolates collected from both colonial and velvet bentgrass fit into clade B. Colony color of isolates within this group generally was similar to the original rose-quartz description of the pathogen. Two (i.e., OpNJ-4 and OpMD-10) out of forty-eight isolates, however, had unusual morphological characteristics (Figure 2). Isolate OpNJ-4 exhibited a gray/buff colony color and was similar in appearance to isolates described in clade C. Isolate OpMD-10, described above, varied from the original description at isolation and may have lost certain colony characteristics during storage.

Clade C included all isolates from MA, NY, and OH, a single isolate from PA, and several MD isolates collected from the University of Maryland Paint Branch Turfgrass Research Facility (PBTRF) in College Park (Figure 5). The PBTRF had been inoculated with various isolates of *O. agrostis*, including OpOH-1, OpMD-9, and OpVA-1. Of the thirteen isolates within clade C, ten were identical based on amplification with

primers *EcoRI*+AG and *MseI*+C. Colony color (gray/buff) generally was identical for isolates separating into this group (Figure 3). Based on the DNA fingerprint using AFLP, colony color generally separated with each clade (Figure 6). Although isolates within each clade generally shared similar colony color (80%), AMOVA revealed that color (n=3: gray, gray/buff, rose-quartz) alone does not indicate genetic similarity among isolates (Table 3). The largest variation occurred within clade A, which seemed to share colony characteristics from both clade B and C.

*O. agrostis* isolates obtained from the same golf course generally were similar. Exceptions to this were observed in isolates collected from P.B. Dye Golf Club located in Urbana, MD and from Honeybrook Golf Club located in Honeybrook, PA. The two isolates collected from P.B. Dye (PBD1 and PBD2) in 1998 separated into clades A and B. Similarly, isolates collected from Honeybrook in 1999 (OpPA-4) and 2000 (OpPA-8) separated into clades A and C, respectively. The broad genetic diversity of *O. agrostis* isolates can be seen in the fingerprints obtained from AFLP analysis of isolates OpMD-48 to OpMD-67 (Figure 4). These isolates were collected on 1 October 2003 from the aforementioned PBTRF research green. As previously noted, between 1999 and 2003, the site had been inoculated with three different isolates including OpVA-1, OpMD-9, and OpOH-1. Of the twenty isolates collected, four separated into clade A, nine into clade B, and seven into clade C, illustrating the diversity of the pathogen within even a small geographic area. Of the isolates used to inoculate the site, OpMD-9 and OpVA-1 grouped into clade B, while the Ohio isolate fit into clade C. Although several isolates collected from the research site on 1 October 2003 grouped into clade A, none of the isolates used to inoculate the area separated into this group. Based on AMOVA analyses,

isolates were not more similar to other isolates when comparisons were based on geographic region alone (n=4: mid-Atlantic, mid-west, northeast, south). Classification based on state origin (n=7: IL, MA, MD, MI, PA, NJ, VA), however, revealed that isolates from the same state had less genetic diversity (Table 3).

Genetic variation among *O. agrostis* isolates based on AFLP fingerprinting is highly diverse. Based on the results of AFLP analysis using primers *EcoRI*+AG and *MseI*+C, *O. agrostis* separated into three distinct clades. The most divergent group, with a similarity of 69%, included thirteen isolates from 6 different locations within 5 different states. Câmara et al. (2000) found similar divergence of the Ohio isolate within a subset of *O. agrostis* isolates. Divergence among isolates within the Ohio group (Clade C), however, was very low with a similarity of 99%, with ten isolates being identical. Previous observations with a single isolate from this group (OpOH-1) indicate many differences between this isolate and others included in this study. The Ohio isolate was the only one to produce pseudothecia on PDA plates, the only one to survive long-term storage on desiccated filter paper, and the only one to show a reduced sensitivity to the fungicide iprodione. Although these only were observations, they likely are associated with the pathogens' ability to enhance its survival. The final two groups were 75% similar, and divergence within clades A and B was high at 77% and 84%, respectively. Although Câmara et al. (2000) reported greater similarity (87%) among a subset of *O. agrostis* isolates (n=12), the analyses also included other *Ophiosphaerella* spp. as outliers, which may have increased the similarity among the *O. agrostis* isolates. Additionally, the aforementioned study only scored bands between 62 and 266 bp, while analyses performed within this study only included isolates of *O. agrostis* and measured

fragments as large as 411 bp. It is likely that differences in the DNA separation technique, subjectivity in scoring DNA fragments, addition of *Ophiosphaerella* spp. other than *O. agrostis*, and possibly other factors resulted in varying levels of genetic diversity among the two studies. Regardless, examination of the dendrogram presented by Câmara et al. (2000) indicates a total of three clades with one, two, and nine isolates in each. Unfortunately, most of the isolates used in the original examination of *O. agrostis* were lost and not included in this study. The isolates that were included (OpVA-1, OpMD-9, OpMD-4, OpMD-5, OpIL-1, OpPA-1 and OpOH-1), however, generally were fit into similar groupings.

**Pseudothecia Production.** Pseudothecia production was monitored using a subset of isolates from the AFLP analysis with varying colony colors (Figure 7). On individual rating dates, there were significant differences among the isolates ( $P = 0.0159$ ). After four days of incubation at 25°C in constant light, pseudothecia began to develop on tall fescues seeds infested with isolates OpMD-9, OpMI-1, OpMA-4, OpNY-1 and OpOH-1 (Figure 8). After four days, no mature ascospores were found within any developing pseudothecia. Six days after incubation (6 DAI), ascospores inside developing pseudothecia generally were immature; however, mature ascospores were found within pseudothecia from isolates OpMD-9, OpMI-1, and OpMA-4. These results were similar to a previous study in which pseudothecia development began in a little as 4 days and mature ascospores were present within one week of incubation (Kaminski et al., 2002). Except for isolates OpNJ-4 and OpNC-1, mature ascospores were found within pseudothecia from all isolates by the final rating date (30 DAI).

The most rapid production of pseudothecia occurred on tall fescue seeds infested with isolates OpMD-9 and OpMI-1 (Figure 8). Except for 6 DAI, the aforementioned isolates produced the greatest number of pseudothecia throughout the experiment. By 18 DAI, pseudothecia production of isolates OpMD-9 and OpMI-1 leveled off as maximum pseudothecia production was approached. Regression analyses performed on ratings between 4 and 18 DAI resulted in the linear equations:  $P = 0.31(\text{DAI}) - 0.53$  ( $R^2 = 0.92$ ;  $P < 0.0001$ ) and  $P = 0.29(\text{DAI}) - 0.60$  ( $R^2 = 0.84$ ;  $P < 0.0001$ ) for isolate OpMD-9 and OpMI-1, respectively, where P = pseudothecia rating on the aforementioned 0 to 5 scale and DAI = days after incubation. Moderate pseudothecia production was observed from isolates OpMA-4, OpOH-1 and OpNY-1. By the final rating date (30 DAI), isolates OpMA-4, OpOH-1 and OpNY-1 had average pseudothecia ratings between 2.3 and 3.8. Both isolates collected from golf courses in NJ (OpNJ-4 and OpNJ-6) exhibited low levels of pseudothecia production. After incubating for 2 weeks, however, the isolate OpNJ-6 generally produced more fruiting bodies than OpNJ-4. Throughout the 30 days of incubation, no pseudothecia developed on seed infested with OpNC-1.

Pseudothecia production from individual isolates generally could be linked with colony color and genetic diversity clades. With the exception of OpNJ-4, isolates belonging to clade B (OpMD-9 and OpMI-1) resulted in the most rapid and greatest number of pseudothecia produced. Isolates OpMD-9 and OpMI-1 were the only isolates in this experiment that exhibited the characteristic rose-quartz colony color when grown on PDA. Although isolate OpNJ-4 grouped into the aforementioned clade based on AFLP fingerprints, it exhibited the greatest variation in colony color, when compared to the other 47 isolates within the genetic clade. Moderate numbers of pseudothecia were

produced with isolates OpMA-4, OpOH-1 and OpNY-1. Separating into clade C, these aforementioned isolates were most closely related in colony color as well as genetic similarity. Representing clade A, isolates OpNJ-6 and OpNC-1 exhibited little or no pseudothecia production. Sharing a similar gray colony color, but belonging to clade A, isolate OpNJ-4 also produced very low levels of pseudothecia. The AMOVA analyses found that there was greater genetic similarity between isolates that had similar pseudothecia production than among all isolates (n=3: high, moderate, low) (Table 3). Since only 8 isolates were used in this experiment, pseudothecia production with additional isolates may help confirm whether sexual reproduction is a large source of variation among *O. agrostis* isolates.

#### CONCLUSION

*O. agrostis* isolates examined in this study separated into three distinct clades. AMOVA analyses indicated that *O. agrostis* collected within each state and pseudothecia production were the best indicators for genetically similarity, and therefore, clade distinction. Colony color generally was similar within clades B and C; while colony color of isolates within clade A appeared to be a mixture of the two aforementioned molecular groupings. Additionally, pseudothecia production of two isolates from clade A generally was reduced or did not occur, while varying levels of pseudothecia were produced with isolates from the remaining groups. Although only isolates within clades B and C were used to inoculate the Paint Branch Turfgrass Research Facility field site, four isolates from clade A were found at this location. The reason for this segregation, however, remains unclear. The pathogen may have been introduced through seed or

ascospores carried to the site via natural air currents. The ability of *O. agrostis* to be distributed via seed is unknown and attempts to determine this using a PCR-based molecular technique were unsuccessful (Kaminski, Chapter VII).

Homothallic filamentous ascomycetes have been shown to have either one or both mating genes (MAT) with two alternative forms (MAT1-1 and MAT1-2) known as idiomorphs (Glass et al., 1990; Pöggeler, 1999). Although the mating genes of *O. agrostis* are unknown, both mating types have been detected in *O. korrae* (Hsiang et al., 2003). Despite having both mating type genes, other homothallic fungi are capable of both selfing and outcrossing. In particular, isolates of *Gibberella zea* (Schwein.) Petch, which carries both MAT1-1 and MAT1-2 in a single nucleus, have been shown to outcross (Lee et al., 2003). Additionally, conversion from heterothallism to homothallism and visa versa has been achieved in vitro by manipulation of the *MAT* genes of *G. zea* and *Cochliobolus heterostrophus* (Lee et al., 2003; Yun et al., 1999). Although *O. agrostis* is a homothallic species, it is unclear if outcrossing among isolates occurs. Currently, varying levels of pseudothecia production (i.e., sexual reproduction) have been observed among isolates of *O. agrostis*. Investigations into the natural diversity of ascospores within a single pseudothecium as well as following successive generations of ascospores beginning with a single ascospore likely would clarify the source of genetic diversity in *O. agrostis*.

Although the actual sexual compatibility of this pathogen remains unknown, *O. agrostis* is a very diverse pathogen. Despite the examination of numerous isolates in this study, the anamorph of *O. agrostis* has not been reported and the pathogen appears to exist exclusively in its teleomorphic state. Within the genus *Ophiosphaerella*, an

anamorph only has been reported for *O. herpotricha* (Webster and Hudson, 1957). This conidial state rarely has been reported and the genetic connection has been questioned (Walker, 1980; Câmara et al., 2000).

Table 1. Isolate designation; cultivar affected; collection location, date received and collector; and color of 77 *Ophiostoma* isolates from the United States.

Isolate <sup>w</sup>	Host cultivar <sup>x</sup>	Location	Date received	Iso. pt <sup>y</sup>	Color <sup>z</sup>
OpIL-1 <sup>RK</sup>	SR 1119	Glencoe, Illinois	18 Dec. 1998	ht	Rose-quartz
OpIL-2 <sup>RK</sup>	SR1119 + L-93 + Providence	Golf, Illinois	8 Dec. 2000	ht	Rose-quartz
OpIL-3 <sup>RK</sup>	L-93	Olympia Fields, Illinois	8 Dec. 2000	ht	Rose-quartz
OpIL-4 <sup>RK</sup>	L-93	Park Ridge, Illinois	1 Aug. 2002	ht	Rose-quartz
OpIL-5 <sup>RK</sup>	L-93	Park Ridge, Illinois	1 Aug. 2002	ht	Rose-quartz
OpMA-1	L-93	West Bridgewater, Massachusetts	27 July 2002	If	Gray/Buf
OpMA-3	Penn A-4	Hingham, Massachusetts	Summer 2003	ht	Gray/Buf
OpMA-4	Penn A-4	Hingham, Massachusetts	Summer 2003	ht	Gray/Buf
OpMI-1	Providence	East Tawas, Michigan	29 Sept. 2000	If	Rose-quartz
OpMI-2	Providence	East Tawas, Michigan	29 Sept. 2000	If	Rose-quartz
OpNC-1 <sup>HW</sup>	Penncross	Laurinburg, North Carolina	11 Aug. 2000	ht	Gray
OACS <sup>BC</sup>	L-93	Millstown, New Jersey	Summer 2001	ht	Gray/Buf
OpNJ-4	L-93	New Brunswick, New Jersey	27 July 1999	ss	Gray/Buf
OpNJ-5	Penn A-4	Northfield, New Jersey	28 Sept. 2000	ht	Gray/Buf
OpNJ-6	Penn A-4	Northfield, New Jersey	28 Sept. 2000	ht	Gray
OpNY-1	L-93	Altamont, New York	11 Aug. 2000	ht	Gray/Buf
OpOH-1	L-93	Chardon, Ohio	21 Oct. 1998	ht	Gray/Buf
OpPA-1	Crenshaw + Southshore	Avondale, Pennsylvania	23 Dec. 1998	ss	Gray/Buf
OpPA-4	L-93	Honeybrook, Pennsylvania	2 Nov. 1999	If	Gray/Buf
OpPA-6	SR1120 + L-93 + Providence	Avondale, Pennsylvania	8 Mar. 2000	sa	Rose quartz
OpPA-8	L-93	Honeybrook, Pennsylvania	15 Aug. 2000	ht	Gray/Buf
OpVA-1	Pennlinks	Sterling, Virginia	10 Sept. 1998	ss	Rose-quartz
OpVA-3	Penn A-4	Virginia Beach, Virginia	15 June 2002	ht	Rose-quartz
OpVA-4	Penn A-4	Virginia Beach, Virginia	15 June 2002	ht	Rose-quartz
#121 <sup>NT</sup>	'Champion' bermudagrass	College Station, Texas	Spring 1998	ht	Rose quartz
HCC1	L-93 + Crenshaw	Hunt Valley, Maryland	21 Oct. 1998	If	Rose quartz
HCC2	L-93 + Crenshaw	Hunt Valley, Maryland	21 Oct. 1998	ss	Rose quartz
OpMD-3	Penncross	Ocean City, Maryland	16 Oct. 1998	ss	Rose-quartz

Isolate <sup>w</sup>	Host cultivar <sup>x</sup>	Location	Date received	Iso. pt <sup>y</sup>	Color <sup>z</sup>
OpMD-4	L-93 + Crenshaw	Upper Marlboro, Maryland	26 Oct. 1998	st	Rose-quartz
OpMD-5	L-93 + Crenshaw	Aberdeen, Maryland	15 Oct. 1998	sa	Rose-quartz
PBD1	Penn-G2	Urbana, Maryland	21 Aug. 1998	ss	Rose-quartz
PBD2	Penn-G2	Urbana, Maryland	21 Aug. 1998	st	Rose-quartz
OpMD-9	Providence	Silver Spring, Maryland	13 Nov. 1998	ss	Rose-quartz
OpMD-10	L-93 + Crenshaw	Upper Marlboro, Maryland	26 Oct. 1998	ss	Beige/Buf
OpMD-12	L-93	Havre de Grace, Maryland	14 Aug. 1999	sa	Gray
OpMD-13	'Bardot' colonial	College Park, Maryland	8 Sept. 2000	If	Rose-quartz
OpMD-14	L-93	Joppa, Maryland	26 July 2001	st	Rose-quartz
OpMD-15	L-93	Joppa, Maryland	26 July 2001	If	Rose-quartz
OpMD-16	Penn A-4	Laytonsville, Maryland	30 Aug. 2001	If	Rose-quartz
OpMD-17	Providence	College Park, Maryland	26 Oct. 2001	ss	Rose-quartz
OpMD-18	Providence	College Park, Maryland	9 Nov. 2001	st	Rose-quartz
OpMD-19	Penncross	Ocean City, Maryland	16 Oct. 1998	If	Rose-quartz
OpMD-21	L-93	College Park, Maryland	20 June 2002	ht	Rose-quartz
OpMD-22	L-93	College Park, Maryland	20 June 2002	ht	Rose-quartz
OpMD-23	L-93	College Park, Maryland	20 June 2002	ht	Rose-quartz
OpMD-26	L-93	College Park, Maryland	28 Oct. 2002	ht	Rose-quartz
OpMD-27	L-93	College Park, Maryland	28 Oct. 2002	ht	Rose-quartz
OpMD-29	L-93	College Park, Maryland	25 Nov. 2002	ht	Rose-quartz
OpMD-34	L-93	College Park, Maryland	25 Nov. 2002	ht	Rose-quartz
OpMD-36	'Bavaria' velvet	College Park, Maryland	25 Nov. 2002	ht	Rose-quartz
OpMD-40	L-93	College Park, Maryland	25 Nov. 2002	ht	Rose-quartz
OpMD-42	L-93	College Park, Maryland	28 Jan. 2003	ht	Rose-quartz
OpMD-43	L-93	College Park, Maryland	26 Mar. 2003	ht	Rose-quartz
OpMD-44	L-93	College Park, Maryland	26 June 2003	ht	Gray/Buf
OpMD-45	L-93	College Park, Maryland	26 June 2003	ht	Gray/Buf
OpMD-46	L-93	College Park, Maryland	26 June 2003	ht	Gray/Buf
OpMD-47	L-93	College Park, Maryland	26 June 2003	ht	Gray/Buf
OpMD-48	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz

Isolate <sup>w</sup>	Host cultivar <sup>x</sup>	Location	Date received	Iso. pt <sup>y</sup>	Color <sup>z</sup>
OpMD-49	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-50	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-51	L-93	College Park, Maryland	1 Oct. 2003	ht	Gray/Buf
OpMD-52	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-53	L-93	College Park, Maryland	1 Oct. 2003	ht	Gray/Buf
OpMD-54	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-55	L-93	College Park, Maryland	1 Oct. 2003	ht	Gray/Buf
OpMD-56	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-57	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-58	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-59	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-60	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-61	L-93	College Park, Maryland	1 Oct. 2003	ht	Gray/Buf
OpMD-62	L-93	College Park, Maryland	1 Oct. 2003	ht	Gray/Buf
OpMD-63	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-64	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-65	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-66	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz
OpMD-67	L-93	College Park, Maryland	1 Oct. 2003	ht	Rose-quartz

<sup>w</sup> Isolates were collected in this study by author unless otherwise noted as follows; NT= Ned Tisserat, HW = Henry Wetzel, BC = Bruce Clarke, and RK = Randy Kane.

<sup>x</sup> All cultivars listed are creeping bentgrass unless otherwise noted.

<sup>y</sup> Isolation of *O. agrostis* were as follows: ht = hyphal tip, lf = leaf, sa = single ascocarp, ss = single spore isolation, st = stolon.

<sup>z</sup> Colony color was assessed after 10 days growth on potato dextrose agar incubated at 25°C in constant darkness.

Table 2. Polymorphic and monomorphic fragments unique to isolates within each of three clades based on unweighted pair-group method with arithmetic mean analyses of amplified fragment length polymorphisms of 77 *Ophiosphaerella agrostis* isolates.

Clade	Locus (bp)	Fragment morphology	No. Isolates	Isolate(s) Designation
A	378	Polymorphic	1	OpMD-55
	376	Polymorphic	1	OpMD-64
	254	Polymorphic	1	OpMD-64
	238	Polymorphic	1	OpMD-64
	203	Polymorphic	5	OACS , OpMD-52, OpMD-63, OpMD-64, OpPA-6
	128	Polymorphic	5	OpMD-55, OpNJ-5, OpNJ-6, OpPA-1, OpPa-4
	107	Polymorphic	2	OACS, OpMD-55
	92	Polymorphic	3	OACS, OpMD-64, OpPA-6
	84	Polymorphic	3	OACS, OpMD-55, OpMD-64
B	403	Polymorphic	1	PBD2
	307	Polymorphic	2	OpMD-66, OpVa-3
	174	Polymorphic	2	OpMD-26, OpMD-27
	117	Polymorphic	13	OpIL-3, OpMI-1, OpMI-2, OpMD-14, OpMD-10, OpMD-15, OpMD-23, OpMD-29, OpMD-42, OpMD-56, OpMD-57, OpMD-59, OpMD-66
	80	Polymorphic	7	OpMD-13, OpMD-26, OpMD-27, OpMD-40, OpMD-66, OpNJ-4, OpVA-4
C	317	Monomorphic	13	All <sup>x</sup>
	297	Monomorphic	13	All
	224	Monomorphic	13	All
	221	Monomorphic	13	All

<sup>x</sup> Fragments of DNA unique to clade C were observed in all isolates (OpMD-44 to OpMD-47, OpMD-51, OpMD-53, OpMD-62, OpNY-1, OpPA-8, OpMA-1, OpMA-3, OpMA-4, and OpOH-1).

Table 3. Analyses of molecular variance of pair-wise distances for *Ophiosphaerella agrostis* isolates based on classes of color, geographic location (state or region), and pseudothecia production.

Class	Source of variation	df	Sum of squares	Mean squares	Total variance (%)	P
Color	Among populations	2	98.0	49.0	20	0.01
	Within populations	74	682.2	9.2	80	
Location	Among populations	6	292.7	48.8	53	0.01
	Within populations	66	492.3	6.5	47	
Geographic region	Among populations	3	83.5	27.8	20	0.01
	Within populations	49	416.0	8.5	80	
Pseudothecia production	Among populations	2	54.0	27.0	63	0.01
	Within populations	5	24.3	4.9	37	

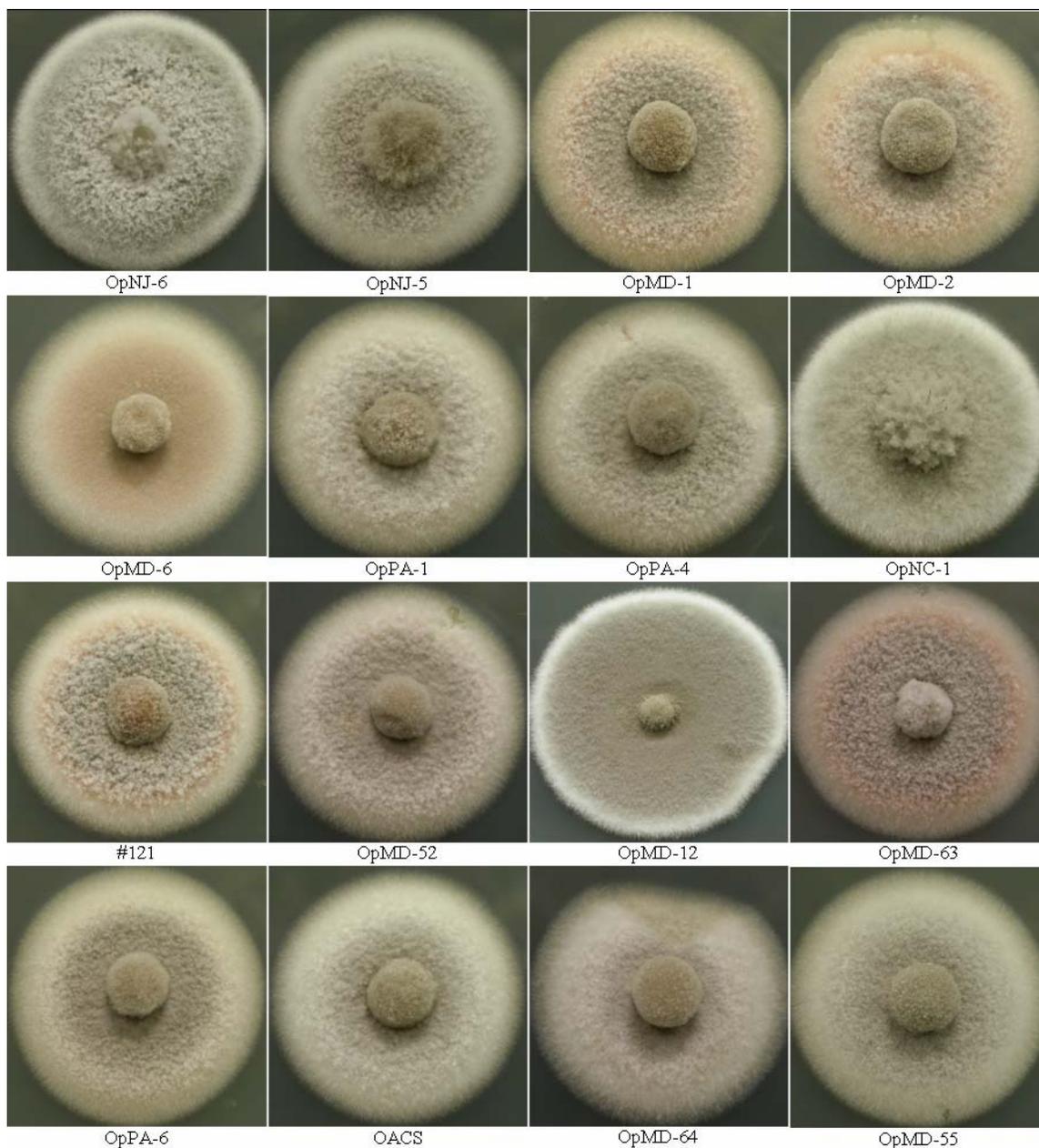


Figure 1. Colony color and cultural morphology of *Ophiosphaerella agrostis* (n=16) isolates grouped into clade A based on amplified fragment length polymorphism fingerprinting analysis using primers *EcoRI* + AG and *MseI* + C.



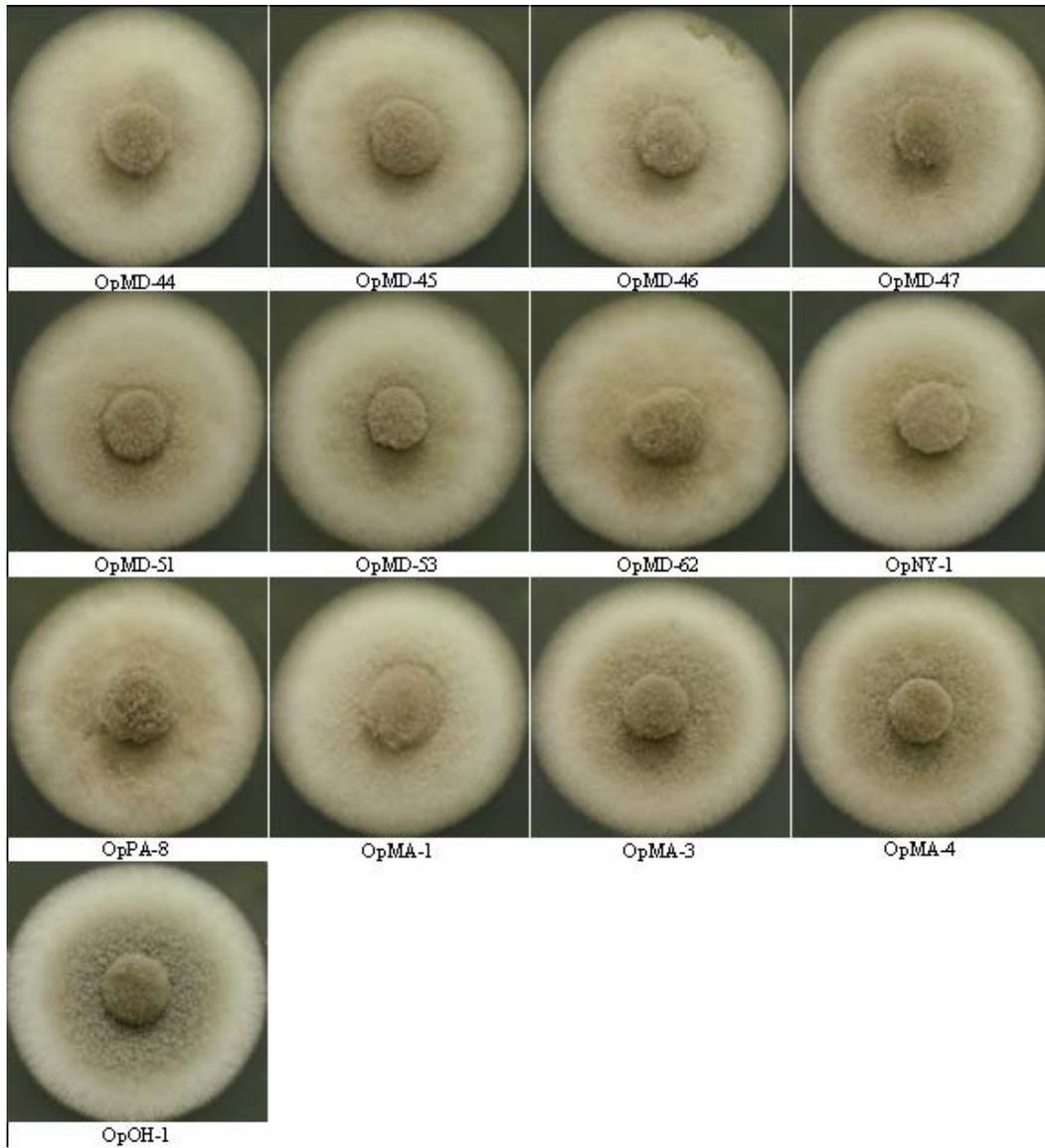


Figure 3. Colony color and cultural morphology of *Ophiosphaerella agrostis* (n=13) isolates grouped into clade C based on AFLP analysis using primers *EcoRI* + AG and *MseI* + C.

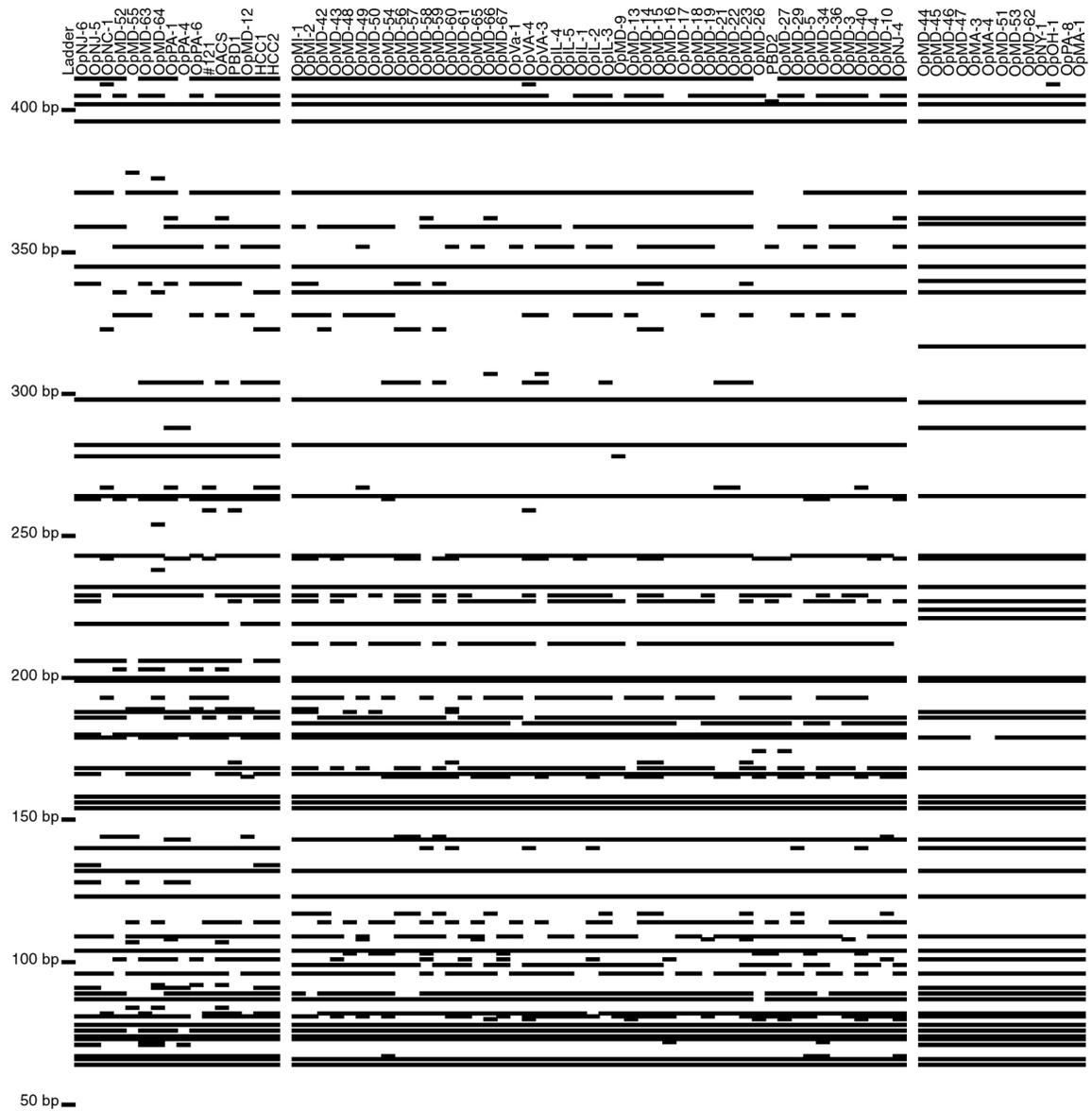


Figure 4. DNA fragment banding pattern of 77 *Ophiosphaerella agrostis* isolates after fluorescent amplified fragment length polymorphism analysis with automated capillary electrophoresis.

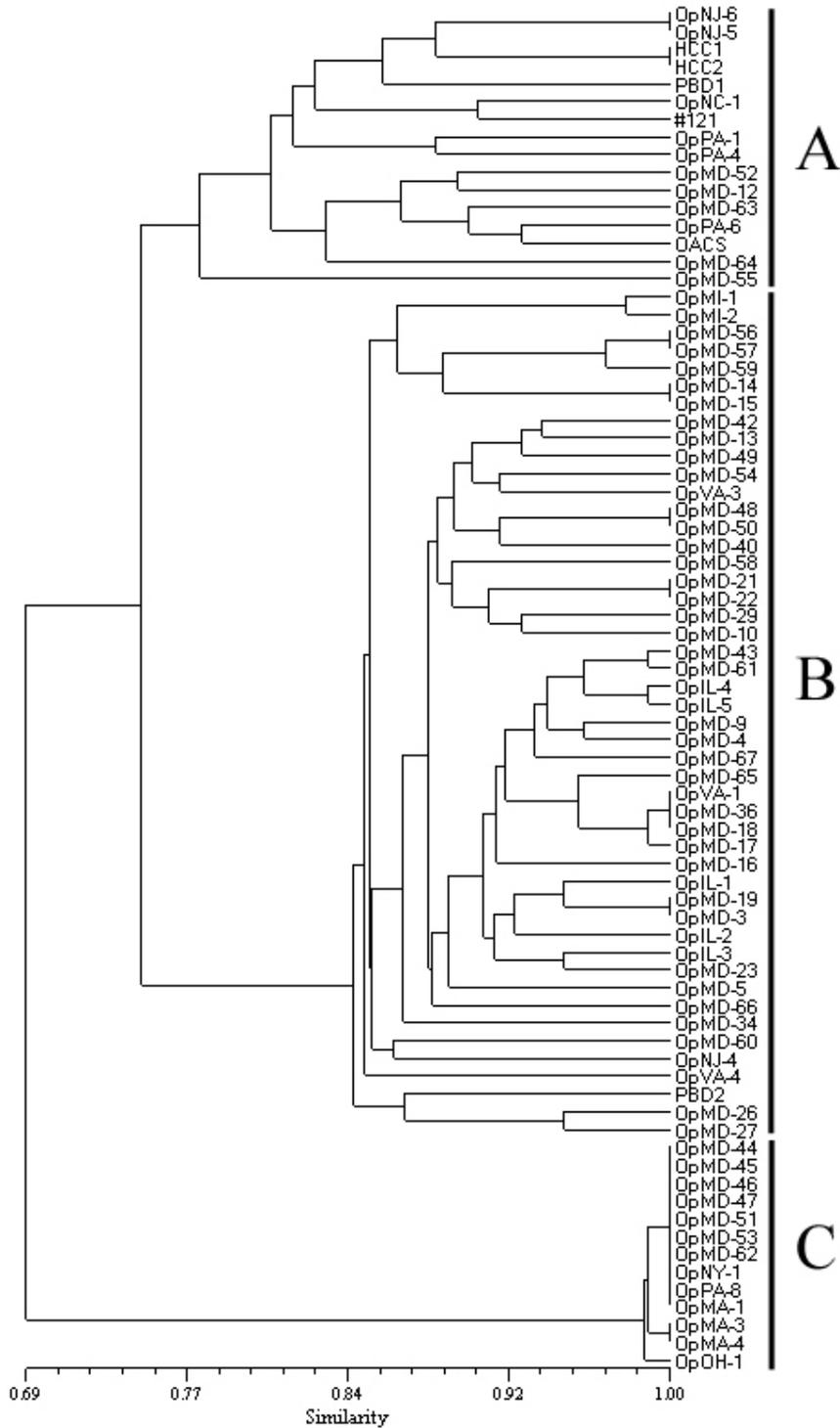


Figure 5. Unweighted pair-group method with arithmetic mean dendrogram of 77 isolates of *Ophiosphaerella agrostis* collected from 21 different locations throughout the USA. Genetic distances were calculated by the SAHN similarity coefficient.

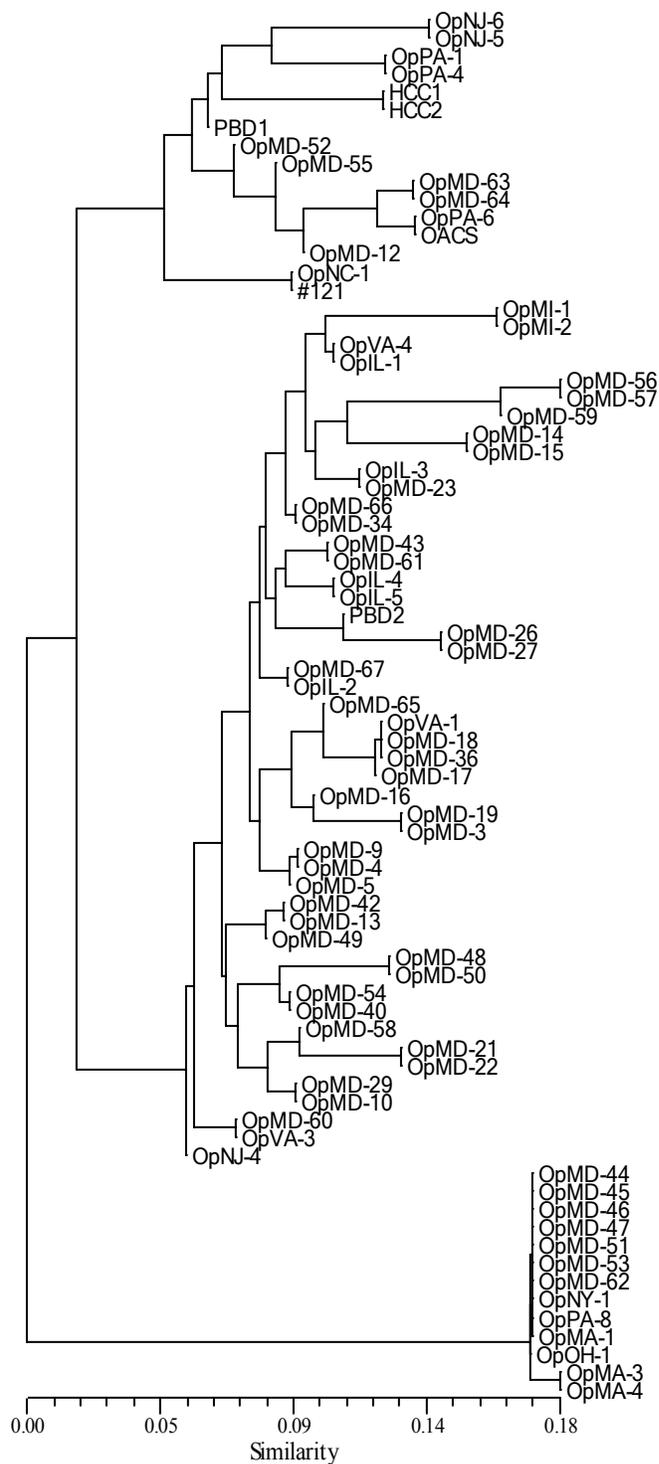


Figure 6. Unweighted pair-group method with arithmetic mean dendrogram of 77 isolates of *Ophiosphaerella agrostis* collected from 21 different locations throughout the USA. Genetic distances were calculated by the Neighbor joining similarity coefficient.

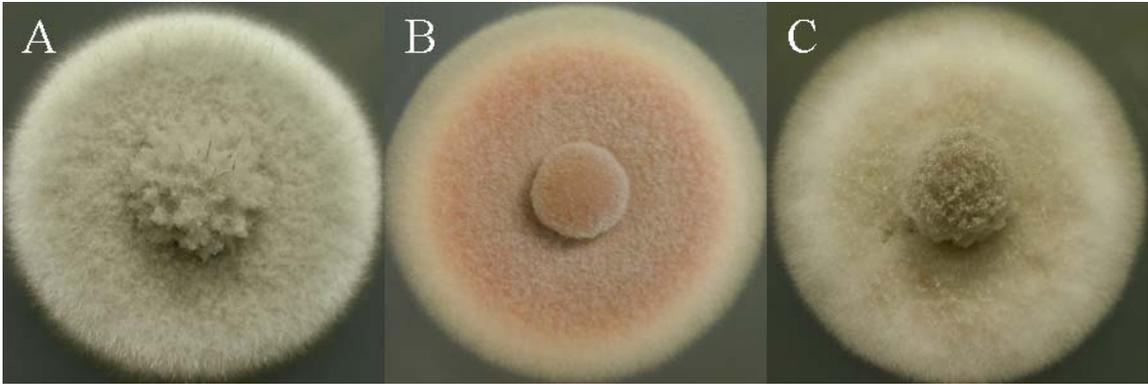


Figure 7. Typical variation in colony color and morphology of *Ophiosphaerella agrostis* isolates separating into each of three clades determined by amplified fragment length polymorphism (AFLP) DNA fingerprinting. Isolates shown include OpNC-1, OpMD-14 and OpPA-8 and represent clades A, B, and C, respectively.

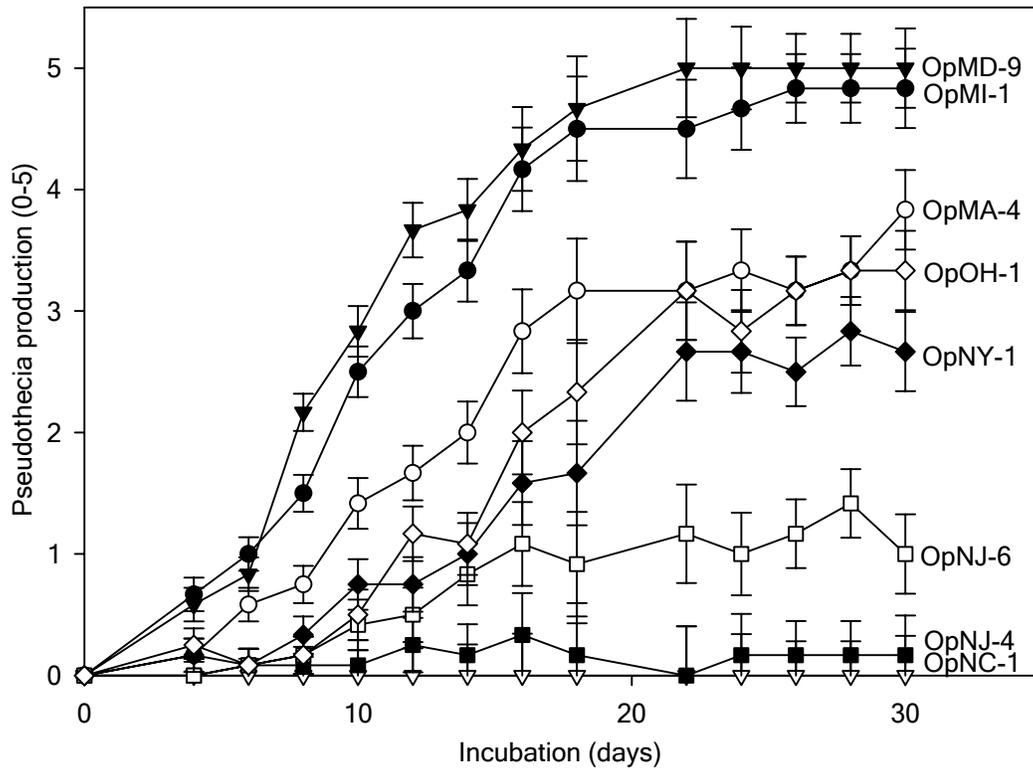


Figure 8. Pseudothecia development from various isolates of *Ophiosphaerella agrostis* after incubation at 25°C in constant darkness for 30 days. Error bars represent standard errors of the differences among isolates on individual rating dates ( $P < 0.05$ ).

## VII. A PCR-BASED METHOD FOR THE DETECTION OF *OPHIOSPHAERELLA AGROSTIS* IN CREEPING BENTGRASS

### SYNOPSIS

Dead spot is a relatively new disease of creeping bentgrass and hybrid bermudagrass and is incited by *Ophiосphaerella agrostis*. Initial symptoms are difficult to diagnose and clinicians generally rely on the presence of pseudothecia or isolation of *O. agrostis* on an artificial medium. The main goal of this study was to develop a PCR-based molecular technique capable of quickly identifying *O. agrostis* within infected creeping bentgrass tissues. Oligonucleotide primers specific for *O. agrostis* were developed based on the ITS1 and ITS2 regions of three previously sequenced isolates of *O. agrostis*. The 22 base-pair (bp) primers amplified a 445 or 446 bp region of 80 *O. agrostis* isolates collected from creeping bentgrass and bermudagrass in 11 states. Primers did not amplify DNA from other common turfgrass pathogens, including three closely related species of *Ophiосphaerella*. Selective amplification of *O. agrostis* was successful from field-infected creeping bentgrass samples and primers did not amplify the DNA of asymptomatic, field-grown creeping bentgrass or hybrid bermudagrass plants. Amplification of purified *O. agrostis* DNA was successful at quantities between 50 nanograms and 5 picograms. The entire process including DNA isolation, amplification and amplicon visualization may be completed within 4 hours. These results indicate the specificity of these primers for assisting in the accurate and timely identification of *O. agrostis* and the diagnosis of dead spot in both bentgrass and bermudagrass hosts.

## INTRODUCTION

Dead spot is a disease of creeping bentgrass (*Agrostis stolonifera* L.), and is caused by *Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum et M.E. Palm (Câmara et al., 2000; Dernoeden et al., 1999). The pathogen first was isolated from a golf course in Maryland in 1998 and since has been isolated from creeping bentgrass in at least 11 states (Kaminski and Dernoeden, 2002). The pathogen also has been isolated from hybrid bermudagrass (*Cynodon dactylon* [L.] Pers. x *C. transvaalensis* Burt-Davy) in Texas and Florida (Kaminski and Dernoeden, 2002; Krausz et al., 2001).

On creeping bentgrass putting greens grown in the mid-Atlantic region of the USA, dead spot symptoms may appear as early as May and disease activity often peaks between July and August. Initially, the disease appears as small, copper or reddish-brown spots approximately 1 to 2 cm in diameter, which slowly increase to approximately 6 to 8 cm throughout the summer months. Initial symptoms are difficult to diagnose and often are mistaken for damage caused by other common turfgrass diseases and pests such as dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett), copper spot (*Gloeocercospora sorghi* Bain & Edgerton ex Deighton), and black cutworms (*Agrostis ipsolon* Hufnagel). Additionally, new disease symptoms may be confused with ball-marks typically found on bentgrass putting greens. Once infection occurs, turfgrass in the center of dead spots dies forming pits or depressions, which adversely affect the playability of the putting surface. Recovery of bentgrass into infected spots is slow and dead spots often remain present throughout the winter until bentgrass growth resumes in

the spring.

A key diagnostic aide used to identify *O. agrostis* is the presence of pseudothecia, which often are found embedded in necrotic leaf tissue and stolons (Kaminski et al., 2002). These sexual fruiting bodies may develop quickly and viable ascospores may be present within one week of initial symptom development. Adding to diagnostic difficulties, pseudothecia are not always present and isolation of *O. agrostis* on an artificial medium often is necessary for a positive laboratory diagnosis. Isolation of the pathogen, however, may take several days to weeks and variation in colony color and morphology among *O. agrostis* isolates can make identification of the fungus difficult (Kaminski et al., 2002).

Polymerase chain reaction (PCR) is a molecular technique receiving increasing attention in the identification of various fungal pathogens (Chiocchetti et al., 2001; Godfrey et al., 2003; Errampalli et al., 2001). Positive identification of diseased plants may be quickly accomplished through the use of species-specific primers. Oligonucleotide primers capable of detecting common turfgrass pathogens present at low concentrations have been developed from various regions of fungal genomic DNA. Harmon et al. (2003) designed primers from the Pot2 transposon of *Magnaporthe grisea* (Herbert) Barr and *M. oryzae* Couch. Other regions of genomic DNA used in the development of species-specific primers include the avenacinase gene from *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *avenae* (E.M. Turner) Dennis and the internal transcribed spacer (ITS) region from *M. poae* Landschoot and Jackson (Bunting et al., 1996; Rachdawong et al., 2002). Conserved sequences within the ITS region have been developed to identify *Rhizoctonia solani* Kühn AG-2-2 as well as

several subsets of this anastomosis group (Carling et al., 2002). Additionally, ITS regions (ITS1 and ITS2) previously were used in the development of primers for three other *Ophiosphaerella* spp. found in association with diseases of turfgrass including *O. herpotricha* J. C. Walker, *O. korrae* Walker and Smith, and *O. narmari* Wetzel, Hulbert and Tisserat (O’Gorman et al., 1994; Tisserat et al., 1994; Wetzel et al., 1999).

To date, disease information collected from golf courses throughout the USA revealed that dead spot generally develops on newly constructed creeping bentgrass putting greens or renovated greens that had been fumigated with methyl bromide (Kaminski and Dernoeden, 2002). Additionally, dead spot found on hybrid bermudagrass occurs on greens that have been overseeded with roughstalk bluegrass (*Poa trivialis* L.), a cool-season turfgrass species often seeded into dormant hybrid bermudagrass putting greens in the southern regions of the USA (Kaminski and Dernoeden, 2002; Krausz et al., 2001). The sudden appearance of this previously undescribed pathogen on newly seeded putting greens in various regions of the USA raises the question of the pathogens’ origin. The role of seed in the spread of *O. agrostis* is unknown. The development of species-specific oligonucleotide primers, however, may be useful in identifying *O. agrostis* in seed.

The main goal of this study was to develop a PCR-based molecular technique capable of identifying *O. agrostis* within infected creeping bentgrass tissues. The objectives of this research therefore were to: 1) develop and test oligonucleotide primers specific to *O. agrostis*, and 2) use species-specific primers to test for the presence of the pathogen in plants and in commercially available creeping bentgrass and roughstalk bluegrass seed.

## MATERIALS AND METHODS

**Fungal Isolates.** Isolates were collected from 11 states between 1998 and 2003. A total of 80 *O. agrostis* isolates from creeping bentgrass were collected from Illinois (IL) (n=5), Maryland (MD) (n=53), Massachusetts (MA) (n=3), Michigan (MI) (n=2), New Jersey (NJ) (n=5), New York (NY) (n=1), North Carolina (NC) (n=1), Ohio (OH) (n=1), Pennsylvania (PA) (n=5), Texas (TX) (n=1), and Virginia (VA) (n=3), (Table 1 & 2). Other common turfgrass pathogens also were collected including *Bipolaris cynodontis* (Marig.) Shoemaker; *Colletotrichum graminicola* (Ces.) G.W. Wils.; *G. graminis* var. *avenae*; *G. sorghi*; *M. poae*; *O. korrae*; *O. narmari*; *O. herpotricha*; *R. solani*; *Rhizoctonia zae* Voorhees; *Rhizoctonia cerealis* Van der Hoeven; and *S. homoeocarpa* (Table 3). All isolates used in this study were supplied by collectors listed in Tables 1 to 3. Prior to DNA extraction, isolates were stored at -20°C in a potato dextrose broth (PDB) or 20% glycerol solution or on potato dextrose agar (PDA) or PDB at 4°C.

**DNA Isolation.** To isolate the DNA from fungal cultures, isolates were grown at room temperature for 7 to 10 days in 100 ml of PDB (24 g L<sup>-1</sup>) on a shaker table (LabLine Orbit Shaker, Lab-Line Instruments Inc., Melrose, IL) set to 90 rpm. Cultures were filtered through Whatman #1 filter paper, rinsed in distilled, deionized water (ddH<sub>2</sub>O) and lyophilized in a Freezemobile 6 (The VirTis Company, Gardiner, NY) for 24 to 48 hours. Freeze-dried mycelial mats were stored at -20°C or immediately prepared for DNA extraction. Approximately 20 to 30 mg of freeze-dried mycelia were placed in

a 1.8 ml microcentrifuge tube (VWR, West Chester, PA) and ground into a fine powder using a micro-pestle (VWR, West Chester, PA). Liquid nitrogen occasionally was used to aid in the grinding process.

The DNA was extracted using Qiagen Mini-Prep Kits (Qiagen Inc., Valencia, CA). A total of 400  $\mu$ l of Buffer AP1 and 4  $\mu$ l of RNase A stock solution were added to pulverized mycelia and vortexed. To lyse cells, the mixture then was incubated for 10 minutes at 65°C, mixing 2 to 3 times during incubation. To precipitate proteins and polysaccharides, 130  $\mu$ l of Buffer AP2 were added to the lysate and incubated on ice for 5 minutes. The tubes then were centrifuged for 5 minutes at 16000 rpm and the lysate was added to the supplied QIAshredder column. The material then was centrifuged for 2 minutes at 16000 rpm. Flow through material was placed in a 1.5 ml microcentrifuge tube and 675  $\mu$ l Buffer AP3/E were added. The DNA was separated from the aforementioned material by centrifugation in a DNeasy mini-spin column. Buffer AW (500  $\mu$ l) was added to the mini-spin column and centrifuged for 1 minute at 8000 rpm. An additional 500  $\mu$ l Buffer AW was added and the spin column membrane was dried by centrifuging for 2 minutes at 16000 rpm. For the final step, 50  $\mu$ l of the preheated (65°C) elution buffer were added, the column centrifuged for 1 minute at 8000 rpm, and the step repeated. This template DNA was later diluted for PCR analysis.

**Development of Species-Specific Oligonucleotide Primers.** Oligonucleotide primers specific for *O. agrostis* were developed based on the ITS1 and ITS2 regions of *O. agrostis* isolates OpOH-1, OpMD-6 and OpVA-1, which had been previously sequenced and deposited in the GenBank database under the accession numbers

AF191550, AF191549 and AF191548, respectively (Câmara et al., 2000) (Figure 1). The primers were OaITS1 (5'-AGCAATACAGCCCAAAGGCCTC-3') and OaITS2 (5'-AAAGGCTTAATGGACGCGAGTG-3'). These primers were chosen based on nucleotide differences when compared to other *Ophiosphaerella* species and were designed to amplify a portion of the ITS1 region, the entire 5.8s rDNA, and a portion of the ITS2 region of all *O. agrostis* isolates (Figure 2). Primers were synthesized by Qiagen Inc.

Genomic DNA was diluted (1 µl template DNA:99 µl ddH<sub>2</sub>O) for PCR reactions. The PCR reactions were run using 1 µl of 10x polymerase buffer (New England BioLabs, Inc. (NEB); Beverly, MA); 0.4 µl 100 mM MgSO<sub>4</sub> (NEB); 0.2 µL 40 µM dNTPs (NEB); 0.4 µl of each 5 µM primer; 0.1 unit Taq polymerase (NEB); and 1 µl of diluted genomic DNA. DNA-grade distilled, deionized water was added to reach a reaction volume of 10 µl. DNA was amplified using an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of 30 seconds of denaturation at 94°C, 45 seconds annealing at 65°C, and 90 seconds elongation at 76°C. Reactions were run in an Eppendorf Mastercycler (Hamburg, Germany), and amplification products were visualized on a 1% or 2% agarose gel stained with ethidium bromide (0.5 to 1.0 µg ml<sup>-1</sup>). Gels were run at 125 V for 30 to 45 minutes. Results were confirmed in a replication run using a different thermal cycler (PTC-0220 DNA Engine Dyad Peltier Thermal Cycler, MJ Research, Inc., Waltham, MA) with the reaction mixture and cycling profile previously described.

**DNA Quantification and Primer Sensitivity.** The DNA concentrations of *O. agrostis* isolates (n=80) were determined using the PicoGreen dsDNA Quantification Kit

(Molecular Probes Inc., Eugene, OR) (Singer et al., 1997). Genomic DNA from each isolate was diluted (1:100) in TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 7.5), and 50 µl of diluted DNA were added to 50 µl of the PicoGreen Reagent (diluted to 1x concentration with TE Buffer). Hence, the final DNA dilution for detection was 1:200 (v/v). Six calibration samples containing DNA stock solutions of previously determined concentrations were used to develop a standard curve. The DNA quantification was performed using a Fluoroskan Ascent Microplate Fluorometer (Thermo Labsystems, Franklin, MA). Prior to quantification, samples were shaken for 10 seconds and incubated for 5 minutes at 28°C. Immediately following incubation, DNA concentrations were quantified at an excitation (485 nm) and emission (538 nm) wavelength specific for the fluorescent dye.

To assess the sensitivity of the developed primers for detecting *O. agrostis*, stock DNA was diluted and PCR performed on known DNA concentrations. Primers developed in this study were tested against DNA quantities of 50 nanograms (ng), 5.0 ng, 0.5 ng, 50 picograms (pg) and 5 pg. Previously isolated DNA from five *O. agrostis* isolates were selected for the sensitivity assay and the experiment was repeated twice.

**Detection of *O. agrostis* in Creeping Bentgrass.** Field samples of creeping bentgrass exhibiting symptoms of dead spot were collected from a research putting green at the Paint Branch Turfgrass Research Facility (PBTRF) located in College Park, MD. Additionally, primers were tested on *O. agrostis* infected creeping bentgrass from Black Rock Country Club located in Massachusetts. The presence of *O. agrostis* was confirmed either by isolation or the presence of pseudothecia and ascospores

characteristic of the species. Samples of asymptomatic plant tissues adjacent to dead spot infection centers were used as a negative control. Finally, healthy hybrid bermudagrass plants were collected from a National Turfgrass Evaluation Program (NTEP, Beltsville, MD) variety trial located at PBTRF and used as a negative control for the primers.

**Identification of *O. agrostis* in Creeping Bentgrass Seed.** Primers were used in an attempt to amplify *O. agrostis* DNA from commercial creeping bentgrass seed listed in Table 4. In addition to bentgrass, primers were tested on ‘Snowbird’, ‘Sabre’, ‘Winterplay’, and ‘Bariviera’ roughstalk bluegrass seed provided by Kevin Morris of the NTEP. Seed DNA were extracted using Qiagen Mini-Prep Kits, with the same procedural specifications previously described with the following modifications. A total of 200 to 300 mg of seed was ground in autoclaved mortars and pestles with liquid nitrogen at the start of the extraction procedure. The DNA extraction generally was replicated twice for each seed sample and amplification with primers OaITS1 and OaITS2 was attempted twice for each DNA sample. For seed collected from Philadelphia Country Club (PCC) and Black Rock Country Club (BRCC), a total of 10 DNA isolations were made and PCR again tested twice per sample. Extracted DNA was confirmed by running a sample aliquot on an agarose gel and visualizing the DNA fragment under UV light. Amplification procedures were identical to those described previously.

## RESULTS AND DISCUSSION

**Fungal Isolation and General Observations.** A total of 80 isolates were tested in this study. Colony morphology varied when grown on PDA and incubated in the dark at 25°C for 10 days. Most isolates collected exhibited a typical rose-quartz or pink colony color previously described (Dernoeden et al., 1999; Câmara et al., 2000; Kaminski et al., 2002). Several other isolates appeared olive-gray or buff and were similar in colony color to the original description of the Ohio isolate (OpOH-1) (Câmara et al., 2000; Kaminski et al., 2002). Isolate OpNC-1 and OpNJ-6 fit into a previously undescribed mycelial color class, and were gray when grown on PDA under the aforementioned conditions. A complete description of most of the isolates tested were previously reported (Kaminski, Chapter VI).

**Development of Species-Specific Oligonucleotide Primers.** The selected primers amplified a putative 445 or 446 bp amplicon in each of the 80 *O. agrostis* isolates tested in this study (Figure 3 and 4). The 446-bp amplicon resulted from amplification of the OpOH-1 isolate, which had an additional 2 nucleotides in the ITS sequences reported by Câmara et al. (2000). It is unknown if other isolates with similar morphology to the aforementioned Ohio isolate contained an additional base pair (bp). The OaITS2 primer developed in this study resided between the two nucleotide differences and therefore amplification of the isolates was not impacted. In addition, 1 bp differences were not detectable when separated on a 1% agarose gel. Primers did not amplify DNA from eleven other turfgrass pathogens tested, including three different *Ophiosphaerella* spp.

(Figure 5). Selective amplification of *O. agrostis* was successful from each of the field-infected creeping bentgrass samples (n=8) (Figure 6). Additionally, primers did not amplify the DNA of asymptomatic, field-grown creeping bentgrass (n=4) or hybrid bermudagrass (n=4) plants. These results indicate the specificity of these primers for assisting in the identification of *O. agrostis* and the diagnosis of dead spot in both bentgrass and bermudagrass species.

**DNA Quantification and Primer Sensitivity.** Quantification of the six calibration samples resulted in the linear equation  $y = 0.090x - 0.017$  ( $R^2 = 0.99$ ); where  $y$  = strength of the PicoGreen emission wavelength (nm) and  $x$  = DNA concentration ( $\mu\text{g ml}^{-1}$ ). The DNA concentrations of 13 isolates were considered to be outside the desired range for detection; therefore, concentrations for these samples were extrapolated from the regression equation. Total DNA extracted from *O. agrostis* isolates (20 to 30 mg freeze-dried mycelium) using the DNeasy DNA isolation kit averaged  $18.03 \text{ ng } \mu\text{l}^{-1}$  (range =  $0.73$  to  $224.26 \text{ ng } \mu\text{l}^{-1}$ ) (Table 5).

Early attempts to amplify portions of *O. agrostis* DNA were erratic when total extracted stock DNA was used in the amplification procedure. Total purified *O. agrostis* DNA (n=5), therefore, was diluted to varying levels to determine the sensitivity to the developed primers. Amplification of purified *O. agrostis* DNA was successful at quantities ranging between 50 ng and 5 pg (Figure 7). Amplification of the 5 isolates generally was detected with all quantities of DNA. Although attempts to amplify the pathogen generally were successful, DNA amplification of isolates OpMD-16 and OpMD-25 resulted in varying inconsistencies. The characteristic amplicon, however,

always was present for the aforementioned isolates when 5 ng DNA was used in the assay. Amplification of the other isolates generally resulted in the presence of the distinctive amplicon at all concentrations analyzed in this study. This PCR-based molecular technique is very sensitive and results were similar to that reported by Harmon et al. (2003) for *Magnaporthe* spp. Amplification was possible with amounts of DNA as low as 5 pg. Regardless of extracted DNA concentration, amplification of *O. agrostis* with primers OaITS1 and OaITS2 were successful when stock DNA from pure cultures or infected creeping bentgrass plants was diluted 1:100.

**Identification of *O. agrostis* in Creeping Bentgrass Seed.** Primers were used in an attempt to amplify DNA of *O. agrostis* from commercially available creeping bentgrass and roughstalk bluegrass seed. Due to the appearance of dead spot in the years following seeding, many golf course managers did not have seed available for testing. A total of 20 seed samples were collected from various locations, however, only seed from PCC and BRCC was known to have been used to seed greens in which dead spot occurred. Amplification of *O. agrostis* DNA only was successful on a single attempt in seed from PCC (data not shown). Attempts to repeat this amplification from the template DNA of the aforementioned sample and from additional DNA extractions of all collected seed were unsuccessful. Additionally, attempts to culture the fungus directly from seeds plated on water agar yielded several unknown fungal species, but *O. agrostis* was not isolated.

Although the pathogen was not consistently detected in seed tested in this study, the role of seed in the spread of *O. agrostis* remains unclear. In this study, very small

quantities of seed were tested for the presence of *O. agrostis*. Seeded at a standard rate of 50 kg ha<sup>-1</sup>, a total of 2.8 kg of bentgrass seed would be needed to establish an average-sized putting green (e.g., 557 m<sup>2</sup>). Based on the quantity of seed tested in this study (400 to 600 mg sample<sup>-1</sup>), only 1.4 x 10<sup>-4</sup> to 2.2 x 10<sup>-4</sup>% of the seed used to establish an average sized putting green was evaluated for the presence of *O. agrostis*. Information on the introduction of *O. agrostis* into the USA is still limited. *O. agrostis* was identified on *Schizostachyum lima* (Blanco) Merr., a bamboo-like plant native to Indonesia that was quarantined by the Animal and Plant Health Inspection Services in 2000 (N.R. O'Neill, personnel communication). The possible role of ornamental grasses in the introduction of the pathogen into USA, however, is unknown.

## CONCLUSION

Dead spot can be difficult to diagnose from field samples if pseudothecia are not present, and often requires isolation of the pathogen to obtain a positive identification. Isolation in pure culture, however, may take several days and variation in colony color makes accurate identification of the pathogen difficult (Kaminski et al., 2002). Primers developed in this study were capable of detecting *O. agrostis* in pure culture and within infected creeping bentgrass in as little as 4 hours. Due to the novelty of this pathogen and the sometimes difficult diagnosis of the disease, these primers will assist diagnostic labs in the identification of dead spot. Molecular techniques, PCR in particular, continue to improve the accuracy and speed of diagnosing plant pathogens. Early diagnosis will assist turf managers in implementing management strategies that help reduce damage caused by dead spot.

Table 1. Isolate designation, cultivar affected, collection location, and date of isolation of *Ophiosphaerella agrostis* isolates collected from Maryland, USA.

Isolate designation	Bentgrass cultivar <sup>x</sup>	City	Isolation Date
HCC1	L-93 + Crenshaw	Hunt Valley	21 Oct. 1998
HCC2	L-93 + Crenshaw	Hunt Valley	21 Oct. 1998
OpMD-3	Penncross	Ocean City	16 Oct. 1998
OpMD-4	L-93 + Crenshaw	Upper Marlboro	26 Oct. 1998
OpMD-5	L-93 + Crenshaw	Aberdeen	15 Oct. 1998
PBD1	Penn G-2	Urbana	21 Aug. 1998
PBD2	Penn G-2	Urbana	21 Aug. 1998
OpMD-9	Providence	Silver Spring	13 Nov. 1998
OpMD-10	L-93 + Crenshaw	Upper Marlboro	26 Oct. 1998
OpMD-12	L-93	Havre de Grace	14 Aug. 1999
OpMD-13	‘Bardot’ colonial	College Park	8 Sept. 1999
OpMD-14	L-93	Joppa	26 July 2001
OpMD-15	L-93	Joppa	26 July 2001
OpMD-16	Penn A-4	Laytonsville	30 Aug. 2001
OpMD-17	Providence	College Park	26 Oct. 2001
OpMD-18	Providence	College Park	9 Nov. 2001
OpMD-19	Penncross	Ocean City	16 Oct. 1998
OpMD-21	L-93	College Park	20 June 2002
OpMD-22	L-93	College Park	20 June 2002
OpMD-23	L-93	College Park	20 June 2002
OpMD-25	L-93	College Park	28 Oct. 2002
OpMD-26	L-93	College Park	28 Oct. 2002
OpMD-27	L-93	College Park	28 Oct. 2002
OpMD-29	L-93	College Park	25 Nov. 2002
OpMD-34	L-93	College Park	25 Nov. 2002
OpMD-36	‘Bavaria’ velvet	College Park	25 Nov. 2002
OpMD-40	L-93	College Park	25 Nov. 2002
OpMD-42	L-93	College Park	28 Jan. 2003
OpMD-43	L-93	College Park	26 Mar. 2003
OpMD-44	L-93	College Park	26 June 2003
OpMD-45	L-93	College Park	26 June 2003
OpMD-46	L-93	College Park	26 June 2003
OpMD-47	L-93	College Park	26 June 2003
OpMD-48	L-93	College Park	1 Oct. 2003
OpMD-49	L-93	College Park	1 Oct. 2003
OpMD-50	L-93	College Park	1 Oct. 2003
OpMD-51	L-93	College Park	1 Oct. 2003
OpMD-52	L-93	College Park	1 Oct. 2003
OpMD-53	L-93	College Park	1 Oct. 2003
OpMD-54	L-93	College Park	1 Oct. 2003

<sup>x</sup> All bentgrass cultivars listed are creeping bentgrass (*Agrostis stolonifera* L.) unless otherwise specified.

Table 1 (con't). Isolate designation, cultivar affected, collection location, and date of isolation of *Ophiosphaerella agrostis* isolates collected from Maryland, USA.

Isolate designation	Bentgrass cultivar <sup>x</sup>	City	Isolation Date
OpMD-55	L-93	College Park	1 Oct. 2003
OpMD-56	L-93	College Park	1 Oct. 2003
OpMD-57	L-93	College Park	1 Oct. 2003
OpMD-58	L-93	College Park	1 Oct. 2003
OpMD-59	L-93	College Park	1 Oct. 2003
OpMD-60	L-93	College Park	1 Oct. 2003
OpMD-61	L-93	College Park	1 Oct. 2003
OpMD-62	L-93	College Park	1 Oct. 2003
OpMD-63	L-93	College Park	1 Oct. 2003
OpMD-64	L-93	College Park	1 Oct. 2003
OpMD-65	L-93	College Park	1 Oct. 2003
OpMD-66	L-93	College Park	1 Oct. 2003
OpMD-67	L-93	College Park	1 Oct. 2003

<sup>x</sup> All bentgrass cultivars listed are creeping bentgrass (*Agrostis stolonifera* L.) unless otherwise specified.

Table 2. Isolate designation; cultivar affected; and collection location, date and collector of various *Ophiosphaerella agrostis* isolates from the USA.

Isolate designation	Cultivar <sup>x</sup>	City, State	Date Received	Collector
OpIL-1	SR 1119	Glencoe, Illinois	18 Dec. 1998	Kane
OpIL-2	SR1119 + L-93 + Providence	Golf, Illinois	8 Dec. 2000	Kane
OpIL-3	L-93	Olympia Fields, Illinois	8 Dec. 2000	Kane
OpIL-4	L-93	Park Ridge, Illinois	1 Aug. 2002	Kane
OpIL-5	L-93	Park Ridge, Illinois	1 Aug. 2002	Kane
OpMA-1	L-93	West Bridgewater, Massachusetts	27 July 2002	Kaminski
OpMA-3	Penn A-4	Hingham, Massachusetts	Summer 2003	Kaminski
OpMA-4	Penn A-4	Hingham, Massachusetts	Summer 2003	Kaminski
OpMI-1	Providence	East Tawas, Michigan	29 Sept. 2000	Kaminski
OpMI-2	Providence	East Tawas, Michigan	29 Sept. 2000	Kaminski
OpNC-1	Penncross	Laurinburg, North Carolina	11 Aug. 2000	Wetzel
OACS	L-93	Englishtown, New Jersey	Summer 2001	Clarke
OpNJ-4	L-93	New Brunswick, New Jersey	27 July 1999	Kaminski
OpNJ-5	Penn A-4	Northfield, New Jersey	28 Sept. 2000	Kaminski
OpNJ-6	Penn A-4	Northfield, New Jersey	28 Sept. 2000	Kaminski
OpNJ-6b	Penn A-4	Northfield, New Jersey	28 Sept. 2000	Kaminski

<sup>x</sup> All cultivars are creeping bentgrass (*Agrostis stolonifera* L.).

Table 2 (con't). Isolate designation; cultivar affected; and collection location, date and collector of *Ophiosphaerella agrostis* isolates from the United States.

Isolate designation	Cultivar <sup>x</sup>	City State	Date Received	Collector
OpNY-1	L-93	Altamont, New York	11 Aug. 2000	Kaminski
OpOH-1	L-93	Chardon, Ohio	21 Oct. 1998	Kaminski
OpPA-1	Crenshaw + Southshore	Avondale, Pennsylvania	23 Dec. 1998	Kaminski
OpPA-4	L-93	Honeybrook, Pennsylvania	2 Nov. 1999	Kaminski
OpPA-6	SR1120 + L-93 + Providence	Avondale, Pennsylvania	8 Mar. 2000	Kaminski
OpPA-7	SR1120 + L-93 + Providence	Avondale, Pennsylvania	8 Mar 2000	Kaminski
OpPA-8	L-93	Honeybrook, Pennsylvania	15 Aug. 2000	Kaminski
OpVA-1	Pennlinks	Sterling, Virginia	10 Sept. 1998	Kaminski
OpVA-3	Penn A-4	Virginia Beach, Virginia	15 June 2002	Kaminski
OpVA-4	Penn A-4	Virginia Beach, Virginia	15 June 2002	Kaminski
#121	'Champion' bermudagrass	College Station, Texas	Summer 1999	Tisserat

<sup>x</sup> All cultivars are creeping bentgrass (*Agrostis stolonifera* L.) or 'Champion' hybrid bermudagrass (*Cynodon dactylon* [L.] Pers. X *C. transvaalensis* Burt-Davy).

Table 3. Isolate designation; pathogen; host species and collector of common turfgrass pathogens used to test the specificity of primers OaITS1 and OaITS2.

Isolate Designation	Genus species	Host species	Collector
#162	<i>Ophiosphaerella korrae</i>	<i>Cynodon dactylon</i>	Tisserat
Lk-5	<i>O. korrae</i>	<i>C. dactylon</i>	Dernoeden
#65	<i>O. herpotricha</i>	<i>C. dactylon</i>	Tisserat
#189	<i>O. herpotricha</i>	<i>C. dactylon</i>	Tisserat
#217	<i>O. herpotricha</i>	<i>C. dactylon</i>	Tisserat
#370	<i>O. narmari</i>	<i>C. dactylon</i>	McCann
BpMD-1	<i>Bipolaris cynodontis</i>	<i>C. dactylon</i>	Kaminski
CgMD-5	<i>Colletotrichum graminicola</i>	<i>Poa pratensis</i>	Kaminski
GgaFR-1	<i>Gaeumannomyces graminis</i> var. <i>avenae</i>	<i>Agrostis stolonifera</i>	Dernoeden
GgaMD-9	<i>G. graminis</i> var. <i>avenae</i>	<i>A. stolonifera</i>	Kaminski
GgaPA-1	<i>G. graminis</i> var. <i>avenae</i>	<i>A. stolonifera</i>	Kaminski
GsMD-1	<i>Gloeocercospora sorghi</i>	<i>A. stolonifera</i>	Kaminski
MpMD-3	<i>Magnaporthe poae</i>	<i>Poa annua</i>	Kaminski
RcCT-1	<i>Rhizoctonia cerealis</i>	<i>P. annua</i>	Kaminski
RsMD-4	<i>R. solani</i>	<i>P. pratensis</i>	Kaminski
RzMD-3	<i>R. zeae</i>	<i>Lolium perenne</i>	Kaminski
ShVA-1	<i>Sclerotinia homoeocarpa</i>	<i>A. stolonifera</i>	Kaminski

Table 4. Creeping bentgrass cultivar, collection site and location, and seedlot number of seed tested for the presence of *Ophiophaearella agrostis*.

Cultivar	Collection Site	City, State	Seedlot #
Crenshaw	Paint Branch Turfgrass Research Facility (PBTRF)	College Park, Maryland	M138-6-CB110
Crenshaw	PBTRF	College Park, Maryland	M138-6-CB111-1
Crenshaw	PBTRF	College Park, Maryland	M33-7-84
Crenshaw	South River Golf Links	Edgewater, Maryland	M33-7-84
L-93	PBTRF	College Park, Maryland	M59-9-811
L-93	Fauquier Springs Country Club	Warrenton, Virginia	M59-0-820-3
L-93	Black Rock Country Club <sup>x</sup>	Hingham, Massachusetts	Unknown
L-93	Compass Pointe Golf Course	Pasadena, Maryland	M2-9-515-1
L-93	Compass Pointe Golf Course	Pasadena, Maryland	M2-9-515-1
L-93	Philadelphia Country Club <sup>x</sup>	Gladwyne, Pennsylvania	Unknown
L-93	Prospect Bay Country Club	Grasonville, Maryland	M59-0-820-2
Penn G-2	PBTRF	College Park, Maryland	L97-9-3
Penn G-2	PBTRF	College Park, Maryland	M33-8-73
Penn G-2	Queenstown Harbor Golf Links	Queenstown, Maryland	L99-0-72
Penncross	Bay Hill Golf Club	Arnold, Maryland	L99-7-40
Penncross	Red Gate Municipal Golf Course	Rockville, Maryland	L114-0-97-17
Princeville	Salt Pond Golf Course	Bethany Beach, Delaware	M2-1-521
PennTrio	Deer Run Golf Club	Berlin, Maryland	M16M-9-25PT
Putter + Cato	Fauquier Springs Country Club	Warrenton, Virginia	LMF0-8-0034
Southshore	Great Hope Golf Course	Westover, Maryland	LA20-8-391322
SR1119	Country Club at Woodmore	Mitchellville, Maryland	JC1-9-11

<sup>x</sup> Seed from Black Rock Country Club and Philadelphia Country Club were the only seed tested in which dead spot was known to have occurred.

Table 5. DNA concentrations of 80 *Ophiosphaerella agrostis* isolates.

Isolate Designation	DNA conc. (ng $\mu\text{l}^{-1}$ )	Isolate Designation	DNA conc. (ng $\mu\text{l}^{-1}$ )	Isolate Designation	DNA conc. (ng $\mu\text{l}^{-1}$ )
OpNC-1	5.88	HCC2	21.91	OpMD-42	23.88
OpNJ-4 <sup>x</sup>	1.90	OpMD-9	16.90	OpMD-43	24.92
OpNJ-5	7.55	OpMD-12	7.47	OpMD-44	4.92
OpNJ-6	42.10	OpMD-13	17.28	OpMD-45	3.21
OpNJ-6b	8.80	OpMD-14	27.40	OpMD-46	5.87
OpNY-1	7.00	OpMD-15	9.42	OpMD-47	3.28
OpOH-1 <sup>x</sup>	2.52	OpMD-16 <sup>y</sup>	31.56	OpMD-48	8.00
OpPA-1	13.52	OpMD-17 <sup>x</sup>	0.73	OpMD-49	4.91
OpPA-4	34.96	OpMD-18	27.61	OpMD-50	18.72
OpPA-6 <sup>y</sup>	29.59	OpMD-19	24.65	OpMD-51	3.12
OpPA-7 <sup>x</sup>	1.36	OpMD-7	8.71	OpMD-52	10.81
OpPA-8 <sup>x</sup>	2.88	OpMD-21	46.32	OpMD-53 <sup>x</sup>	2.92
OpVA-1	35.90	OpMD-22	30.14	OpMD-54	6.18
OpVA-4	17.71	OpMD-23	13.76	OpMD-55	5.54
OpVA-3 <sup>y</sup>	46.70	OpMD-25 <sup>y</sup>	99.26	OpMD-56 <sup>x</sup>	2.84
OpIL-4	36.26	OpMD-26	4.53	OpMD-57	5.15
OpIL-5	7.74	OpMD-27	27.62	OpMD-58	8.32
#121	6.66	OpMD-29	5.77	OpMD-59	4.07
OACS <sup>x</sup>	1.95	HCC1	20.72	OpMD-60	7.11
OpIL-1	16.30	OpMD-34	13.50	OpMD-61	4.34
OpIL-2	7.25	OpMD-36	4.20	OpMD-62 <sup>x</sup>	2.03
OpIL-3	32.00	OpMD-3 <sup>x</sup>	3.14	OpMD-63 <sup>x</sup>	2.99
OpMA-1 <sup>x</sup>	224.26	OpMD-40	21.49	OpMD-64	6.41
OpMI-1	14.81	PBD1	18.56	OpMD-65	11.87
OpMI-2 <sup>x</sup>	1.58	PBD2	5.30	OpMD-66	6.59
OpMA-3	67.06	OpMD-4	10.95	OpMD-67	6.26
OpMA-4 <sup>y</sup>	50.27	OpMD-10	4.45		

<sup>y</sup> Isolates used to determine polymerase chain reaction amplification sensitivity for detecting *Ophiosphaerella agrostis*.

<sup>x</sup> Quantification was outside the computer generated range; therefore, DNA concentrations were determined from the linear equation from the DNA standards ( $R^2=0.99$ )

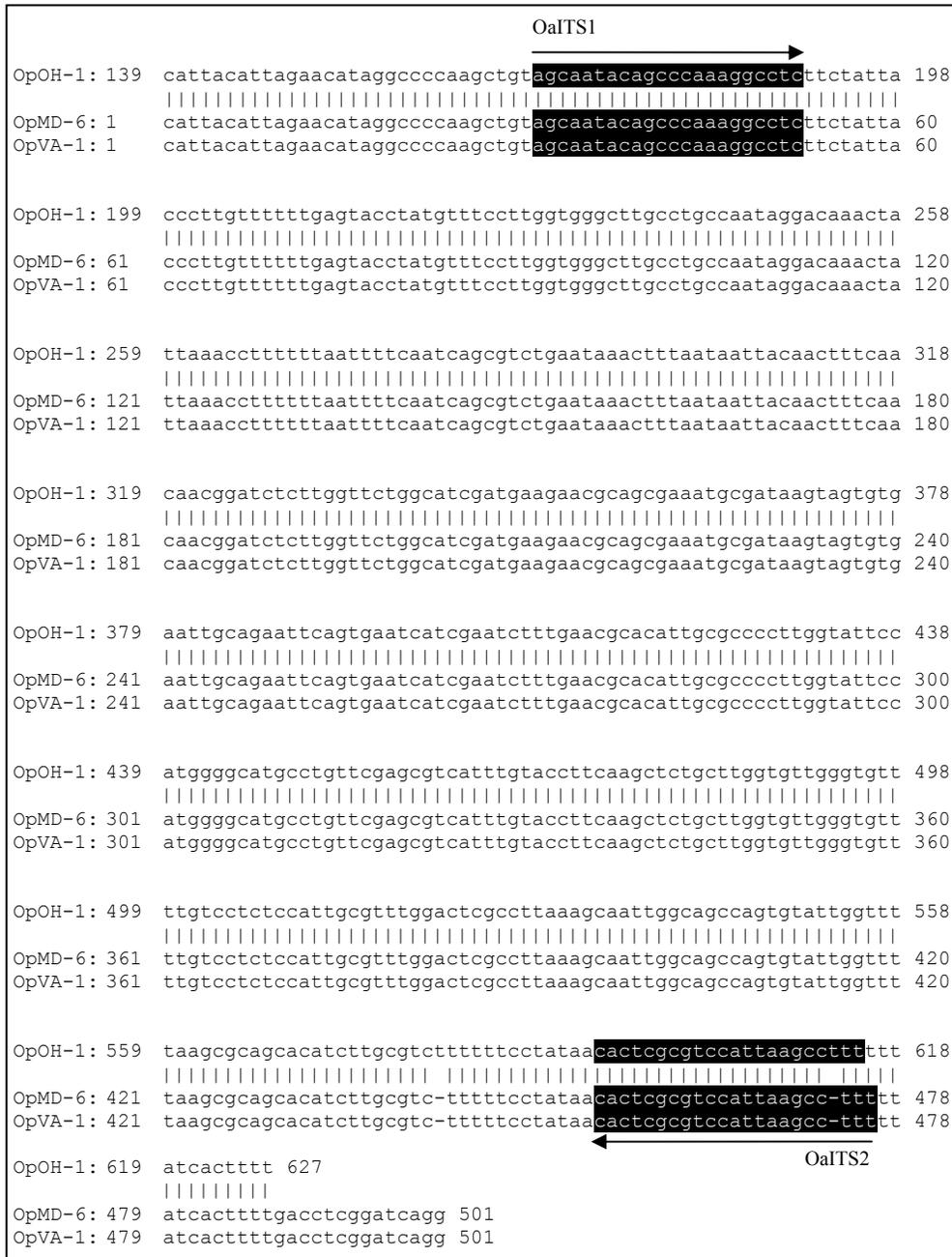


Figure 1. Oligonucleotide primers specific to *Ophiostoma agrostis* (OaITS1 and OaITS2) were developed based on the ITS regions of isolates OpOH-1, OpMD-6 and OpVA-1, previously sequenced by Câmara et al., 2000.

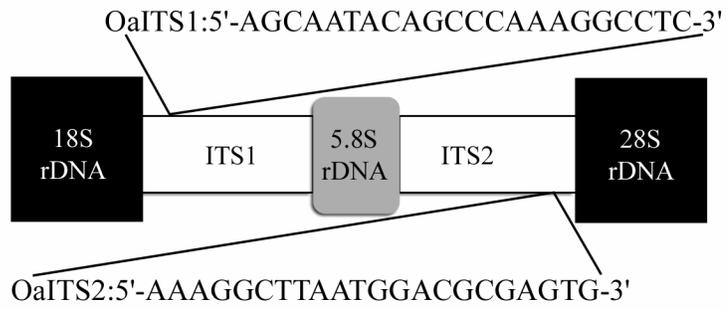


Figure 2. Internal transcribed spacer region depicting location of primers OaITS1 and OaITS2 specific for *Ophiosphaerella agrostis*. The primer pair amplified a 445 to 446 bp region of all 80 *O. agrostis* isolates tested.

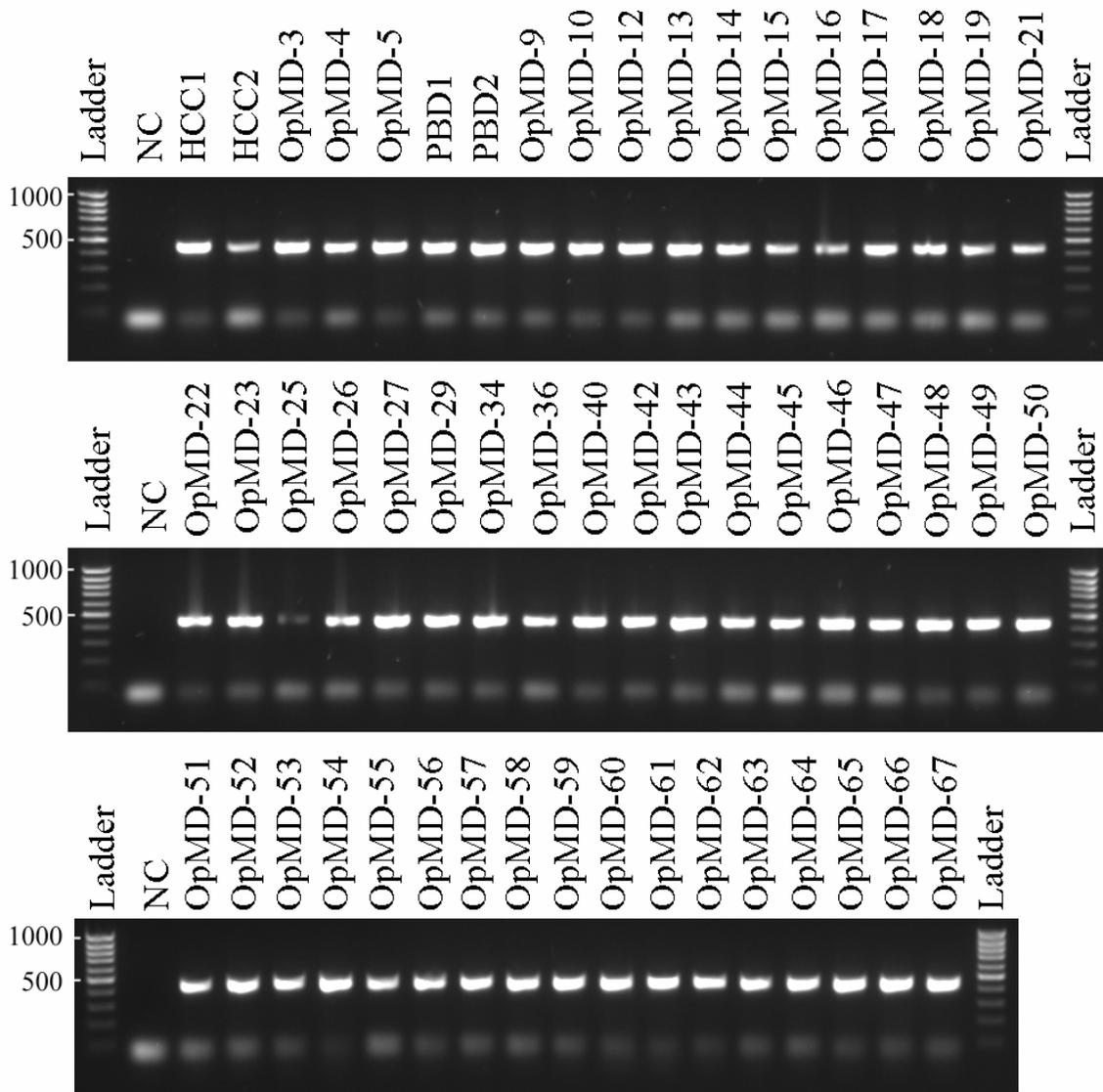


Figure 3. Polymerase chain reaction (PCR) product amplified from the ITS region of DNA from (n=53) *Ophiosphaerella agrostis* isolates collected in Maryland, USA. A negative control (NC) and a 100 bp ladder are shown.

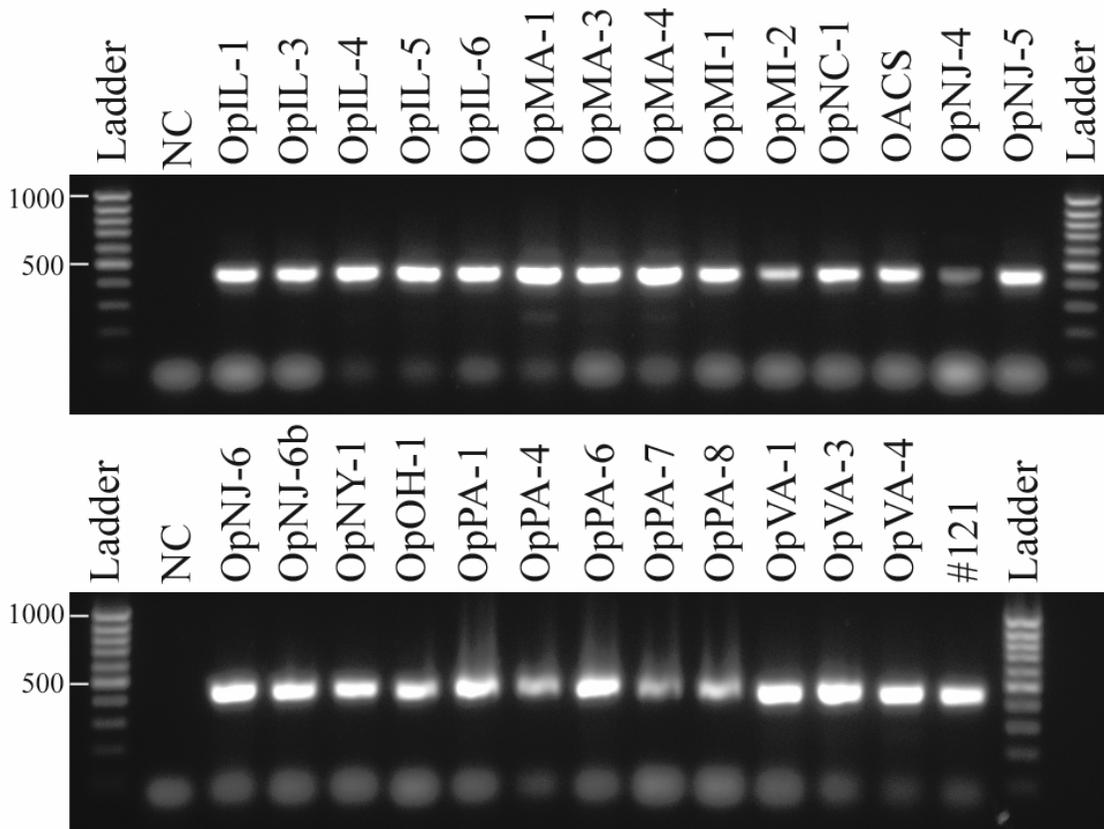


Figure 4. Polymerase chain reaction (PCR) product amplified from the ITS region of DNA from (n=27) *Ophiosphaerella agrostis* isolates from 10 states. A negative control (NC) and a 100 bp ladder are shown.

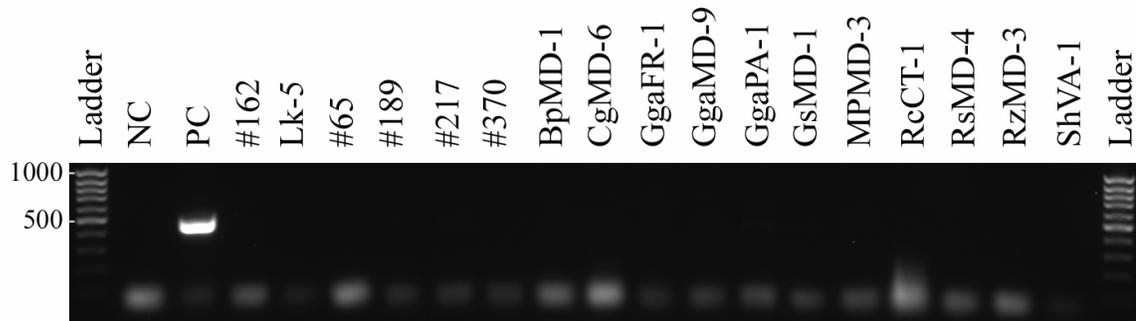


Figure 5. Polymerase chain reaction (PCR) amplification of fungal DNA from various turfgrass pathogens. From left to right: 100 bp ladder, negative control (NC); positive control (PC; OpVA-4); *Ophiostphaerella korrae* (#162, Lk-5); *O. herpotricha* (#189,#217); *O. narmari* (#370); *Bipolaris cynodontis* (BpMD-1); *Colletotrichum graminicola* (CgMD-6); *Gauemannomyces graminis* var. *avenae* (GgaFR-1, GgaMD-9, GgaPA-1); *Gloeocercospora sorghi* (GsMD-1), *Magnaporthe poae* (MpMD-3), *Rhizoctonia cerealis* (RcCT-1); *R. solani* (RsMD-4); *R. zae* (RzMD-3); *Sclerotinia homoeocarpa* (ShVA-1); and a 100 bp ladder.

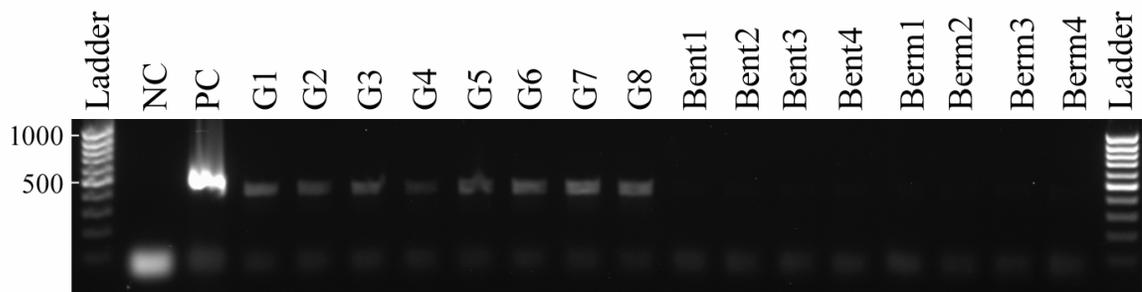


Figure 6. Polymerase chain reaction (PCR) amplification of fungal DNA from field-infected creeping bentgrass. From left to right: 100 bp ladder; negative control (NC); positive control (PC; OpVA-4); *Ophiosphaerella agrostis*-infected bentgrass plants (G1-G8), asymptomatic creeping bentgrass (Bent1-Bent4); hybrid bermudagrass (Berm1-Berm4) plants; and a 100 bp ladder.

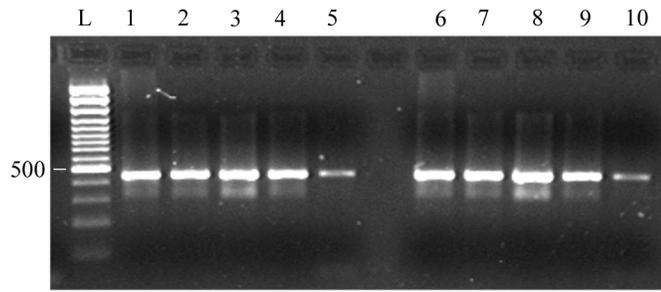


Figure 7. Amplification of *Ophiosphaerella agrostis* at various DNA dilutions. From left to right: ladder (L); OpPA-6 (1-5); and OpVA-3 (6-10) at 50 ng, 5 ng, 0.5 ng, 50 pg, and 5 pg DNA.

## VIII. EPILOGUE: A ROAD MAP TO FUTURE DEAD SPOT RESEARCH

### INTRODUCTION

In 1998, Dernoeden et al. (1999) discovered a new disease of creeping bentgrass (*Agrostis stolonifera* L.) incited by an unidentified species of *Ophiosphaerella*. Through morphological and molecular study, it was shown that the pathogen constituted a new species, *Ophiosphaerella agrostis* Dernoeden, M.P.S. Câmara, N.R. O'Neill, van Berkum et M.E. Palm (Câmara et al., 2000). The disease commonly is referred to as dead spot. Research on various biological and genetic aspects of *O. agrostis* and epidemiology and management of dead spot was conducted at the University of Maryland between 1998 and 2003. A synthesis of these results and suggestions for future dead spot research are presented herein.

### EPIDEMIOLOGY

Dead spot only has been observed on creeping bentgrass and bermudagrass (*Cynodon dactylon* [L.] Pers. x *C. transvaalensis* Burt-Davy) grown on sand-based root zones. There are no reports of the disease in turf grown on native soil. Active dead spot infection centers generally appear in areas with full sun and good air circulation. In particular, *O. agrostis* infection centers often appear initially along ridges and on mounds and south-facing slopes of putting greens. These areas are particularly prone to higher soil temperatures and often are the first to exhibit drought symptoms. Dead spot typically develops between 1 and 2 years following bentgrass establishment, however, outbreaks of the disease have been observed on creeping bentgrass that was less than 1 year old and as old as 6 years of age (Kaminski and Dernoeden, 2002).

Dead spot is most severe during the first or second year of symptom expression and the disease rarely recurs in areas previously infected by the pathogen (Kaminski and Dernoeden, 2002). The decline phase typically lasts from 1 to 3 years after the first year of disease expression, with the number of infection centers per green normally decreasing in subsequent years. Turf recovers very slowly, as stolons growing into dead patches often become infected and die. Dead spot is most prevalent on newly constructed putting greens, but the disease also may appear on putting greens established following fumigation with methyl bromide. In a pilot field study, the disease did not recur following fumigation with dazomet (tetrahydro-3,5,-dimethyl-2H-1,3,5-thiadiazine-2-thione) (Kaminski, unpublished). Evidently, methyl bromide more effectively reduces populations of microbes that in some way antagonize or compete with *O. agrostis*. A slower decline occurs with take-all (*Gauemannomyces graminis* (Sacc.) Arx and D. Olivier var. *avenae* (E. M. Turner) Dennis) in *Agrostis* turf in response to a build-up of bacterial antagonists (Smiley et al., 1992; Smith et al., 1989). The speculated antagonists responsible for dead spot decline are unknown. *O. agrostis* antagonists may reproduce more rapidly and/or be more competitive than those responsible for the decline of *G. graminis* var. *avenae*. For this reason, *O. agrostis* may serve as a model pathogen for examining the impact of soil microbial antagonists on natural disease suppression. Identification and enumeration of populations of known microbes (e.g., fluorescent *Pseudomonas* spp.) or total microbial populations following initial construction of sand-based putting greens as well as following methyl bromide fumigation may provide some insight into their influence on dead spot decline. Emerging techniques that aid in the

identification and quantification of microbial communities (e.g., Biolog<sup>®</sup>, Inc., Hayward, CA) may provide a useful tool for studying decline phenomena.

In addition to an increase in microbial populations, a reduction in inoculum also may lead to a decrease in disease severity. In a three year cultivar study, dead spot was severe in the first and third year, but only limited disease was observed in the second year. Although monthly air and soil temperatures during all three years of the field study generally were similar (i.e., 1 to 4°C difference for each month), inoculum levels varied each year. Ascospores were not quantified in year one, however, damage from *O. agrostis* was considered severe and numerous pseudothecia were observed within infection centers. In the second year following establishment, a reduction in the number of new infection centers as well as the number of pseudothecia produced resulted in low levels of ascospores. For the final year of the study, an adjacent portion of the research green was fumigated with methyl bromide and disease levels again were extremely and moderately severe in both the fumigated and re-inoculated cultivar study, respectively. The number of infection centers and pseudothecia developing in year three resulted in a greater number of ascospores released, when compared to year two. Environmental conditions appear to play an important role in the development of disease symptoms, but the pathogen likely is most damaging where soil microbial populations are low and ascospore levels are high. The maturity of the turf also likely influences the incidence and severity of dead spot. Growth chamber studies, in which bentgrass plants of varying maturity are inoculated with varying concentrations of ascospores, may reveal differences in the ability of the pathogen to infect plants and elicit dead spot symptoms. An additional aspect of such a study should include an assessment of the influence of

fumigated versus non-fumigated soil on dead spot severity.

An important aspect of the biology of *O. agrostis* is the release and alighting of ascospores on bentgrass tissues. Ascospores can be forcefully released from pseudothecia and disseminated by wind or water. Dead spots generally only coalesce under severe pressure, suggesting that wind is the primary mechanism of dispersal. It is likely, however, that a majority of the ascospores released into wind currents are blown off-site and would not come into contact with a susceptible host. Ascospores that ooze from pseudothecia in the presence of water likely have the ability to infect bentgrass stolons, roots and leaves within or along the periphery of dead spot infection centers. Visual observations of the distribution of dead spot infection centers revealed that new infection centers most often develop within close proximity (1 to 30 cm or greater) of older infection centers. Based on the aforementioned observation, however, it appears possible that splashing rain or irrigation water may carry large numbers of ascospores to other plants. The rain-splash mechanism of ascospore dispersal also suggests that a critical mass of ascospores may be necessary to cause infection and subsequent symptom expression.

On creeping bentgrass grown in the mid-Atlantic region, dead spot is most severe between mid-June and late August. During this period, patch diameter as well as the number of pseudothecia produced per patch increase at a linear rate. In growth chamber studies, pseudothecia developed when incubated under constant light and were not produced when incubated in darkness (Kaminski et al., 2002). In field studies, pseudothecia began to develop when day length increased to 14 hours. Although light is an important factor for the development of pseudothecia, the influence of photoperiod on

their development remains unclear. Examination of diseased hybrid bermudagrass turf from putting greens in Texas and Florida, however, revealed the presence of numerous pseudothecia as early as March. Therefore, in addition to light, the accumulation of heat may play an important role in the development of pseudothecia. In a Maryland study, pseudothecia production generally began to increase at a linear rate in mid-to-late June. Air and soil temperatures increase earlier in the year in the southern United States, when compared to the mid-Atlantic region. If the accumulation of heat were important to their development, pseudothecia likely would begin to develop later (i.e., June) in the mid-Atlantic region, when compared to Florida and Texas (i.e., March). Controlled experiments designed to examine the influence of temperature and photoperiod on *O. agrostis*-infested tall fescue/wheat bran mix would elucidate their role and possible interaction on pseudothecia development.

Dead spot symptoms can appear as early as May in inoculated sites, however, the development of natural disease symptoms likely occurs following prolonged periods when soil temperatures are  $\geq 20^{\circ}\text{C}$ . Indeed, laboratory studies revealed that a 12 to 28 day incubation period at a constant temperature (20 to  $30^{\circ}\text{C}$ ) was required for dead spot reactivation in naturally infected, winter-dormant plants (Kaminski et al., 2002). A model that utilized soil temperatures of  $\geq 20^{\circ}\text{C}$  to predict the appearance of new *O. agrostis* infection centers (incidence) generally made false predictions during late spring. In the autumn months, however, soil temperature generally declined dramatically ( $21^{\circ}\text{C}$  in September to  $14^{\circ}\text{C}$  in October) and few new infection centers were observed after early October. In a three year field study, dead spot was severe in the initial year following establishment and following fumigation. In those years when disease pressure

was severe (2000 and 2002), dead spot symptoms first appeared in either early June (2000) or early May (2002). In the 2000 study site, the area was inoculated the previous October, while inoculation of the study area used to monitor dead spot in 2002 occurred in March 2002. Despite nearly a one month difference in the appearance of new infection centers in the spring of 2000 and 2002, a linear increase in the expansion of new spots did not begin in either year until 218 and 213 total degree days (biofix date = 1 May; base temperature = 15°C). Additionally, when inoculation occurred in the autumn, *O. agrostis* infection centers immediately began to increase in size when they first appeared in late spring (8 June). When bentgrass was inoculated in the spring, however, new infection centers appeared, but remained relatively small in size for approximately one month prior to the linear increase in patch diameter. These observations indicate that there is an influence of inoculation timing on the appearance of dead spot symptoms, but they also support the view that naturally developing symptoms appear later in the year (mid-to-late June) following the accumulation of degree days. Due to the differences in inoculation timing, a cumulative degree-day (DD) model was not accurate in predicting the development of initial infection centers. The linear increase phase for patch diameter and pseudothecia development, therefore, may be representative of the environmental conditions influencing natural symptom expression and peak dead spot activity. As previously noted, natural dead spot symptoms develop between 213 and 218 cumulative DD. Hence, while a DD model for predicting the appearance of the initial infection centers in the spring was highly variable, the DD model predicting the start of the linear growth phase (i.e., peak period of disease) of the disease was more precise. Validation of the DD model for predicting the linear growth phase of dead spot, however, would

require the collection of environmental data from various geographic regions and should be conducted under conditions in which inoculation occurs in the autumn so the pathogen overwinters and the disease is allowed to develop more naturally.

Dead spot is a polycyclic disease and the importance of *O. agrostis* ascospores in the spread and development of the disease was examined. Ascospores of *O. agrostis* were observed to be forcefully ejected through ostioles of pseudothecia or exuded *en masse* in the presence of water (Kaminski et al., 2002). In field and growth chamber studies, ascospores rarely were released when pseudothecia were dry. In the field, ascospores were released in large numbers at dawn and dusk, and also during precipitation events. Ascospore release events occurred when periods of low relative humidity coincided with periods of leaf wetness. As indicated by a growth chamber study, ascospores only were captured when pseudothecia contained some level of moisture, but generally were not released from dry fruiting bodies. During the morning hours in a field study, relative humidity was observed to sharply decrease while the canopy remained wet. Conversely, as dew and guttation fluid began to form in the evening hours, relative humidity remained low (~60%). In both instances, a moisture gradient would have been created in which dry atmospheric air presumably pulled water from saturated pseudothecia. The loss of moisture from saturated pseudothecia presumably caused a disruption of the two-layered asci, which resulted in the forceful discharge of ascospores. This likely occurs from contraction of bitunicate asci, which have a rigid outer layer (ectoascus) and an elastic inner layer (endoascus). As the moisture available within a pseudothecium decreases, the ectoascus layer ruptures allowing the elastic endoascus to extend towards the ostiole, rupture, and forcefully

release its ascospores (Alexopoulos et al., 1996). The importance of declining atmospheric moisture levels on ascospore release was further supported by a growth chamber study where it was shown that a sharp decrease (100% to ~50%) in relative humidity resulted in the immediate discharge of ascospores. While it is clear that some level of moisture is needed for ascospore release, it appears that lower levels of relative humidity in conjunction with moist or saturated pseudothecia are essential for forceful ascospore release. It is possible, however, that high levels of relative humidity which saturate pseudothecia with water may result in an oozing rather than ejection of ascospores into the air. This may explain the lack of ascospores found in air samples collected from pseudothecia in a growth chamber where relative humidity was raised rapidly from ~50% to 100%. The rapid increase in relative humidity and resulting saturation of pseudothecia may have prevented the forceful release of ascospores, however, pseudothecia were not monitored for *en masse* ascospore release at this time. Future growth chamber studies designed to elucidate the impact of precipitation events during periods of varying levels of relative humidity would further clarify the mechanism of *O. agrostis* ascospore release. The study would include the application of water from simulated rainfall events to mature pseudothecia being maintained at a constant level of relative humidity ranging in increments from high (100%) to low (50%) relative humidity. To assess the impact of high levels of constant relative humidity on the *en masse* release of ascospores, the concentration of ascospores within the free water accumulating around mature pseudothecia could be determined through pipette and hemocytometer quantification techniques. Additionally, the impact of rain splash on the dissemination of ascospores could be assessed by quantifying the concentration of

ascospores collected within Petri dishes placed at varying distances from the inoculum source.

Regardless of whether ascospores are forcefully discharged or ooze from pseudothecia, the initiation of their release is rapid ( $\leq 1$  hour) and may continue as long as free moisture is present and relative humidity is below some unknown critical level. In the laboratory, ascospores were observed to germinate in as little as 2 hours (Kaminski et al., 2002). During the early hours of incubation, ascospores generally germinated in larger numbers in the presence of light and bentgrass leaves or roots. Using an ascospore suspension, ascospore germination and infection were observed to occur within 24 hours, but the infection process may occur more rapidly (Kaminski et al., 2002). Up to four germ tubes may develop from an individual ascospore. Each germ tube either can produce an appressorium or continue to grow as hyphae. Appressoria are capable of directly penetrating leaves and roots, while hyphae or germ tubes may enter open stomates on leaves. Tissue penetration from multiple appressoria from the same ascospore was observed in the lab. Leaf surface exudates appear to be an important factor in rapid germination and subsequent infection of leaves by *O. agrostis*. Although ascospores can germinate quickly (i.e.,  $\leq 2$  hours), a majority of the ascospores germinated within 8 to 12 hours in water in the lab (25°C). Based on lab results, it appears likely that ascospores released in nature and prior to an extended period of leaf wetness are more likely to complete the infection process. In the aforementioned study, percent ascospore germination was  $\leq 36\%$  after four hours of incubation at 25°C. In a field study, large numbers of ascospores were collected between 0700 and 1000 hours and 1900 and 2300 hours. The bentgrass canopy, however, generally was dry by 1000 or

1100 hours leaving only a short period for germination and infection. Therefore, only small numbers of ascospores released in the morning or during short irrigation cycles (15 to 30 minutes) are likely to cause infection due to the rapid drying (1 to 2 hours) of the bentgrass canopy. A majority of successful *O. agrostis* infections likely occur following the release of ascospores at dusk, prior to extended periods of leaf wetness during the evening hours.

Although unknown, the field environmental conditions necessary for ascospore germination and penetration of bentgrass leaves likely include prolonged periods of leaf wetness and average daily air temperatures  $\geq 22^{\circ}\text{C}$ . The importance of leaf wetness duration and temperature on the incidence and severity of several plant pathogens has been widely studied (Carisse and Kushalappa, 1992; Evans et al., 1992; Schuh and Adamowicz, 1993; Sullivan et al, 2002; Wu et al., 1999). The requirement for some minimum temperature for infection by *O. agrostis* to occur is supported by the observation that the large numbers of ascospores released in May and again in September, did not coincide with the appearance of similarly large numbers of new infection centers. While average air temperatures in May and September (2000 to 2002) ranged between 17 and 21 $^{\circ}\text{C}$ , air temperatures between June and August ranged between 22 and 26 $^{\circ}\text{C}$ . The aforementioned temperatures, as well as extended periods of leaf wetness, are common at night on creeping bentgrass putting greens in Maryland throughout the summer. Elucidation of the environmental conditions necessary for *O. agrostis* ascospores to infect tissue would provide a better understanding of the disease cycle. Growth chamber studies in which ascospore-inoculated creeping bentgrass was exposed to varying hours of leaf wetness and temperatures and then incubated at varying

post-inoculation temperatures would better define both the conditions necessary for infection to occur as well as the conditions necessary for disease symptoms to appear. Additionally, inoculation using varying ascospore concentrations and direct placement of inoculum on various tissues (i.e., leaves, collar region, roots/stolons, etc.) would help determine the relative susceptibility of the various tissue types to infection. Results from the aforementioned studies would provide insight into the infection process. Furthermore, this new information would allow for the reexamination of data from the three year Maryland field study in which the environmental conditions surrounding the development and spread of dead spot were recorded.

Although ascospores often are released in abundance, leaf spots rarely are observed on bentgrass plants. Dead spot symptoms generally include reddish-brown or discolored leaves and darkened leaf tissue at the collar region of older leaves. Based on these observations, it appears likely that most ascospores alighting on bentgrass leaves are washed down into the collar (i.e., intersection of leaf and ligule) and potentially between the leaf sheaths, where they germinate and infect the leaf, sheath or both. Following infection of bentgrass leaves, the pathogen apparently moves downward, colonizing tissue as it progresses toward the roots and stolons. It appears that *O. agrostis* enters a latent phase of several days between the time when infection is initiated and when dead spot symptoms first appear. It is likely that during this period, the pathogen continues to spread along the surfaces of the plant as hyphae and hyphal mats may be seen on stem bases and more commonly on nodal regions of bentgrass stolons of infected plants. Infection of these tissues does occur since simple hyphopodia and direct penetration have been observed. While traditional histological techniques may be useful

in the examination of the infection process, these techniques often are time consuming and require familiarity with the pathosystem. For instance, examination of the infection process in potted plants often results in the necessity to differentiate fungal mycelia from both the target pathogen as well as other fungi that commonly contaminate the growing medium. Even when growing media are sterilized, these opportunistic fungi quickly reestablish themselves. If infection is successful with the target pathogen, the pathogen can be stained for visual examination. This process usually kills the organism, resulting in a snap-shot of one particular phase of the infection process. Emerging molecular techniques (e.g., green fluorescence protein [GFP] transformation), however, allow for the visual observation of the infection process as well as the movement of the pathogen throughout the plant in real time and without disturbance to either the plant or the pathogen. Following insertion of a fluorescence gene, the pathogen will emit a fluorescent ‘glow’ when exposed to various excitation wavelengths such as ultraviolet light. *O. agrostis* isolates transformed with the GFP protein would allow for the monitoring of a single ascospore as it germinates, infects the host and moves throughout the plant without death of either the plant or pathogen.

During environmental conditions favorable for dead spot, plants survive for short periods following infection during what may be termed the “latent phase” of the disease. Symptoms appear when the pathogen colonizes large areas of the plant and/or the plant is weakened by some other environmental stress (e.g., heat or drought). Small dead spots (approximately 1 to 2 cm in diameter) generally appear in the field between 4 and 10 days following a large release of ascospores. The length of this latent period (i.e., after infection, but before the appearance of symptoms) likely is dependent upon

environmental conditions on days following infection. While extended periods of leaf wetness likely are necessary for infection to occur, dead spot symptoms appear to be influenced by environmental conditions that promote plant stress (i.e., heat and drought). The environmental conditions defined below that influence epidemics and pseudothecia production help to better characterize the latent period between infection and symptom expression.

Conditions that influence dead spot epidemics were defined by a multiparameter model including elevated air ( $AT_{Max} \geq 27^{\circ}C$ ) and soil ( $ST_{Mean} \geq 18^{\circ}C$ ) temperatures; low relative humidity ( $RH_{Mean} \leq 80\%$ ); shortened periods of leaf wetness ( $LWD \leq 14$  hours); and high levels of solar radiation ( $SOL_{Mean} \geq 230 W m^{-2}$ ). The model (i.e., EFI) accurately predicted 37 out of 40 dead spot epidemics. It should be noted, however, that the models for predicting dead spot incidence and severity were developed in Maryland and validation in other geographic regions has not been performed. In field situations, pseudothecia can be found within necrotic tissues when infection centers first appear. In growth chamber studies, pseudothecia developed within 4 days following incubation in constant light on media consisting of tall fescue seed and wheat bran (Kaminski et al., 2002). Combining the information obtained from the growth chamber study and field observations, it is likely that pseudothecia formed by the time field-symptoms first appear had begun to develop at least four days prior to the appearance of new infection centers. Hence, the latent period between infection and symptom expression can occur in as little as three days under ideal environmental conditions that favor the pathogen.

Mature ascospores develop in pseudothecia within 7 days of incubation at 25°C in a growth chamber. Pseudothecia development within necrotic bentgrass tissues occurred between late-June and early September in Maryland and corresponded with mean soil temperatures between 24 and 26°C in all three years. This process of ascospore release, infection, symptom expression and the subsequent production of pseudothecia, and maturity and release of new ascospores occurred in a cyclic pattern, which was repeated about every 12 days. In the field, major ascospore release events occurred as few as 9 days apart. Accounting for the release of ascospores and subsequent infection (possibly  $\leq$  24 hours), the appearance of dead spot symptoms (3 to 10 days following ascospore release), and the development of mature ascospores (3 to 4 days following appearance of mature pseudothecia), the entire cycle from ascospore release to the development of new ascospores may occur within 6 to 14 days. This timeline is consistent with field observations in which large numbers of ascospores were released on average every 11 (2001) to 12 (2002) days. While it is apparent that the release of *O. agrostis* ascospores and the development of dead spot symptoms occur in a cyclic fashion, it is unlikely that this cycle is dictated by anything more than the necessary time-line required between ascospore release and the development of new ascospores. It is likely, however, that the time required to accomplish this reproductive process is influenced by the previously described environmental conditions associated with infection, symptom expression and pseudothecia development. Controlled growth chamber experiments designed to assess the influence of temperature, relative humidity, soil moisture, and other factors on the development of dead spot symptoms would more accurately define the disease cycle.

## VARIOUS MOLECULAR ASPECTS OF *O. AGROSTIS*

Dead spot often is confused with other common turfgrass diseases or maladies. While the presence of pseudothecia often serves as a quick and accurate diagnostic aid, these sexual fruiting bodies are not always present in infected samples. To aid in the identification of the pathogen and diagnosis of the disease, species-specific primers were developed. Each primer in the set was designed from previously sequenced internal transcribed spacer (ITS) regions of *O. agrostis* (Câmara et al., 2000). Both primers (OaITS1 and OaITS2) consist of 22 nucleotides and amplify a 445 or 446 bp DNA fragment specific to *O. agrostis* and they do not amplify the DNA of the three other known *Ophiosphaerella* species found in turf. Additionally, the primers did not amplify the DNA from several other pathogens commonly associated with various turfgrass diseases. The primers were capable of detecting the pathogen both from pure cultures as well as from field-infected creeping bentgrass. The entire process including DNA isolation, amplification, and visualization can be completed in approximately four hours. Therefore, these primers would be beneficial to diagnostic labs that are equipped to perform PCR, and especially valuable to those having little or no experience in diagnosing dead spot.

In general, plant pathogens that reproduce solely by asexual means exhibit very little genomic diversity. On the other hand, higher levels of genetic diversity would be expected from fungi that undergo sexual recombination. Additionally, many plant pathogens with a polycyclic disease cycle initiate disease by sexual means (e.g. ascospores) and then produce asexual structures (e.g. conidia), which serve as the sole source of secondary inoculum. An unusual characteristic of *O. agrostis*, however, is its

lack of a known anamorph combined with its ability to produce numerous pseudothecia throughout the summer months. Ascospores within pseudothecia serve as the secondary source of inoculum. The exclusivity of the sexual stage of *O. agrostis* in nature likely adds to the genetic diversity of the species. Initial DNA fingerprinting of *O. agrostis* isolates collected in 1998 revealed an 87% or greater similarity among isolates from five different states (Câmara et al., 2000). Based on the DNA fingerprinting of 77 isolates collected between 1998 and 2003 from eleven states, the species was separated into three distinct clades with  $\geq 69\%$  similarity. Variation in the results of the two studies likely was due to differences in the AFLP techniques utilized (fluorescent versus polyacrylamide gel) and the larger number of isolates examined (12 versus 77).

In the examination of the 77 *O. agrostis* isolates, three clades were formed. Isolates examined within each clade generally had varying levels of pseudothecia production and varying colony colors when grown on PDA. In general, colony color was similar within clades B and C; while colony color of isolates within clade A appeared to be a mixture of the two aforementioned molecular groupings. Pseudothecia production of two isolates from clade A generally was low or did not occur, while moderate and high levels of pseudothecia were produced with isolates from clades B and C, respectively. Although *O. agrostis* is a homothallic species, it is unclear if outcrossing among strains occurs. On a bentgrass research putting green located in College Park, MD, isolates separating into all three clades were recovered despite inoculation of the site with isolates from only two (i.e., B and C) of the clades. The reason for this segregation, however, remains unclear. Different strains of the pathogen may have been introduced through seed or ascospores carried to the site naturally by air currents. Other possible

explanations would be mycelial fusion between different *O. agrostis* strains and therefore the combination of varying genotypic information during meiosis. Homothallic, filamentous ascomycetes have been shown to have either one or two mating genes (MAT) with two alternative forms (MAT1-1 and MAT1-2) known as idiomorphs (Glass et al., 1990; Pöggeler, 1999). Although the mating genes of *O. agrostis* are unknown, both mating types have been detected in *O. korrae* (Hsiang et al., 2003). Despite having both mating type genes, other homothallic fungi are capable of both selfing and outcrossing. In particular, isolates of *Gibberella zeae* (Schwein.) Petch, which carry both MAT1-1 and MAT1-2 in the same nucleus, have been shown to outcross (Lee et al., 2003). Additionally, recent studies involving the differential deletion of each idiomorph within *G. zeae* resulted in a self-sterile strain (Lee et al., 2003). In other words, the homothallic *G. zeae* was modified through molecular processes to behave as a strictly heterothallic fungus. The ability of the mutated strain of *G. zeae* to outcross with tester isolates was retained, indicating the need for both MAT idiomorphs to be self fertile. Currently, varying levels of pseudothecia production (i.e., sexual reproduction) have been observed among isolates of *O. agrostis*. Future work involving the characterization of mating type genes of *O. agrostis* may help to elucidate the variability of sexual reproduction among strains. Amplification and sequencing of these MAT genes may be useful for genetic analyses of pathogenicity, diversity and other characteristics of the pathogen.

Sexual reproduction plays an important role in the survival and pathogenicity of many plant pathogens. The ability to sexually reproduce is an evolutionary process that often results in increased pathogen fitness and survival. Genetic diversity of isolates also

may lend important implications into the introduction of the pathogen into different geographic regions. Populations that maintain high levels of similarity within a specific geographic region, and also are similar to a select group of isolates from a completely different geographic area, may indicate that the pathogen was introduced into both regions from the same or similar genetic source. For example, *O. agrostis* isolates within clade C were extremely similar ( $\geq 99\%$ ), when compared to isolates within clade A (77%) and B (84%). Despite this similarity, isolates within clade C were collected from various regions of the United States including Maryland, Massachusetts, New York, Ohio and Pennsylvania. Results from these and future analyses may indicate that the pathogen was introduced from a similar source, such as from seed grown in the same region. To determine the genetic diversity of *O. agrostis*, single ascospore isolates should be collected from successive generations from a subset of isolates from each of the three clades and AFLP performed. This information may reveal the inherent diversity of a single ascospore over multiple generations. If the diversity of successive generations is high, the pathogen likely undergoes a natural divergence during self fertilization. More likely, however, is that multiple generations from a single ascospore would retain a higher genetic similarity and thus suggest that the high levels of genetic diversity among isolates occurred from outcrossing among *O. agrostis* strains. To account for the diversity resulting from meiosis of a single *O. agrostis* ascospore, multiple ascospores should be obtained from a single pseudothecium. The DNA fingerprinting of the isolates obtained from each ascospore may reveal natural variation in multiple ascospores developing from a single, self-fertile strain of the pathogen. Genetic differences among isolates collected from these processes will help to clarify the natural diversity of the

species and the possible introduction of different *O. agrostis* strains into various geographic regions.

In addition to differences in the DNA fingerprinting among *O. agrostis* isolates, previous research revealed differences in rDNA sequences in the internal transcribed spacer (ITS) region. The ITS region is conserved within species of a particular genus and sequences from this region are perhaps the most widely used in the reclassification of fungi based on molecular, rather than morphological characteristics. The validity of this approach, however, has been questioned and multiple gene sequencing may be necessary to accurately categorize both new and existing pathogens into its correct genus and species. Sequencing of three *O. agrostis* isolates revealed a single base pair (bp) insertion of thymine nested between a cytosine and a five thymine repeat in two different locations of the ITS2 region in an atypical isolate (OpOH-1) (Câmara et al., 2000). Sequence variation in these regions may reveal further differences among isolates separating into each of the three clades and potentially clarify the placement of these isolates within this newly described species. Based on the aforementioned DNA fingerprinting, a total of 12 additional isolates exhibited strong similarities to isolate OpOH-1. The entire group (clade C), however, only was 69% similar to the other *O. agrostis* isolates (n=65) examined. The strong similarity among a small subset of isolates may indicate possible divergence to a new species of *Ophiosphaerella* other than *O. agrostis*. Therefore, further examination of ITS and glycer-aldehyde-3-phosphate dehydrogenase (GPD) sequences from additional isolates should be investigated.

## DEAD SPOT CONTROL AND MANAGEMENT

Little is known about cultural and chemical strategies for managing dead spot. A few studies, however, have identified differences in disease severity among bentgrass cultivars, fungicides, and various nitrogen sources (Kaminski and Dernoeden, 2002; Towers et al., 2000; Wetzal and Butler, 2000; Wetzal and Butler, 2001). Kaminski and Dernoeden (2002), reported that *O. agrostis* was capable of infecting the three common *Agrostis* species grown on golf courses including creeping, velvet (*Agrostis canina* L.) and colonial (*Agrostis capillaris* L.) bentgrasses. Variation in cultivar susceptibility was reported among bentgrass cultivars. Newer bentgrass cultivars ('L-93' and the Penn 'A' and 'G' series), generally exhibited the most susceptibility and the older cultivars (i.e., 'Penncross' and 'Pennlinks') the least.

When applied after peak disease activity, various nitrogen (N) sources (ammonium sulfate; IBDU, isobutylidene diurea; SCU, sulfur coated urea; urea; Ringer Greens Super; and methylene urea) aided in the recovery of bentgrass from dead spot. In this study, the disease was allowed to naturally develop throughout the summer and no control measures were implemented prior to application of the fungicide iprodione and the various fertilizer treatments. It was observed that large infection centers (6 to 10 cm in diameter) were not capable of completely recovering, regardless of treatment. Hence, none of the fertilizers provided for complete turf recovery prior to winter. Plots treated with ammonium sulfate, however, resulted in a more rapid turf recovery, when compared to the slow-release IBDU fertilizer. These results indicated that quick release N-sources may be more beneficial in aiding bentgrass recovery, but that larger infection centers likely will remain present for extended periods, regardless of N-source.

In a second field study, the impact of five, water-soluble N-sources ( $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , urea, and 20-20-20) on dead spot severity and recovery were examined. Anecdotal information obtained from Dr. R. White at Texas A&M indicated that the application of ammonium sulfate may prevent and/or reduce the occurrence of dead spot in bermudagrass. When applied approximately one month (28 June 2002) after infection centers first were observed, however, the aforementioned N-sources failed to prevent new infection centers from occurring. In fact, the number of infection centers among all treatments increased from an average of 21 to 93 infection centers plot<sup>-1</sup> between 27 June and 10 August 2002. All of the N-sources reduced disease severity prior to winter, when compared to unfertilized plots. In year two of this second field study, dead spot did not recur in plots receiving  $(\text{NH}_4)_2\text{SO}_4$ , but new infection centers were observed in plots treated with nitrate or urea-based fertilizers. The number of dead spot infection centers was positively correlated with pH, and disease incidence appeared to be favored by a pH greater than 6.0 and 6.6 in the mat (0 to 2.5 cm) and underlying soil (2.6 to 5.0 cm), respectively. Ammonium-based N fertilizers have been shown to reduce the severity of several turfgrass diseases, presumably due to its ability to acidify soil. These results suggest that the acidifying ammonium sulfate may reduce dead spot when applications are initiated during establishment and several months in advance of the time when initial symptoms would appear. Conversely, increases in the severity of some turfgrass diseases have been attributed to the application of alkaline-reacting fertilizers such as  $\text{Ca}(\text{NO}_3)_2$  and  $\text{NaNO}_3$ . Although not statistically different from the other N-sources evaluated, applications of  $\text{KNO}_3$  resulted in a general reduction in dead spot in the first year, but dead spot symptoms recurred in these plots in the second year. The full

impact of these fertilizers on dead spot incidence and severity only may be realized following their repeated application during establishment (i.e., prior to symptom expression). Monitoring increases or decreases in soil pH accorded to the different N-sources and relating them to dead spot incidence and severity would be most informative.

In the second study, the initial soil  $K_2O$  levels were low, and it remains unclear if the addition of K from sources other than  $KNO_3$  would result in an overall reduction of the disease. Further research on the impact of K in reducing dead spot would provide a better understanding of cultural dead spot suppression methods. Various K-sources and rates should be examined for their impact on dead spot severity. It would be informative to apply  $K_2SO_4$  (0-0-50) or  $KCl$  (0-0-60) with various N-sources to determine the impact of N + K versus the impact of N or K alone on dead spot severity. These studies may reveal an additive or synergistic effect on the reduction of dead spot from the application of N (e.g., ammonium sulfate versus  $NaNO_3$ ) in conjunction with K. A potential reduction in disease simply from the addition of K in K-deficient soils also should be assessed.

Additionally, growth chamber and field experiments assessing the impact of soil type and varying pH levels would help elucidate their role on dead spot incidence and severity. Greenhouse studies should examine the effect of sand-based soils (calcium versus silicate sands) modified to varying levels of pH on dead spot severity. While it is clear that various nitrate or ammonium-based N-sources change soil and mat pH after repeated applications, the direct impact of pH is unknown. Due to the elevated pH levels of water used to irrigate many golf courses, acidifying agents occasionally are used to reduce irrigation water pH. Therefore, studies also should be conducted to address the

impact of irrigation water pH on the disease. The repeated application of acidifying fertilizers (i.e., ammonium sulfate) may reduce the pH to levels unfavorable for bentgrass growth. In these instances, turfgrass managers may apply lime to ameliorate a low soil pH condition. The effect of lime or other alkaline-reacting materials applied alone or in combination with ammonium sulfate is unknown and requires further investigation.

Due to the limited occurrence of dead spot and its rapid decline, few fungicide efficacy studies have been conducted. The ability of the fungicide iprodione to control dead spot has provided varying results. In North Carolina, iprodione was shown to reduce dead spot severity when applied both preventively and curatively (Wetzel and Butler, 2000). In a New Jersey study, however, preventive applications of iprodione provided only fair control of dead spot (Tower et al., 2001). Iprodione applied after the peak period for dead spot activity in Maryland had passed (i.e., September) resulted in little or no reduction in dead spot severity. After this peak period in the Maryland study, however, few new infection centers appeared and increases in patch diameter generally did not occur. Therefore, the Maryland study indicated that iprodione, and perhaps other fungicides, only are likely to be effective when applied preventively or just after the initial symptoms are observed. In the mid-Atlantic region, a preventive fungicide program for new putting green constructions would begin in May, prior to the time when symptoms are most likely to appear. Applying fungicides during the period of increasing patch diameter and pseudothecia development (i.e., June through August), would likely result in reduced or poor dead spot control. According to Wetzel and Butler (2000), weekly applications of urea in conjunction with early-curative fungicides reduced the number and diameter of dead spot infection centers. When applied weekly, however,

urea alone did not significantly reduce dead spot severity, when compared to the untreated control (Wetzel and Butler, 2000). Hence, in an early curative fungicide program, chemicals need to be applied early enough to prevent new infections and nitrogen should be tank-mixed with fungicides to aid in the recovery of existing dead spots. The interaction of various fungicides and fertilizers needs to be investigated further to properly develop control programs directed toward the most effective management of dead spot.

APPENDIX A. Nitrogen Source Impact on Dead Spot (*Ophiosphaerella agrostis*)  
Recovery In Creeping Bentgrass

MATERIALS AND METHODS

***Autumn Recovery-Study I***

In 2000, turf was mowed with a walk-behind mower (Toro 500, Toro Inc., Minneapolis, MN) to a height of 5.1 mm at least 3 times wk<sup>-1</sup>. In 2001, mowing was resumed on 3 April. The height of cut was lowered from 4.6 to 4.0 mm by 6 June. The area was vertical mowed using a Toro 1000 walk-behind mower equipped with a groomer (Toro Inc., Minneapolis, MN) on 25 May and 10 June 2000. Due to mechanical damage from the vertical cutting the mowing height was raised back to 4.6 mm on 12 July. Approximately 2000 kg ha<sup>-1</sup> of sand topdressing (i.e., dried construction mix) were applied on 15 December 1999 and 29 March, 5 April, 3 and 22 June, and 21 August 2000. On 18 April 2001, the entire area was aerated to a depth of 10 cm using 6.4 mm diameter hollow tines and topdressed with approximately 10,000 kg of dried construction mix ha<sup>-1</sup>. Thereafter, light applications of topdressing (i.e., 2000 kg ha<sup>-1</sup>) were made on 25 and 31 May and 8 June 2000. The site was treated with 590 and 490 kg of agricultural limestone (CaCO<sub>3</sub>) ha<sup>-1</sup> on 19 November 1999 and 29 March 2000, respectively.

To control sod webworm (*Crambus* spp.) and black cutworm (*Agrotis ipsilon* Hufnagel), 5.7 kg a.i. ha<sup>-1</sup> chlorpyrifos [0,0-diethyl O-(2,5,6-trichloro-2-pyridyl) phosphorothioate] and 0.08 kg a.i. ha<sup>-1</sup> fluvalinate [(RS)-  $\alpha$ -cyano-3-3-phenoxybenzyl(R)-2-[2-chloro-4-(trifluoromethyl)anilino]-3-methyl-butanoate] were applied on 8 May and 19 July 2001, respectively. To control localized dry spot (LDS),

6.2 L ha<sup>-1</sup> of Aqueduct™ (50% nonionic polyols) (Aquatrols, Inc., Cherry Hills, NJ), was applied on 29 June 2001. Sprayable materials were applied through a CO<sub>2</sub> pressurized sprayer as described previously and granulars through a rotary spreader.

**Dead spot severity.** Dead spot severity also was rated on a visual 0 to 100 percent linear scale where 0 = no dead spot present and 100 = entire plot covered with dead spot. Damage caused by dollar spot was assessed in October and November 2000 and again in August 2001. In 2000, dollar spot was rated by counting the number of *S. homoeocarpa*-infection centers plot<sup>-1</sup>. On 7 August 2001, dollar spot severity was rated on a visual 0 to 100 percent linear scale where 0 = no dollar spot present and 100 = entire plot covered with dollar spot.

### ***Curative Management and Recovery-Study II***

For curative control of northern masked chaffer (*Cyclocephala borealis* Arrow) and black cutworm (*Agrotis ipsilon* Hufnagel), 4.3 kg a.i. ha<sup>-1</sup> chlorpyrifos [0,0-diethyl O-(2,5,6-trichloro-2-pyridyl) phosphorothioate] + 2.4 kg a.i. ha<sup>-1</sup> isofenphos (1-methylethyl 2-[ethoxy (1-methylethyl) amino] phosphinothioyl] oxy] benzoate) and 5.7 kg a.i. ha<sup>-1</sup> chlorpyrifos were applied on 28 June 2002 and 16 August 2002, respectively. On 2 May 2003, 0.76 kg a.i. ha<sup>-1</sup> deltamethrin ((S)-alpha-cyano-3-phenoxybenzyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) was applied to control sod webworm (*Crambus* spp.). To manage localized dry spot, 92 kg ha<sup>-1</sup> of LescoWet® (2-butoxyethanol, polyoxyethylene, polypropoxypropanol) granular (Lesco, Inc., Sebring, FL) and 51 L ha<sup>-1</sup> LescoFlo were applied on 16 September 2002 and 6 May 2003; respectively. Due to periodic epidemics of dollar spot (*Sclerotinia homoeocarpa* F.T.

Bennett), fungicides were applied throughout the study. On three dates (13 September 2002 and 14 May and 9 June 2003), however, fungicide applications that impact dead spot severity were mistakenly applied to the site. On 13 September 2002, propiconazole (0.38 kg a.i. ha<sup>-1</sup>) (1-[2,4-dichlophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole ) was applied to control dollar spot. Chlorothalonil (8.3 kg a.i. ha<sup>-1</sup>) + propiconazole (0.19 kg a.i. ha<sup>-1</sup>) was applied on 14 May and 9 June 2003. On 29 July 2003, triadimefon (1.5 kg a.i. ha<sup>-1</sup>) + myclobutanil (0.37 kg a.i. ha<sup>-1</sup>) (a-butyl-a-(chlorophenyl)-1H-1,2,4-triazole-1-propanenitrile) were applied. On 8 August 2003, 1.5 kg a.i. ha<sup>-1</sup> triadimefon were applied to control dollar spot.

**Turfgrass quality and color.** In 2002 and summer 2003, turfgrass quality was rated approximately every two weeks on the 0 to 10 visual scale previously described. Again, damage caused by dead spot was not included in overall quality ratings. Between 6 June and 23 September 2003, color was rated biweekly on a visual 0 to 10 scale where 0 = brown or dead turf, 8.0 = minimal acceptable color for a creeping bentgrass putting green, and 10 = dark green turf. In addition to dead spot, ratings were made on several other pests and stress problems. Take-all patch and brown patch were rated visually on a linear 0 to 100% scale as previously described. Dollar spot was rated by counting the number of infection centers per plot. Additionally, analyses were performed to determine the correlation between mat mass and mat depth and surface firmness and mat depth and mass. Regression lines for mat measurements (uncompressed and compressed) also were compared using the method described by Neter and Wasserman (1974).

**pH and Soluble Salts.** Hydrogen concentration [H<sup>+</sup>] also was measured to monitor short-term changes in pH in the top 2.5 cm of the soil. Measurements of [H<sup>+</sup>]

were made between 15 and 22 September 2003 following the application of 12 kg N ha<sup>-1</sup> on 13 September. Soluble salts only were measured in plots receiving Ca(NO<sub>3</sub>)<sub>2</sub>, KNO<sub>3</sub>, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and measurements were made between 15 and 22 September 2003. For electrical conductivity measurements, samples were processed as described for pH with the following modifications. A total of 12.5 grams of thatch were placed into each cup and 25 ml ddH<sub>2</sub>O added. Samples immediately were placed on a shaker table (LabLine Orbit Shaker, Lab-Line Instruments Inc., Melrose, IL) and shaken at 90 rpm. After shaking for 10 min, samples were removed and sediment was allowed to settle for 20 minutes. Electrical conductivity was measured using a Model 72 conductivity meter (Engineered Systems and Designs, Newark, DE).

**Thatch depth and Mass.** In the second year of study II, the influence of the fertilizer treatments on thatch depth and mass as well as firmness of the putting surface were measured. For thatch measurements, three core samples (1.9 cm diam.) plot<sup>-1</sup> were taken on 29 August 2003 and 17 October 2003. Thatch depth was measured by either measuring uncompressed thatch and by compressing thatch between fingers as described by Callahan et al. (1997). Samples collected on 17 October then were processed immediately for thatch biomass analysis. To determine biomass in thatch, green leaf tissues as well as sand and roots below the thatch were removed. Samples then were dried for 1 week at 40°C and weighed. Samples were placed in a 550°C ashing oven overnight and re-weighed. Thatch biomass was calculated as dry weight minus ashed weight. Putting surface firmness was measured using a penetrometer (Lang Penetrometer, Gulf Shores, AL). Measurements were made by slowly applying pressure to the penetrometer until penetration through the thatch layer occurred. A total of five

measurements were made plot<sup>-1</sup>. All penetrometer readings were converted to a force measurement (kPa) using the calibration scale provided by the manufacturer. For all measurements, sub-sample measurements were averaged for each experimental unit and used for the statistical analysis.

## RESULTS

### *Autumn Recovery-Study I*

**Dead spot incidence.** After fumigation with methyl bromide in 2001, study site I showed delayed onset and reduced severity from dead spot in 2002, when compared to an adjacent site previously not used for study (Figure 1). On 18 July 2002, the adjacent area surrounding the Study Site I had approximately 42 infection centers  $2.3 \text{ m}^{-2}$ , while plots in study site I only had an average of 10 to 16 infection centers  $2.3 \text{ m}^{-2}$  (Table 1). Unfortunately, due to application of N to the declining untreated control plots of study I, the impact of fertilizer level could not be determined. The reasons for the suppression of dead spot in study I site, when compared to an adjacent area, is unknown.

**Dollar spot.** *Sclerotinia homoeocarpa* infection centers were present between October and November 2000. On all rating dates, there was no fertilizer by fungicide interactions (Table 2). There were, however, significant main effects from fertilizer treatment (29 November 2000) and iprodione (20 October 2000 and 7 August 2001). On 20 October, pressure from dollar spot was severe. Split-plots treated with iprodione resulted in excellent dollar spot control (2 infection centers  $\text{plot}^{-1}$ ), while those not receiving iprodione had an average of 22 infection centers  $\text{plot}^{-1}$  (Table 3). In an effort to reduce the damage caused by dollar spot, the entire study area was treated with triadimefon and fenarimol + chlorothalonil on 20 October 2000 and 10 November 2000, respectively. The application of fungicides to the non-iprodione sub-plots negated the main effect of fungicide on dollar spot. On 29 November, however, the effect of fertilizer treatment on the number of *S. homoeocarpa* infection centers became significant. The untreated control had the greatest number of infection centers  $\text{plot}^{-1}$ .

There were no differences in the number of *S. homoeocarpa* infection centers among plots treated with IBDU, Ringer Lawn Restore and the untreated control. Ammonium sulfate, methylene urea, sulfur coated urea, and urea reduced *S. homoeocarpa* infection centers, when compared to the aforementioned treatments. Despite the fact that no additional fungicides were applied in 2001, sub-plots treated with iprodione exhibited significantly less dollar spot ( $P=0.0040$ ), when compared to the untreated sub-plots on 7 August 2001. On the aforementioned date, percent dollar spot in iprodione-treated plots versus the untreated plots was 0.8 and 2.2%, respectively. It is unclear if the repeated applications of iprodione resulted in extended reduction of the disease or if the application of fenarimol only to the untreated control plots in September somehow resulted in resurgence of the pathogen in 2001.

### ***Curative Management and Recovery-Study II***

**Dead spot severity.** On 23 August 2002, creeping bentgrass plots treated with  $KNO_3$  had the lowest percent area affected by dead spot (2.6%), when compared to the unfertilized control (6.8%) (Table 4). There were no differences, however, between plots treated with  $KNO_3$  and all other N-source and Mn-alone treatments on 23 August 2002. Percent of plot area affected by dead spot increased in unfertilized and Mn-alone plots until mid September and leveled off between 10 and 12 percent disease plot<sup>-1</sup> on the final rating date (31 October). In plots receiving N, the greatest percent of plot area affected by dead spot occurred between late-August and early-September. Some recovery occurred in plots treated with N in mid-September, but was followed by a recurrence of

active disease in late-September. No new infection centers appeared after 30 September, and recovery of diseased turf occurred slowly during the autumn.

**Turfgrass quality and color.** A reduction in turfgrass color in plots treated with ammonium sulfate resulted in poor overall quality during spring 2003 (Table 5). Color, therefore, was rated separately and removed from overall quality ratings during the summer months. Between July and early-August, bentgrass color improved in ammonium sulfate plots and was similar among treatments receiving N. For the remainder of the study, few significant differences in color existed among plots in which N was applied. Except for ammonium sulfate-treated plots, however, all fertilized plots had excellent color between 21 August and the final rating date (23 September).

On 6 June 2003, creeping bentgrass color was poor in plots treated with ammonium sulfate. The darkest bentgrass color was observed in plots treated with either of the nitrate N-sources. There were no differences, however, among the aforementioned treatments and urea, urea + Mn, and 20-20-20. On all rating dates, color and quality were very poor in plots in which no N was applied.

**pH, soluble salts and thatch.** Mass of the organic matter within the mat also was measured (Table 6). Mass was greatest in plots treated with ammonium sulfate. Mass in the aforementioned treatment, however, was similar to thatch mass in plots treated with urea and urea+Mn. Plots not receiving N (Mn and untreated control) had the lowest thatch mass. Thatch mass and thatch depth were highly correlated ( $P=0.0001$ ), regardless of thatch depth measurement technique (Figure 2). Pearson correlations for uncompressed thatch and compressed thatch were  $r=0.85$  and  $r=0.86$ , respectively.

Regression analysis of compressed versus uncompressed thatch measurements also was significant ( $P < 0.0001$ ). The equation of the regression line was  $y = 1.069x + 0.609$ , where  $y$  = compressed thatch measurements and  $x$  = uncompressed thatch measurements (Figure 3).

To examine the effect of applying  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_3$ , and  $(\text{NH}_4)_2\text{SO}_4$  on mat pH, measurements were made following fertilizer applications on 13 and 27 September 2003. On both dates, N was applied at a rate of  $12 \text{ kg ha}^{-1}$ . No fertilizer was applied during the month of August and pH measurements on 29 August were used as a base measurement for comparison purposes. On 15 September (2 days after fertilizer applications), mat pH levels in plots receiving  $\text{KNO}_3$  and  $\text{Ca}(\text{NO}_3)_2$  increased by 0.38 and 0.29 pH units, respectively (Table 7). Conversely, the pH within the mat of plots treated with ammonium sulfate, decreased from 5.57 on 29 August to 4.95 on 15 September. When compared to 29 August, mat pH in plots treated with  $\text{Ca}(\text{NO}_3)_2$  or  $\text{KNO}_3$  remained elevated for 6 to 12 days. However, the sharp decrease in mat pH observed in plots treated with ammonium sulfate only was sustained for a short duration and within 4 days of application mat pH levels were similar to those observed on 29 August. Electrical conductivity measured during this same period revealed few differences among treatments. Soluble salts recorded in the mat of the three fertilizer treatments ranged from 98 to  $271 \text{ mg kg}^{-1}$  and differences only were significant on 22 September 2003 (Table 8). On the aforementioned date, soluble salts in the mat from plots treated with ammonium sulfate were significantly lower than those measured in plots treated with either nitrate N-source.

Mat pH again was monitored following application of fertilizer treatments on 27 September. Plots receiving urea and the unfertilized plots also were included in the analyses. On all rating dates following N application, mat in plots treated with  $\text{KNO}_3$  and ammonium sulfate had the highest (pH=6.78 to 7.18) and lowest (pH=5.10 to 5.65) pH, respectively (Table 9). Calcium nitrate-treated plots generally had mat pH levels similar to those observed in plots receiving  $\text{KNO}_3$ . On four out of five rating dates, plots treated with urea had lower mat pH levels, when compared to  $\text{Ca}(\text{NO}_3)_2$ -treated plots. The unfertilized plots had mat pH levels between 6.40 and 6.56, which generally were similar to the plots treated with  $\text{Ca}(\text{NO}_3)_2$  and urea.

Thatch depth was measured at the end of the study on 29 August and 17 October 2003 (Table 6). On 29 August, measurements on uncompressed thatch revealed few differences in thatch depth. The greatest thatch depth was observed in plots receiving urea+Mn and 20-20-20 (24.0 to 24.2 mm). However, there were no differences among any treatments in which N was applied (21.6 to 23.7 mm). The shallowest thatch depth was observed in unfertilized plots (19.9), but thatch depth in those plots were not different from plots treated with  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_3$ , urea, or Mn. On 17 October, depth of thatch was measured using either the uncompressed or compressed method. Despite the measurement technique, plots treated with ammonium sulfate had the greatest thatch depth (24.8 and 26.5 mm). These depths, however, were not significantly different than those found in plots treated with any other N-source (24.3 to 26.5 mm).

The necessary pressure required to break through the thatch layer with a penetrometer was used as a measure of surface firmness. Based on results using the Lang Penetrometer, fertilizer significantly affected surface firmness ( $P=0.0001$ ). Plots treated

with ammonium sulfate were most firm (0.705 kPa) (Table 6). There was no surface firmness difference, however, between the aforementioned treatment and plots treated with  $\text{Ca}(\text{NO}_3)_2$  (0.611 kPa). The pressure needed to break through the thatch layer was least in unfertilized and Mn alone plots (0.395 and 0.433 kPa, respectively). The firmness of the remaining treatments was 0.522 to 0.705 kPa.

**Insect pests, disease and other disorders.** On 26 June 2003, plots treated with urea, urea + Mn, and 20-20-20 had high levels (12.5 to 15%) of take-all (Table 10). Although not significantly different from the aforementioned treatments,  $\text{Ca}(\text{NO}_3)_2$ -treated plots had moderate (4.8%) take-all damage. Similarly, low to moderate levels (1.8 to 2.8%) of take-all also were observed in plots treated with  $\text{KNO}_3$  and ammonium sulfate. By 12 July, all N treated plots had take-all levels ranging from 9% to 18%, and there were no differences among treatments.

Dollar spot, brown patch and yellow spot (etiology unknown) were monitored in the study area between June and August. On all rating dates, there were no differences among fertilizers for any disease; however, plots treated with ammonium sulfate were not visibly affected by brown patch or yellow spot on any rating date (Table 10).

Appendix A. Table 1. Number of dead spot infection centers as influence by six N-sources, College Park, 2000.

Fertilizer	Dead spot infection centers plot <sup>-1</sup>					
	2000					2002
	13 Sept	6 Oct	3 Nov	29 Nov	AUDPC	18 July
				no.		
Ammonium sulfate	30 <sup>x</sup>	20	14	12	16	17
IBDU	25	29	20	19	20	15
Methylene urea	23	18	13	13	14	14
Ringer Greens Super	28	22	20	16	18	18
SCU	20	17	10	9	12	11
Urea	27	20	13	14	15	10
Untreated	33	42	32	33	30	16

<sup>x</sup> Means were not significantly different ( $P<0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 2. Source of variation for *Sclerotinia homoeocarpa* infection centers as influence by six N-sources and iprodione, College Park, 2000 to 2001.

Rating date	Source	NDF	DDF	Type III F	Pr>F
20 October 2000	Fertilizer	6	39	0.83	NS <sup>x</sup>
	Fungicide	1	39	27.43	***
	Fert x Fung	6	39	0.93	NS
29 November 2000	Fertilizer	6	39	3.25	*
	Fungicide	1	39	0.60	NS
	Fert x Fung	6	39	0.97	NS
7 August 2001	Fertilizer	6	39	0.24	NS
	Fungicide	1	39	9.38	**
	Fert x Fung	6	39	0.35	NS

<sup>x</sup> \* = Significant at  $P=0.05$ , \*\* = Significant at  $P=0.01$ , \*\*\* = Significant at  $P=0.001$ , NS = Not significant.

Appendix A. Table 3. Number of *Sclerotinia homoeocarpa* infection centers plot<sup>-1</sup> and percent dollar spot in creeping bentgrass as influence by six N-sources and iprodione, College Park, 2000 to 2001.

Source of Variation	Dollar spot		
	2000		2001
	20 Oct	29 Nov	7 Aug
Fertilizer	Infection Centers		%
Ammonium sulfate	9 a <sup>x</sup>	12 b	1.8 a
IBDU	18 a	19 a	1.9 a
Methylene urea	14 a	13 b	1.4 a
Ringer Greens Super	12 a	15 a	1.0 a
SCU	3 a	9 b	1.4 a
Urea	15 a	13 b	1.5 a
Untreated	13 a	33 a	1.4 a
Fungicide			
Iprodione	2 b <sup>y</sup>	15 a	0.8 b
Untreated	22 a	17 a	2.2 a

<sup>x</sup> Only ratings in which a significant main effect occurred are shown.

<sup>y</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 4. Impact of various N-sources and manganese on percent of plot area damaged by dead spot in an 'L-93' creeping bentgrass putting green, College Park, 2002.

Treatment <sup>z</sup>	Dead spot plot <sup>1</sup>									
	2 Aug	10 Aug	16 Aug	23 Aug	4 Sept	17 Sept	30 Sept	20 Oct	31 Oct	
										%
Ca(NO <sub>3</sub> ) <sub>2</sub>	3.0 a <sup>y</sup>	2.9 a	4.6 a	3.8 ab	3.5 b	3.3 b	3.8 b	3.1 b	2.9 b	
KNO <sub>3</sub>	1.4 a	1.3 a	2.0 a	2.1 b	2.6 b	1.9 b	2.5 b	2.9 b	2.3 b	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3.3 a	2.9 a	4.1 a	4.4 ab	2.8 b	2.4 b	2.6 b	1.4 b	1.0 b	
Urea	2.8 a	2.4 a	3.1 a	3.3 ab	3.4 b	2.5 b	3.8 b	3.0 b	2.5 b	
Urea + MnSO <sub>4</sub>	2.5 a	2.6 a	3.5 a	3.5 ab	4.0 b	2.6 b	3.1 b	3.3 b	2.4 b	
MnSO <sub>4</sub>	2.9 a	2.9 a	4.6 a	5.1 ab	8.1 a	10.5 a	11.5 a	10.3 a	11.0 a	
Nutriculture	2.3 a	2.6 a	4.0 a	3.4 ab	2.6 b	2.8 b	3.1 b	2.4 b	2.0 b	
Untreated	3.6 a	3.6 a	5.4 a	6.8 a	8.6 a	11.5 a	11.8 a	11.0 a	12.3 a	
P > F	0.3019	0.4212	0.2331	0.0482	0.0001	0.0001	0.0001	0.0001	0.0001	

<sup>z</sup> Nitrogen treatments were applied as follows: 5 kg N ha<sup>-1</sup> on 28 June; 5, 12, 19 and 27 July; and 2 10 and 17 August and 12 kg N ha<sup>-1</sup> on 26 August; 4, 13 and 30 September; and 21 October 2002.

<sup>y</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 5. Impact of various N-sources and manganese on creeping bentgrass color in an 'L-93' creeping bentgrass putting green, College Park, 2003

Treatment <sup>z</sup>	Color							
	6 June	12 July	29 July	7 Aug	21 Aug	29 Aug	13 Sept	23 Sept
	0-10 scale <sup>y</sup>							
Ca(NO <sub>3</sub> ) <sub>2</sub>	8.1 a <sup>x</sup>	8.0 a	7.6 a	8.3 a	7.8 ab	8.3 a	8.5 ab	8.4 a
KNO <sub>3</sub>	8.3 a	7.9 a	7.1 a	8.1 a	8.3 ab	8.1 a	8.3 ab	8.3 ab
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	7.1 b	8.4 a	8.0 a	8.1 a	7.6 b	7.9 a	7.0 b	7.0 b
Urea	7.8 ab	7.8 a	7.6 a	8.0 a	8.4 ab	8.5 a	8.5 ab	8.6 a
Urea + MnSO <sub>4</sub>	7.8 ab	7.9 a	7.6 a	8.4 a	8.0 ab	8.1 a	8.3 ab	8.1 ab
MnSO <sub>4</sub>	1.5 c	2.6 b	2.8 b	2.8 b	3.5 c	4.0 b	4.6 c	3.3 c
Nutriculture	7.5 ab	7.6 a	7.5 a	8.3 a	8.8 a	8.8 a	9.1 a	8.6 a
Untreated	1.5 d	2.4 b	2.6 b	2.0 b	2.3 d	2.9 b	3.4 c	2.5 c

<sup>z</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Creeping bentgrass color was assessed visually on a 0 to 10 scale, where 0 = brown or dead turf, 8 = minimum acceptable color for a bentgrass putting green, and 10 = dark green turf.

<sup>x</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test ( $P = 0.0001$ ).

Appendix A. Table 6. Impact of various N-sources and manganese on thatch/mat accumulation in an 'L-93' creeping bentgrass putting green, 2003

Treatment <sup>y</sup>	Thatch/mat		Thatch/mat		Firmness
	Uncompressed <sup>z</sup>		compressed	17 Oct	16 Oct
	29 Aug	17 Oct	17 Oct		
	mm			g	kPa
Ca(NO <sub>3</sub> ) <sub>2</sub>	22.9 abc <sup>x</sup>	24.3 ab	22.3 abc	0.475 b	0.611 ab
KNO <sub>3</sub>	21.6 abc	25.0 ab	22.3 abc	0.488 b	0.599 b
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	23.7 ab	26.5 a	24.8 a	0.616 a	0.705 a
Urea	22.7 abc	25.3 ab	23.3 a	0.541 ab	0.522 bc
Urea + MnSO <sub>4</sub>	24.0 a	25.3 ab	22.5 ab	0.548 ab	0.576 b
MnSO <sub>4</sub>	20.4 bc	19.0 c	17.8 c	0.365 c	0.433 cd
Nutriculture	24.2 a	25.0 ab	22.8 ab	0.508 b	0.522 bc
Untreated	19.9 c	20.3 bc	18.3 bc	0.389 c	0.395 d
P > F	0.0018	0.0007	0.0006	0.0001	0.0001

<sup>z</sup> Thatch/mat was measured by either compressing thatch between fingers gently or uncompressed.

<sup>y</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>x</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 7. Impact of ammonium sulfate and nitrate nitrogen on the pH (0-2.5 cm depth) in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>z</sup>	29 Aug <sup>y</sup>	15 Sept	17 Sept	19 Sept	22 Sept	25 Sept
	pH					
Ca(NO <sub>3</sub> ) <sub>2</sub>	6.61 a <sup>x</sup>	6.90 b*	6.83 a*	6.76 a	6.83 b*	6.61 a
KNO <sub>3</sub>	6.76 a	7.14 a*	7.05 a*	6.95 a*	7.05 a*	6.69 a
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.57 b	4.95 c*	5.22 b	5.38 b	5.70 c	5.42 b

<sup>z</sup> Nitrogen treatments were applied at 12 kg N ha<sup>-1</sup> on 13 September 2003.

<sup>y</sup> Ratings taken on 29 Aug were used as the base pH for comparison purposes. For individual nitrogen treatments, significant differences ( $P < 0.05$ ) between initial pH (29 Aug) and pH on various rating dates are indicated by the symbol '\*'.  
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<sup>x</sup> For individual rating dates, treatment means followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's adjusted least significant difference test ( $P \leq 0.0001$ ).

Appendix A. Table 8. Impact of ammonium sulfate and nitrate nitrogen on the electrical conductivity of the mat (0-2.5 cm depth) in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>z</sup>	Soluble salts			
	15 Sept	17 Sept	19 Sept	22 Sept
	mg kg <sup>-1</sup>			
Ca(NO <sub>3</sub> ) <sub>2</sub>	152 a <sup>y</sup>	193 a	203 a	190 a
KNO <sub>3</sub>	157 a	267 a	271 a	165 a
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	144 a	244 a	203 a	98 b

<sup>z</sup> Nitrogen treatments were applied at 12 kg N ha<sup>-1</sup> on 13 September 2003.

<sup>y</sup> Means in a column followed by the same letter are not significantly different (P<0.05) according to Tukey's adjusted least significant difference test.

Appendix A. Table 9. Impact of ammonium sulfate, urea and nitrate nitrogen sources on the pH (0-2.5 cm depth) in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>z</sup>	27 Sept	29 Sept	1 Oct	3 Oct	6 Oct	9 Oct
	pH					
Ca(NO <sub>3</sub> ) <sub>2</sub>	6.74 a <sup>y</sup>	6.99 ab	6.99 ab	6.76 b	6.78 ab	6.86 ab
KNO <sub>3</sub>	6.78 a	7.15 a	7.18 a	7.09 a	7.10 a	7.00 a
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.65 b	5.54 d	5.21 d	5.26 d	5.11 c	5.10 d
Urea	- <sup>x</sup>	6.56 c	6.53 c	6.51 c	6.46 b	6.40 c
Untreated	6.70 a	6.75 bc	6.80 bc	6.60 bc	6.55 b	6.71 b

<sup>z</sup> Nitrogen treatments were applied at 12 kg N ha<sup>-1</sup> on 27 September 2003.

<sup>y</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's adjusted least significant difference test ( $P = 0.0001$ ).

<sup>x</sup> pH not measured.

Appendix A. Table 10. F values for the recovery of *Ophiosphaerella agrostis*-infected creeping bentgrass treated with six N-sources based the number of on infection centers plot<sup>-1</sup>, 2000.

Rating date	Source	NDF	DDF	Type III F	Pr>F
13 September 2000	Fertilizer	6	39	0.37	NS
	Fungicide	1	39	0.28	NS
	Fert x Fung	6	39	0.20	NS
6 October 2000	Fertilizer	6	39	1.79	NS
	Fungicide	1	39	0.30	NS
	Fert x Fung	6	39	0.38	NS
3 November 2000	Fertilizer	6	39	1.69	NS
	Fungicide	1	39	0.05	NS
	Fert x Fung	6	39	0.11	NS
29 November 2000	Fertilizer	6	39	2.11	NS
	Fungicide	1	39	1.12	NS
	Fert x Fung	6	39	0.42	NS
AUDPC 2000	Fertilizer	6	39	1.95	NS
	Fungicide	1	39	0.18	NS
	Fert x Fung	6	39	0.13	NS
15 May 2001	Fertilizer	6	39	2.03	NS
	Fungicide	1	39	2.43	NS
	Fert x Fung	6	39	0.51	NS
18 June 2001	Fertilizer	6	39	4.42	** <sup>x</sup>
	Fungicide	1	39	0.21	NS
	Fert x Fung	6	39	0.17	NS
24 July 2001	Fertilizer	6	39	8.23	***
	Fungicide	1	39	0.02	NS
	Fert x Fung	6	39	0.14	NS
AUDPC 2000	Fertilizer	6	39	1.95	NS
	Fungicide	1	39	0.18	NS
	Fert x Fung	6	39	0.13	NS

<sup>x</sup> \*\* = Significant at  $P=0.01$ , \*\*\* = Significant at  $P=0.001$ , NS = Not significant.

Appendix A. Table 11. F values for the recovery of *Ophiosphaerella agrostis*-infected creeping bentgrass treated with six N-sources based on percent recovery, 2000.

Rating date	Source	NDF	DDF	Type III F	Pr>F
6 October 2000	Fertilizer	7	18	5.32	** <sup>x</sup>
	Fungicide	1	20	1.54	NS
	Fert x Fung	6	20	1.20	NS
3 November 2000	Fertilizer	7	18	5.03	**
	Fungicide	1	20	1.78	NS
	Fert x Fung	6	20	1.46	NS
29 November 2000	Fertilizer	7	18	6.52	***
	Fungicide	1	20	0.00	NS
	Fert x Fung	6	20	2.58	NS
15 May 2001	Fertilizer	7	18	2.11	NS
	Fungicide	1	20	0.25	NS
	Fert x Fung	6	20	0.97	NS
18 June 2001	Fertilizer	7	18	11.89	***
	Fungicide	1	20	0.00	NS
	Fert x Fung	6	20	1.13	NS
24 July 2001	Fertilizer	7	18	13.61	***
	Fungicide	1	20	1.51	NS
	Fert x Fung	6	20	0.45	NS

<sup>x</sup> \*\* = Significant at  $P=0.01$ , \*\*\* = Significant at  $P=0.001$ , NS = Not significant.

Appendix A. Table 12. Source of variation for creeping bentgrass quality ratings as influence by six N-sources and iprodione, College Park, 2000 to 2001.

Rating date	Source	NDF	DDF	Type III F	Pr>F
Autumn	Fertilizer	7	18	251.19	*** <sup>x</sup>
	Fungicide	1	21	0.40	NS
	Fert x Fung	6	21	0.78	NS
Winter	Fertilizer	7	18	268.94	***
	Fungicide	1	20	0.20	NS
	Fert x Fung	6	20	0.04	NS
Spring	Fertilizer	7	18	472.13	***
	Fungicide	1	20	0.04	NS
	Fert x Fung	6	20	0.03	NS
Summer	Fertilizer	7	18	84.46	***
	Fungicide	1	20	0.06	NS
	Fert x Fung	6	20	0.05	NS

<sup>x</sup> \*\* = Significant at  $P=0.01$ , \*\*\* = Significant at  $P=0.001$ , NS = Not significant.

Appendix A Table 13. Impact of various N-sources and manganese on dead spot incidence in an 'L-93' creeping bentgrass putting green, 2002.

Treatment <sup>x</sup>	<i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>									
	27 June	5 July	12 July	19 July	27 July	2 Aug	10 Aug	16 Aug	23 Aug	
Ca(NO <sub>3</sub> ) <sub>2</sub>	24 <sup>y</sup>	43	45	61	89	96	98	95	80	
KNO <sub>3</sub>	19	33	34	47	67	73	72	53	61	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	21	42	50	60	83	87	91	81	73	
Urea	21	38	43	59	73	87	90	84	77	
Urea + MnSO <sub>4</sub>	22	41	48	60	81	90	104	90	80	
MnSO <sub>4</sub>	19	33	42	61	77	84	86	88	82	
20-20-20	22	41	48	61	85	90	98	74	78	
Unfertilized	23	42	46	66	90	96	105	99	96	
P > F	0.9916	0.8615	0.9445	0.9778	0.7527	0.8866	0.5399	0.5102	0.6623	

<sup>x</sup> Nitrogen treatments were applied as follows: 5 kg N ha<sup>-1</sup> on 28 June; 5, 12, 19 and 27 July; and 2 10 and 17 August and 12 kg N ha<sup>-1</sup> on 26 August; 4, 13 and 30 September; and 21 October 2002.

<sup>y</sup> There were no significant differences among treatments between 27 June and 23 August 2002.

Appendix A Table 13 (con't). Impact of various N-sources and manganese on dead spot incidence in an 'L-93' creeping bentgrass putting green, 2002.

Treatment <sup>z</sup>	<i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>					
	4 Sept	17 Sept	30 Sept	20 Oct	31 Oct	AUDPC <sup>y</sup>
				no.		
Ca(NO <sub>3</sub> ) <sub>2</sub>	60 bc <sup>x</sup>	52 b	55 b	44 b	35 b	62 ab
KNO <sub>3</sub>	42 c	33 b	47 b	38 b	29 b	47 b
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	56 bc	40 b	41 b	17 b	15 b	52 b
Urea	57 bc	46 b	57 b	42 b	30 b	57 ab
Urea + MnSO <sub>4</sub>	56 bc	45 b	53 b	44 b	33 b	59 ab
MnSO <sub>4</sub>	85 ab	89 a	84 a	78 a	68 a	74 ab
20-20-20	54 bc	43 b	47 b	35 b	25 b	57 ab
Unfertilized	99 a	97 a	96 a	94 a	75 a	86 a

<sup>z</sup> Nitrogen treatments were applied as follows: 5 kg N ha<sup>-1</sup> on 28 June; 5, 12, 19 and 27 July; and 2 10 and 17 August and 12 kg N ha<sup>-1</sup> on 26 August; 4, 13 and 30 September; and 21 October 2002.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 27 June to 31 October 2002.

<sup>x</sup> Means in a column followed by the same letter are not significantly different (P<0.05) according to Tukey's protected least significant difference test.

Appendix A. Table 14. Impact of various N-sources and manganese on dead spot incidence in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>z</sup>	<i>Ophiostherella agrostis</i> infection centers plot <sup>1</sup>									
	26 Mar	2 May	19 May	30 May	6 June	12 June	26 June	12 July	29 July	
Ca(NO <sub>3</sub> ) <sub>2</sub>	38 abc <sup>x</sup>	45 abc	44 bc	37 b	31 b	31 b	26 b	24 b	15 b	
KNO <sub>3</sub>	29 c	35 c	31 c	29 b	23 b	23 b	23 b	22 b	9 b	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	27 c	28 c	23 c	23 b	21 b	21 b	19 b	18 b	14 b	
Urea	41 abc	36 bc	35 c	37 b	27 b	27 b	23 b	24 b	17 b	
Urea + MnSO <sub>4</sub>	36 bc	41 abc	39 b	32 b	32 b	32 b	23 b	21 b	10 b	
MnSO <sub>4</sub>	56 ab	59 a	61 ab	63 a	58 a	58 a	49 a	50 a	51 a	
20-20-20	31 c	37 bc	26 c	25 b	23 b	23 b	21 b	5 b	1 b	
Unfertilized	61 a	55 ab	70 a	70 a	62 a	62 a	54 a	52 a	58 a	

<sup>z</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 12 and 26 June; and 11 July; 5 kg N ha<sup>-1</sup> on 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 26 March to 23 September 2003.

<sup>x</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 14 (con't.) Impact of various N-sources and manganese on dead spot incidence in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>z</sup>	<i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>							AUDPC <sup>y</sup>
	7 Aug	21 Aug	29 Aug	13 Sept	23 Sept	no.		
Ca(NO <sub>3</sub> ) <sub>2</sub>	11 b <sup>x</sup>	11 b	5 b	3 b	9 b			24 b
KNO <sub>3</sub>	6 b	9 b	6 b	6 b	11 b			19 b
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	6 b	7 b	8 b	3 b	1 b			16 b
Urea	6 b	10 b	6 b	4 b	6 b			21 b
Urea + MnSO <sub>4</sub>	6 b	9 b	5 b	4 b	6 b			21 b
MnSO <sub>4</sub>	47 a	42 a	37 a	26 a	37 a			50 a
20-20-20	1 b	1 b	1 b	0 b	1 b			13 b
Unfertilized	52 a	51 a	41 a	37 a	43 a			55 a
P > F	0.0001	0.0001	0.0001	0.0001	0.0001			0.0001

<sup>z</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 26 March to 23 September 2003.

<sup>x</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 15. Impact of various N-sources and manganese on the reactivation of *Ophiosphaerella agrostis* in an 'L-93' creeping bentgrass putting green, 2003

Treatment <sup>z</sup>	<i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>					
	21 Aug	13 Sept	23 Sept	27 Sept	7 Oct	AUDPC <sup>y</sup>
Ca(NO <sub>3</sub> ) <sub>2</sub>	2.8 a <sup>x</sup>	2.3 ab	6.5 ab	12.5 a	9.0 ab	5.3 a
KNO <sub>3</sub>	1.8 a	3.8 a	9.8 a	15.3 a	13.3 a	6.9 a
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.0 b	0.0 b	0.0 c	0.0 b	0.0 c	0.0 c
Urea	1.8 a	1.5 ab	4.5 abc	9.3 ab	5.8 bc	3.6 ab
Urea + MnSO <sub>4</sub>	1.5 ab	2.3 ab	4.3 bc	6.8 ab	5.8 bc	3.4 abc
MnSO <sub>4</sub>	0.0 b	0.0 b	0.0 c	0.0 b	0.0 c	0.0 c
20-20-20	0.0 b	0.0 b	1.0 c	6.3 ab	5.8 bc	1.7 bc
Unfertilized	0.0 b	0.0 b	0.0 c	0.0 b	0.0 c	0.0 c
P > F	0.0001	0.0049	0.0001	0.0.0001	0.00001	0.0001

<sup>z</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 21 August to 7 October 2003.

<sup>x</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 16. Impact of various N-sources and manganese on dead spot recovery in an 'L-93' creeping bentgrass putting green, College Park, 2003.

Treatment <sup>z</sup>	Dead spot recovery			
	19 May	6 June	12 June	7 Aug
	0-5 scale <sup>y</sup>			
Ca(NO <sub>3</sub> ) <sub>2</sub>	2.6 b <sup>x</sup>	1.9 b	1.6 b	0.5 b
KNO <sub>3</sub>	1.9 bc	1.1 bc	1.1 bc	0.4 b
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.5 c	0.8 c	0.5 c	0.2 b
Urea	2.4 bc	1.5 bc	1.4 bc	0.4 b
Urea + MnSO <sub>4</sub>	2.4 bc	1.9 b	1.4 bc	0.4 b
MnSO <sub>4</sub>	4.6 a	4.6 a	4.4 a	3.6 a
Nutriculture	2.0 bc	1.3 bc	1.0 bc	0.1 b
Untreated	4.9 a	4.6 a	4.5 a	4.0 a

<sup>x</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dead spot severity was rated visually on a 0 to 5 scale; where 0 = infection centers completely healed, 3.0 = bare spots with visible bentgrass encroachment and 5 = bare spots with no visible bentgrass encroachment.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 17. Impact of various nitrogen-sources and manganese on total disease (AUDPC) in an 'L-93' creeping bentgrass putting green, 2002 and 2003.

Treatment	<i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>	
	AUDPC <sup>y</sup>	
	2002	2003
	no.	
Ca(NO <sub>3</sub> ) <sub>2</sub>	62 ab <sup>z</sup>	24 b
KNO <sub>3</sub>	47 b	19 b
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	52 b	16 b
Urea	57 ab	21 b
Urea + MnSO <sub>4</sub>	59 ab	21 b
MnSO <sub>4</sub>	74 ab	50 a
20-20-20	57 ab	13 b
Unfertilized	86 a	55 a

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 27 June to 31 October 2002 and 26 March to 23 September 2003.

<sup>z</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 18. Impact of various N-sources and manganese on the reactivation of *Ophiosphaerella agrostis* in an 'L-93' creeping bentgrass putting green, 2003

Treatment <sup>z</sup>	<i>Ophiosphaerella agrostis</i> infection centers plot <sup>-1</sup>					
	21 Aug	13 Sept	23 Sept	27 Sept	7 Oct	AUDPC <sup>y</sup>
Ca(NO <sub>3</sub> ) <sub>2</sub>	2.8 a <sup>x</sup>	2.3 a	6.5 ab	12.5 a	9.0 a	5.3ab
KNO <sub>3</sub>	1.8 ab	3.8 a	9.8 a	15.3 a	13.3 a	6.9a
Urea	1.8 ab	1.5 a	4.5 ab	9.3 a	5.8 a	3.6ab
Urea + MnSO <sub>4</sub>	1.5 ab	2.3 a	4.3 ab	6.8 a	5.8 a	3.4ab
20-20-20	0.0 b	0.0 a	1.0 b	6.3 a	5.8 a	1.7b
P > F	0.0113	0.0571	0.0045	0.1140	0.0603	0.0075

<sup>z</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Dates used to calculate standardized area under the disease progress curve (AUDPC) values were 21 August to 7 October 2003.

<sup>x</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

Appendix A. Table 19. Impact of various N-sources and manganese on various diseases and disorders in an 'L-93' creeping bentgrass putting green, 2003.

Treatment <sup>x</sup>	Dollar Spot		Take All Patch		Brown patch		Yellow spot	
	12 June	29 July	26 June	12 July	7 Aug	7 Aug	21 Aug	21 Aug
	No. spots plot <sup>-1</sup>							
	%							
Ca(NO <sub>3</sub> ) <sub>2</sub>	9 a <sup>y</sup>	4 a	4.8 abc	9 a	2.8 a	2.8 a	0.5 a	0.5 a
KNO <sub>3</sub>	15 a	10 a	2.8 bc	10 a	2.0 a	2.0 a	0.3 a	0.3 a
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5 a	1 a	1.8 c	14 a	0.0 a	0.0 a	0.0 a	0.0 a
Urea	7 a	3 a	14.0 ab	17 a	4.3 a	4.3 a	1.8 a	1.8 a
Urea + MnSO <sub>4</sub>	9 a	6 a	15.0 a	18 a	0.8 a	0.8 a	3.0 a	3.0 a
MnSO <sub>4</sub>	- <sup>z</sup>	-	-	-	-	-	5.8 a	5.8 a
20-20-20	15 a	5 a	12.5 abc	12 a	0.5 a	0.5 a	4.0 a	4.0 a
Untreated	-	-	-	-	-	-	0.3 a	0.3 a
P > F	0.2203	0.0599	0.0043	0.6328	0.0527	0.0527	0.2009	0.2009

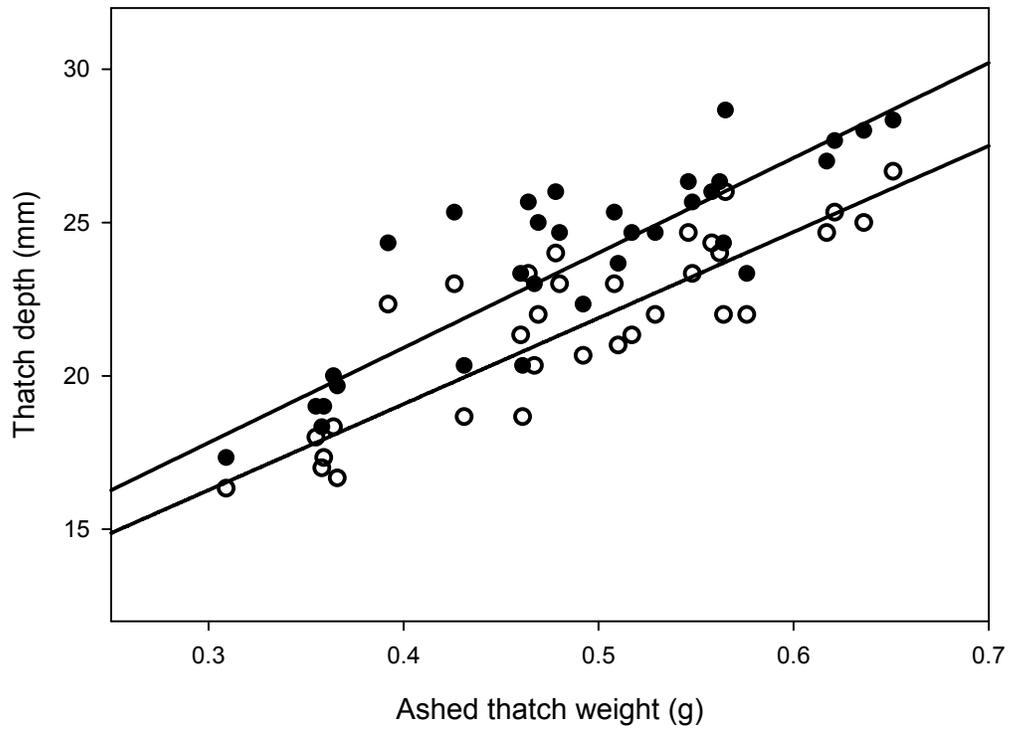
<sup>x</sup> Nitrogen treatments were applied as follows: 12 kg N ha<sup>-1</sup> on 29 April; 14 and 30 May; 12 and 26 June; 11 July, 5 kg N ha<sup>-1</sup> applied 18 and 28 July; 12 kg N ha<sup>-1</sup> on 13 and 27 September; and 24 kg N ha<sup>-1</sup> on 19 November 2003.

<sup>y</sup> Means in a column followed by the same letter are not significantly different ( $P < 0.05$ ) according to Tukey's protected least significant difference test.

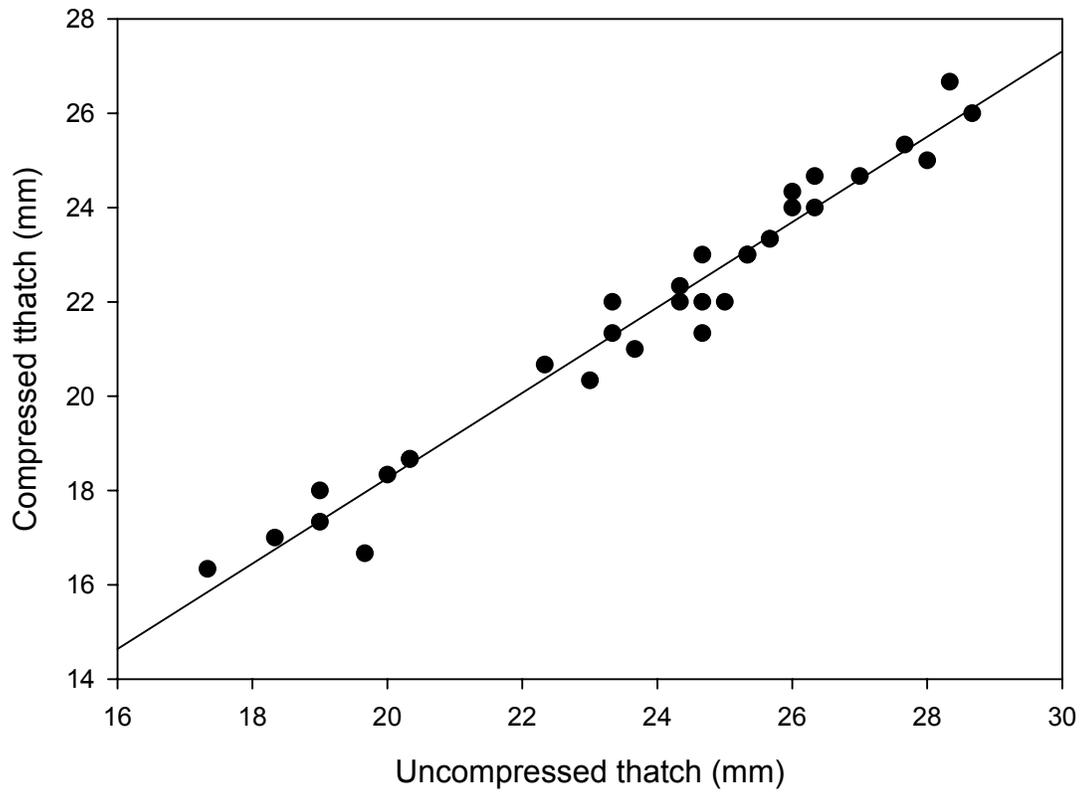
<sup>z</sup> Ratings were not made due to poor turfgrass quality and the inability to visually rate various disease symptoms.



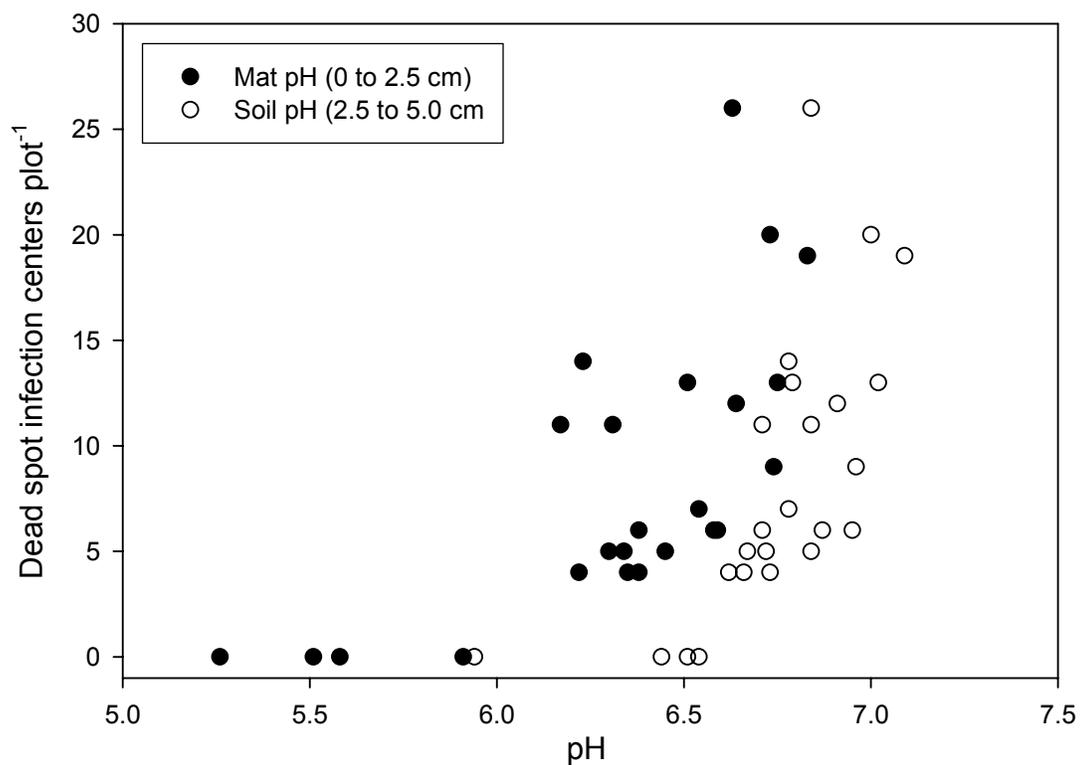
Appendix A. Figure 1. After fumigation, the autumn recovery study site showed delayed onset and reduced severity of dead spot in 2002.



Appendix A. Figure 2. Relationship between thatch depth and thatch weight using either a compressed (○) or uncompressed (●) thatch measurement technique.



Appendix A. Figure 3. Regression analysis of thatch depth using two different measuring techniques (compressed and uncompressed).



Appendix A. Figure 4. Relationship between the development of new dead spot infection centers and mat (●) and soil (○) pH. Dead spot infection centers were counted on 27 September 2002 and pH measured on 29 August 2002.

## APPENDIX B. ENVIRONMENTAL MONITORING AND DEVELOPMENT OF A PREDICTIVE MODEL FOR THE ONSET OF DEAD SPOT SYMPTOMS IN CREEPING BENTGRASS.

### INTRODUCTION

Due to the recent and rapid development of this new turfgrass disease, information with regards to dead spot management is very limited. Field studies have demonstrated that all major *Agrostis* spp. used on golf course putting greens are susceptible to dead spot (Kaminski and Dernoeden, 2002). Based on limited fungicide tests, current chemical management strategies for dead spot include the preventive application of fungicides on a 14-day interval (Towers et al., 2000; Wetzel and Butler, 2000; Wetzel and Butler, 2001). Unfortunately, little or no information is available regarding the timing of initiating a preventive management program. Because active disease symptoms may be present for an extended period of time, fungicide applications often begin in late-April or early-May and may last through the autumn months. Once dead spot symptoms occur on creeping bentgrass, disease management is difficult and fungicides must be applied on a 7 to 10 day interval. Due to the slow recovery of dead spot infected bentgrass, infection centers may still remain throughout the summer months despite repeated fungicide applications. For the aforementioned reasons, preventive management practices generally result in a reduction in the overall use of fungicides for controlling dead spot.

## MATERIALS AND METHODS

Mowing resumed 27 March 2000, and the height of cut was gradually lowered to 5.1 mm beginning on 17 May. In 2001, mowing was resumed on 3 April, and the height of cut was lowered and maintained between 4.0 and 4.6 mm. The area was vertical mowed using a Toro 1000 walk-behind mower equipped with a groomer on 25 May and 10 June 2000. Approximately 2000 kg ha<sup>-1</sup> of a sand topdressing (i.e., dried construction mix) was applied on 15 December 1999 and 29 March, 5 April, 3 and 22 June, and 21 August 2000. In 2001, applications of topdressing (2000 kg ha<sup>-1</sup>) were made on 25 and 31 May and 8 June.

A mix of tall fescue (*Festuca arundinacae* Schreb.) seed and wheat (*Triticum aestivum* L.) bran (50/50% v/v) inoculum was prepared as described by Kaminski and Dernoeden (2002). Briefly, was prepared by soaking tall fescue seeds in tap water overnight. Seeds then were rinsed three times and mixed with wheat bran (v/v), placed in 1L flasks, and autoclaved for 1 h on two consecutive days. Mycelia plugs from the edge of an actively growing colony (OpVA-1) were removed and placed on the surface of the cooled seed/bran mix. Flasks then were incubated in a dark growth chamber (I30BLL, Percival Scientific, Inc., Perry, IA) at 25°C for at least 16 d. Inoculum was mixed every 2 to 3 d to promote aeration and to allow mycelium to become evenly distributed throughout the medium. The site was inoculated by placing 0.5 g at the soil surface in a grid pattern spaced approximately every 0.9 meters.

In 2000, the area received 110 kg N ha<sup>-1</sup> from urea between 15 May and 8 September 2000. On 27 September, 49 kg N ha<sup>-1</sup> from urea was applied to the entire area. The final fertilizer application was made with 24 kg N ha<sup>-1</sup> using the

aforementioned starter fertilizer (19N-25P<sub>2</sub>O<sub>5</sub>-5K<sub>2</sub>O) on 3 November 2000.

Microdochium patch (*Microdochium nivale* (Fr.) Samuels and I. C. Hallett) was active in the site in April and May 2000. Therefore, on 1 June, a single application of 9.2 kg a.i. ha<sup>-1</sup> chlorothalonil (tetrachloroisophthalonitrile) + 9.2 kg a.i. ha<sup>-1</sup> iprodione (3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide) was applied.

During 2000, the area was fertilized similar to the area used in study year 1. On 27 April 2001, plots received 49 kg N ha<sup>-1</sup> (20N-20P<sub>2</sub>O<sub>5</sub>-20K<sub>2</sub>O) and an additional 12 kg S and 29 kg K ha<sup>-1</sup> from K<sub>2</sub>SO<sub>4</sub>. The area was treated with foliar applications of low rates of water-soluble fertilizers (5-6 kg N ha<sup>-1</sup>) approximately every 1 to 3 wk with urea, ammonium phosphate, and potassium nitrate between 17 May and 13 August 2001.

During the period (i.e., 17 May to 13 August) the area received a total of 76 kg N ha<sup>-1</sup>, 34 kg P ha<sup>-1</sup>, and 41 kg K ha<sup>-1</sup>. Fertilizer (12N-24P<sub>2</sub>O<sub>5</sub>-14K<sub>2</sub>O) was applied at 24 kg N ha<sup>-1</sup> on 4 and 12 September 2001. The final fertilizer application in 2001 was made with 49 kg N ha<sup>-1</sup> from urea. Due to mechanical injury from topdressing and mowing, several micronutrients also were applied either pre-packaged with the various fertilizers or in supplemental nutrient applications to enhance recovery (i.e., Scott's Starter Fertilizer<sup>®</sup>, Spray Gro<sup>®</sup>, Nutriculture<sup>®</sup>, Lesco Chelated Iron Plus<sup>®</sup>, Lesco Micronutrients<sup>®</sup>, Astron<sup>®</sup>, Jack's Classic Interiorscape Special<sup>®</sup>, Macrosorb<sup>®</sup> and Quelant<sup>™</sup>-Ca) on 19 June, 13, 17 and 18 July, 13 August and 21 September. Micronutrients applied to the area in 2001 included boron (B), calcium (Ca), copper (Cu), Iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), sulfur (S) and zinc (Zn) and totaled 0.084, 0.846, 0.359, 2.44, 1.16, 1.16, 0.004, 5.62 and 0.391 kg ha<sup>-1</sup>, respectively.

Triadimefon (2.4 kg a.i. ha<sup>-1</sup>) was applied on 4 and 22 May and 8 June and 0.8 kg a.i. ha<sup>-1</sup> tebuconazole (H-1,2,4-triazole-1-ethanol  $\alpha$ -[2-(4-chlorophenyl)-ethyl]- $\alpha$ -(1,1-dimethylethyl)) was applied on 11 September in an effort to reduce dollar spot (*Sclerotinia homoeocarpa* F. T. Bennett) and *Rhizoctonia* blight (*Rhizoctonia solani* Kühn). To control sod webworm (*Crambus* spp.) and black cutworm (*Agrotis ipsilon* Hufnagel), 5.7 kg a.i. ha<sup>-1</sup> chlorpyrifos [0,0-diethyl O-(2,5,6-trichloro-2-pyridyl) phosphorothioate], 0.08 kg a.i. ha<sup>-1</sup> fluvalinate [(RS)-  $\alpha$ -cyano-3-3-phenoxybenzyl(R)-2-[2-chloro-4-(trifluoromethyl)anilino]-3-methyl-butanoate], and 6.7 kg a.i. ha<sup>-1</sup> chlorpyrifos were applied on 8 May, 1 August, and 11 September 2001, respectively. Wetting agents were applied as needed to control localized dry spot (LDS) beginning in June 2001. LescoWet<sup>®</sup> (2-butoxyethanol, polyoxyethylene, polypropoxypropanol) granular (Lesco, Inc., Sebring, FL) was applied at 215 kg a.i. ha<sup>-1</sup> on 20 June. Subsequent wetting agent applications included 6.2 L ha<sup>-1</sup> of Aqueduct<sup>™</sup> (50% nonionic polyols) (Aquatrols, Inc., Cherry Hills, NJ), 6.2 L ha<sup>-1</sup> of Primer<sup>®</sup> (95% polymeric polyoxyalkylenes, 5% oxoalkenyl hydroxy polyoxylakane diyl) (Aquatrols, Inc, Cherry Hills, NJ) and 6.2 L ha<sup>-1</sup> of LescoWet Flowable between 16 July and 1 August.

On 11 May 2002, 25 kg N ha<sup>-1</sup> was applied. Urea was applied at 12 kg N ha<sup>-1</sup> on 24 and 31 May, 20 June, 8 July, 2 August and 6 September 2002. The final urea application (25 kg N ha<sup>-1</sup>) was on 11 October. Similar to the previous 2 years, various pesticides were applied to the study site in an effort to minimize damage from other pests. To control dollar spot, 9.2 kg a.i. ha<sup>-1</sup> chlorothalonil was applied on 1 May and 2.4 kg a.i. ha<sup>-1</sup> triadimefon was applied on 24 May, 14 June and 6 September. To control black cutworm 6.7 kg a.i. ha<sup>-1</sup> chlorpyrifos was applied on 18 May.

Each year, the area was subjected to routine golf course maintenance including vertical mowing, aeration and topdressing. Except for chlorothalonil, all fungicides applied between 2000 and 2002 were chosen because of their limited impact on dead spot severity (Towers et al., 2000). Granular pesticides were applied with a rotary spreader, while all sprayable materials were applied either with a CO<sub>2</sub> pressurized (262 kPa) sprayer equipped with an 8010 flat fan nozzle calibrated to deliver 407 L ha<sup>-1</sup> water.

*Ophiosphaerella agrostis ascospores.* In 2001 and 2002, ascospore release was monitored using a Burkard 7-day volumetric spore sampler (Burkard Manufacturing Co. Ltd., Rickmansworth Hertfordshire, United Kingdom). The Burkard trap samples 0.6 m<sup>3</sup> air hour<sup>-1</sup> through an orifice (14 x 2 mm) placed 45 cm above the turfgrass canopy. Ascospores in the sampled air were trapped on melinax tape coated with a silicone/hexane mixture (5:1 wt/v). Estimations of hourly ascospore release were determined as described by the manufacturer. Briefly, the coated melinax tape rotates at a rate of 2 mm hour<sup>-1</sup>; therefore, each 2 mm x 14 mm section of tape represents 1 hour of spore collection. However, because the orifice is 2 mm wide the total number of ascospores collected within any given hour may have been deposited during a 2 hour period (4 mm section). Spores deposited along a narrow linear traverse (100 µm) across the 14 mm wide opening will have been deposited within a single hour. Ascospore measurements along the narrow traverses, therefore, may be regarded as the mean number of spores collected for an hour. Ascospore data were collected between 10 June and 31 October and 14 May and 31 October in 2001 and 2002, respectively. Hourly estimations of ascospore release were summed and total daily ascospores (SPORES) collected during the 24 hour and 7 day period (SPORE7Day) prior to 800 hours were

used for statistical analyses.

## RESULTS

Spearman and Pearson correlations of the environmental and disease variables are shown in Tables 1 and 2, respectively. Descriptive statistics for all spore data is shown in Table 3. BDSAny was weakly correlated with SPORE 7Day ( $r = 0.37$ ), while other disease variables were not significantly or were weakly correlated with spore data ( $r \leq .151$ ) (Table 3). The highest  $\chi^2$  value (136) occurred from the combination of STMean  $\geq 20^\circ\text{C}$  and cumulative spore release for the 7 days prior to symptom development  $\geq 115$  (Table 4). This model accurately predicted dead spot symptom expression on 215 of 238 days (90.3%).

Appendix B. Table 1. Spearman correlation coefficients and significance levels for the environmental variables from creeping bentgrass in 2000-2003, College Park, MD.

	RH90	RH75	RH60	RH	RH48	RH72	RH	ST	ST	ST	AT	AT	AT	AT	Spore	Spore	LWD	Rain	Rain	SOL	WS	WS	SOL	WS	SOL	
	Mean	Mean	Mean	Mean	Mean	Mean	Min	Max	Min	Max	Mean	Min	Max	Mean	Daily	Daily		48	48	Mean	Mean	Mean	Mean	Mean	Max	Max
RH90	-.829	***	***	***	***	***	***	***	***	***	***	NS	***	***	NS	***	***	***	***	***	***	***	***	***	***	***
RH75	.829	-	***	***	***	***	*	***	***	***	***	NS	***	***	NS	***	***	***	***	***	***	***	***	***	***	***
RH60	.641	.871	-	***	***	***	***	***	***	***	NS	***	**	NS	NS	***	***	***	***	***	**	***	***	***	***	***
RH Mean	.854	.937	.890	-	***	***	***	***	***	***	NS	*	***	NS	NS	***	***	***	***	***	***	***	***	***	***	***
RH48 Mean	.749	.849	.818	.903	-	***	***	***	***	***	NS	NS	***	NS	NS	***	***	***	***	***	***	***	***	***	***	***
RH72 Mean	.666	.751	.722	.798	.941	-	***	**	NS	***	NS	NS	***	NS	NS	***	***	*	***	***	***	***	***	***	***	***
RH Min	.553	.768	.889	.856	.775	.673	-	NS	***	**	NS	***	**	NS	NS	***	***	***	***	***	NS	***	***	***	***	***
ST Mean	-.135	-.102	.026	-.043	-.025	-.025	.070	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
ST Min	.011	.110	.238	.161	.153	.118	.299	.933	-	***	***	***	***	***	***	***	***	***	***	***	*	***	***	***	***	***
ST Max	-.196	-.226	-.123	-.166	-.126	-.102	-.113	.763	-.763	***	***	***	***	***	***	***	***	*	***	***	***	***	***	***	***	***
AT Mean	-.229	-.159	-.019	-.117	-.122	-.131	.052	.904	.829	.841	-	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
AT Min	-.048	.064	.195	.107	.059	.020	.285	.829	.842	.700	.922	-	***	***	***	***	***	***	***	***	***	***	***	***	***	***
AT Max	-.236	-.240	-.142	-.199	-.184	-.176	-.122	.886	.745	.904	.931	.766	-	***	***	***	***	NS	NS	***	***	***	***	**	***	***
Spore Daily	-.110	-.094	-.067	-.037	-.011	-.034	.038	.380	.405	.312	.300	.346	.258	-	***	***	***	NS	NS	***	***	***	**	***	***	***
LWD	.547	.622	.534	.602	.506	.428	.490	-.311	-.154	-.369	-.373	-.214	-.414	-.203	***	***	-	***	***	***	***	***	***	***	***	***
Rain	.207	.251	.269	.268	.169	.093	.296	.161	.241	.100	.134	.212	.077	.067	***	***	.309	-	***	***	NS	*	*	*	*	
Rain48	.214	.232	.241	.250	.238	.166	.258	.187	.253	.130	.114	.184	.062	.142	***	***	.224	.760	-	*	NS	NS	NS	NS	NS	
SOL Mean	-.465	-.610	-.593	-.595	-.592	-.521	-.592	.390	.146	.551	.384	.187	.485	.225	***	***	-.601	-.153	-.105	-	NS	NS	NS	NS	NS	
WS Mean	-.340	-.203	-.112	-.207	-.197	-.165	-.021	-.165	-.112	-.225	-.132	-.131	-.199	.178	***	***	-.165	.059	.049	.065	-	NS	NS	NS	NS	
WS Max	-.253	-.218	-.204	-.238	-.229	-.197	-.178	-.149	-.148	-.146	-.145	-.213	-.117	.112	***	***	-.175	.090	.075	.157	.714	-	***	***	***	
SOL Max	-.404	-.540	-.508	-.513	-.516	-.460	-.521	.398	.174	.560	.376	.193	.480	.272	***	***	-.528	-.101	-.063	.930	.078	.216	-	-	-	
BDS 93 m <sup>2</sup>	-.181	-.111	-.050	-.132	-.152	-.180	0.009	.601	.627	.480	.606	.601	.508	.333	***	***	-.223	.131	.144	.205	-.060	-.144	.186	***	***	
Significance	***	NS	NS	**	***	***	NS	***	***	***	***	***	***	***	***	***	***	***	**	***	NS	**	***	***	***	
BDSAny	-.105	-.054	-.012	-.054	-.070	-.098	.055	.588	.598	.507	.557	.550	.478	.376	***	***	-.139	.174	.192	.204	-.087	-.117	.202	***	***	
Significance	*	NS	NS	NS	NS	NS	NS	***	***	***	***	***	***	***	***	***	**	***	***	***	NS	*	***	***	***	
BDSMajor	-.207	-.206	-.204	-.242	-.243	-.259	-.159	.284	.268	.231	.340	.312	.301	.083	***	***	-.229	-.011	-.006	.153	-.036	-.124	.122	***	***	
Significance	***	***	***	***	***	***	***	***	***	***	***	***	***	NS	***	***	***	NS	NS	**	NS	*	*	*	*	



Appendix B. Table 3. Descriptive statistics of *Ophiosphaerella agrostis* ascospore data collected on a creeping bentgrass putting green in College Park, MD , 2001 and 2002.

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
SPORES (no.)	313	28.3	63.6	8868	0.0	634.0
SPORES7Day (no.)	295	198.4	233.6	58524	0.0	1419.0

Appendix B. Table 4. Chi square ( $\chi^2$ ) analyses of individual and combined environmental variables.

Disease Parameter	Dead spot Incidence		$\chi^2$ (Accuracy)
	Yes	No	
A:			
STMean $\geq$ 20°C	204	50	135
STMean < 20°C	30	115	(79.9%)
B:			
SporesSum7D $\geq$ 115 <sup>y</sup>	132	10	70
SporesSum7D < 115	49	59	(76.4%)
A + E:			
STMean $\geq$ 20°C and SporesSum7D $\geq$ 115 <sup>x</sup>	162	14	136 (90.3%)
STMean < 20°C and SporesSum7D < 115	9	53	

<sup>x</sup> Spore data only was collected during the second (2001) and third (2002) study years.

APPENDIX C. INCIDENCE AND SEVERITY OF DEAD SPOT AND PSEUDOTHECIA  
DEVELOPMENT AND OVERWINTERING OF *OPHIOSPHAERELLA AGROSTIS* IN CREEPING  
BENTGRASS.

Appendix C. Table 1 Mean air and soil temperature between 2000 and 2002, College Park, MD.

Month	Air temperature			Soil temperature		
	2000	2001	2002	2000	2001	2002
	°C					
May	19	17	17	20	19	18
June	23	23	23	24	24	24
July	22	22	26	24	25	25
August	23	25	25	24	26	25
September	19	18	21	21	21	22
October	13	13	13	14	14	15

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