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DOLLAR SPOT FUNGUS *SCLEROTINIA HOMOEOCARPA* PRODUCES OXALIC ACID

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ABSTRACT

Dollar spot, caused by *Sclerotinia homoeocarpa*, is one of the most devastating diseases of turfgrass worldwide. Many fungi belonging to the genus *Sclerotinia* produce oxalic acid along with pectolytic cell wall-degrading enzymes. A series of *in vitro* experiments showed the relationships among temperature, pH, mycelial growth and acid production. Mycelial growth and acid production were most abundant when *S. homoeocarpa* was grown between 20 and 30 °C. Acid production by *S. homoeocarpa* appeared to be dependent upon the pH of the environment in which it was grown. High performance liquid chromatography analysis of spent broth revealed the presence of oxalic acid. Thus, as reported in other species of *Sclerotinia*, oxalic acid is produced by *S. homoeocarpa*. This is the first published report describing the production of oxalic acid by *S. homoeocarpa*.

Keywords: Defense response, mycelia, pH, temperature, turfgrass

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INTRODUCTION

Dollar spot, caused by *Sclerotinia homoeocarpa* F. T. Bennett, is one of the most serious diseases of turfgrass (Couch, 1995; Smiley et al., 2005; Vargas, 2005). Fungi in the genus *Sclerotinia* include some of the most devastating plant pathogens known and cause disease loss on many economically important crops. Dollar spot is widespread in the temperate climates of North America, Europe, and Australia and affects most species of turfgrass (Viji et al, 2004). Upon infection, tissue necrosis leads to the characteristic dollar spot symptoms that appear as tan, circular spots, roughly 2-5 cm in diameter on close-cut turfgrass and larger and more diffuse on higher-cut turfgrass (Viji et al, 2004). Optimal temperatures for the development of dollar spot have been reported to be between 15 and 30 °C. *Sclerotinia homoeocarpa* has been reported to survive as stromata and as dormant mycelia in infected plant tissue, thatch and clippings. Plant-to-plant spread throughout the growing season is through mycelial infection of adjacent plants and unintentional transport by humans, animals, mowers, and other equipment (Couch, 1995; Smiley et al., 2005; Vargas, 2005). Recent work by Horvath et al., (2007), however, showed that secondary spread of the pathogen was limited during a given growing season. The disease typically is managed through the combined use of an adequate fertility program and cultural practices designed to minimize the duration of prolonged leaf wetness. Dollar spot management in intensively cultivated turfgrass is heavily dependent on the timely applications of fungicides (Couch, 1995; Smiley et al., 2005; Vargas, 2005).

Other pathogenic fungi belonging to the genus *Sclerotinia* such as *S. sclerotiorum*, *S. rolfsii, S. trifoliorum* and *S. minor* all produce oxalic acid (Maxwell and Lumsden, 1970; Bateman and Beer, 1965; Pierson and Rhodes, 1992; Livingstone et al, 2005). Oxalic acid in these fungi has been shown to play a key role in pathogenesis and fungal development (Godoy et al. 1990; Lumsden 1979). In 1965, Bateman and Beer concluded from their studies of infected bean hypocotyls that oxalic acid production by *S. rolfsii* functions to create an acidic environment favorable for polygalacturonase activity. Numerous researchers have demonstrated that oxalic acid also complexes with calcium in the cell wall leading to the rapid degradation of pectic substances and necrosis of host tissues (Bateman and Beer, 1965; Kurian and Stelzig, 1979; Smith et al., 1986).

The importance of oxalic acid in pathogenesis by *S. sclerotiorum* has been further substantiated in studies using either mutants unable to produce oxalic acid (Godoy et al, 1990) or by using genetically modified plants designed to express oxalate oxidase (Livingstone et al., 2005). In studies by Godoy et al. (1990), an oxalic acid-deficient mutant of *S. sclerotiorum* was nonpathogenic as compared to the wild type. The introduction and expression of a barley oxalate oxidase gene (accession no. Y14203) in peanuts resulted in the transgenic plants showing enhanced resistance to Sclerotinia blight caused by *S. minor* (Livingstone et al., 2005).

Molecular studies related to the role of oxalic acid in plant pathogenesis are limited. Cessna et al. (2000) showed that the defensive oxidative burst in tobacco was suppressed by oxalic acid produced by *S. sclerotiorum* through the inhibition of peroxide production. Guimarães and Stolz (2004) showed that oxalate production by *S. sclerotiorum* leads to guard cell dysfunction and foliar dehydration. Most recently, oxalic acid secreted by *S. sclerotiorum* was shown to elicit an apoptotic-like response in the plants during

development of disease leading the authors to conclude that oxalic acid is a key pathogenicity factor (Kim et al, 2008).

The goals of this research were to determine whether *S. homoeocarpa* produces oxalic acid and if so, the influence culture conditions have on its production. An abstract citing oxalic acid production by *S. homoeocarpa* was previously reported by DaRoche and Hammerschmidt (2003).

MATERIALS AND METHODS

Sclerotinia homoeocarpa isolate B1, originally isolated from leaf tissue with symptoms of dollar spot was used in this study. This isolate was recovered from creeping bentgrass (*Agrostis stolonifera* L.; syn = *A. palustris* Huds.) obtained from The Ohio State University's Turfgrass Research & Education Facility, Columbus, OH. Diseased leaf tissue was surface disinfested for 1 min in a 3% sodium hypochlorite solution, rinsed twice in sterile water, and placed on acidified potato dextrose agar prepared by adding 0.75 ml of 85% lactic acid (Fisher Scientific, Fair Lawn, NJ) per 1 liter of PDA (Difco, Becton Dickinson and Company, Sparks, MD) after autoclaving. Culture plates were incubated at 25 °C and *S. homoeocarpa* was purified by making repeated hyphal tip transfers as required. Identification of *S. homoeocarpa* was based on cultural morphology and microscopic observation as originally described by Bennett (1937).

Assessment of *S. homoeocarpa* growth and *in vitro* acid production.

The influence of temperature and pH on the growth and acid production by *S. homoeocarpa* was assessed. The influence of temperature and pH on the growth of *S. homoeocarpa* was assessed by growing the fungus on PDA (39 g/L) adjusted to pH 4.0 or pH 6.0 with either 85 % lactic acid or 1 M potassium hydroxide (Fisher Scientific, Pittsburgh, PA), respectively. Agar plugs (5-mm in diameter) containing actively growing *S. homoeocarpa* mycelium were transferred to each of these media (three replicates per treatment) and incubated for 9 days in growth chambers set to provide constant temperatures of 5, 10, 15, 20, 25, 30 and 35 °C (no illumination). Mycelial growth was determined by measuring colony diameters every 24 hours. These data were subsequently used to calculate area under the *S. homoeocarpa* growth curves (Steadman et al, 1994). One-way analysis of variance (ANOVA) was used to assess differences among the area under the growth curve values. Differences among treatment means were determined using Fisher's protected least significance difference (LSD) at P=0.05. This experiment was performed twice.

Acid production by *S. homoeocarpa* was assessed using a slightly modified version of the procedure described by Steadman et al. (1994) in which the pH indicator bromophenol blue (Bb) (Sigma, St. Louis, MO) was added to PDA (50 mg/L) adjusted to either pH 4.0 or pH 6.0. A color change from purple to yellow is indicative of acid production on this medium. Agar plugs (5-mm in diameter) of actively growing *S. homoeocarpa* mycelium were transferred to each of these media (three replicates per treatment) and incubated as described previously. Mycelial growth on the bromophenol blue amended PDA was determined by measuring colony diameters every 24 hours. The impact of bromophenol blue on the growth of *S. homoeocarpa* was determined by comparing growth on PDA+Bb versus growth on PDA at the different temperatures evaluated. Because *S. homoeocarpa* produced cleared zones that varied in size and shape as compared to *S. sclerotiorum,* it was not possible to quantify acid production by measuring the diameter of the cleared zones as described previously

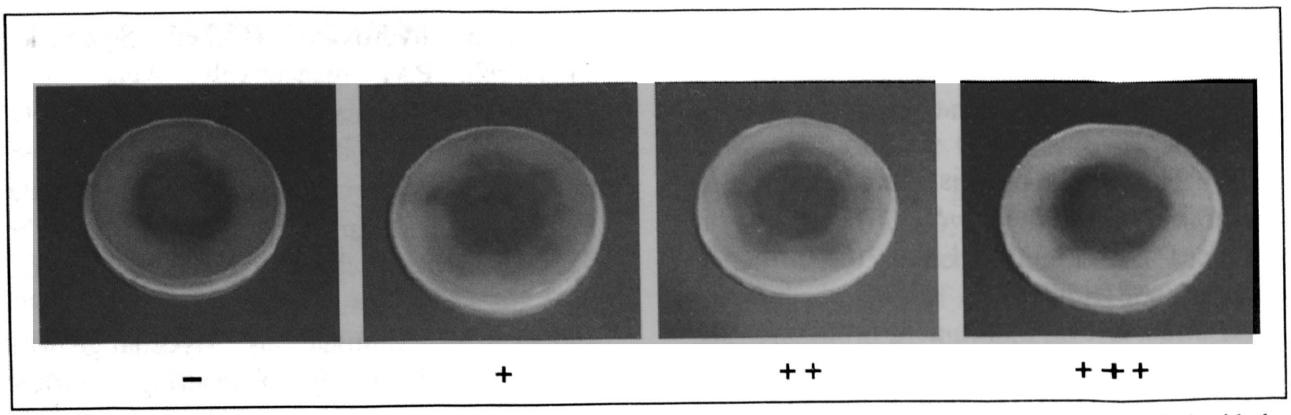

Figure 1. Acid production was assessed by growing *Sclerotinia homoeocarpa* on potato dextrose agar amended with the pH indicator bromophenol blue. A color change from purple to yellow is indicative of acid production on this medium. Acid production was assessed using the following qualitative visual rating scale in which: '-' = no acid production; '+' = minimal acid production; '++' = moderate acid production; and '+++' = maximum acid production.

by Steadman et al. (1994). Instead, a qualitative visual rating scale was used to assess acid production in which: '-' = no acid production; '+' = minimal acid production; '++' = moderate acid production; and '+++' = maximum acid production (Fig. 1). This experiment was performed twice.

Identification and quantification of oxalic acid using HPLC.

Potato dextrose broth (PDB) (Difco, Becton Dickinson and Company, Sparks, MD) was used to grow *S. homoeocarpa* isolate B1. Each flask of PDB (24 g/L) was adjusted to an initial pH of either 4.0 or pH 6.0, inoculated with three 5-mm diameter plugs of actively growing *S. homoeocarpa* and incubated at 25 °C for 17 days. Non inoculated PDB was used as a control. The pH of the PDB in each flask was measured at 0, 3, 6, 9, 12, 15 and 17 days after inoculation with an Accumet pH meter (Fisher Scientific, Pittsburgh, PA). After 17 days, the spent broth was filtered through Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK) and analyzed for the presence of oxalic acid via HPLC analysis. HPLC analyses were performed using modifications of two previously described methods (Martz et al., 1990; Jordan et al., 1996). In summary, injections of 20 ul were made onto an analytical C18 reverse phase column (LiChrosorb RP-18 10 u, 250 mm X 4.6 mm, Alltech Associates, Deerfield, IL). Elution was isocratic in HPLC grade water (Fisher Scientific, Pittsburgh, PA) at a flow rate of 2.0 ml/min and oxalic acid was detected at 210 nm. Oxalic acid was identified by co-elution (and spiking) with an oxalic acid standard (Sigma, St. Louis, MO). Oxalic acid was quantified using a standard curve of concentrations from 0.1 mM to 5 mM. This experiment was performed twice. Three replicates of each treatment per experimental trial were used.

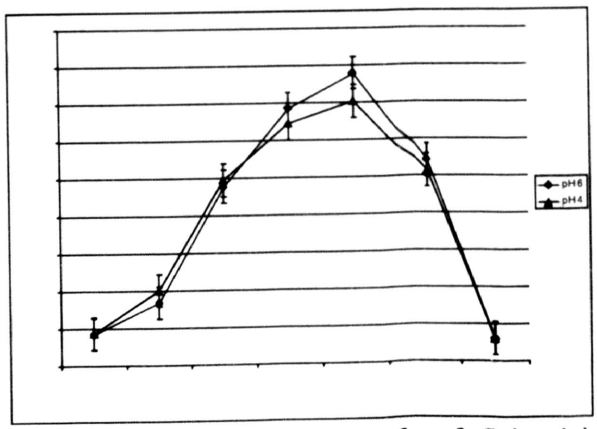

Figure 2. Mean mycelial growth of Sclerotinia homoeocapra on potato dextrose agar measured after 96 hours at different temperatures. Mycelial colony diameter (mm) was measured at each temperature. Vertical bars represent standard errors (n=3).

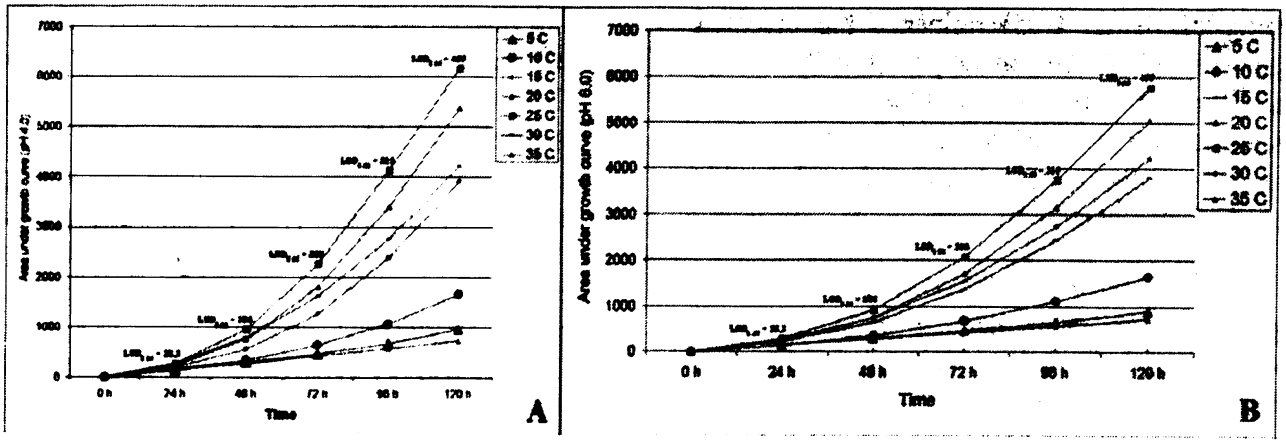

Figure 3. Growth curves showing the impact of temperature and pH on the growth of *Sclerotinia homoeocarpa*. Figures 3A and 3B represent growth on PDA adjusted to an initial pH 4.0 and pH 6.0, respectively. (n=3 per treatment).

RESULTS AND DISCUSSION

We performed time course studies to evaluate the impact of temperature and pH on the growth of *S. homoeocarpa* on PDA and PDA amended with bromophenol blue. Our temperature optima for the *in vitro* growth of *S. homoeocarpa* B1 is consistent with that generally stated in the literature for this pathogen (Couch, 1995; Smiley et al., 2005; Vargas, 2005). Although *S. homoeocarpa* grew at 5 and 10 °C, maximum growth occurred between 15 and 30 °C (Fig. 2 and 3). Growth of *S. homoeocarpa* B1 declined sharply between 30 and 35 °C. Although our experiments were monitored for 9 days (216 hours) post inoculation, mycelial growth at 15, 20, 25 and 30 °C had completely filled the Petri plates within 120 hours (Fig. 3) making it impossible to compare growth or acid production past this time. The initial pH (4.0 versus 6.0) of the PDA did not

Table 1. Influence of temperature and pH on the production of acid by *Sclerotinia homoeocarpa*.

	Acid Production†													
	5 °C		10 °C		15 °C		20 °C		25 °C		30 °C		35 °C	
Time	pH 4	pH 6	pH 4	pH 6	pH 4	pH 6	pH 4	pH 6	pH 4	pH 6	pH 4	pH 6	pH 4	pH 6
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	+	-	-
48 h	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	-	-	-	-	-	-	-	-	-	-	-	+	-	-
72 h	-	-	-	-	-	-	-	+	-	+	-	+	-	-
	-	-	-	-	-	-	-	+	-	+	-	+	-	-
	-	-	-	-	-	-	-	+	+	+	+	++	-	-
96 h	-	-	-	-	-	-	-	+	+	++	+	++	-	-
	-	-	-	-	+	+	+	++	+	++	+	++	-	-
	-	-	-	-	-	-	++	+++	++	++	+	+++	-	-
120 h	-	-	-	-	-	+	++	+++	+++	+++	+	+++	-	-
	-	-	-	-	+	++	+++	+++	+++	+++	+	+++	-	-

†Acid production was assessed by growing *Sclerotinia homoeocarpa* on potato dextrose agar amended with the pH indicator bromophenol blue. A color change from purple to yellow is indicative of acid production on this medium. Acid production was assessed using the following qualitative visual rating scale in which: '-' = no acid production; '+' = minimal acid production; '++' = moderate acid production; and '+++' = maximum acid production.

significantly (P=0.05) influence the mycelial growth of *S. homoeocarpa* (Fig. 2). The addition of bromophenol blue also did not significantly (P=0.05) impact mycelial growth at any of the temperatures evaluated in this study (data not shown).

In so far as we know, this is the first peer-reviewed report of acid production by *S. homoeocarpa*. Acid production by *S. homoeocarpa* as assessed on PDA amended with bromophenol blue, was highest between 20 and 30 °C (Table 1). Acid production was not detected at 5, 10 or 35 °C and might be explained as a growth-dependent phenomenon since acid production was correlated with mycelial growth. Bateman and Beer (1965), however, working with liquid cultures of *S. sclerotiorum* and who quantified both fungal biomass and oxalic acid concentrations, reported that oxalate production was not directly related to the total amount of mycelium, but rather based on the quantity and quality of the carbon source upon which the fungus was grown.

Although growth was not significantly impacted by the pH, acid production by *S. homoeocarpa* did appear to be pH-dependent occurring 24-48 hours earlier in PDA adjusted to an initial pH of 6.0 (Table 1). In a time course experiment in which *S. homoeocarpa* was grown in PDB adjusted to an initial pH of

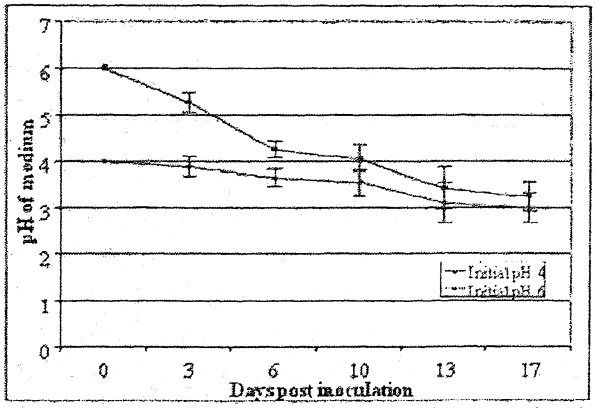

Figure 4. Acidification of potato dextrose broth inoculated with *Sclerotinia homoeocarpa*. Vertical bars represent standard errors (n=3).

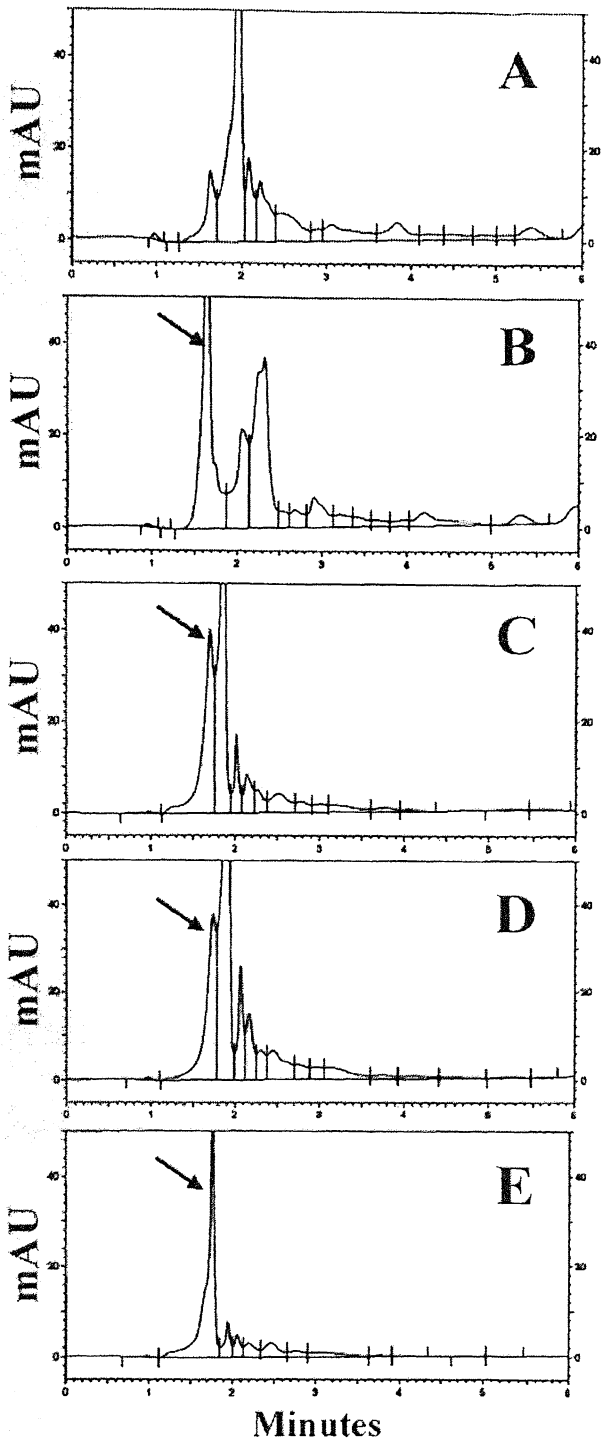

Figure 5. Identification of oxalic acid produced by *Sclerotinia homoeocarpa* grown in potato dextrose broth using HPLC. Arrows indicate the location of the internal oxalic acid control spike (210 nm). Fig. 5A = PDB only (no oxalic acid); Fig. 5 B = PDB + 1mM oxalic acid; Fig. 5C = PDB (pH 6.0) + *S. homoeocarpa*; Fig. 5D = PDB (pH 4.0) + *S. homoeocarpa*; and Fig. 5E = PDB (pH 4.0) + *S. homoeocarpa* + oxalic acid spike.

either 4.0 or 6.0, the decrease in pH was more dramatic in the PDB adjusted to pH 6.0 (Fig. 4). Maxwell and Lumsden (1970) showed that pH of the growth media was a primary factor in regulating oxalic acid accumulation by *S. sclerotiorum* in a glucose-sodium succinate medium. The production of acid and its role in creating an environment ideal for polygalacturonase activity was demonstrated by Bateman and Beer (1965) in their work with *S. rolfsii* and bean hypocotyls.

Analysis of spent culture filtrates through HPLC analysis confirmed that the predominant acid produced and secreted into the medium was oxalic acid (Fig. 5). Oxalic acid secretion by other species of *Sclerotinia* appears to be an essential determinant of their pathogenicity. In addition, oxalic acid acts as an elicitor of plant programmed cell death during the development of diseases caused by *S. sclerotiorum* (Dutton and Evans, 1996; Zhou and Boland, 1999; Kim et al., 2008). Given the results of this study, we speculate that oxalic acid produced and secreted by *S. homoeocarpa* may play a key role in host pathogenesis similar to that reported for other fungi in the genus *Sclerotinia* (Godoy et al., 1990; Dutton and Evans, 1996; Zhou and Boland, 1999).

CONCLUSIONS

Although, there are reports of oxalic acid production by other members of genus *Sclerotinia*, the detailed role of oxalic acid in the host pathogenesis is still unclear. We conclude that oxalic acid is produced by *S. homoeocarpa*. This is a significant finding due to the lack of knowledge on this particular species and how it causes disease. Oxalic acid may play an important role in turfgrass pathogenesis by *S. homoeocarpa* similar to its role in other fungi in this genus. Understanding the molecular mechanisms involved in oxalic acid secretion and suppression of host responses may ultimately lead to the development of more effective disease management strategies.

REFERENCES

Bateman, D.F. and Beer, S.V. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*, Phytopathology 55:204–211.

Bennett, F.T. 1937. Dollar spot disease of turf and its causal organism *Sclerotinia homoeocarpa* n. sp. Ann. Appl. Biol. 24:236-257.

Cessna, S.G., Sears, V.E., Dickman, M.B. and Low, P.S. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. The Plant Cell 12:2191-2200.

Couch, H.B. 1995. Diseases of turfgrasses, 3rd ed. Krieger publishing co., Malabar, FL.

Da Rocha, A.B. and Hammerschmidt, R. 2003. Increase in oxalate oxidase activity in bentgrass response to *Sclerotinia homoeocarpa* and chemical treatment. Dept. of Plant Pathology, Michigan State University, East Lansing, MI 48824 USA. Publication No. P-2004-0009-NCA.

Dutton, M.V. and Evans, C.S. 1996. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42:881–895.

Guimarães, R. and Stotz, H. 2004. Oxalate Production by *Sclerotinia sclerotiorum* Deregulates Guard Cells during Infection. Plant Physiol. 136(3):3703–3711.

Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 37:179–191.

Horvath, B.J., Kravchenko, A.N., Robertson, G.P. and Vargas, Jr. J.M. 2007. Geostatistical analysis of dollar spot epidemics occurring on a mixed sward of creeping bentgrass and annual bluegrass. Crop Science 47:1206-1216.

Jordan, C.R., Dashk, W.V. and Highley, T.L. 1996. Detection and quantification of oxalic acid from the brown-rot decay fungus, *Postia placenta*. Holzforschung 50:312-318.

Kim, K.S., Min, J.Y. and Dickman, M.B. 2008. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. Mol. Plant Microbe In. 21:605-612.

Kurian, P. and Stelzig, D.A. 1979. The synergistic role of oxalic acid and endopolygalacturonase in bean leaves infected by *Cristulariella pyramidalis*. Phytopathology, 69, 1301-1304.

Livingstone, D.M., Hampton, J.L., Phipps, P.M. and Grabau, E.A. 2005. Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. Plant Physiol. 137:1354–1362.

Lumsden, R.D. 1979. Histology and physiology of pathogenesis in plant diseases caused by *Sclerotinia* species. Phytopathology 69:890-896.

Martz, F.A., Weiss, M.F. and Belyea R.L. 1990. Determination of oxalate in forage by reverse-phase High Pressure Liquid Chromatography. J. Dairy Sci. 73:474-479.

Maxwell, D.P. and Lumsden, R.D. 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60:1395-1398.

Pierson, P. and Rhodes, L. 1992. Effect of culture medium on the production of oxalic acid by *Sclerotinia trifoliorum*. Mycologia 84(3):467-469.

Smiley, R.W., Dernoeden, P.H. and Clarke, B.B. 2005. Compendium of Turfgrass Diseases. Third Edition, APS Press.

Smith, V.L., Punja, Z.K. and Jenkins, S.F. 1986. A histological study of infection of host tissue by *Sclerotium rolfsii*. Phytopathology 76:755-759.

Steadman, J.R., Marcinkowska, J. and Rutledge, S. 1994. A semi-selective medium for isolation of *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 16:68-70.

Vargas, J.M. 2005. Management of turfgrass diseases. 3rd edition, John Wiley and Sons, Inc., New Jersey.

Viji, G., Uddin, W., O'Neill, N.R., Mischke, S., and Saunders, J.A. 2004. Genetic diversity of *Sclerotinia homoeocarpa* isolates from turfgrasses from various regions in North America. Plant Disease 88:1269-1276.

Zhou, T., and Boland, G.J. 1999. Mycelial growth and production of oxalic acid by virulent and hypovirulent isolates of *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 21:93–99.