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BIOLOGY AND PATHOGENICITY FACTORS OF *RUTSTROEMIA FLOCCOSUM* AND THE EFFECTS OF LIGHTWEIGHT ROLLING ON DOLLAR SPOT DISEASE INCIDENCE IN CREEPING BENTGRASS PUTTING GREENS.

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By

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ABSTRACT

BIOLOGY AND PATHOGENICITY FACTORS OF *RUTSTROEMIA FLOCCOSUM* AND THE EFFECTS OF LIGHTWEIGHT ROLLING ON DOLLAR SPOT DISEASE INCIDENCE IN CREEPING BENTGRASS PUTTING GREENS.

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A study to investigate the effects of light-weight rolling on the reduction of dollar spot was conducted between 2008 and 2009. Treatments rolled in the afternoon exhibited similar disease reduction as treatments rolled in the morning when compared to the control. A twice day^{-1} rolling treatment exhibited significantly less disease than all other treatments and resulted in greater rootzone soil volumetric water content (VWC) in both 2008 and 2009, when compared to the non-rolled control. Rolled treatments exhibited increases in fatty acid abundances associated with common bacteria when rootzone soil phospholipid fatty acids (PLFA) were analyzed. Furthermore, a general trend towards higher total bacterial PLFA abundances was present in rolled treatments. These results suggest that daily, season-long, light-weight rolling on putting greens may contribute to dollar spot reductions through the alteration of microbial populations in the upper rootzone. Additionally, afternoon rolling treatments suggest mechanisms other than dew and guttation removal to be responsible for disease reductions. *R. floccosum* isolates were examined for oxalic acid (OA) and cell wall degrading enzymes (CWDE) production. Non-virulent isolate Sh12B displayed high levels of CWDEs and OA production to a lesser extent when compare to virulent isolates. This suggests that OA and CWDEs may not be sole pathogenicity factors necessary for *R. floccosum* infection on turfgrass.

To my loving parents, Patricia and Paul for all that you have done to help shape the person that I am today; I can never thank you enough.

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LITERATURE REVIEW

DOLLAR SPOT

Causal Agent. Dollar spot is a disease of turfgrass found throughout the world (Smiley et al., 1992). It is caused by the fungal pathogen *Rutstroemia floccosum* syn. *Sclerotinia homoeocarpa* F.T. Bennet (Smiley et al., 1992) and is considered the most economically important disease on golf courses nationwide. More money is spent to manage dollar spot than any other pathogen on golf courses in the United States (Vargas, 2005). When grown in vitro, the fungus exhibits a characteristic white mat of fast-growing, fluffy mycelium, which turns to a gray or brown as the culture ages. After 15-30 days of growth in culture, the white mycelium can be accompanied by planes of dark stroma (Smiley et al., 1992).

Symptoms. The name dollar spot was given to the disease due to the symptoms it produces on the host turfgrass tissue, about the size of a silver dollar (Vargas, 2005). The disease appears on putting greens and low mown turf as small, straw colored patches, less than 6 cm, and commonly 1-3 cm, in diameter. In taller grass, like home lawns and athletic fields, the patches may reach 15 cm in diameter. As the disease progresses and conditions favorable for development persist, individual spots may often coalesce into irregularly shaped patches, destroying large areas of turf. Lesions on individual leaf blades are distinguished by pale, bleached, water soaked bands, bound by a tan or reddish-brown margin at the outer edge of the lesion in bentgrass, Ketucky bluegrass, fine-leaf fescue, zoysiagrass, and bermudagrass. During periods of leaf wetness, such as early morning hours when heavy dew and guttation fluid are present, the active mycelium

is often visible as white, cottony or cobwebby growth (Smiley et al., 1992; Vargas, 2005; Beard and Tani, 1997). Mycelia disappear as the leaves dry.

Disease Cycle. *Rutstroemia floccosum* can survive as dormant mycelia in previously infected tissue. The disease is spread primarily by mowers, maintenance equipment, and people carrying mycelia and infected plant tissue on shoes or other equipment. The mycelium rapidly invades healthy leaf tissue and extends into moisture-saturated air spaces surrounding infected leaves when the turfgrass environment favors activity of the fungus. Mycelial infection is thought to occur through cut ends of leaves or through stomata on leaf blades (Smiley et al., 1992). Although infertile aborted apothecial primordial have been produced by the fungus in culture and fertile apothecia have been observed from *Festuca* turf in field conditions (Baldwin and Newell, 1992), neither sexual nor asexual spores are considered important in the epidemiology of the disease (Fenstermacher, 1979; Jackson, 1973)

Epidemiology. Dollar spot is known to infect many species of turfgrass (Smiley et al., 1992). In the northern U.S., it is most damaging to creeping bentgrass (*Agrostis palustris* Huds.), annual bluegrass (*Poa annua*), colonial bentgrass (*Agrostis tenuis*), and fine-leaf fescues (*Festuca spp.*) (Vargas, 2005). The disease can be prevalent on turfgrass throughout the U.S. from the latter part of the spring through the end of the fall. Most epidemics occur in July and again in late August through September (Smiley et al., 1992). Conditions that favor the development and infection of dollar spot are temperatures of 15-30 C. Many different vegetative compatibility groups exist that cause the disease at various temperature and humidity regimes (Vargas, 2005). Guttation fluid is thought to be an ideal food source for the fungus. Guttation water is a fluid rich in

carbohydrates and amino acids that is exuded from the tip of the grass blade through hydathodes when turgor pressure builds up in the plant (Vargas, 2005). Dollar spot is more severe under conditions of low nitrogen fertility, dry soils, and/or water stress. The fungus can survive as mycelia and stroma on leaf tissue when conditions for infection are unfavorable.

DOLLAR SPOT MANAGEMENT

Cultural. Dollar spot management is often an integrated plan implemented by golf course superintendents, and turf managers alike. Cultural, chemical, and biological control techniques are all used in the management of this disease. Methods of cultural control such as fertilization, irrigation, and cultivation are often effective in reducing the amount of diseased turfgrass, and can aide in the reduction of chemical inputs. Maintaining high nitrogen fertility, especially when applied at times of high disease pressure, reduces disease (Vargas, 2005). Maintaining proper soil moisture levels, thus avoiding plant drought stress, is thought to also contribute to disease control. Couch and Bloom (1960) found low soil moisture to be important in the development of dollar spot, and Howard and Smith reported more dollar spot in seasons with less rainfall (cited in Vargas, 2005).

One of the most frequently used techniques in the cultural management of dollar spot is the removal of dew or plant exudates known as guttation fluid from the leaf blades in the early morning hours after mowing. Prolonged leaf wetness duration (LWD) increases the severity of dollar spot and other fungal diseases by providing a favorable environment for fungal penetration of leaf tissues (Huber and Gillespie, 1992; Williams et al., 1996; Gross

et al., 1998; Walsh, 2000; Uddin et al., 2003). Walsh (2000) found that the minimum LWD for the development of dollar spot for one isolate of *S. homoeocarpa* on creeping bentgrass (*Agrostis palustris* Huds.) was 12 h at 17.5°C. A longer LWD was required for infection when the temperature was lower. Dew or guttation removal is widely implemented in the turfgrass industry, particularly on golf course putting greens, and is thought to inhibit fungal mycelium growth by reducing LWD or the amount of food source available for the fungus. Rolling, whipping, dragging, or syringing are among some of the techniques used by turf managers to accomplish adequate removal or dispersion of the moisture. Many studies have elucidated the benefits of early morning mowing, syringing, and other dew removal techniques in order to decrease LWD (Williams and Powell, 1995; Ellram et al, 2007) ultimately reducing dollar spot incidence. Williams and Powell (1995) noted that guttation droplets secrete from wound exudates and these droplets are rich in nutrients that pathogens may use during hyphal growth. Release of these exudates may be exacerbated in the early dawn hours due to a combination of a fresh wound being produced by mowing and that turgor pressure may be high at this time. Nikolai (2005) proposed the process of dew/guttation removal as an underlying mechanism behind dollar spot reduction among other hypotheses such as altered soil microbial populations, and plant phytoalexin defense responses (Nikolai, 2002).

Chemical. While cultural management of dollar spot is relatively effective, the most efficient dollar spot management can be obtained when good cultural practices are combined with a sound fungicide program. Contact fungicides, like chlorothalonil, are effective at controlling dollar spot for short (7-14 days) periods of time. Resistance to

contact fungicides has not been reported and is unlikely to develop due to the multi-site mode of action these fungicides exhibit (Golembiewski et al., 1995). Effective systemic fungicides include propiconazole, fenarimol, iprodione and vinclozolin, among others. Systemic fungicides are able to give longer periods of control than contact fungicides, but have a much greater likelihood of becoming ineffective due to pathogen resistance developing. Resistance can be defined as reduced efficacy and shortened control intervals of a previously sensitive fungal population. Since 1972, resistance has been reported to many major systemic fungicide classes, which include the benzimidizoles (Warren, 1974), dicarboximides (Detweiler et al., 1983), and the demethylation inhibitors (DMI's) (Golembiewski et al., 1995). *R. floccosum* resistance to demethylation inhibitors was confirmed only 11 years after this family of fungicides was introduced to manage this disease (Golembiewski et al., 1995). Today, demethylation inhibitors are ineffective against dollar spot on many golf courses where this fungicide family has been used extensively (Vargas, 2005).

Biological. The introduction of the biological control agent *Pseudomonas aureofaciens* strain Tx-1 sold as Spot-less (Turf Science Laboratories Inc., National City, CA) for use in managing dollar spot has been a successful addition to the cultural and chemical means available to manage dollar spot on golf courses (Dwyer, 1999). To date, *P. aureofaciens* has been only mildly successful as a biological agent used in field situations for the control of dollar spot. Satisfactory disease suppression during years of light to moderate dollar spot infection has been shown with composted materials containing *Enterobacter cloaca* (Nelson et al., 1991, Nelson et al., 1992) as well.

EXTRACELLULAR ENZYME AND ACID PRODUCTION BY *RUTSTROEMIA FLOCCOSUM*

While many products are available to control the dollar spot disease, much remains unknown about the true life cycle and epidemiology of the fungus. One key to disease control is having a keen understanding of the infection process of the pathogen of interest. While a great deal of effort and research has been dedicated to dollar spot control and temporal variations (i.e. vegetative compatibility groups), infection mechanisms are yet to be elucidated. Knowledge of these mechanisms and pathogenicity factors are important, and can play critical roles in the development of novel, effective methods of control.

Infection Mechanisms. Plant pathogens must overcome many obstacles in order to successfully infect their host. Some bacteria, for example, enter their hosts through wounds or natural openings such as hydathodes in the plant. Other microorganisms may be able to infect by means of mechanical pressure created by specialized structures that aide in the facilitation of microbial entry (Agrios, 1988). Plant cells are surrounded by rigid walls in which a multitude of polysaccharides with specific structures are interconnected (Baur et al., 1973; Burke et al., 1974; Wilder and Albersheim, 1973). These interconnected polysaccharides form initial barriers which serve as primary defense mechanisms against invading pathogens. The evolutionary struggle between pathogen and host most likely gave rise to cell wall degrading enzymes being secreted by pathogens in order to aide in infection and overall virulence.

Cell Wall Degradation. Many pathogens achieve host infection by disrupting the plant cell wall (Albersheim et al., 1969). Plant cell wall degradation results from the action of pathogen-produced enzymes that are capable of cleaving specific linkages in the wall matrix of their host (Albersheim et al., 1969). Extracellular proteins secreted by fungi and bacteria are often able to macerate plant tissues and degrade plant cell wall components. Pathogens must thus contain all of the enzymes corresponding to the types of glycosidic linkages present in the cell wall polysaccharides of their target host. Multiple enzymes can be sequentially secreted by a single fungal pathogen when grown on isolated host cell walls (English et al., 1970; Jones et al., 1972). This observation is logical when considering the array of polysaccharides associated with different plant cell walls. Some pathogens can control the concentration and variety of mono- and polysaccharide degrading enzymes they produce depending on the environment they are growing in and the amount and type of substrate available (Albersheim et al., 1969). Extensive research involving the elucidation of enzymes capable of degrading major constituents of creeping bentgrass and determining whether specificity exists within the dollar spot pathogen has not been conducted.

Plant Cell Wall Composition. The composition of plant cell walls varies significantly from one cell type to another, one species to another, and between accessions within species (Hazen et al., 2003). For instance, a typical dicotyledonous plant cell wall contains around 30% cellulose, 30% hemicellulose, 35% pectin and 1–5% structural protein. By contrast, a typical grass species contains around 25% cellulose, 55% hemicellulose and only 10% pectin (Cosgrove, 1997). The extent of cell wall disruption often varies among plant pathogens and relies upon the secretion of

extracellular enzymes. Soft rot pathogens, for instance, are able to separate individual cells causing severe tissue maceration (Mount et al., 1970; Stephens and Wood, 1975). Other pathogens are limited to mycelia penetration through the cell wall, which aides in the infection process (Weinhold and Motta, 1973).

Oxalic Acid. Many *Sclerotinia* species are capable of producing compounds and enzymes which may contribute to the virulence on their particular host. Oxalic acid (OA) is thought to play multiple roles in the virulence of *Sclerotinia* species and has been shown to be required for the pathogenicity of a multitude of species which infect a wide range of hosts (Maxwell and Lumsden, 1970; Noyes and Hancock, 1981; Marciano et al., 1983; Godoy et al., 1990; Dutton and Evans, 1996; Zhou and Boland, 1999). Since many of the pathogens in this genus produce cell wall degrading enzymes, which are optimally active at a low pH, OA is thought to play a major role in lowering the pH of the apoplast during the infection process (Bateman and Beer, 1965). Direct toxicity of OA in the plant is also thought to be a factor in weakening the host, facilitating greater invasion of the pathogen (Noyes and Hancock, 1981). Other theories as to what role OA plays in pathogen virulence include the chelation of Ca^{2+} by the oxalate anion, which is thought to compromise the integrity of the host cell wall as well as inhibit Ca^{2+}-dependent defense responses (Bateman and Beer, 1965). In the case of *Sclerotinia sclerotiorum,* the oxidative burst of the host plant is suppressed by OA through the inhibition of H_2O_2 production, most likely via the blocking of a signaling step in the oxidase assembly/activation stream, in tobacco and soybean cells (Cessna et al., 2000).

Beaulieu (2008) showed that the dollar spot pathogen *R. floccosum* (*Sclerotinia Homoeocarpa*) indeed produces oxalic acid by analysis of secretions via gas

chromatography. However, concentrations of secreted OA were substantially lower than other *Sclerotinia* species such as *S. Sclerotiorum*. While the dollar spot pathogen may no longer be considered a member of the *Sclerotinia* genus (Powell, 1998) the production of oxalic acid is a commonality that cannot be ignored with regard to the elucidation of infection mechanisms and virulence.

Hypovirulence. *Sclerotinia* species infect a wide range of hosts and are responsible for extensive economic loss due to their widespread distribution. Hypovirulence has been reported to occur in species such as *S. sclerotiorum, S. minor*, and *S. homoeocarpa* (*R. floccosum*) (Boland, 2004). Hypovirulence refers to the reduced ability of selected isolates within a population of a fungal plant pathogen to infect, colonize, kill and (or) reproduce on susceptible host tissues (Elliston, 1982). Often times these isolates may be associated with various phenotypic characteristics such as reduced growth rate or sporulation, and altered colony morphology or color. Hypovirulence in Sclerotinia spp. is often due to the infection by fungal viruses and associated double-stranded RNA elements (Boland, 2004). In the case of *S. homoeocarpa* (*R. floccusom*), the causal agent of dollar spot, hypovirulence has been associated with the presence of double-stranded ribonucleic acid (dsRNA) (Zhou and Boland, 1997). However, some isolates detected as hypovirulent have been variable in expression of the phenotype, or were not associated with detectable concentrations of dsRNA.

Presence of the hypovirulence associated virus *Ophiostoma* mitovirus 3a (OMV3a) in numerous strains of hypovirulent *S. homoeocarpa* has been reported, however many isolates testing positive for the presence of the virus do not display the hypovirulent phenotype, leading to the presumption of possible latent infection by the

OMV3a virus (Melzer et al., 2003). Conclusive evidence has been difficult to ascertain due to inconsistencies in the determination of the true mechanism contributing to hypovirulence in the dollar spot fungus.

Hypovirulent isolates of various plant pathogens have been exploited to better understand pathogenicity factors and virulence. Pathogenesis by fungi in the genus *Sclerotinia* has been associated with the production of aforementioned cell wall degrading enzymes (Hancock, 1967; Lumsden 1976, 1979; Lumsden, 1969) as well as oxalic acid (Maxwell and Lumsden 1970). Oxalic acid has been confirmed as a pathogenicity determinant in *S. sclerotiorum* by using oxalic-acid-deficient mutants (Godoy et al. 1990). Double stranded RNA-associated hypovirulence in isolates of *S. scerotiorum* was shown to be associated with reduced and delayed production of oxalic acid, in comparison to virulent isolates (Zhou and Boland, 1999). Mechanisms associated with dsRNA hypovirulent strains of the dollar spot fungus in relation to pathogenicity factors have yet to be elucidated, particularly with regard to oxalic acid and extracellular enzyme production.

LIGHTWEIGHT ROLLING

Turfgrass as a commodity is subject to unique agricultural management practices compared to most cash crops. Many cultural techniques implemented in the maintenance of high quality turfgrass are not applicable with other families of managed plants. The ability of some turfgrasses to withstand not only frequent (daily) mowing, but low (< 1 inch) mowing heights, as well as heavy traffic volumes are some of the defining characteristics of this classification of plant. On a typical golf course, the putting greens

are the most intensely managed areas. Golf course superintendents and turf managers alike have been experimenting with different techniques to improve the quality of their greens for centuries. One commonly used cultural practice that has been found to be beneficial in numerous aspects of management is rolling. Lightweight rolling is most often used to increase ball roll speed and distance on highly maintained putting greens. The rollers, weighing from 200 to 1000 lbs (91 to 454 kg), are manufactured in many different shapes and sizes and can have a significant affect on the playability of a putting green.

Green Speeds. The Stimpmeter is a device used to measure the speed of a golf course putting green by applying a known force to a golf ball and measuring the distance travels in feet. Designed by golfer Edward Stimpson, Sr. in 1935, the Stimpmeter, is an angled track that releases a golf ball at a known velocity so that the distance it rolls on a putting green's surface can be measured. The further a golf ball rolls, the "faster" the green is considered. It was first used by the USGA during the 1976 U.S. Open at Atlanta and was made available to golf course superintendents in 1978 (Radko, 1977). Since the inception of the Stimpmeter, golf course superintendents have used a variety of cultural and chemical practices in an attempt to obtain firmer, faster greens; rolling being one of them (Throssell, 1986). The implementation of these practices is often thought to be detrimental to the health and quality of the turfgrass on the putting green (Kussow, 1998; Stier, 2006), especially during times of heat stress.

Rolling History. In the 1920's, numerous publications addressed roller weight, frequency, compaction and soil texture (Harban, 1922; Piper and Oakley, 1921; Anonymous, 1926) without coming to any valid conclusions. Rolling had been a

common practice on most golf courses at that time, mainly for the purpose of improving surface uniformity. Shortly thereafter, the practice of frequent rolling ceased, as turfgrass research showed a link between high levels of soil compaction and turf root growth (DiPaola and Hartwiger, 1994). The negative connotations around rolling persisted within the turfgrass industry until the 1990's (Hartwiger, 1996). Some turfgrass scientists believed that rolling may be causing unwanted damage to a healthy stand of turf, in addition to the near century old concerns regarding soil compaction. These same scientists felt the need to investigate the potential for above ground turfgrass problems associated with continual season-long turf rolling and the possibility that pathogens may invade crushed tissues, leading to diseased turf (Beard, 1994).

Disease Suppression. With demand for faster putting green speeds increasing, with continued emphasis on turfgrass quality, rolling has become a major component of putting green maintenance. During a rolling experiment designed to compare green speed, Nikolai et al. (2001) also noticed that morning rolling (3 times per week) not only increased green speeds but decreased the incidence of dollar spot significantly on creeping bentgrass maintained at putting green height. After conducting the study for several years, with all results yielding significant dollar spot reduction, many theories began to arise as to why and how rolling was inhibiting the development of this prolific turfgrass disease. Many of the theories revolved around the belief that rolling, which was conducted immediately after morning mowing, was removing excess dew or guttation water exuded by the plant and serving as a nutrient source for *R. floccosum*.

The benefits of rolling have proven to be numerous and because experiments involving rolling have shown to reduce dollar spot disease on creeping bentgrass greens

(Nikolai et al., 2001), many hypotheses to explain this phenomenon have been proposed. Dispersion of dew and guttation fluid (Nikolai et al., 2001), enhancement of phytoalexin production, increased surface water holding capacity, and microbial changes in the upper rootzone have all been proposed as possible factors responsible for reduced dollar spot incidence, although the actual mechanism(s) remains unknown (Nikolai, 2005). Routine rolling can produce a more prostrate turf canopy and limit the gradual elevation of plant crowns at the thatch–soil interface during the growing season (Beard, 2002). These effects could reduce the amount of leaf blade and leaf sheath tissue removed or damaged at low mowing heights. This could also enhance photosynthetic capacity because the youngest leaf blades, which would be most often removed by mowing, are the most photosynthetically active (Youngner, 1969). Additionally, maintaining the position of crowns lower in the mat layer may reduce plant exposure to high temperature stress because temperatures are often greatest just below the surface of dense, short-mowed turf (Beard, 1973).

MICROBIAL POPULATIONS IN TURFGRASS ROOTZONES

The rhizosphere (as well as the phyllosphere) is an infection epicenter where soil borne plant pathogens encounter their host and establish a parasitic relationship. The rhizosphere is also where a complex community of both soil microflora and microfauna can interact with pathogens, as well as the host, and influence the outcome of infection of the plant. The rhizosphere can be considered a battlefield, where complex communities of microbial players interact with one another, an environment where pathogenic and

beneficial microorganisms constitute groups that have a major influence on plant growth and development (Lynch, 1990).

Agricultural management practices can have significant impacts on the size and activity of soil microbial communities (Bolton et al., 1985; Fraser et al., 1988; Kirchner et al., 1993; Powlson et al., 1987). Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in key processes such as soil structure formation, decomposition of organic matter, toxin removal, and nutrient cycling. In addition, many microorganisms have been found to play key roles in suppressing soilborne plant diseases, promoting plant growth, and changing natural vegetation (Doran et al., 1996).

Suppressive Soils. A suppressive soil can be defined as one with a broad array of antibiosis-related functions, some of which associated with the suppression of plant pathogens (van Elsas et al., 2008) The mechanisms by which soils are suppressive to different pathogens, although not well understood, can often involve biotic and/or abiotic factors. These factors may often vary with the pathogen as well as host plant in the environment. The main agents responsible for soil suppressiveness are thought to be microbial in nature, due to the observation that sterilization by autoclaving, stem pasteurization, and irradiation has rendered soils more conducive to pathogens (Malajczuk, 1983). The mechanisms by which these microorganisms make soil suppressive can be divided into several categories: nutrient competition, microbial antagonism, parasitism, and systemic induced resistance (Raaijamkers et al., 2009).

Competition. Competitive colonization of the rhizosphere and successful establishment in the root zone is a prerequisite for effective biocontrol, regardless of the

mechanism(s) involved (Weller, 1988; Raaijmakers et al 1995). Competition for nutrients can often times be an effective biocontrol mechanism, particularly competition for organic compounds which are necessary for reactivation of propagules and subsequent proliferation of rhizosphere-dwelling pathogens (Paulitz et al 1992; Van Dijk and Nelson 2000; Fravel et al. 2003). Competition for micronutrients, essential for growth and activity of the pathogen, can take place as well.

Antagonism. Representatives of a range of bacterial (*Pseudomonas*, *Burkholderia*, *Bacillus*, *Serratia*, many Actinomycetes) and fungal (*Trichoderma*, *Penicillium*, *Gliocladium*, *Sporidesmium*, nonpathogenic *Fusarium* spp.) genera have been identified as antagonists of soilborne plant pathogens. One of the most important groups containing antagonistic microorganisms is the group containing fluorescent Pseudomonads. Several antibiotic-producing *Pseudomonas* spp. were isolated from soils suppressive to diseases such as take-all of wheat, black rot of tobacco and *Fusarium* wilt (Keel et al. 1996; Tamietti et al. 1993; Weller et al. 1988). Naturally occurring root-associated fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-DAPG were numerously populated in take-all-suppressive soil and are key components of specific suppression of *Gaeumanonmyces graminis* var. *tritici* (Raaijmakers and Weller, 1998; Raaijmakers et al., 1997). This suppressive activity was lost when 2,4-DAPG-producing *Pseudomonas* spp. were eliminated and, conversely, conducive soil gained suppressiveness to take-all when 2,4-DAPG-producing *Pseudomonas* strains were introduced. Biocontrol microorganisms may adversely affect the population density, dynamics, and metabolic activities of pathogens.

Induced Systemic Resistance. Along with biocontrol activity of microorganisms in the rhizosphere, several microbial groups can have a direct positive effect on plant growth and health. Phytostimulatory and biofertilizing microbes can promote plant health by making the plant "stronger". Many rhizosphere microorganisms can induce a systemic response in the plant as well, resulting in the activation of plant defense mechanisms (Pieterse et al., 2003). Induced systemic resistance (ISR) does not necessarily confer complete resistance, but rather protects the plant from various types of pathogen infection. The capacity to convey this type of ISR has been identified in a wide range of bacteria (Van loon et al., 1998; Haas and Defago, 2005), not only in greenhouse experiments, but under field conditions as well (Zehnder et al., 2001; Pieterse et al., 2003).

Microbial Populations in Soil. Several studies have identified trends within the microbial activity of soil related to and responsible for the suppression of pathogens. For example, van Os & van Ginkel (2001) showed a clear relationship between the suppression of *Pythium* root rot in bulbous *Iris* and soil microbial biomass and activity. Their findings showed that high microbial biomass and activity induced the suppression of *Pythium* growth and development in the soil. Relationships have been found between microbial diversity and root disease suppression as well (Nitta, 1991; Workneh and van Bruggen, 1994). Rovira & Wildermuth (1981) indicated that the microbiota in a "rich" soil tends to reduce the severity of attack by many soilborne plant pathogens, or, in other words, soils higher in microbiota content and diversity tend to show trends towards general disease suppression. Tippett (1978) provided an example of the importance of the soil microbiota on the level of suppressiveness. By adding soil containing large

quantities of microorganisms to microbially deficient soil, they were able to eliminate *P. cinnamoni*. Many antagonistic microorganisms are naturally present in soil and exert a certain degree of biological control over plant pathogens, regardless of human activities. However, this level of natural control is often insufficient for consistent, reliable disease-free cropping. Researchers are, therefore, attempting to enhance the effectiveness of antagonists, thus increasing suppressiveness (Hoitink et al., 2003).

Management of the biotic and abiotic properties of a soil is an important approach in promoting the activity and diversity of beneficial microorganisms. Cultural practices have been proposed as means to decrease soil inoculum levels, or increase suppressiveness, thus limiting the densities and activities of rhizosphere pathogens. Disease suppression has been obtained through crop rotation (Cook et al., 2002), as well as other practices such as intercropping (Schneider et al., 2003), residue destruction (Baird et al., 2003), organic amendments (Tilston et al., 2002), and tillage management practices (Sturz et al., 1997; Pankhurst et al., 2002).

With public perception and environmental stewardship at the forefront of turfgrass management concerns, alternative options for management of diseases are becoming highly desired in the turfgrass industry. Dollar spot is considered to be the most important disease on turfgrass, particularly in the northeastern regions of the United States (Vargas, 2005). If used as a tool in the management of dollar spot, rolling could potentially provide major economical benefits to golf course superintendents as well as boast significant conservational benefits. However, literature regarding *R. floccosum* suppression relating to rolling is sparse, particularly regarding mechanisms involved in

disease reductions. A multi-faceted approach was taken in an attempt to elucidate particular hypotheses regarding the rolling effect on dollar spot.

R. floccosum infection mechanisms, including enzyme, and toxin production were studied. Understanding specific plant-pathogen interactions and mechanisms related to pathogen virulence is important in elucidating novel methods of disease control. These have gone relatively uninvestigated with regard to the dollar spot pathogen on turfgrass. Rolling hypotheses and *R. floccosum* infection and virulence are discussed in the chapters that follow.

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CHAPTER ONE

DOLLAR SPOT REDUCTION THROUGH LIGHTWEIGHT ROLLING ON CREEPING BENTGRASS PUTTING GREENS

ABSTRACT

A study to investigate the effects of light-weight rolling on the reduction of dollar spot was conducted at the Hancock Turfgrass Research Center on the campus of Michigan State University in 2008 and 2009. Creeping bentgrass (*Agrostis palustris*) plots subjected to daily (5 days week^{-1}) rolling treatments resulted in significantly less disease when compared to a non-rolled control. Morning rolling treatments and afternoon rolling treatments were implemented to investigate the effects of dew and guttation fluid removal and dispersal. Treatments rolled in the p.m. (after dew and guttation naturally dissipated) exhibited similar disease reduction as treatments rolled in the a.m. (while dew and guttation fluid were present) when compared to the control. Cumulative effects of rolling were investigated with treatments rolled twice (2x) day^{-1}. The 2x rolling treatment exhibited significantly less disease than all other treatments and resulted in significantly higher upper rootzone soil volumetric water content means in both 2008 and 2009, when compared to the control. These results suggest that mechanisms other than dew and guttation dispersal may be responsible for disease reduction due to the observed dollar spot inhibition from afternoon rolling. Additionally, data suggests that season-long lightweight rolling twice day^{-1} may have an effect on upper rootzone volumetric water content in sand based creeping bentgrass putting greens.

INTRODUCTION

Turfgrass is subject to unique agricultural management practices compared to those of other agronomically important crop systems. Many cultural techniques implemented in the maintenance of high quality turfgrass are not applicable with other species of managed plants, even within the family *Gramineae*. One commonly used cultural practice on golf course turf is lightweight rolling. Lightweight rolling is most often implemented to increase ball roll speed and distance during putting as well as to enhance surface uniformity on intensively maintained putting greens. The rollers, typically weighing from 200 to 1000 lbs (91 to 454 kg), are manufactured in many different shapes and sizes, and can have a significant affect on the playability of a putting green thus having dramatic impacts on the game of golf.

The rolling of putting greens has been common on most golf courses for decades, and prior research has led to even more questions regarding its effects on turfgrass and underlying soil. Many negative associations with rolling have persisted within the turfgrass industry (Hartwiger, 1996). Theories that rolling may be causing unwanted damage to a healthy stand of turf, as well as concerns regarding soil compaction issues, have existed since the 1920's. Turf scientists have debated over the need to investigate the potential for above ground turfgrass problems associated with continual, season-long turf rolling and the possibility that pathogens may invade crushed tissues, leading to diseased turf (Beard, 1994).

Nikolai et al. (2001) conducted a multiple year rolling study at Michigan State University, aimed at the effects of rolling and fertility on putting green speed, disease incidence, and soil properties in different root zone mixes. This study gave rise to

surprisingly significant data regarding numerous aspects of plant management. Morning rolling (3 times per week) was found to dramatically decrease the incidence of the fungal disease dollar, spot caused by *Rutstroemia floccosum,* on creeping bentgrass maintained at putting green height, thus improving overall turfgrass quality. These results were observed in several years of the study. Since that time, others have observed similar results with lightweight rolling. Inguagiato et. al. (2009) observed slight reductions in turfgrass anthracnose, caused by *Colletotrichum cereale*, on plots rolled every other day. These results were contrary to the previous suggestions that rolling enhances disease severity (Dernoeden, 2000; Smiley et. al., 2005).

The findings of decreased disease occurrence through rolling spurred many hypotheses and theories with regard to possible suppression mechanisms. Many of the theories stem from the belief that rolling, which typically is conducted immediately after morning mowing, removes excess dew or guttation fluid exuded by the plant. This moisture is thought to serve as a nutrient rich food source for foliar feeding pathogens like *R. floccosum* (Williams and Powell, 1995). Dew removal and reduced leaf wetness duration (LWD) are widely accepted techniques used to reduce dollar spot incidence on turfgrass. Many studies have revealed the benefits of early morning mowing, syringing, and other dew removal techniques in order to decrease LWD (Williams and Powell, 1995; Ellram et al, 2007), ultimately reducing dollar spot incidence.

If used as a tool in the management of dollar spot, rolling could have substantial economic and environmental benefits. However, the literature is sparse on rolling studies, particularly on the topic of disease suppression mechanisms.

The objective of this study was to test specific hypotheses regarding rolling and its effect on dollar spot incidence on creeping bentgrass putting greens. Different rolling treatments were implemented for the duration of this study in order to generate data used to draw conclusions related to each hypothesis. The first objective was to investigate the hypothesis that morning rolling contributed to the removal or dispersal of secondary dew/guttation fluid after mowing, and that the reduction of disease is directly related to this practice. Other hypotheses related to soil microbial populations and plant defense responses induced by rolling are addressed in forthcoming chapters. Phenomena related to lightweight turfgrass rolling, dew/guttation removal, and dollar spot reductions were examined. Investigations into possible cumulative effects of repeated daily rolling were also undertaken in order to elucidate the potential for expedited disease reduction.

MATERIALS AND METHODS

Field research was conducted from June through October in 2008 and 2009 at the Hancock Turfgrass Research Center on the campus of Michigan State University, East Lansing, Michigan (42°43'48" N, 84°28'35" W). A research putting green, constructed in 2005, consisting of a mixed sward of creeping bentgrass (*Agrostis stolonifera cv 'Independence'*) and annual bluegrass (*Poa annua* L.) was used.

A 60 x 60 ft (18.3 x 18.3 m) research area was divided into twelve 7 x 12 ft (2.13 x 3.65 m) evenly spaced plots. Prior to initiating the study, each plot was randomly assigned a rolling treatment. Alleys of three feet separated each plot so that the roller had adequate space to stop without impeding into neighboring plots. All plots were mowed in the morning between 6 and 7 a.m. before morning rolling treatments were

implemented. Rolling treatments were carried out 5 days week^{-1} (Monday-Friday) and were as follows: 1) control (no rolling), 2) rolled once (1x) in the a.m. immediately after mowing, 3) rolled once (1x) in the p.m. when turf was dry or dew and guttation water had dissipated, and 4) rolled twice (2x) in the a.m. immediately after mowing. The morning rolling treatments were implemented between 7:00 and 8:30 a.m. and the afternoon rolling between 1:00 and 2:00 p.m. Rolling of plots was conducted with a Tru-Turf ride-on greens roller, model RS48-11B (Tru-Turf Pty. Ltd, Molendinar, Queensland, AUS), with a 39 inch (99 cm) roll swath weighing 562 pounds (255 kg) without an operator. A single rolling treatment consisted of rolling across the plot using multiple passes in opposite directions to ensure complete coverage of the plot with minimal overlap of rolled areas. Once a single rolling pass was made, the process was repeated immediately on plots rolled twice per day. To ensure study uniformity, the rolling treatment was the only practice that differed among plots. All other cultural and chemical practices remained constant among treatments for the duration of the study.

The root zone soil consisted of a sand based 80:20 (sand:peat v/v) mix constructed to USGA recommendations (USGA, 1993). Sand topdressing was applied to the entire research area on a light, frequent (bi/tri-weekly) basis throughout the growing season in order to simulate typical golf course putting green maintenance practices. No vertical mowing or core cultivation occurred on the research plots during the course of the study in order to minimize turfgrass and soil disruption. Fungicides were not applied to any of the research plots during the duration of the study in order to encourage dollar spot disease infection. Insecticides and herbicides were applied as needed, and were applied uniformly over the entire study area.

Nitrogen fertility was applied at a rate of 24.4 kg N ha^{-1} mo^{-1} (0.5 lbs N 1000 ft. $sq.^{-1}$ mo^{-1}) during the growing season from April to September of each year. Irrigation was applied via four Rain Bird Eagle irrigation heads, model 750 (Rain Bird Distribution Co., Azusa, CA), which were located at the corners of the research area. Irrigation was applied as needed in order to keep the turf stand healthy and free of wilting symptoms. Plots were mowed at a height of 0.156 inches (3.96 mm) six days $week^{-1}$ with a walk-behind Toro Greensmaster 1000 greens mower (Toro Co., Bloomington, MN). and clippings were collected throughout the study.

Dollar spot disease counts were taken when disease was active and symptoms occurred (generally once per week or bi-weekly) by counting the number of individual infection centers per plot. Spots greater than 3 cm in diameter were counted as one infection center. Larger, coalescing spots were broken down into smaller spots when rating and considered to be multiple infection centers. All ratings were taken in the morning before mowing, when conditions were conducive to counting infection centers.

Quality ratings were taken on a regular basis throughout the growing seasons (generally once per month from May – October). Quality was rated on a scale of 1- 9 where 1=dead/necrotic, 6=acceptable, and 9=excellent. Quality ratings were based on a combination of characteristics such as color, density, uniformity, and playability. Ratings of 6 and above were regarded as acceptable turf for a creeping bentgrass putting green.

Percent volumetric water content (%VWC) was measured using a FieldScout TDR 300 Soil Moisture Meter (Spectrum Technologies Inc., Plainfield, IL) with probe rods at a depth of 1.5 inches (3.81 cm). Twenty measurements were taken at random locations in each plot and averaged in order to obtain a representative %VWC for each

plot on each measurement date. All volumetric water content measurements were taken at least one full day (24 hours) after a rain event, with-holding irrigation, in order to ensure consistent VWC ratings.

The experimental design was a randomized block design with three replications. Analysis of variance was performed on observational measurements in order to determine significant effects, followed by Fisher's protected Least Significant Difference (LSD) if differences were found at the probability level (P) < 0.05. Treatment differences were analyzed using the Proc GLM procedure of the Statistical Analysis System (SAS Institute, 2009), and when appropriate, means were separated using Fisher's LSD procedure at the 0.05 level of probability unless otherwise noted.

RESULTS AND DISCUSSION

Dollar spot disease incidence data were collected on a total of 15 dates between 2008 and 2009 (Tables 1.01 and 1.02, respectively). Three dates (18-Sep, 7-Oct, 20-Oct) in 2008 resulted in significant treatment effects on dollar spot, with the twice rolled (2x) a.m. treatment having less dollar spot than the control. Treatments rolled 1x day^{-1}, either in the morning or afternoon, were not significantly different from one another on any of the dates recorded (P > 0.05). In 2008, no significant treatment effect for either 1x rolling treatments were found compared to the control; and neither of the 1x treatments were significantly different from the 2x treatment according to Fischer's LSD (P > 0.05).

While differences were not significant on individual measuring dates, combined seasonal dollar spot means from 2008 indicated a significant rolling effect on dollar spot incidence in both the 1x p.m. and 2x a.m. treatments (Figure 1.01). The 1x a.m.

treatment was not significantly different from any other treatment ($P > 0.05$). This lack of statistical separation is likely due to one replication within the 1x a.m. treatment having uncharacteristically higher dollar spot incidence than the other two replications. The high dollar spot counts throughout the season on that particular plot resulted in a much higher seasonal mean, thus rendering the 1x a.m. treatment statistically similar to the control.

In 2009, dollar spot ratings were taken on nine separate occasions (Table 1.02). Disease pressure was much higher in 2009 than in 2008, resulting in an average increase in disease incidence of 2.72 times between the two yearly means. Dollar spot incidence occurred earlier in the season and at much higher levels throughout the season as indicated by the number of infection centers recorded. The 2x a.m. treatment had significantly less dollar spot incidence than the control on all but the first two rating dates in 2009 (Table 1.02). When dollar spot incidence was most severe (September and October), all rolling treatments resulted in significantly lower disease counts compared to the control. July 31, was the only rating date where significant differences between 2x a.m. and either of the 1x rolling treatments were observed (Table 1.02). However, the 2x a.m. rolling treatment showed a trend towards lower dollar spot incidence. The 1x a.m. and 1x p.m. treatments were not significantly different from one another on any observation date, but both had a significant effect on dollar spot when compared to the control on four of the nine rating dates (25-Aug, 3-Sep, 22-Sep, 17-Oct) (Table 1.02).

Table 1.01. Means and LSD comparisons for treatment effects of rolling on dollar spot disease incidence on different dates in 2008.

	No. of Dollar spot infection centers 2008					
Treatment	7/11	7/28	9/1	9/18 †	10/7	10/20
Not Rolled (Control)	11.00	11.33	33.00	56.00 a	52.33 a	54.00 a
1x a.m.	7.67	12.00	32.67	36.67 ab	33.33 ab	32.67 ab
1x p.m.	4.00	5.67	17.00	23.67 ab	28.67 ab	27.33 ab
2x a.m	3.00	2.33	6.33	6.00 b	2.00 b	1.33 b
Significance	NS	NS	NS	*	*	*
LSD (0.05)	19.87	16.93	36.46	43.04	39.50	36.87

NS, *, ** Not significant and significant at the 0.05 statistical probability level respectively.

† Means followed by the same letter in a column are not significantly different according to Fisher's LSD (P > 0.05).

Table 1.02. Means and LSD comparisons for treatment effects of rolling on dollar spot disease incidence at different dates in 2009.

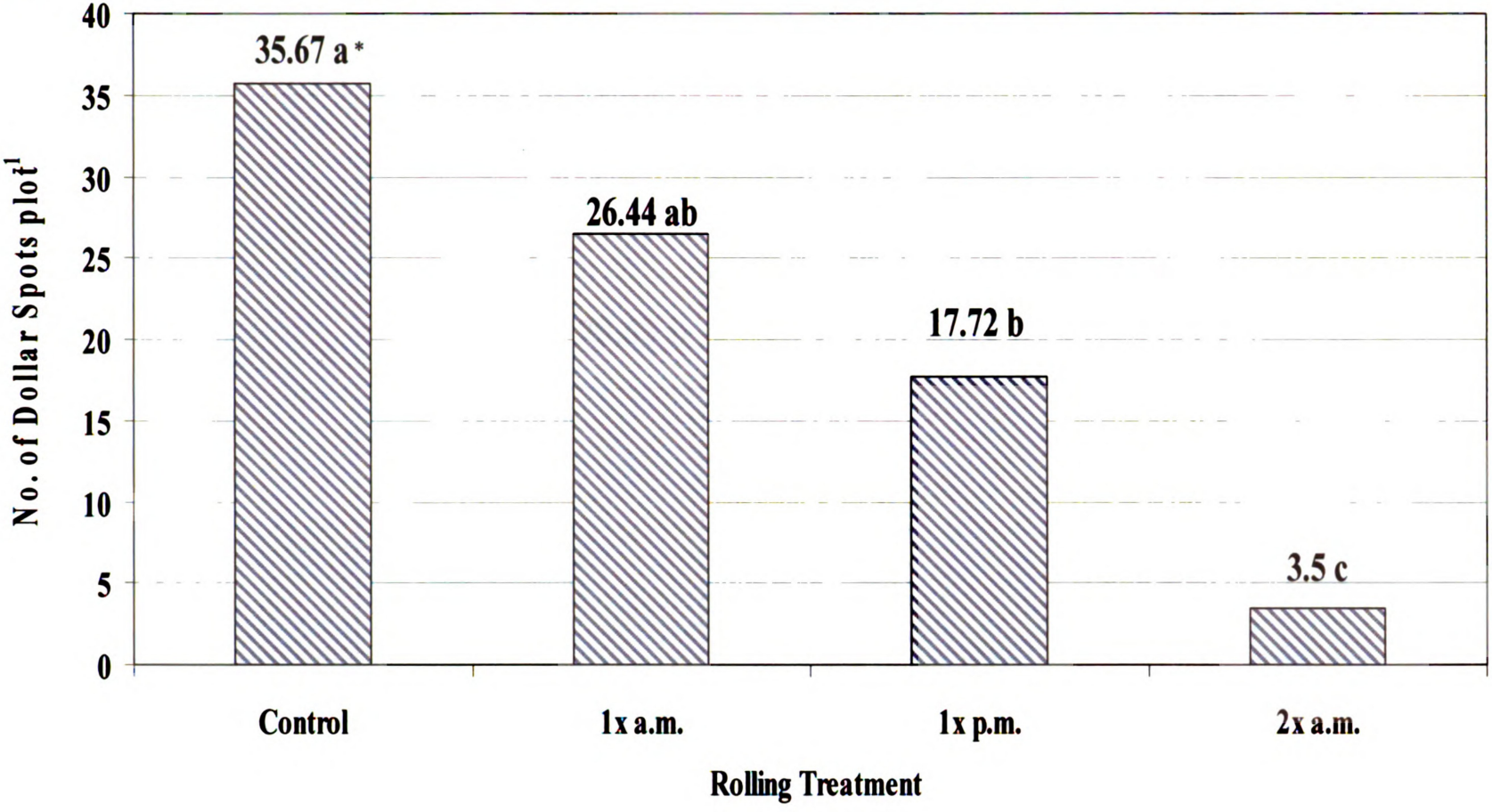
	No. of dollar spot infection centers 2009								
Treatment	6/19	6/25	7/1 †	7/17	7/31	8/25	9/3	9/22	10/17
Not Rolled (control)	38.67	41.00	55.33 a	60.00 a	103.33 a	243.67 a	292.67 a	301.33 a	291.33 a
1x a.m.	26.33	32.67	35.67 ab	22.00 ab	70.67 a	111.33 b	123.00 b+	125.67 b+	115.33 b+
1x p.m.	27.67	29.00	35.33 ab	32.33 ab	74.67 a	105.67 b	128.33 b+	121.67 b+	108.66 b+
2x a.m	6.33	7.33	9.33 b	3.33 b	13.67 b+	38.00 b+	42.67 b++	39.00 b++	31.33 b++
Significance	NS	NS	*	*	*	*	*	*	*
LSD (*0.05)	56.74	60.63	40.24	41.12	49.37	107.96	97.47	86.98	86.18

NS, * Not significant and significant at the 0.05 statistical probability level respectively.

† Means followed by the same letter in a column are not significantly different according to Fisher's LSD (P > 0.05).

+ Mean is different from control at lower probability level; + represents one lower probability level. Probability levels in order: 0.05, 0.01, 0.001, 0.0001.

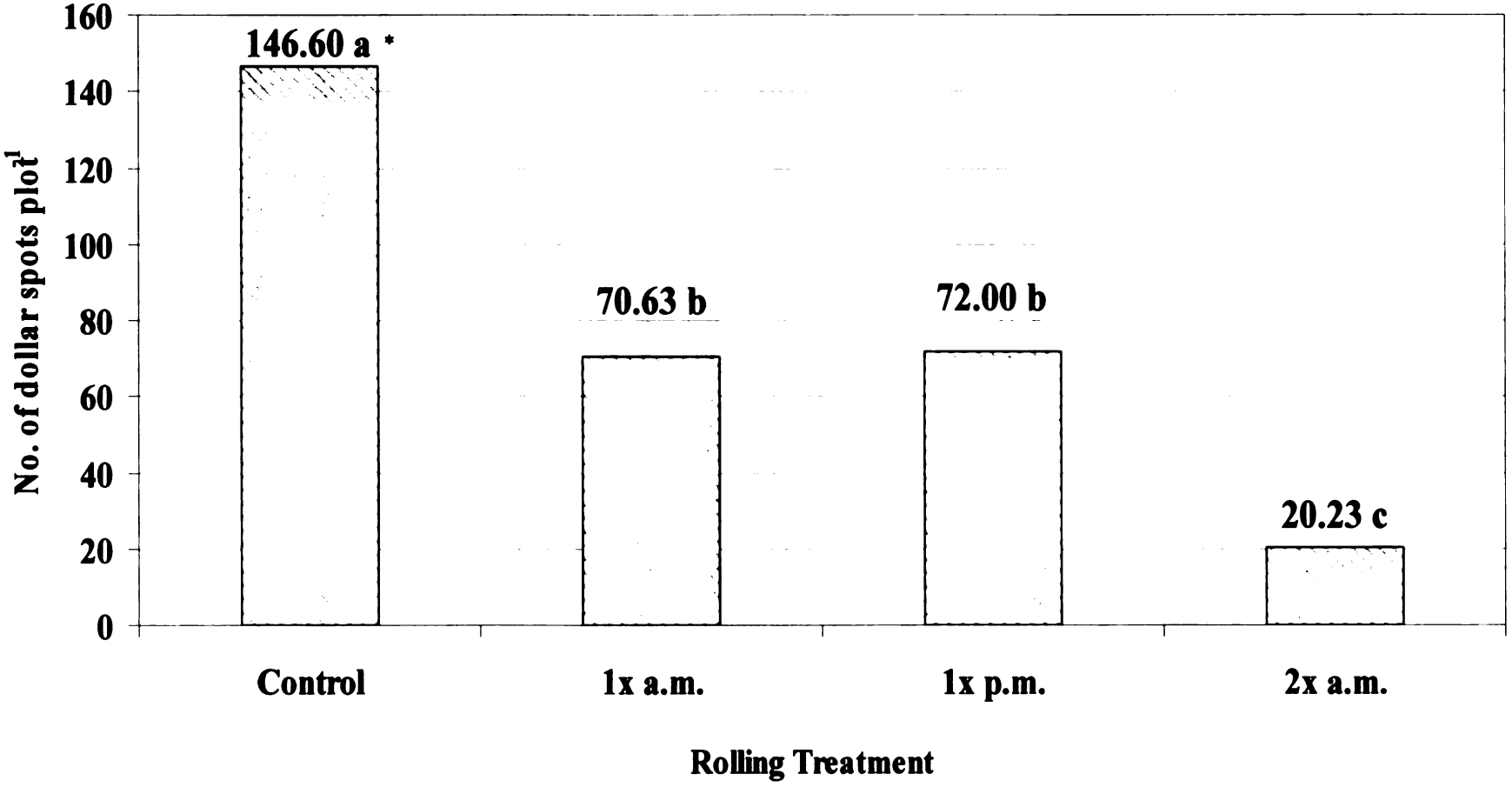
Figure 1.01. Seasonal dollar spot means[z] among different rolling treatments on creeping bentgrass (*Agrostis palustris Huds.*) putting green plots in 2008.



[z] Values are means of 6 different measurement dates and three replications per date.

* Treatment means followed by the same letter are not significantly different according to Fisher's LSD (P > 0.05).

Figure 1.02. Seasonal dollar spot means[z] among different rolling treatments on creeping bentgrass (*Agrostis palustris Huds.*) putting green plots in 2009.



[z] Values are means of 9 different measurement dates and three replications per date.

* Treatment means followed by the same letter are not significantly different according to Fisher's LSD (P > 0.05).

These data support earlier observations reported by Nikolai et al., (2001) who found that dollar spot severity was reduced on rolled plots with every dollar spot outbreak that was recorded over a six-year period.

Combined 2009 seasonal dollar spot means indicate a significant treatment effect on dollar spot in all rolling treatments (Figure 1.02). Additionally, the 2x a.m. treatment resulted in significantly less dollar spot compared to all other treatments ($P < 0.05$). Both the 1x a.m. and 1x p.m. treatment resulted in significantly lower dollar spot incidence compared to the control ($P < 0.05$), and no significant differences were observed among the two 1x rolling treatments ($\alpha = 0.05$).

In 2008, seven quality ratings were taken and are reported in Figure 1.03. Turfgrass quality was rated on a scale of 1-9, with 9 signifying excellent quality, 6 and above acceptable, and 1 indicating necrotic or dead turf. All rating dates resulted in a significant effect on quality for the 2x a.m. rolling treatment in 2008 ($P < 0.05$) compared to the control. The 1x rolling treatments (a.m. and p.m.) had significant effects on turfgrass quality when compared to the control at three observation dates (5-Jul, 26-Jul, 10-Oct). No differences in turfgrass quality were observed among the two 1x rolling treatments at any date in 2008 ($P > 0.05$). The 2x a.m. treatment had significantly better turfgrass quality than the 1x a.m. treatment on three dates (5-Jul, 26-Jul, 10-Oct) in 2008 ($P < 0.05$). Additionally the 2x a.m. treatment resulted in significantly better turfgrass quality compared to the 1x p.m. treatment on two dates (5-Jul, 26-Jul) in 2008 ($P < 0.05$).

When a seasonal mean was calculated for 2008 turfgrass quality, both 1x rolling treatments were significantly better than the control while the 2x a.m. treatment, which

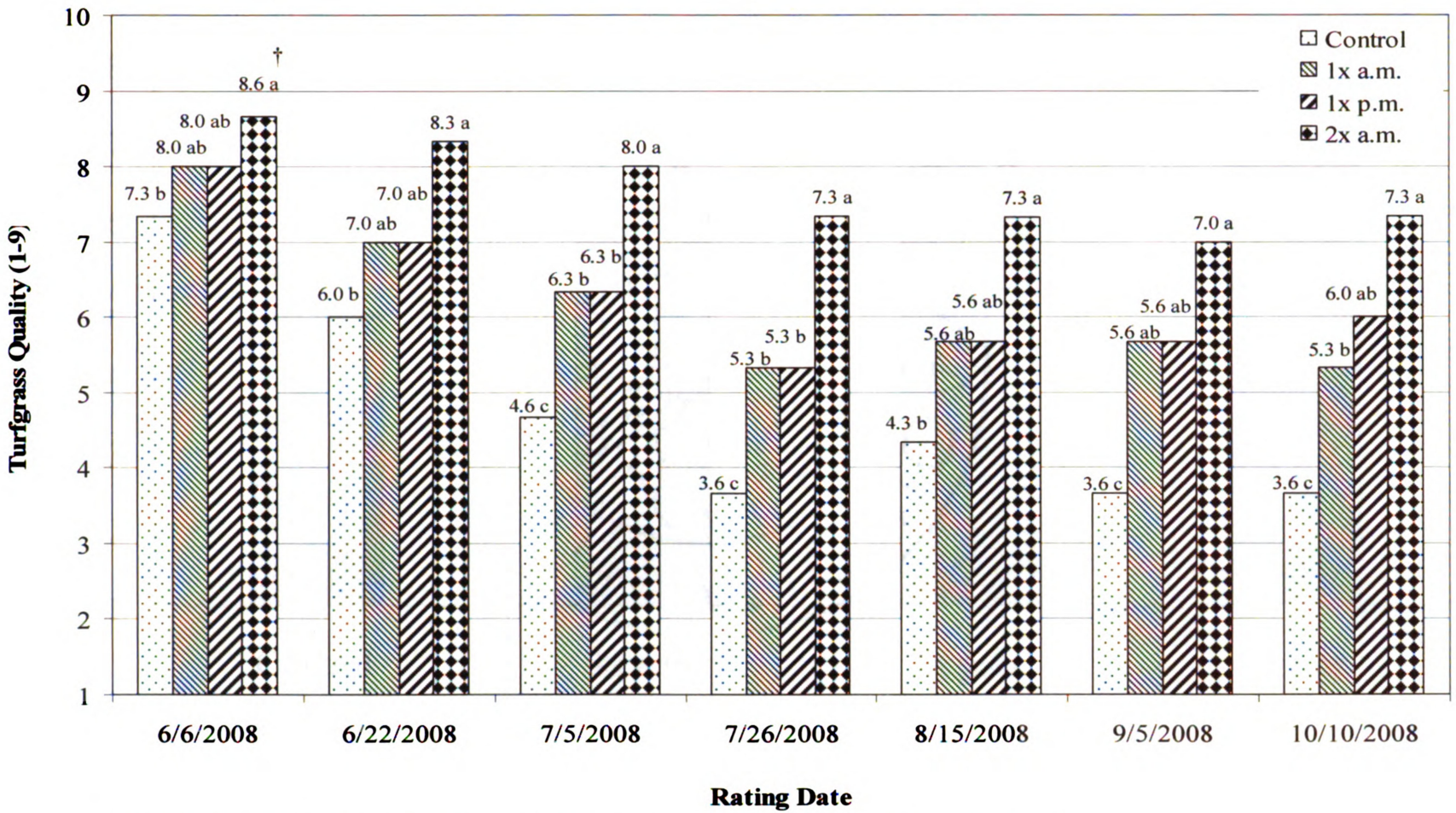
averaged a rating of 7.7, resulted in significantly better turfgrass quality than all other treatments (Figure 1.04).

In 2009, quality ratings were taken on 7 different dates and are reported in Figure 1.05. The 2x a.m. treatment resulted in significantly better turfgrass quality compared to the control on six of the seven rating dates. The 1x a.m. treatment was significantly better than the 1x p.m. treatment on only one occasion early in the growing season (19-Jun); the rest of the rating dates resulted in statistically similar ratings for both 1x rolling treatments. Both 1x rolling treatments resulted in significant treatment effects on the final four rating dates in 2009 (Figure 1.05).

Quality ratings from all rating dates in 2009 were averaged in order to get seasonal turfgrass quality means for treatments, which are reported in Figure 1.06. Both 1x treatments resulted in significantly better turfgrass quality compared to the control ($P < 0.05$), but were not significantly different from one another ($P > 0.05$). The 2x a.m. treatment resulted in significantly better turfgrass quality compared to all other treatments ($P < 0.05$).

Percent volumetric water content (%VWC) of the top 1.5 inches (3.81 cm) of soil was measured in 2008 and 2009 on six and four occasions, respectively. In 2008, significant treatment effects were observed on three reading dates, data is reported in Figure 1.07. The 2x a.m. treatment had significantly higher %VWC compared to the control on three different occasions in 2008 (13-Jul, 10-Sep, and 17-Oct). The 1x rolling treatments (1x a.m. and 1x p.m.) were not significantly different from one another on any rating date, and only resulted in a significant treatment effect on one measurement date.

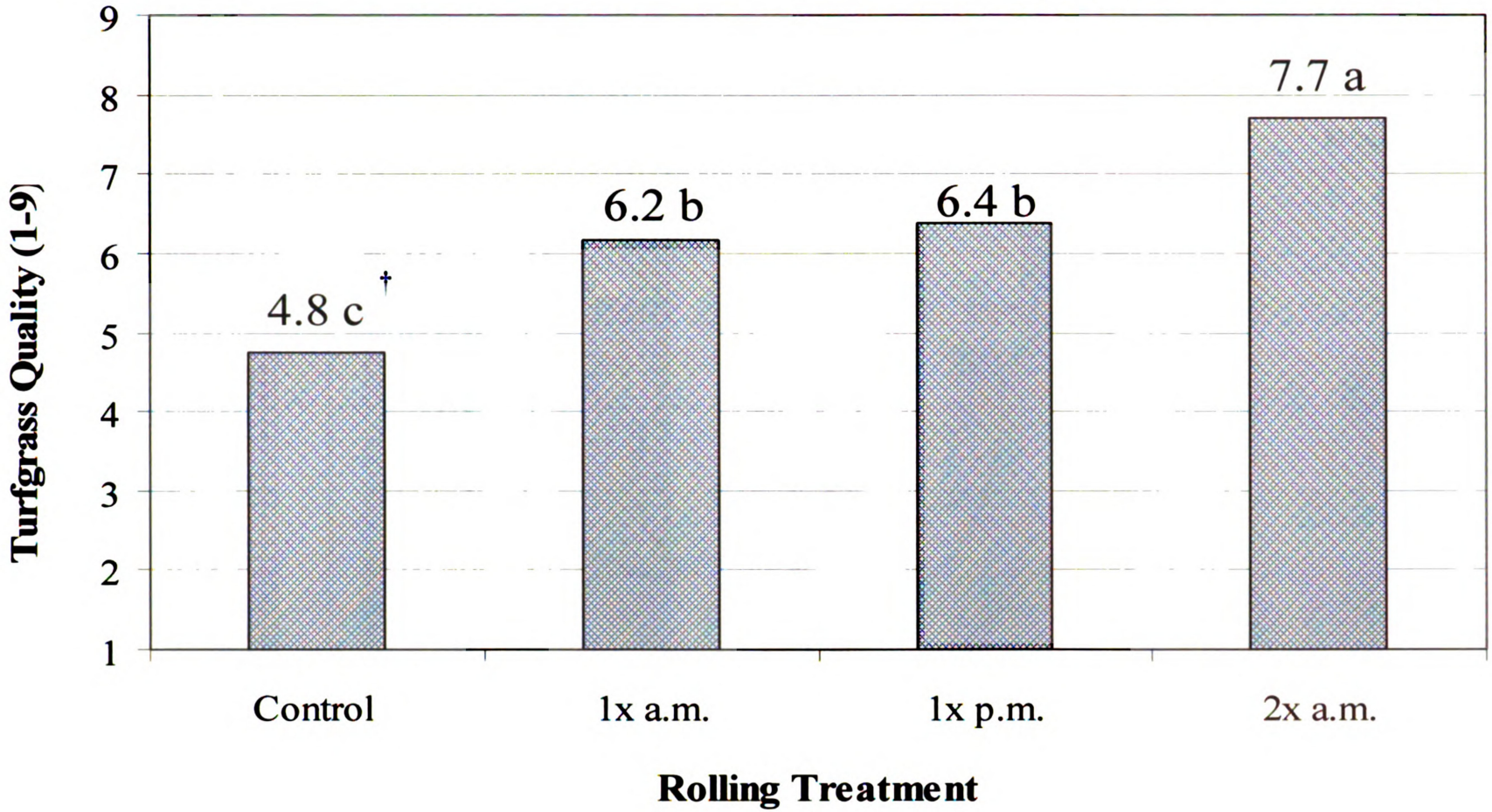
Figure 1.03. Quality ratings* on creeping bentgrass (*Agrostis palustris Huds)* turf plots for rolling treatments on different dates in 2008.



* Quality based on a 1-9 rating scale where 9 = excellent turf, 6 = acceptable, and 1 = dead or necrotic turf.

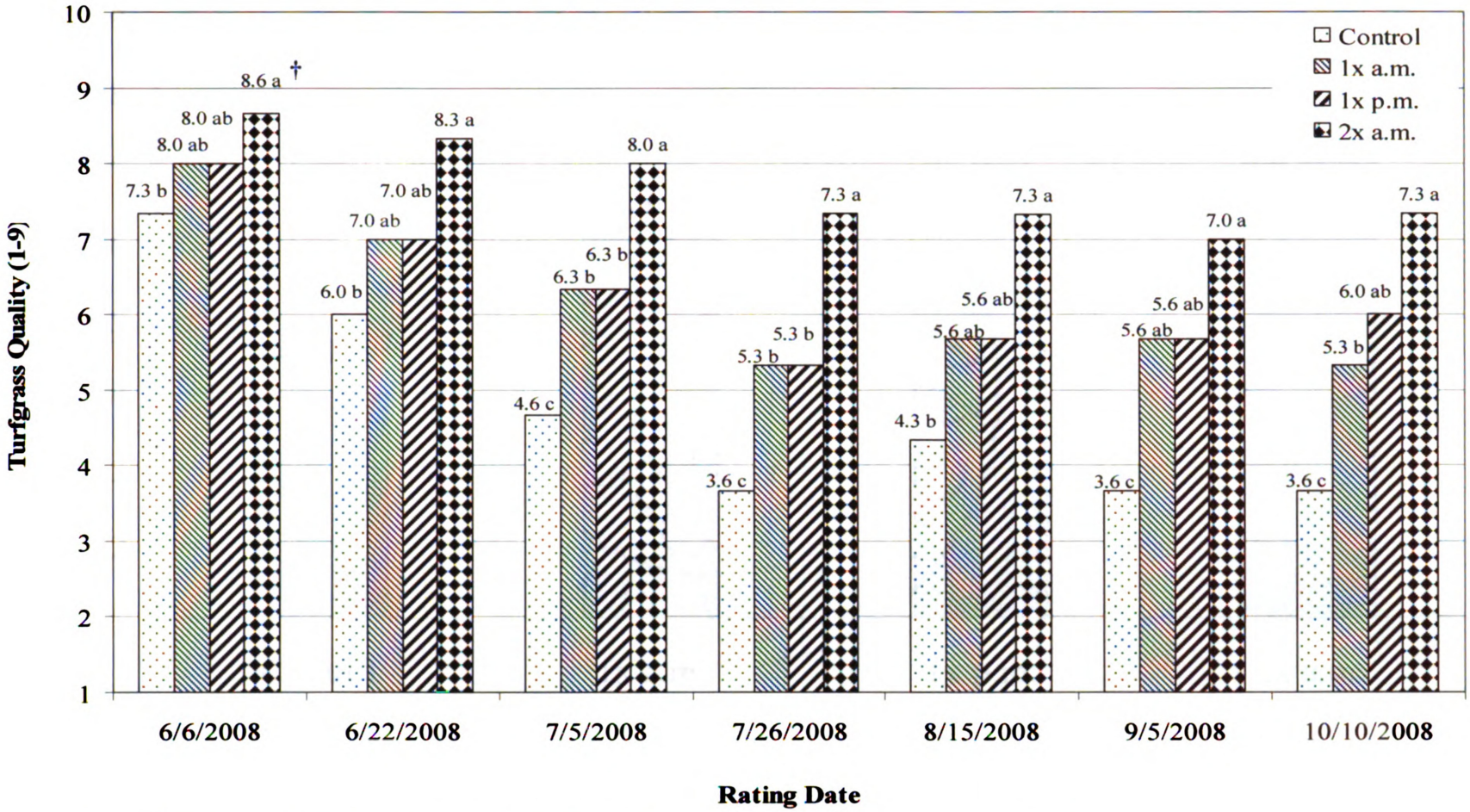
† Within rating dates, means followed by the same letter are not significantly different according to Fisher's LSD (P > 0.05).

Figure 1.04. Seasonal turfgrass quality ratings * for rolled treatments on creeping bentgrass (*Agrostis palustris Huds.)* putting green plots in 2008.



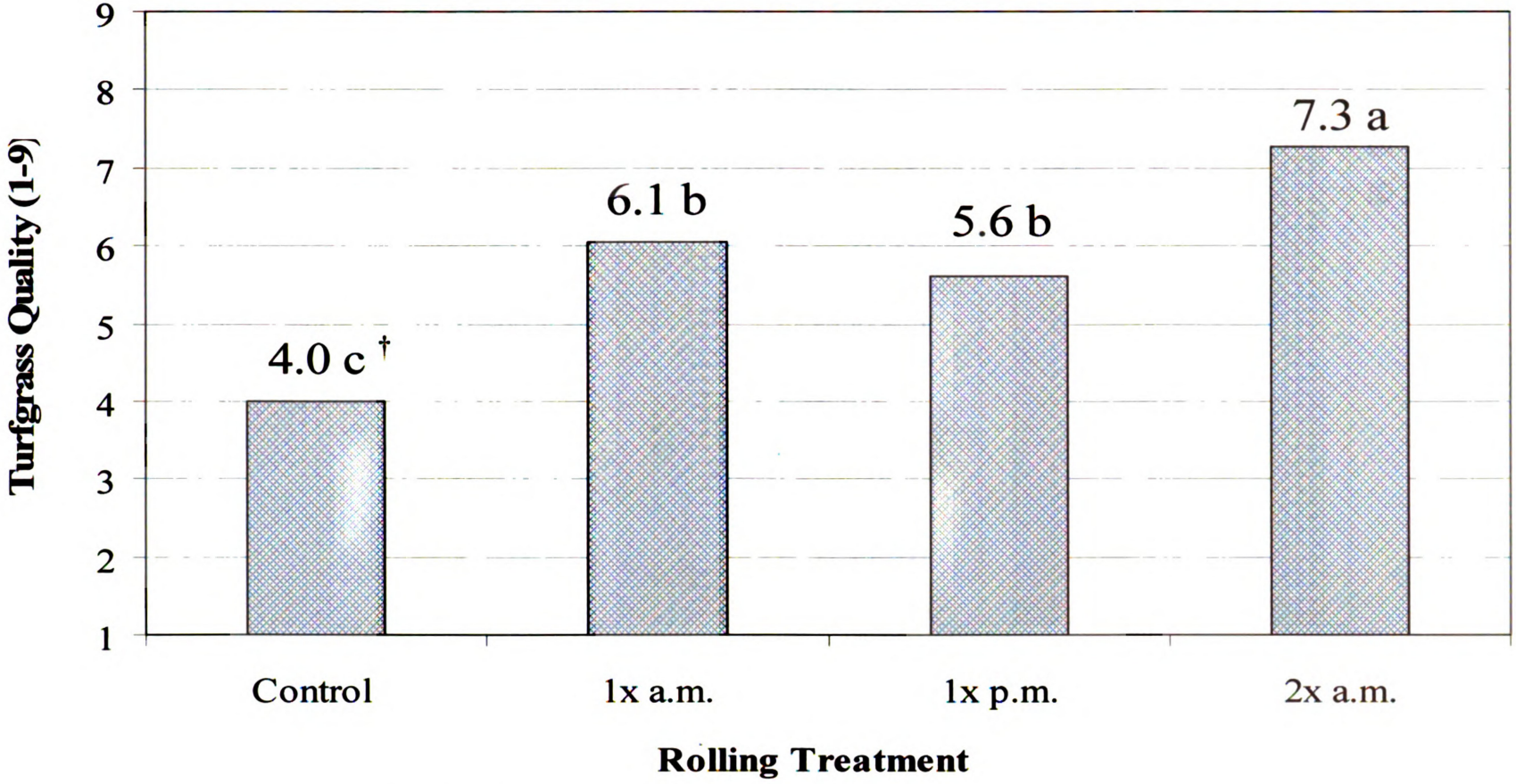
* Quality based on a 1-9 rating scale where 9 = excellent turf, 6 = acceptable, and 1 = dead or necrotic turf.
† Within rating dates, means followed by the same letter are not significantly different according to Fisher's LSD (P > 0.05).

Figure 1.05. Quality ratings* on creeping bentgrass (*Agrostis palustris Huds)* turf plots for rolling treatments on different dates in 2009.



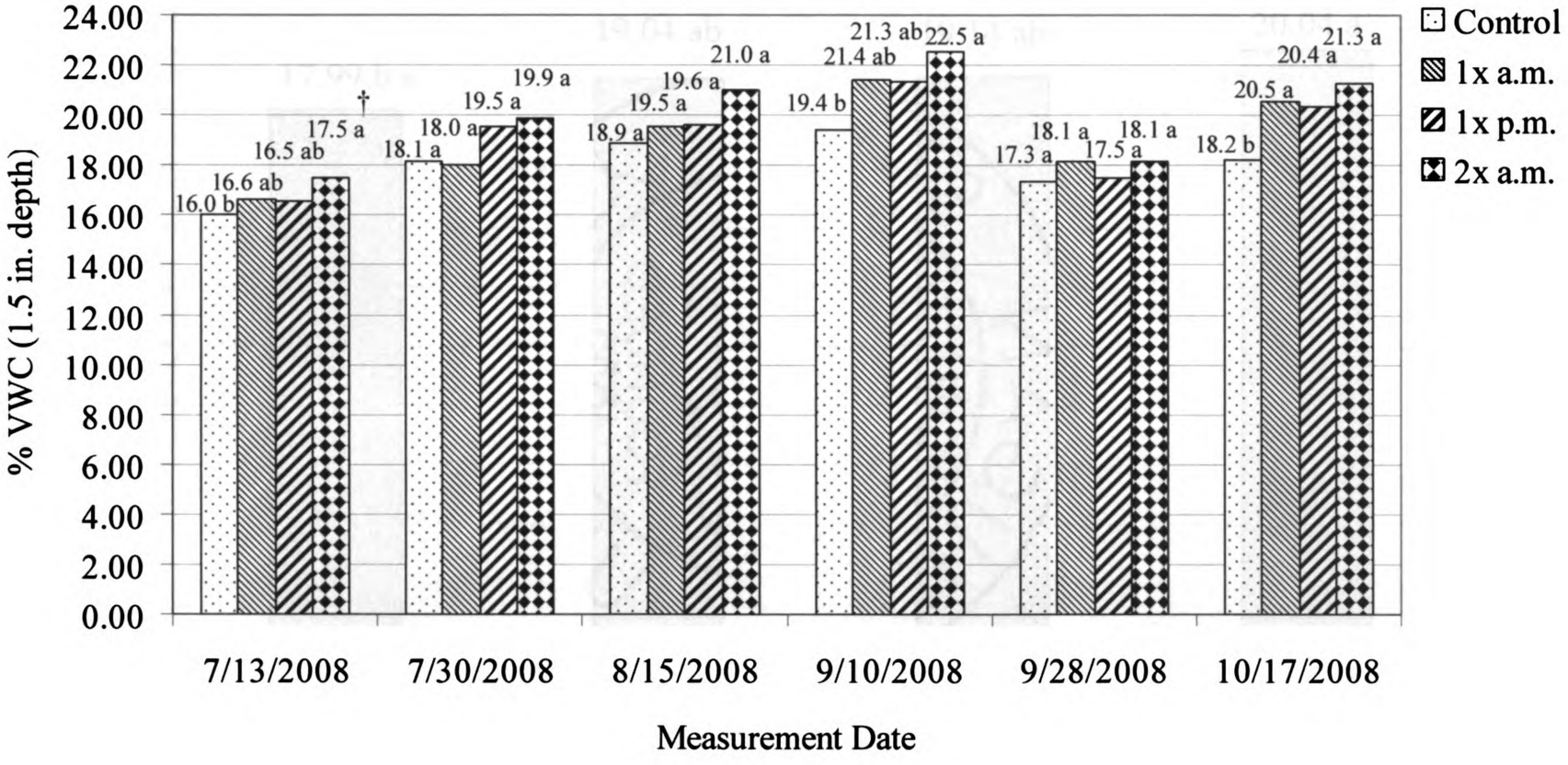
* Quality based on a 1-9 rating scale where 9 = excellent turf, 6 = acceptable, and 1 = dead or necrotic turf.
† Within rating dates, means followed by the same letter are not significantly different according to Fisher's LSD (P > 0.05).

Figure 1.06. Seasonal turfgrass quality means * for rolled treatments on creeping bentgrass (*Agrostis palustris Huds.)* putting green plots in 2009.



* Quality based on a 1-9 rating scale where 9 = excellent turf, 6 = acceptable, and 1 = dead or necrotic turf.
† Within rating dates, means followed by the same letter are not significantly different according to Fisher's LSD (P > 0.05).

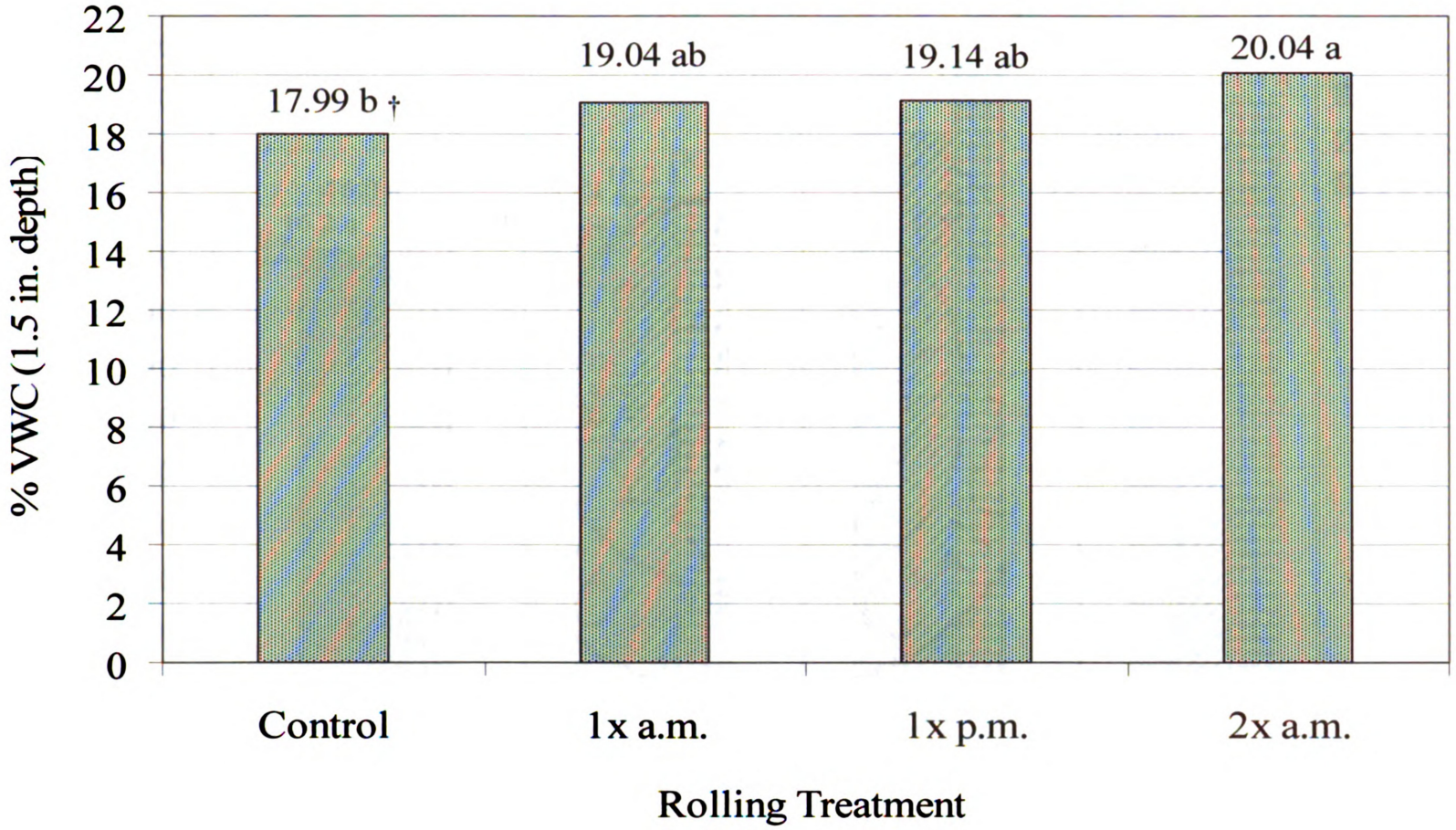
Figure 1.07. Percent volumetric water content (VWC) means* for rolling treatments on creeping bentgrass (*Agrostis palustris Huds.*) plots in 2008.



* Treatment means are averages of (20) %VWC readings per plot, and three replications per treatment.

† Within rating dates, means followed by the same letter are not statistically different according to Fisher's LSD (0.05).

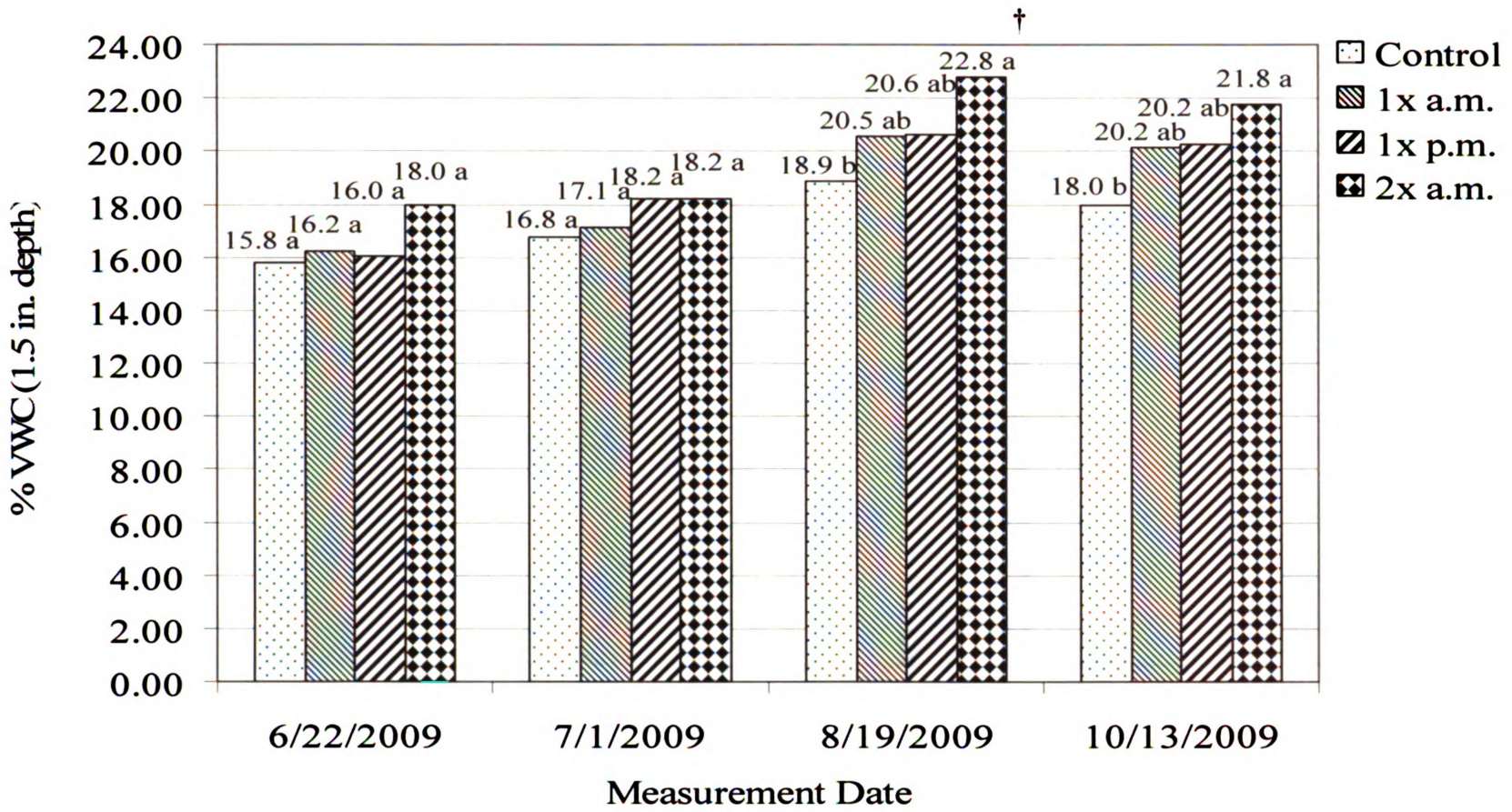
Figure 1.08. Seasonal percent volumetric water content (%VWC) means* for rolling treatments on creeping bentgrass (*Agrostis palustris Huds.)* plots in 2008.



* Seasonal %VWC treatment means are the average of six different measurement dates in 2008.

† Treatment means followed by the same letter are not statistically different according to Fisher's LSD (P > 0.05).

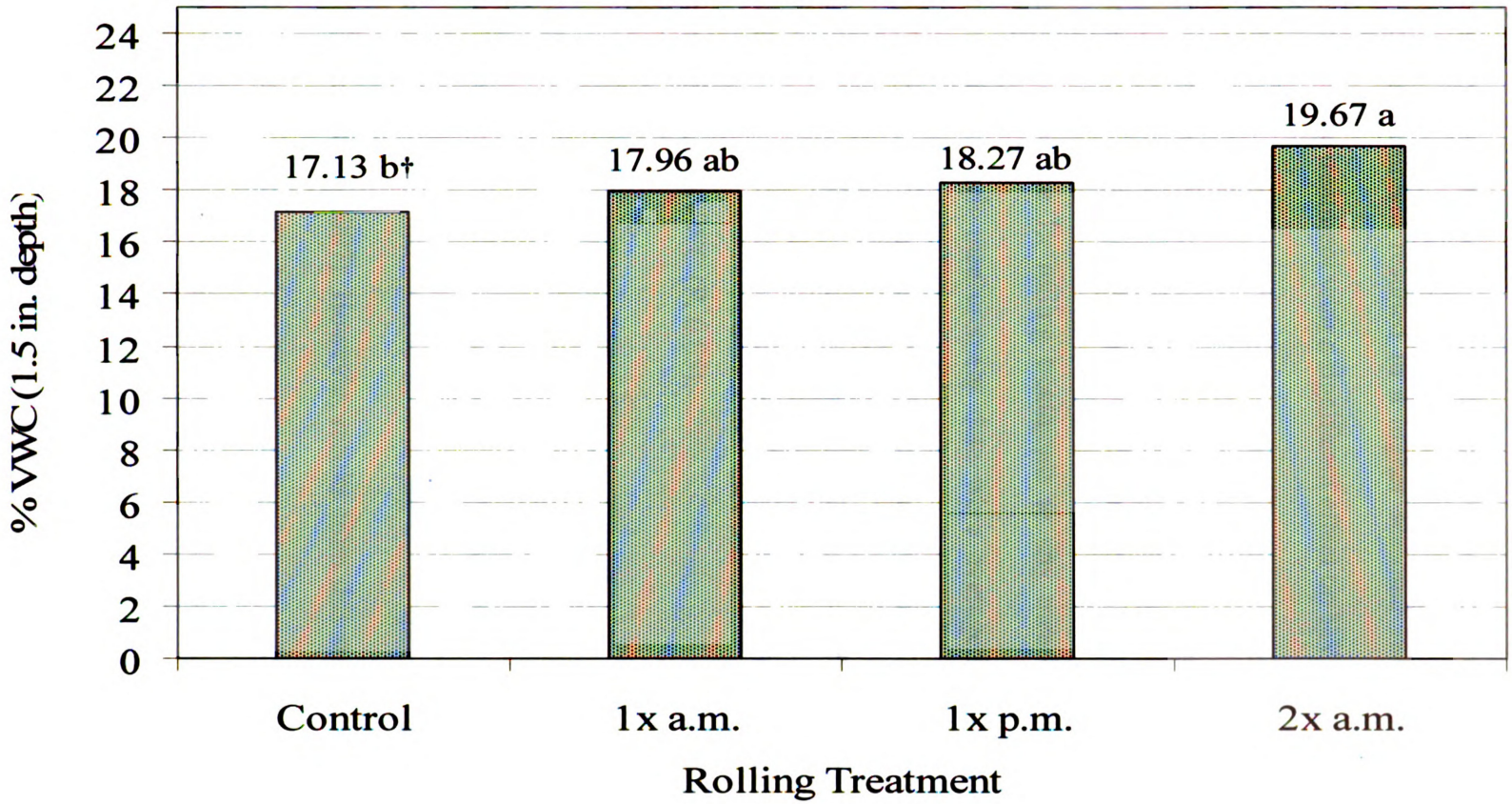
Figure 1.09. Percent volumetric water content means* for rolling treatments on creeping bentgrass (*Agrostis palustris Huds.)* plots in 2009.



* Treatment means are averages of 20 readings per plot, and three replications per treatment.

† Within rating dates, means followed by the same letter are not statistically different according to Fisher's LSD ($P > 0.05$).

Figure 1.10. Seasonal percent volumetric water content (%VWC) means* for rolling treatments on creeping bentgrass (*Agrostis palustris Huds.)* plots in 2009.



* Seasonal %VWC treatment means are the average of four different measurement dates in 2009.

† Treatment means followed by the same letter are not statistically different according to Fisher's LSD (P > 0.05).

The final measurement date (17-Oct) resulted in a significant treatment effect on %VWC for all rolled treatments. Treatment means for individual dates were combined to obtain a seasonal %VWC mean for treatments in 2008 and are reported in Figure 1.08. The 2x a.m. rolling treatment resulted in a higher seasonal %VWC compared to the control and was the only treatment with a significant effect on %VWC ($P < 0.05$).

Two of the four dates in 2009 in which VWC measurements were taken resulted in a significant treatment effect on %VWC in the 2x a.m. treatment, (90-Aug and13-Oct); results are reported in Figure 1.09. No significant treatment effects on VWC were observed with either of the 1x rolling treatments at any measurement date in 2009. Treatment means from each measurement date were averaged in order to obtain a seasonal %VWC mean for treatments; results are reported in Figure 1.10. Similar to 2008, only the 2x a.m. rolling treatment was significantly different from the control ($P<0.05$), resulting in a 3.35% increase in %VWC. Neither of the 1x rolling treatments had a significant effect on %VWC (P. 0.05).

CONCLUSIONS

It is widely accepted that the removal and dispersal of dew from turfgrass by early morning mowing plays a significant role in reducing dollar spot disease (Williams and Powell, 1995; Ellram et al., 2007). However, results from this study indicate alternative mechanisms in disease control. Many questions remain regarding lightweight rolling, particularly concerning the mechanisms involved in dollar spot reductions. A common theory revolves around dollar spot incidence being reduced on rolled plots due to the removal or dispersal of excess moisture on the turfgrass canopy after initial mowing. The

moist, humid environment is thought to not only serve as a microclimate conducive for fungal growth and infection, but guttation exuded from freshly mown turfgrass is thought to supply a nutrient-rich food source to the dollar spot pathogen, *Rutstroemia floccosum* (Williams and Powell, 1995).

In an attempt to challenge the notion that dollar spot reduction is solely due to the removal of dew and guttation fluid by morning rolling, one rolling treatment was implemented in the morning hours, while dew was still present on the plant canopy, and one in the afternoon hours, after surface moisture had naturally evaporated. Both morning and afternoon rolling treatments resulted in significant reductions in disease. No significant differences were found between the 1x a.m. treatment and the 1x p.m. treatment in either year of the study. These results confirm the observation that daily rolling of turfgrass plays a role in disease suppression but challenge the hypothesis that dew and guttation removal from early morning rolling is the underlying mechanism contributing to dollar spot reduction on creeping bentgrass putting greens. Afternoon rolling treatments proved to have a statistically equal treatment affect on dollar spot reduction as morning rolling treatments. Additionally, no statistical differences existed between the afternoon and morning treatments. This suggests that, aside from the physical removal/dispersal of surface moisture, possible biological or physiological mechanisms could be involved in the reduction of dollar spot on creeping bentgrass putting greens when daily lightweight rolling is taking place.

Rolling five days week^{-1}, regardless of the time of day, consistently resulted in lower disease incidence, as well as superior turfgrass quality ratings, in 2008 and 2009. In order to investigate whether or not rolling has cumulative effects on dollar spot

reduction, the 2x a.m. treatment was implemented. This 2x day^{-1} rolling treatment resulted in consistently lower dollar spot observations compared to both the control and the 1x rolled treatments, as well as higher turfgrass quality ratings in both years of the study. Greater reductions in dollar spot counts, significantly better turfgrass quality, and high probabilities of treatment effects on plots rolled 2x day^{-1}, particularly at the conclusion of the second year, are indications of a possible cumulative effect rolling may be having on disease suppression and turfgrass health. These results are consistent with Nikolai et al. (2001) in the respect that greater differences with regard to disease occurrence, existed between rolled and non-rolled plots as the study progressed for multiple years. Contrary to previous rolling research, Horvath et al. (2009) observed no differences in dollar spot severity on rolled and non-rolled plots of creeping bentgrass. However, rolling treatments were implemented 2-3 times $week^{-1}$ and results were based on six weeks of data collection. Cumulative effects of rolling that have been observed herein as well as in Nikolai et al. (2001) are based on multiple years of data collection, significant treatment effects were typically a result of season long rolling, not observed until later observation dates in the season, or until seasonal averages were obtained.

Average volumetric water content was significantly higher in the 2x a.m. treatment in both years of the study, and both 1x rolling treatments trended towards higher %VWC when compared to the control. These observations not only suggests that rolling may be contributing to to greater water holding capacity in the upper root zone of the turfgrass canopy, but support observations by Couch and Bloom (1960) and Liu et al. (1995), where higher soil moisture resulted in reduced dollar spot development and incidence. Reports by Danneberger (1989), Hamilton et al. (1994), and Hartwiger et al.

(2001) indicated rolling did not increase soil compaction of putting greens constructed with a high sand content root zone. While this may be true, rolling may be contributing to a decrease in pore size in the top 1.5 in. (3.81 cm) of the root zone. Smaller pores equate to a greater attractive force by which water can be held. Additionally, volumetric water content can be defined as $\theta = \frac{V_w}{V_T}$, where V_w is the volume of water and V_T is the total volume associated with the soil (i.e. soil volume + water volume + void space). If pore size is decreased due to slight compaction in the upper (top 3.81 cm) root zone, a reduction in void space takes place, thus lowering V_T in the equation. This may be responsible for increasing θ or the total volumetric water content measurements in rolled treatments.

The phenomenon by which rolling inhibits disease incidence was investigated. Rolling had a significant effect on dollar spot incidence in both years of this study. Morning and afternoon rolling treatments had similar effects on dollar spot reduction compared to the control. These similarities between morning and afternoon rolling suggest mechanisms other than secondary dew/guttation removal may be responsible for dollar spot reductions. Many theories still remain, and future rolling research should be conducted in order to investigate the potential biological or physiological impacts associated with turfgrass rolling. This study also revealed a trend towards cumulative effects of rolling on turfgrass health and quality. Multiple rolling applications per day resulted in substantially better turfgrass quality with less disease when compared to treatments rolled once per day. Continued research investigating this effect, particularly in afternoon or dew/guttation free settings should be pursued. The mechanism by which rolling is detrimental to dollar spot incidence is most likely multi-faceted, involving

aspects of soil physics, microbial ecology, plant physiology, and plant pathology or epidemiology. In the forthcoming chapter, one particular mechanism regarding microbial populations is discussed.

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CHAPTER TWO

TURFGRASS ROOTZONE MICROBIAL RESPONSES TO ROLLING

ABSTRACT

Relative abundance of microorganism community populations is useful information for estimating the effects of particular management strategies on rhizosphere soil (REF). A two-year rolling study was conducted during the growing seasons of 2008 and 2009 and relative abundance of soil microorganisms under a creeping bentgrass (*Agrostis palustris*) putting green was elucidated via phospholipid fatty acid (PLFA) analysis (REF). Treatments rolled twice in the morning (2x a.m.) and once in the morning (1x a.m.) both showed significant increases in particular PLFA abundance when compared to the control ($P < 0.05$) all of which are bacterial biomarkers. When combined to form specific PLFA taxonomic biomarker groups, relative abundance was found to be higher in all rolled treatments compared to the non-rolled control, while fungal and other PLFA relative abundance remained relatively constant. A trend towards higher bacterial abundance in rolled treatments suggest a potential biological effect of lightweight rolling possibly attributed to the aforementioned increase in volumetric water content observed in rolled treatments (Chapter 1). Increases in bacterial relative abundance may play a role in dollar spot (*Rutstroemia floccosum*) reductions due to various mechanisms of fungal disease suppression often observed in many soil dwelling bacterial organisms.

INTRODUCTION

The rhizosphere is a complex community of soil microflora and microfauna. Along with facilitating plant infection and disease, many microorganisms play beneficial roles in the root zone of a turfgrass stand. Microbial communities have been implicated in suppressing soilborne plant diseases, promoting plant growth, and in changing vegetation (Doran et al., 1996). Several studies have elucidated the significance of microbial activity in soil related to and responsible for the suppression of plant pathogens. For example, relationships have been found between microbial diversity and root disease suppression (Nitta, 1991; Workneh and van Bruggen, 1994). Rovira & Wildermuth (1981) indicated that the microbiota in a "rich" soil tends to reduce the severity of attack by many plant pathogens or, in other words, soils higher in microbiota content and diversity show tendencies towards general disease suppression.

Bacterial populations in the soil and rhizosphere have long been of interest to agronomists and plant scientists, partially due to the observation that antagonism and competition between bacteria and many important plant pathogens can be exploited as possible control mechanisms. An active microbial population performs and plays a role in many beneficial activities and processes such as organic matter decomposition, assisting in plant nutrient acquisition, availability and recycling, and plant pathogen suppression (Sylvia et al., 1997). Thus, turfgrasses grown in rootzones containing lower microbial populations may be less healthy and possibly more easily affected by turfgrass pathogens, resulting in an overall lower quality turfgrass than those grown in the presence of higher microbial populations (Hodges, 1990; Couch, 1995). In turfgrass systems, successful suppression of fungal diseases such as dollar spot (*Rutstroemia floccosum*) and

anthracnose (*Colletotrichum cereale*) has been achieved both *in vitro* and with field applications of an antibiotic producing *Pseudomonas spp.* (Powell, 1993; Uddin and Viji, 2002). These bacterial metabolites are thought to contribute to the antagonistic effects on plant pathogenic fungi.

Bolton et al., (1985), Fraser et al., (1988), Kirchner et al., (1993), and Powlson et al., (1987) have documented associations between agricultural management practices and microbial community make-up and activities. Particular management practices in different systems may alter the composition and structure of soil microbial communities. This has been documented in agricultural soils by examining either whole soil microbial communities or specific functional groups (Ka et al., 1995; Lundquist et al., 1999; Webster et al., 2002; Clegg et al., 2003). Microbial populations fluctuate, and their community structure and composition is highly sensitive to change with intense management (Donnison et al., 2000). Disease suppressiveness has been achieved through crop rotation (Cook et al., 2002), intercropping (Schneider et al., 2003), residue destruction (Baird et al., 2003), organic amendments (Tilston et al., 2002), and tillage management practices (Sturz et al., 1997; Pankhurst et al., 2002). All of these cultureal practices contribute to altering the microbial community in a way that favors plant health and hinders disease development.

A useful approach to estimating the fungal and bacterial abundance in soil is to measure chemical components that are specific for microbial groups. One such approach relies on bacteria and fungi having different fatty acid compositions in their phospholipids (Hardwood and Russell, 1984; Tunlid and White, 1992). Phospholipid fatty acids (PLFAs) are widely accepted as biomarkers that indicate viable microbial

biomass and provide a microbial community 'fingerprint' (Vestal and White, 1989; Zelles, 1999). Changes in soil microbial communities under many different experimental applications have been successfully measured using PLFA analysis. Fatty acid patterns have been used to distinguish microbial fluxes between different types of cultivation (Zelles et al. 1995), fertilizer treatments (Guo and Wang, 2009), pH gradients (Baath and Anderson 2003), agricultural management systems, seasons, soil types (Bossio et al. 1998), and changes in the soil microbial community due to pollution (Baath et al., 1992). A quantitative measure of microbial communities can be obtained using PLFA analysis as opposed to most current DNA-based fingerprinting methods (Muyzer et al., 1993; Kowalchuk et al., 1997).

While many studies have shown the direct effect that high populations of microbes have on plant pathogens in the rhizosphere, much remains unknown with regard to their disease suppression mechanisms. Numerous species within several genera of bacteria have been reported to be effective biological control agents for root and foliar diseases on turfgrass. The mechanisms of biological control are thought to occur mainly through the process of antagonism. Antagonism can be defined as active opposition that results from the production of substances by one organism that are toxic to other organisms. Such substances can be classified as antibiotics that cause lysis or death of microbial competitors (Rovira and Wildermuth, 1981). Additionally, competition for food, oxygen, and space is often considered a form of antagonism or a distinct mechanism of biological inhibition all together (Baker and Cook, 1974).

The purpose of this study was to determine the effects of lightweight rolling of creeping bentgrass putting greens in relation to soil microbial populations. Results of

dramatic decreases in disease incidence caused by the fungal pathogen *R. floccosum,* have been outlined in the previous chapter. Microbial community assessment was explored in order to test the hypothesis that rolling is having an effect on the community dynamics, possibly related to disease suppression. Microbial community composition was estimated using PLFA analysis of root zone samples from treatment plots in order to make comparisons among rolled and non-rolled turf stands.

MATERIALS AND METHODS

Field research was conducted at the Hancock Turfgrass Research Center on the campus of Michigan State University, East Lansing, Michigan, on an experimental putting green constructed in 2005 and seeded with creeping bentgrass (*Agrostis stolonifera cv 'Independence'*).

Research plots were 7 ft x 12 ft (2.13 m x 3.65 m) arranged in a randomized block design with three replications for each rolling treatment. Prior to initiating the study, each plot was randomly assigned a rolling treatment. Mowing took place between 6:00 and 7:00 a.m. and was implemented on all plots prior to any rolling. Each rolling treatment was carried out 5 days week^{-1} (Monday-Friday) and were as follows: 1) Control (no rolling), 2) rolled once (1x) in the a.m. immediately after mowing, 3) rolled once (1x) in the p.m. when dew and guttation water had dissipated, and 4) rolled twice (2x) in the a.m. immediately after mowing. The morning rolling treatments were implemented between 7:00 and 8:30 a.m., and the afternoon rolling between 1:00 and 2:00 p.m. Rolling of plots was conducted with a Tru-Turf ride-on greens roller, model RS48-11B, with a 39 inch (99cm) roll swath weighing 562 pounds (255kg) without an

operator. To ensure study uniformity, rolling was the only treatment that differed in each plot.

The root zone mix consisted of a sand based 80:20 (sand:peat v/v) mix constructed to USGA recommendations (USGA, 1993). Sand topdressing was applied to the entire research area on a light, frequent (bi/tri-weekly) basis throughout the growing season in order to simulate typical golf course putting green maintenance practices. No vertical mowing or core cultivation occurred on the research plots during the course of the study in order to minimize turfgrass and soil disruption. Fungicides were not applied to any of the research plots during the duration of the study. Insecticides and herbicides were applied only on an as needed basis, and were applied uniformly over the entire study area when necessary.

Soil samples from plots were taken at a depth of 1 in (2.54 cm) with a 1 in (2.54 cm) diameter soil probe. Ten cores were randomly taken from each plot on September 25, 2009. Top growth, including turfgrass leaves, stems, roots, and debris was removed from each core by cutting with a sterile razor blade just below the thatch/soil interface. Cut ends were gently shook so that loose soil that remained in thatch and roots would be included in the sample. Unused top growth was discarded. The remaining upper rootzone soil was homogenized by vigorous mixing in plastic bags. Thirty grams from each of the representative, homogenized soil samples was measured and placed into separate 3 1/8 X 5 1/2 inch paper coin envelopes before being lyophilized. Freeze dried soil samples from each plot were ground using a pestle and mortar until they passed through an ATM #40 (425 μm) U.S. standard testing mesh sieve (Advantech Manufacturing Inc., New Berlin, WI). Ten grams of the ground material from each sample were stored in 2 oz. Nasco

Whirl-Pak bags at -20° C prior to their shipment to the University of Wisconsin, Madison, Department of Soil Science for PLFA analysis by Dr. Teri Balser's laboratory staff.

Lipids were extracted from 3 g (dry weight) subsamples using a modified Bligh and Dyer (1959) technique as described in Balser and Firestone (2005). Samples were analyzed using a Hewlett-Packard Agilent 6890A gas chromatograph (GC) (Agilent Tech. Co., Santa Clara, CA) equipped with an Agilent Ultra-2 (5% phenyl)-methylpolysiloxane capillary column (25m by 0.2mm by 0.33μm) and flame ionization detector (FID). Peaks were identified using a mix of known FAME standards and comparing retention times or estimated chain lengths (ECL) from each sample output to a naming table. These naming tables have ECL's for hundreds of known lipids and a "naming window", which is the accepted amount that an unknown can vary from the ECL and still be assigned a particular name. The fatty acid analyses were carried out by an MIDI Sherlock microbial identification system (Version 4.5, MIDI, Newark, NJ).

Lipid data were converted from raw peak area to nmol/g soil in order to facilitate statistical analysis. Peak area was converted to μg carbon (C) using conversion factors from internal standards run on the GC. Conversion factors are equal to μg C of the standard divided by the area of the standard on the GC and are typically the averages of two internal standard lipids, 9:0 and 19:0 in order to capture the response of both short- and long-chain lipids. This number was then divided by the unit weight soil in each sample (approximately 3g), and finally converted to μmol C g soil^{-1} using the molecular weight of each lipid.

Processed lipid data are expressed as abundance ($nmol_{lipid}\ g_{soil}^{-1}$), mole fraction ($nmol_{lipidx}\ nmol_{totallipid}^{-1}$,) (0-1)), or mole percent (mole fraction*100, (0-100%)). Mole fraction and mole percent are normalized by the total biomass in a sample and are thus measures of the relative abundance of any given lipid. Mole fraction is appropriate for use in ordination analyses (after transformation, e.g. arcsine, square-root), while mole percent (mole fraction multiplied by 100) is an easy-to-interpret value. Lipid abundances are the absolute amount of a given lipid extracted per gram of soil. Because the quantity of lipid per cell is reasonably constant, and the lipid extraction is highly quantitative (i.e. close to 100% extraction efficiency), abundance is, in effect, an estimate of microbial biomass. Total abundance is total biomass, and the abundance of key indicators reflects the biomass of the group it represents (T. Balser, pers. comm.).

Fatty acid nomenclature used in this study is as follows: total number of carbon atoms:number of double bonds, followed by the position of the double bond (ω) from the methyl end of the molecule. *Cis* and *trans* geometries are indicated by the suffixes *c* and *t*, respectively. The prefixes *a* and *i* refer to anteiso- and iso-branching, respectively. Methyl groups on the tenth carbon atom from the carboxyl end of the molecule are indicated by. The positions of the hydroxyl (OH) groups are noted when necessary, while *cy* indicates cyclopropane fatty acids (Bossio et al., 2006).

Each PLFA value is represented by the mean of three soil extraction replications. Particular individual fatty acids extracted from samples have been used as signature indicators for various taxonomic groups of microorganisms.

Combinations of particular PLFAs were considered to be representative of groups of organisms of interest in the soil. The PLFAs 18:1ω9c, 18:2ω6c, and 18:3ω6c were

used to represent soil fungi (Myers et al., 2001 and Vestal and White, 1989). Gram-positive (gm+) bacteria were represented by *i*15:0, *a*15:0, 15:0, *i*16:0, 17:0, i17:0 and *a*17:0, while 16:1ω7c, cy17:0, cy19:0 and 18:1ω9t were used to indicate gram-negative (gm−) bacteria (Ratledge and Wilkinson, 1988 and Zogg et al., 1997). Total bacteria were represented by the combined gm− and gm+ bacterial indicators (Ratledge and Wilkinson, 1988 and Zogg et al., 1997).

Treatment effects on PLFA relative abundance were analyzed using the PROC Mixed procedure in the SAS software (SAS Institute Inc. 2009, Cary, NC, USA). Analysis of variance (ANOVA) followed by Dunnett's test, for the comparison of treatment means to the control for individual fatty acid mole percentages, were carried out. Combined fatty acids representing groups of microorganisms were also subjected to ANOVA as well as mean comparisons based on Dunnett's test. A principal component analysis (PCA) of the arcsine transformed mole fraction PLFA data was carried out with the correlation matrix.

RESULTS AND DISCUSSION

Mole percent is considered an indication of relative abundance in relation to total PLFA abundance. As FID response from gas chromatography is proportional to molecular mass, results were expressed as molar percentages. Thirty fatty acids were identified for use in data analysis. Individual fatty acid relative abundances were compared in order to investigate possible differences due to season long lightweight rolling treatments. All PLFAs used in analyses were evaluated for normal distribution before being subjected to significance testing. Significant differences in particular fatty

acid mole percentages existed between rolled and control treatments in rootzone soils of the creeping bentgrass putting green. These PLFA indicators, mole percentages, and references, are reported in Table 2.01.

Of the thirty microorganism indicator PLFAs extracted, eight were significantly higher in mole percentage in the 2x a.m. rolled treatment than in the untreated control ($P \leq 0.10$). These included the straight chain saturated fatty acids 12:0, 14:0, 15:0, 18:0, 19:0cy, 20:0, 9:0 (bacterial indicators) and the monounsaturated fatty acid 18:1ω9t (actinomycete indicator).

Other significant differences in individual PLFAs existed between the 1x a.m. treatment and the control ($P \leq 0.10$). These included cyclopropane fatty acids 17:0cy and 19:0cy, straight chain saturated fatty acids 18:0, 20:0, and 9:0, and the monounsaturated fatty acid 18:1 w7c (all bacterial indicators); the polyunsaturated fatty acid 18:2ω6c (saprophytic fungi indicator); and the methyl branched fatty acid *i*17:1 and monounsaturated fatty acid 18:1ω9t (both actinomycete indicators).

Table 2.01. Relative abundance (mol %) of PLFA indicators in turfgrass root zones.

	Rolling Treatment				
PLFA	**Control**	**1x a.m.**	**1x p.m.**	**2x a.m**	**PLFA Biomarker†**
12:0	0.7446	0.7797	0.8962	1.0465 **	Bacteria
14:0	0.9251	1.0461	1.0315	1.1277 **	Bacteria
***i*14:0 3OH**	0.3279	0.4073	0.3889	0.4011	Unknown
14:1ω5c	0.2097	0.2225	0.2189	0.1428	(Gram -) bacteria
15:0	0.4878	0.5458	0.5477	0.5591 *	Bacteria
a15:0	1.1085	1.4009	1.3757	1.3671	(Gram +) bacteria
i15:0	2.4031	2.9951	2.8863	2.9857	(Gram +) bacteria
16:0	15.949	15.599	15.540	15.714	Bacteria and fungi
***i*16:0**	0.5792	0.7681	0.7288	0.7313	(Gram +) bacteria
***i*16:1 H**	0.5080	0.5875	0.6872	0.4279	(Gram -) bacteria
16:1ω5c	36.410	31.143	32.753	31.896	Arbuscular mycorrhizae
16:1ω7c	2.6360	3.0052	2.9218	2.9093	Gram- bacteria
16:1ω9c	0.4481	0.5341	0.5392	0.5150	Unknown
17:0	0.1997	0.2736	0.2506	0.1780	Bacteria
***a*17:0**	0.3455	0.4717	0.4321	0.4319	(Gram +) bacteria
17:0cy	0.5650	0.77704 *	0.7137	0.7324	Gram - /anaerobes
***i*17:0**	0.3455	0.4717	0.4321	0.4319	(Gram +) bacteria
17:1ω8c	0.4281	0.5226	0.6033	0.5518	(Gram -) bacteria
18:0	0.6418	0.9294 *	0.7805	0.8558 **	Bacteria
18:1ω5c	0.4178	1.2252	0.8376	0.4048	Unknown
18:1ω9c	7.5882	7.4477	7.5651	8.2416	Saprophytic or ectotrophic fungi
18:1ω9t	0.2344	0.29724 *	0.2694	0.3214 **	Actinomycetes
18:3ω6c	0.5733	0.6938	0.6054	0.6865	Saprophytic/ ectotrophic fungi
19:0	0.1188	0.1500	0.1235	0.1483	Bacteria
19:0cy	0.5445	0.8611 **	0.7305	0.7731 *	Gram - / anaerobic bacteria
20:0	0.2255	0.33765 **	0.2714	0.3068 *	Bacteria
9:0	0.2318	0.28886 *	0.2576	0.2904 *	Bacteria
***i*17:1**	1.2652	1.6838 *	1.5674	1.5392	Actinomycetes
18:2ω6c	3.6361	4.0150	3.1897	3.6111	Saprophytic fungi
18:1ω7c	4.9095	6.0582 *	5.2525	5.8397	(Gram -) bacteria

*, ** Means within the same row are significantly higher than the control according to Dunnett's test at the $P \leq 0.10$ and 0.05 probability levels respectively.

† PLFA biomarker indicators according to: Bossio et al., 1998; Ratledge and Wilkinson, 1988; Zogg et al., 1997; Frostegård and Bååth, 1996; Myers et al., 2001; Vestal and White, 1989; Turpeinen et al., 2004; T. Balser, personal Communication, 2009.

The 1x p.m. rolling treatment resulted in no significant differences in any of the 30 indicator PLFAs when compared to the control, but remained higher in relative abundance than the non-rolled control in most of the bacterial indicators. The 1x p.m. treatment also did not stray statistically from the other rolling treatments in any of the individual PLFA relative abundances. Conversely, the untreated control had significantly higher proportions of the PLFA 16:1ω5c (Arbuscular mycorrhizae indicator) compared to the 1x a.m. rolling treatment.

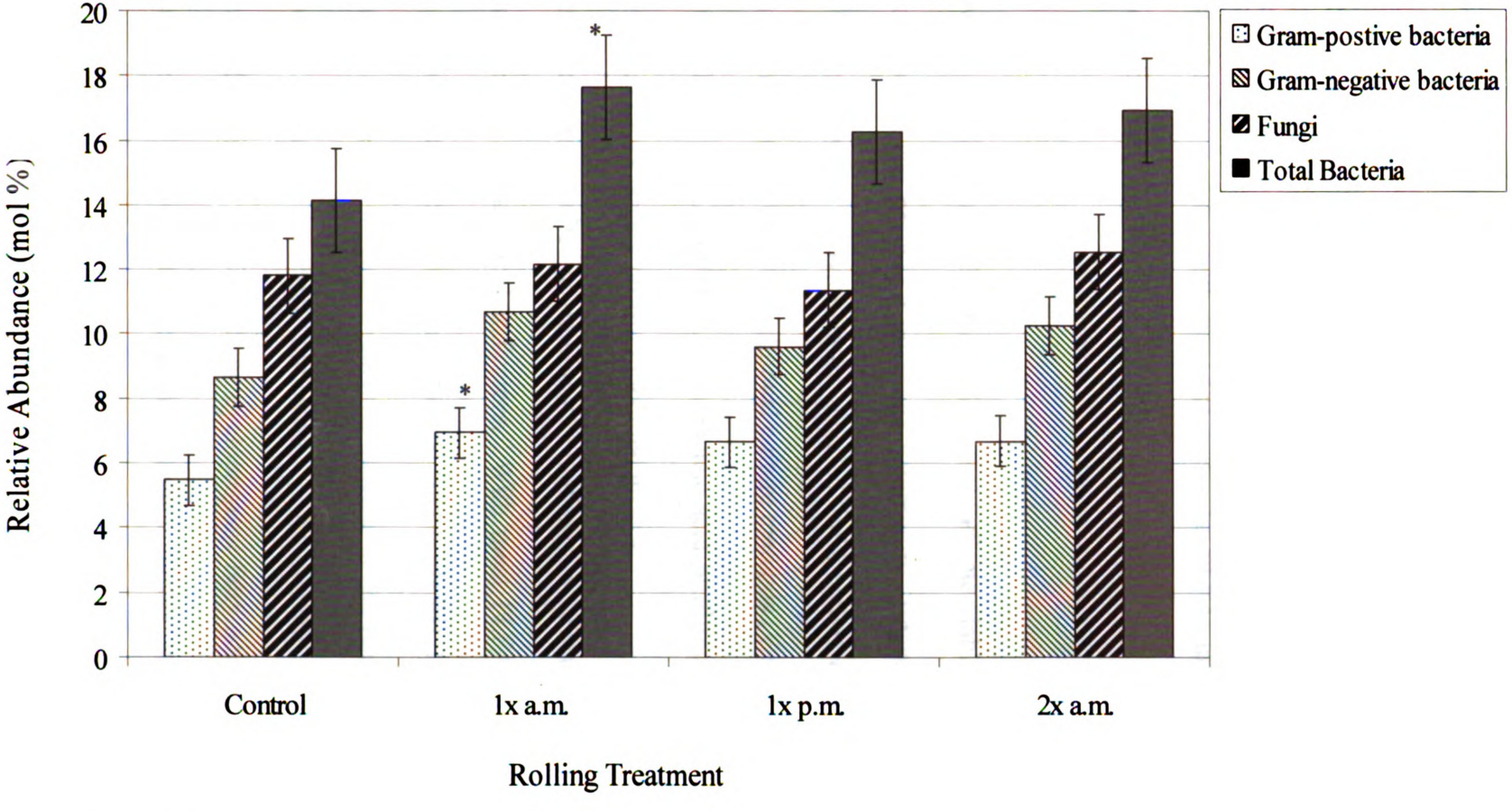
Commonly used combinations of PLFAs were analyzed and used to represent taxonomic groups of interest in the root zone soil samples and make comparisons among rolling treatments, PLFA combinations are reported in Table 2.02. These combinations rendered significant differences among treatments as indicated in Figure 2.01. Gram-negative bacterial PLFA relative abundances were significantly higher ($P < 0.10$) in plots rolled 1x a.m. (10.70%) compared to the control (8.66%), with a standard error (SE) of 0.8941. Treatments 1x p.m. and 2x a.m. resulted in 0.963% and 1.599% higher gram negative bacteria PLFA abundances than the control respectively. Gram-positive bacterial PLFAs were not statistically different in any treatment but were slightly higher in all rolled treatments than the control.

Total bacterial PLFA mole percentages were significantly higher ($P < 0.10$) in the 1x a.m. rolled treatment compared to the non-rolled control. A trend towards higher total bacterial PLFA indicators existed in all rolled treatments when compared to the control with a difference of 2.15% and 2.82% in the 1x p.m. and 2x a.m. treatments respectively.

Table 2.02. PLFA markers used for taxonomic microbial groupings.

Taxonomic group	**PLFA group**	**Specific PLFA markers**	**References**
PLFA biomarkers			
Bacteria	Multiple groups	*i*15:0, *a*15:0, 15:0, *i*16:0, 17:0, *i*17:0, *a*17:0, 16:1ω7c, 17:0cy, 19:0cy, 18:1ω7c.	Bossio et al., 1998; Ratledge and Wilkinson, 1988; Zogg et al., 1997; Frostegård and Bååth, 1996.
Gram-positive bacteria	Branched PLFAs	*i*15:0, *a*15:0, 15:0, *i*16:0, 17:0, *i*17:0, *a*17:0.	Ratledge and Wilkinson, 1988; Zogg et al., 1997.
Gram-negative bacteria	Cyclopropyl and mono PLFAs	16:1ω7c, 17:0cy, 19:0cy and 18:1ω7c.	Ratledge and Wilkinson, 1988; Zogg et al., 1997.
Fungi	Polyunsaturated PLFAs	18:1ω9c, 18:2ω6c and 18:3ω6c.	Myers et al., 2001; Vestal and White, 1989.

Figure 2.01. PLFA group abundance (mol %)† in turfgrass root zones subjected to different rolling treatments.



† Vertical bars represent standard error (SE) of mean.
* Indicates a significant difference in relative abundance from the control using Dunnet's test ($P \leq 0.10$).

Total fungal PLFA mole percentages were not significantly different among treatments; additionally, no trends towards higher fungal PLFAs were observed in rolled treatments.

Results from a principal component analysis (data not shown) were insignificant when discriminating rolling treatments by PLFA profile. No definite shifts in microbial community structure or patterns with regard to particular PLFAs under different rolling treatments were observed.

CONCLUSIONS

PLFAs are widely accepted as biomarkers that indicate viable components of soil microbial biomass and can provide a microbial community "fingerprint" (Liang et al., 2008). PLFA analysis can provide more detailed information of the "active" soil microbial community compared with traditional culture methods (Vestal and White 1989; Yao et al., 2000; Liang et al., 2008). Unlike nucleic acid profiling where there is high species specificity, PLFAs largely profile functional groups.

Rolled treatments resulted in significantly higher relative abundance of particular PLFAs across a range of fatty acid indicators. With most PLFAs being statistically similar among treatments, increases in the saturated fatty acids 9:0, 12:0, 14:0, 15:0, 18:0, and 20:0 in rolled treatments suggests a potential increase in soil bacteria, as these are all listed as being common bacterial indicators. Additionally, increases in the monounsaturated fatty acid 18:1ω7c and the cyclopropane fatty acid 17:0cy, both listed as being common in gram-negative bacteria, suggest further increases in bacterial abundance. However, care should be taken when connecting individual PLFAs to

specific microbial groups; it is, perhaps, better to look at the trends in groups of fatty acids.

Combining PLFAs into common taxonomic indicator groups such as gram-negative bacteria, gram-positive bacteria, total fungi and total bacteria allows for a more representative analysis as opposed to individual fatty acid comparisons. There was evidence of an increased relative abundance of total bacterial PLFAs in rolled treatments when compared to the not rolled control. This general trend towards higher bacterial proportions could be due to the aforementioned increase in volumetric water content in rolled plots (see chapter 2 of this thesis). Low water availability can inhibit microbial activity by lowering intracellular water potential, thus reducing hydration and activity of enzymes. In solid matrices such as soil, low water content may also reduce microbial activity by restricting substrate supply (Stark and Firestone, 1995). Therefore, higher soil water contents could be contributing to higher bacterial proportions and activities.

Fungal PLFAs were not significantly different among treatments, indicating consistent PLFA extraction among samples and, therefore, low sampling error. Fungi are filamentous, enabling them to bridge air gaps between the thin water films that occur in soil pore spaces under conditions of soil desiccation (Killham, 1994). It would follow then that, fungi would be expected to better withstand lower soil water contents compared to bacteria, thus possibly contributing to the similarities in fungal relative abundance among treatments with different volumetric water contents under different rolling treatments. Additionally, dollar spot incidence has been found to be more severe in soils exhibiting lower moisture contents (Couch and Bloom, 1960; Liu et al., 1995), adding to

the notion that fungal pathogens, such as *R. floccosum,* are better suited to dehydrated conditions than bacteria.

Table 2.03 includes bacterial organisms found to have biological control qualities on turfgrass pathogens. While extensive, this table is only a small portion of bacterial species isolated from soil with biological control capabilities on plant patghogens. Biological control mechanisms can be thought of as a series of traits expressed synchronously, in a controlled sequence. Many non-pathogenic soil microorganisms can effectively colonize foliage as well as roots in soil and allow protection of these tissues from pathogen infection (Nelson, 1992). Of the traits most common to soil microbes, five have been consistently linked with biocontrol: 1) production of metabolites such as antibiotics or inhibitory volatiles, 2) microbial resource competition, 3) hyperparasitism, 4) induction of systemic plant resistance, 5) Rhizosphere competence (Nelson, 1998). Increased volumetric water contents in turfgrass root zones continually rolled throughout the season may be contributing to a favorable environment which is more suited for bacterial populations than dryer soils. These bacteria may, perhaps, be having profound effects on dollar spot incidence by inhibiting the growth and proliferation of *R. floccosum* in the turfgrass root zone via the aforementioned mechanisms. The mechanisms discussed above are all subject to further investigation and discussion with regard to dollar spot reductions and bacterial populations.

Preliminary research has indicated possible increases in bacterial relative abundance in turfgrass root zones subjected to continual season long rolling regimens. While this groundwork has elucidated interesting trends related to disease reductions,

Table 2.03. Bacterial organisms with antagonistic effects against common turfgrass pathogens.

Organism	Disease Controlled	Mechanism	References
Pseudomonas aureofaciens	Dollar spot, leaf spot, summer patch	Antibiotic production	Powell et al. 2000; Vargas, 1999
Pseudomonas fluorescens	Dollar spot, leaf spot, take-all patch	Antibiotic production	Austin et al., 1977; Baldwin et al., 1991; Hodges et al., 1994; Rodriguez et al., 1997
Pseudomonas aeruginosa	Dollar spot, gray leaf spot, brown patch	PGPR	Viji et al., 2000
Pseudomonas lambergii	Dollar spot, leaf spot	Antagonism	Hodges et al., 1994
Pseudomonas putida	Take all patch	Antagonism	Wong and Baker, 1981; Wong and Baker, 1984; Wong and Baker, 1985
Unidentified *Pseudomonas spp.*	Pythium blight	General suppression	Nelson and Craft, 1992
Enterobacter cloacae	Dollar spot, pythium blight, summer patch	Direct inhibition	Nelson and Craft, 1991; Nelson and Craft, 1992; Thompson et al., 1996
Xanthomonas maltophilia	Summer patch, spring leaf spot	Antagonism	Kobayashi and El-Barrad, 1996; Kobayashi et al., 1995; Zhang and Yuen, 1999
Xanthomonas campestris	Drechslera leaf blight	Antagonism	Austin et al., 1977
Serratia marcescens	Summer patch	Antagonism	Kobayashi et al., 1995
Bacillus spp.	Summer patch	Antagonism	Kobayashi et al., 1995
Streptomyces spp.	Dollar spot, spring leaf spot, brown patch, pythium root rot	Antagonism	Hodges et al., 1994; Reuter et al., 1991; Zhang and Yuen, 1999

much research is still required in order to draw any substantial conclusions. PLFA profiles can be used to fingerprint the structure of soil microbial communities and measure their biomass (Bossio and Scow 1998; Bossio et al., 1998). These methods are free of the distortion associated with the requirements for quantitative removal of microbes from surfaces or the selectivity associated with growth on artificial media (White 1988; Bossio & Scow 1998). PLFA measurement can provide detailed information about the structure of the active microbial community because only lipids from living organisms are measured (Vestal & White 1989). As a result, PLFA profiles can be useful in predicting and manipulating physical and chemical factors in soils to sustain long-term pathogen suppression (Chen et al., 1988).

It must be noted that microbial populations, particularly biomass and metabolic activities, in soil environments are extraordinarily dynamic, dependent on factors such as temperature, moisture, radiation, and atmosphere, and can change drastically throughout the growing season and even over the course of one day (F. Dazzo, personal communication, March 1, 2010). Population fluxes occur between areas within close proximity to one another as well. While the study area examined was maintained relatively consistently throughout (with exception to rolling treatments), many spatial discrepancies can result with regard to the abovementioned environmental factors. For this reason, relative abundance was chosen for comparative analysis in order to limit the misinterpretation of population estimates. By measuring the amount PLFAs belonging to particular taxonomic groups in relation to the total amount of PLFAs extracted, conclusions could be drawn regarding population affects due to rolling with minimal concern to variations due to sampling parameters.

Perhaps, future rolling studies can implement multiple soil sampling dates for PLFA analysis during a growing season. Additionally, incorporating molecular-based techniques by which community diversity and qualification can be elucidated should be considered. Identifying key microorganisms associated with cultural practices such as rolling and microbial disease suppression is crucial in understanding the intricacies in disease control mechanisms. This initial study is meant to serve as a foundation for future projects aimed at elucidating soil microbial characteristics linked to turfgrass disease suppression.

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CHAPTER THREE

DOLLAR SPOT REDUCTION THROUGH LIGHTWEIGHT ROLLING ON CREEPING BENTGRASS PUTTING GREENS

ABSTRACT

Hypovirulent and virulent isolates of *Rutstroemia floccosum* were compared for mycelial growth, oxalic acid and cell wall degrading enzyme production. Virulence of isolates VCG-B, VCG-C, and CS was confirmed while a known hypovirulent strain, Sh12B, was unable to cause disease symptoms. Isolates were grown for 14 days on potato dextrose agar (PDA) amended with the acid indicator dye bromophenol blue. All isolates were capable of changing the media from purple to yellow indicating acid production, with VCG-B showing statistically less of a color change than the three other isolates. Oxalic acid (OA) concentrations were measured via HPLC in culture filtrates grown in potato dextrose broth (PDB) for 15 days. Virulent isolates produced significantly higher amounts of OA than isolate Sh12B. The cell wall degrading enzymes cellulase and pectin methylesterase were assayed in culture filtrates grown on isolated creeping bentgrass cell walls. All isolates exhibited pectin methylesterase activity, with isolate Sh12B displaying the highest activity relative to mycelia dry weight. Cellulase activity was highest in isolate Sh12B as well relative to mycelia dry weight. Mycelial growth of the hypovirulent isolate was less than the virulent isolates in all experiments; however enzyme activity and oxalic acid production remained steady. These results suggest that oxalic acid and extracellular enzymes are produced by hypovirulent strains of *R. floccosum*, indicating that virulence may not be solely determined by the production of these particular compounds.

INTRODCTION

Association between oxalic acid (OA) production and pathogenicity has been reported for a number of phytopathogenic fungi (Bateman and Beer, 1965; Kritzman et al., 1977; Magro et al., 1984; Marciano et al., 1983; Godoy et al., 1990). Extensive research has documented the importance of OA production, particularly by *Sclerotinia* spp. as a factor in pathogenesis. However, the role of additional factors such as extracellular enzymes remains debated (Callahan and Rowe, 1991). Mutants of *Sclerotinia sclerotiorum* that are deficient in the ability to synthesize OA are non-pathogenic, whereas revertant strains that regain their OA biosynthetic capacity exhibit normal virulence (Godoy et al., 1990.)

Mechanisms by which OA secretion might enhance virulence of certain pathogens have long been investigated. Oxalic acid appears to be involved in pathogenesis by lowering pH in advance of infected tissues to enhance the activity of extracellular enzymes produced by the pathogen (Hodgkinson 1977; Dutton and Evans 1996). Other theories center around OA being directly toxic to the plant, thus weakening the plant, facilitating further invasion (Noyes and Hancock, 1981). This occurs by the chelation of cell wall Ca^{2+} by the oxalate anion which is thought to compromise the function of Ca^{2+}-dependent defense responses (Bateman and Beer, 1965).

Variation in production of OA in liquid medium by hypovirulent and virulent strains of *Cryphonectria parasitica* (Murr.) Barr has been reported (McCarroll, 1978). Virulent strains produced twice as much calcium oxalate as hypovirulent strains. Hypovirulence is a phenotype associated with reduced virulence in fungal plant pathogens, and has been associated with several mechanisms of action (Bharathan and

Tavantzis, 1990; Boland, 1992; Elliston, 1982; Zhou and Boland, 1997). The dollar spot pathogen *Rutstroemia floccosum* (Syn. *Sclerotinia homoeocarpa*) produces oxalic acid in vitro, though to a much lesser extent than other *Sclerotinia* members such as *S. sclerotiorum* and *S. trifoliorum* (Beaulieu, 2008). Very little is known regarding the pathogenesis of *R. floccosum*, and whether or not OA is an important factor related to turfgrass infection. Understanding the mechanisms of action by which hypovirulence influences the fungal pathogen with regard to OA and extracellular enzyme production, may give valuable insight into the role that oxalic acid plays in the infection process. Elucidating the role that OA plays in *R. floccosum* pathogenicity, allows for investigations into novel methods of dollar spot control related to plant defense mechanisms. For instance, the expression of oxalate oxidase in transgenic plants has been shown to be successful in conveying resistance to a diverse array of pathogens particularly those that produce OA (Thompson et al. 1995; Liang et al., 2001; Hu et al., 2003). Since many pathogens produce OA as a toxin (as mentioned above), and it is thought to play a major role in pathogenesis, the overall breakdown of OA by oxalate oxidase may be detrimental to the pathogen, as well as beneficial to the plant with the generation of H_2O_2 possibly playing a role in defense signaling.

Within the context of this thesis, different mechanisms related to dollar spot reduction as a result of lightweight rolling were investigated. One hypothesis not discussed in these chapters centered on the idea that rolling simulates a wounding effect in turfgrass plants, which subsequently induces an enzymatic response. Two enzymes of interest (oxalate oxidase, and peroxidase) were assayed for in grass tissues from various rolling treatments (data not shown). Unsuccessful attempts investigating plant oxalate

oxidase activity led to an assessment of pathgoenicity factors in the dollar spot pathogen *R. floccosum*. The purpose of this study, therefore, was to examine *in vitro* oxalate production by virulent and hypovirulent strains of the fungus and to clarify the effect of hypovirulent agents on OA and enzyme production. The goal of this research was to examine whether oxalic acid and extracellular secreted cell wall degrading enzymes are important in the infection of creeping bentgrass (*Agrostis palustris*) by *R. floccosum*.

MATERIALS AND METHODS

Fungal Isolates.

Isolates of *R. floccosum* used in this study are summarized in Table 3.01. All isolates were grown on potato dextrose agar (PDA) (Becton Dickinson, Sparks, MD), and subcultured onto fresh PDA plates using colonized agar plugs (5 mm diam.) 7-10 days prior to each test. The hypovirulent strain Sh12B, was obtained from Dr. Greg Boland at the University of Guelph, ON, Canada. The presence of dsRNA and hypovirulent characteristics in strain Sh12B is discussed in Zhou and Boland (1996).

Table 3.01. *Rutstroemia floccosum* isolates studied and their source of obtainment.

Isolate	Source
Vegetative compatibility strain B (VCG-B)	Dr. Joseph M. Vargas Jr., Michigan State University. East Lansing, MI
Vegetative compatibility strain C (VCG-C)	Dr. Joseph M. Vargas Jr., Michigan State University. East Lansing, MI
Common Strain (CS)	Dr. Joseph M. Vargas Jr., Michigan State University. East Lansing, MI
Hypovirulent *Sclerotinia homoeocarpa* (Sh12B)	Dr. Greg J. Boland, University of Guelph. Guelph, Ontario

Isolate Virulence Testing

Virulence for each isolate was tested under greenhouse conditions by inoculating 9 cm diameter cups of 10 wk old creeping bentgrass (*Agrostis palustris* cv. Crenshaw) with 6 mm colonized agar plugs of actively growing *R. floccosum* isolates. Each virulence trial was replicated four times for each of the four isolates tested. Inoculated plants were kept in enclosed chambers surrounded by plastic in a greenhouse maintained at approximately 26-28 C with an approximate 13 hr photoperiod in order to encourage humidity; cups were arranged in a completely randomized design.

Disease ratings were taken by measuring the advancing fungal mycelium and subsequent infected area on each plant on a daily basis. Advancing mycelium radiated from the center inoculation point, causing subsequent infection of turfgrass in a circular pattern. Infected turfgrass was rated by measuring the area of bleached, water soaked patches on the inoculated cups. Mean disease measurements were used for statistical comparisons among dollar spot isolates.

Oxalic Acid production on Bromophenol Blue amended Potato Dextrose Agar.

In order to compare different strains of *R. flocossum* and their ability to produce acid when grown in vitro, a slightly revised method from Steadman et al. (1994) was used. Fungal isolates growing on potato dextrose (PDA) agar for 10 days were transferred to PDA amended with 0.05g/l bromophenol blue (BB) adjusted to pH 6.0 with 1M NaOH. Bromophenol blue is an indicator of acid production due to its rapid and visible response indicated by a characteristic yellow color change when pH is below 3. Approximately 20 ml of BB amended PDA (BBPDA) was aseptically pipetted into 100 x 15 mm polystyrene Petri-plates. One plug (5mm) of agar containing each isolate was

inoculated onto the center of a BBPDA plate and placed under fluorescent light (photoperiod of 12 h) at room temperature (20-22 C), and observed for oxalic acid production for 14 days. Each strain grown on BBPDA was replicated 4 times and the experiment was repeated twice. Measurement of fungal radial growth was taken daily, along with color change measurements taken visually against a 5000K fluorescent light Porta-Trace (Gagne Inc.). Color change ratings were given on a scale of 0-5 with 0 indicating no color change and 5 indicating a complete yellowing of the plate. Mean color change and fungal radial growth measurements were used for statistical comparisons.

Growth and acid production in potato dextrose broth medium

Mycelial growth and production of oxalic acid were evaluated by culturing individual isolates in potato-dextrose broth (PDB). One 5 mm diameter agar plug from actively growing margins of 7-day-old colonies was transferred to 50 ml liquid medium in 125-ml Erlenmeyer flasks. Inoculated flasks were incubated on a rotary shaker at 100 rpm at 20-22 C.

Mycelial dry weights were determined by vacuum filtration of culture contents through a layer of pre-weighed Whatman No.1 filter paper in order to separate mycelium from culture filtrates. Dry weight of mycelium was determined after filter paper with mycelium was dried in a 80 C oven for 72 h.

Approximately 3 ml of culture filtrate from each treatment was collected and measured for pH on five different sampling days, collected samples were maintained at -20 C until analysis. Concentrations of oxalic acid in PDB filtrates were determined after 15 days of growth in PDB. Extracts were analyzed on a Waters XBridge C18 3.5 um

column (Waters Corp., Milford, MA) high pressure liquid chromatograph (HPLC)/ mass selective detector (MSD) for oxalic acid. The column diameter and length were 3/0x50 mm, and the system was run at ambient temperature. The mobile phase was run at 0.25 ml/min which started at 80 % 0.1 % formic acid (A) and 20 % 0.1 % formic acid in acetonitrile (B) and was held there for one minute. A gradient began moving to 10 % A at four minutes. This was held for 5 minutes and then back to original flow. The MSD was monitoring ions 46 and 45 daltons in electrospray positive mode. 10µl from each sample was used in analysis, and values are reported as µg oxalic acid ml sample^{-1}.

Growth and production of extracellular enzymes on isolated creeping bentgrass cell walls.

In order to investigate the fungal secretion of cell wall degrading enzymes by *R. floccosum*, cell walls from creeping bentgrass (*Agrostis palustris* cv. 'Crenshaw') were isolated and used as the sole carbon source in a minimal growing medium for *R. floccosum* isolate inoculation.

Cell walls were prepared according to English et al., (1971) by grinding the frozen tissue to a fine powder in liquid nitrogen with the aid of a mortar and pestle. This frozen powder was then ground in approximately 2.5 volumes (v/w) of cold 100 mM postassium phosphate buffer, pH 7.0. The insoluble material was collected on Whatman No. 1 filter paper by suction filtration. The residue was resuspended in 1 volume of cold buffer, before re-filtration. This washing procedure was repeated four times, using 1 volume of buffer each time. Buffer washes were followed by one wash with 1 volume of cold distilled water to remove salts. The residue was then suspended in 2.5 volumes of a cold mixture of chloroform and methanol (1:1 v/v) and ground with a mortar and pestle.

The insoluble material was collected on Whatman No. 1 filter paper, washed three times with 1 volume of the chloroform-methanol mixture at room temperature, then washed three times with 1 volume of acetone at room temperature. The residue remaining after the acetone extraction, which constitutes the cell walls used in this study, was air dried and stored at room temperature. Immediately preceding use, the cell walls were placed in a dessicator to remove residual water.

The medium for liquid culture was prepared according to a modified protocol from Anderson (1978). In one liter the medium contained 0.25g K_2HPO_4, 0.25 g NH_4NO_3, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.15 ml 2% FeCl. In 125 ml Erlenmeyer flasks 50 ml of medium was added along with 0.5 g of cell wall material, flasks were then autoclaved at 121 C for 20 min.

Cell wall amended media flasks were inoculated with one colonized agar plug (5 mm diam.) from 7-10 day old isolates growing on PDA. Inoculated flasks were incubated on a rotary shaker at 100 rpm at 20-22 C for 14 days. Filtrates were obtained from the liquid cultures by passage of the suspensions through Whatman No. 1 filter paper via suction filtration. Mycelia was weighed after drying at 80 C for 72 h. Isolates were replicated four times, and liquid filtrates were stored at -20 C until enzyme assays were performed.

Enzyme assays were performed on culture filtrates for cellulase and pectin methylesterase activities following modified procedures described in Downie et al. (1998) Hagerman et al. (1985) and Taylor and Secor (1988). Pectin-containing medium was prepared by mixing 0.1% (w/v) pectin, and 1% (w/v) Type II agarose (Sigma Chemical Co., St. Louis, MO) in 0.2 M phosphate buffer adjusted to pH 5.3. The mixture

was dissolved by heating to a boil while stirring. 18 ml of the hot mixture was aseptically dispensed into 100 X 150-mm sterile petri dishes using a sterile 25 ml pipette.

Carboxymethylcellulose (CMC) containing medium was prepared using the same procedure but substituting 0.1% CMC for the pectin. Once agar plates cooled, a cork borer was used to punch 3 holes, 5 mm in diameter and approximately 1.7 cm apart, in the solidified medium. The holes were arranged in three rows, in a 3 x 3 x 3 pattern and the wells were filled with (35 µl) with standard, control, or filtrate solutions. The assay was incubated at 28 C for 17 hours.

Pectin containing gels were developed after incubation by flooding the assay plate with 10 ml of 0.05% (w/v) ruthenium red (Sigma Chemical Co.) in water for 30 min at 25 C. Excess dye was removed by washing the plate several dimes with deionized water. The diameters of the resulting rings or halos of activity were measured against a 5000K fluorescent light Porta-Trace (Gagne Inc., Johnson City, NY).

The cellulose containing plates were developed in a similar fashion using 1% (w/v) Congo red (Sigma Chemical Co.) in water to stain the plates. The stain was removed after 15 min and plates were de-stained for 15 min with 1 M NaCl in 0.2 M phosphate buffer. Enzyme activity was assessed by measuring ring diameter similar to the pectin containing plates, and values were standardized by dividing halo diameter by mycelia dry weight from each isolate. Assays for each isolate were replicated three times, and experiments were repeated twice. Mean ring diameters were used for statistical comparisons among isolates using the PROC GLM procedure in SAS.

RESULTS AND DISCUSSION

Virulence.

Isolates VCG-B, VCG-C, and CS initiated dollar spot symptoms on cups of creeping bentrgrass ranging from 7.10 to 8.00 cm in diameter after 16 days of incubation (Table 3.02). Some differences existed among these isolates with VCG-C displaying significantly higher virulence ratings than the other isolates at 6, 8, 11, 13, and 16 days after inoculation (DAI). Isolate Sh12B failed to cause disease symptoms, and resulted in no significant difference from the control at any DAI. These results confirmed the hypovirulence phenotype of the strain Sh12B. Mean virulence measurements resulted in no statistical differences among isolates VCG-B, VCG-C, and CS. However, all three of the aforementioned isolates resulted in statistically higher virulence ratings ($P < 0.05$) than the control and isolate Sh12B; neither isolate resulted in any disease observation.

Table 3.02. Disease ratings of *R. floccosum* isolates on swards of creeping bentgrass (*Agrostis palustris* cv. Crenshaw)*.

	Days After Inoculation Disease Rating (cm)						
Isolate	5†	6	8	11	13	16	**Mean**
VCG-B	1.07 a	1.17 b	2.90 b	4.03 b	5.02 b	7.10 b	3.55 a
VCG-C	1.27 a	1.43 a	4.70 a	5.37 a	6.37 a	8.00 a	4.52 a
CS	1.07 a	1.17 b	3.40 b	4.17 b	4.70 b	7.43 b	3.67 a
Sh12B	0.00 b	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 b
Control	0.00 b	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 b

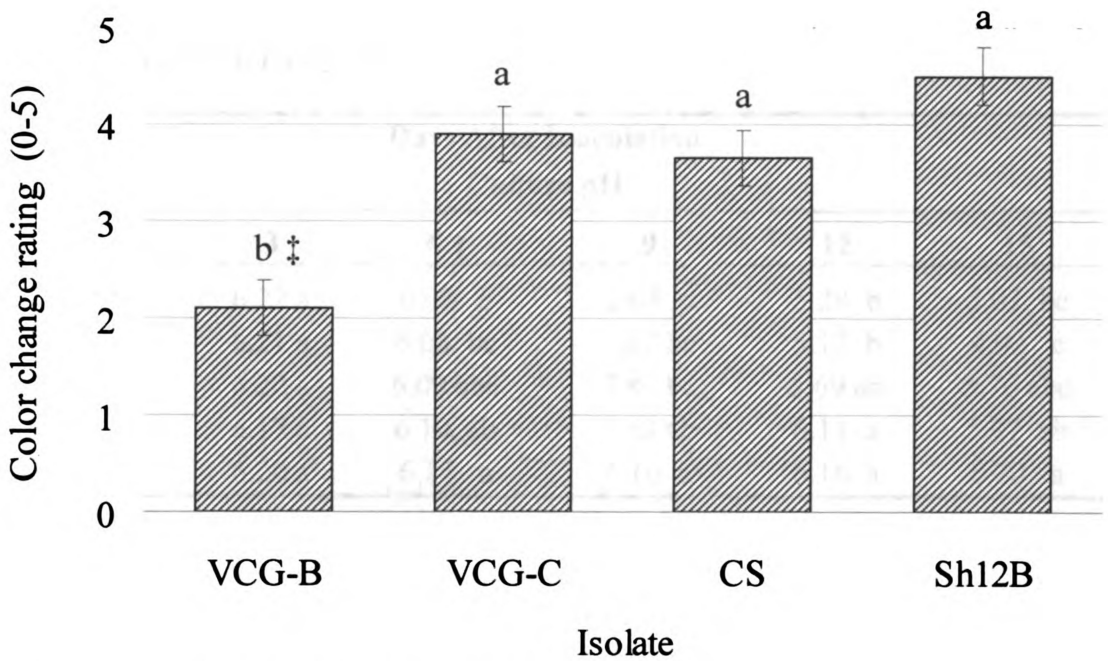
* Means are average disease ratings from four replications.
† Means followed by different letters are significantly different according to Fisher's LSD ($P < 0.05$).

Oxalic acid production on bromophenol blue amended PDA.

Acid production in vitro was measured by observing the characteristic color change of PDA amended with the acid indicator dye bromophenol blue. All isolates

displayed some degree of acid production, with evident color changing of the media from purple to yellow. Figure 3.01 reports mean color change ratings for the four dollar spot isolates tested after 15 days of growth on BBPDA. Isolate VCG-B resulted in significantly lower color change ratings after 15 days of growth compared to all other isolates tested ($P < 0.05$).

Figure 3.01. Mean* media color change ratings† from acid production of four *R. floccosum* isolates grown on bromophenol blue-amended potato dextrose agar for 15 days.



* Values are means of 8 replications from two separate assays.
† Color change ratings were given on a scale from 0-5 with 0 indicating no color change, and 5 indicating a uniform change of color in the media from purple to yellow.
‡ Means followed by different letters indicate a significant difference according to Fisher's LSD ($P < 0.05$). Error bars represent the standard error (SE) of the mean

Growth and acid production in PDB

Oxalic acid production was measured in vitro by growing dollar spot isolates in potato dextrose broth. Culture pH was measured throughout the duration of the experiment every three days. Table 3.03 gives mean culture pH measurements. All isolates displayed similar culture pH after 3 days of growth, however after 6 days, VCG-B had significantly lower pH than both the control and isolate Sh12B. After 15 days of growth, both VCG-B and VCG-C cultures were significantly lower in pH than the control; while VCG-C was significantly lower than isolate Sh12B as well ($P < 0.05$).

Table 3.03. Mean culture pH measurements* of four *R. floccosum* isolates growing in potato dextrose broth for 15 days.

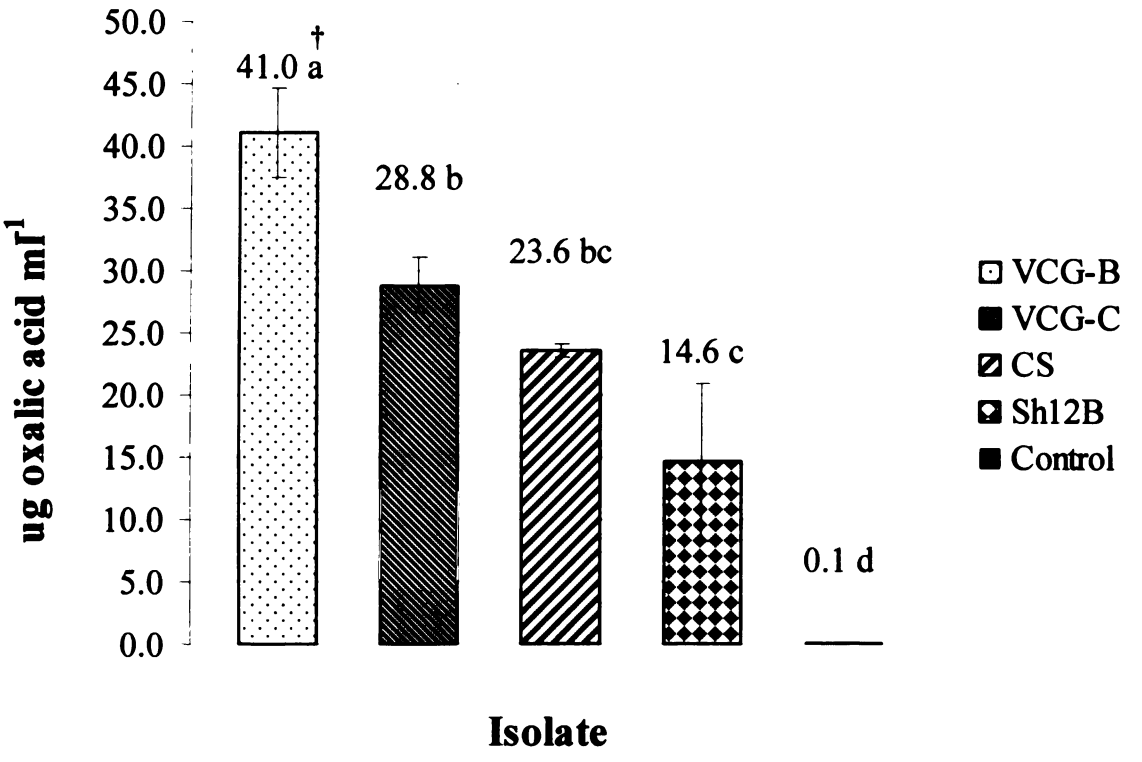
	Days After Inoculation Culture pH				
Isolate	**3**	**6 †**	**9**	**12**	**15**
VCG-B	6.22 a	6.02 c	5.64 c	5.29 b	5.07 bc
VCG-C	6.21 a	6.03 bc	5.67 bc	5.17 b	4.92 c
CS	6.22 a	6.09 abc	5.81 bc	5.69 ab	5.76 abc
Sh12B	6.17 a	6.10 ab	5.93 ab	6.11 a	5.97 ab
Control	6.20 a	6.15 a	6.16 a	6.16 a	6.20 a

* Each pH measurement represents the mean of four replications.
† Means followed by different letters indicate a significant difference in pH according to Fisher's LSD ($P < 0.05$).

Oxalic acid levels were measured in culture filtrates after 15 days of growth. All isolates displayed some level of OA production in liquid broth media. VCG-B displayed the highest levels followed by the other virulent isolates VCG-C and CS (Figure 3.02). The hypovirulent isolate Sh12B displayed variable levels of oxalic acid production

among replications, yet was able to produce OA at levels significantly lower than the other isolates (Figure 3.02). When OA production was standardized to the dry weight of fungal mycelia in each flask, hypovirulent isolate Sh12B produced significantly higher levels of OA than VCG-B (figure 3.03). While not statistically significant, Sh12B had higher OA production relative to mycelial growth than isolates VCG-C and CS as well (figure 3.03).

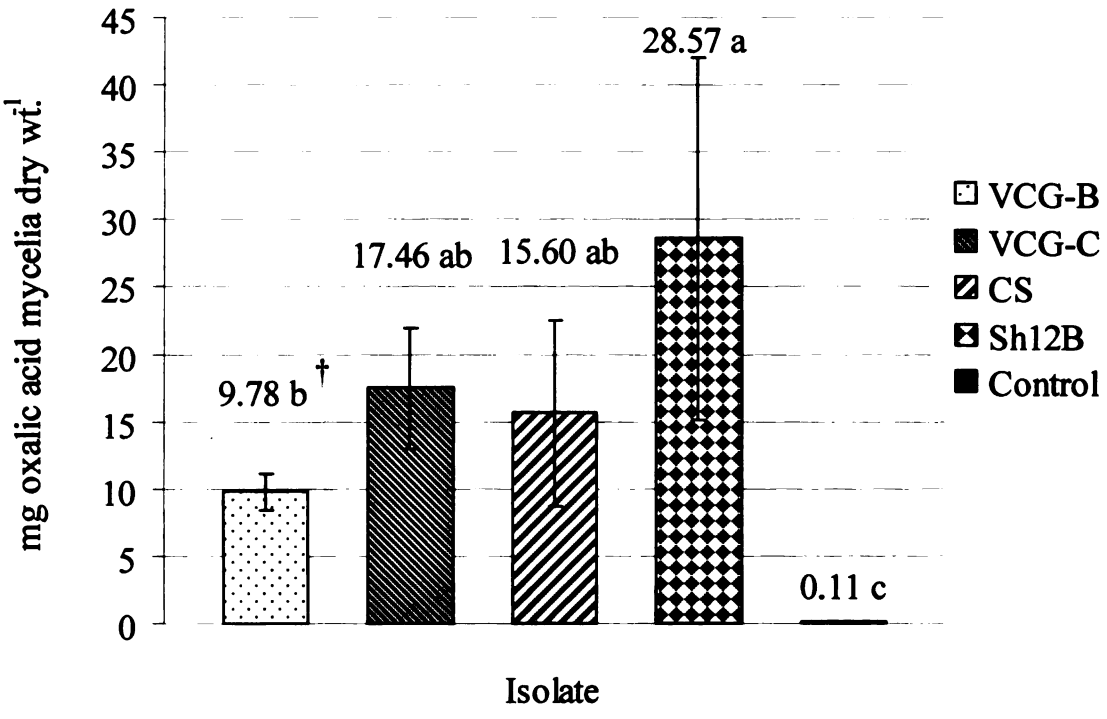
Figure 3.02. Oxalic acid production* of *R. floccosum* isolates grown in potato dextrose broth for 15 days.



* Means represent the average of 4 replications

† Means followed by different letters are significantly different according to Fisher's LSD ($P < 0.05$). Error bars represent the standard error (SE) of the mean.

Figure 3.03. Oxalic acid production related to mycelial dry weight* of *R. floccosum* isolates grown in potato dextrose broth for 15 days.

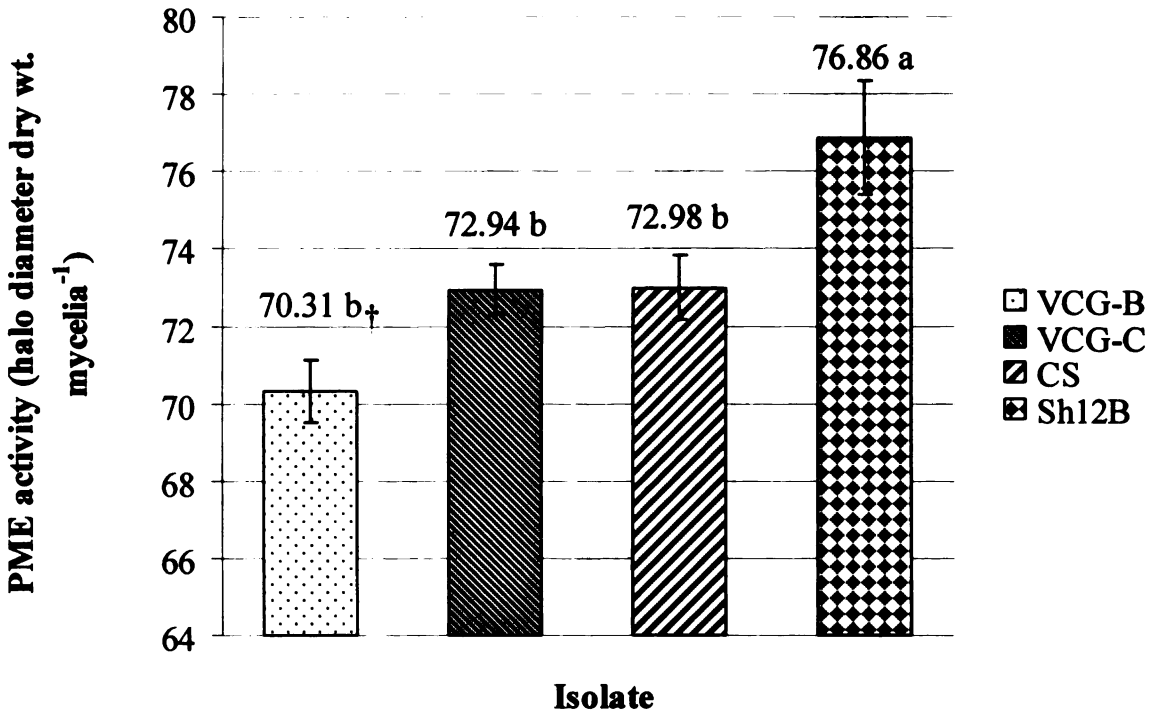


* Means represent the average of 4 replications
† Means followed by different letters are significantly different according to Fisher's LSD ($P < 0.05$). Error bars represent the standard error (SE) of the mean.

Growth and production of extracellular enzymes on isolated creeping bentgrass cell walls.

Isolates were assayed for their ability to produce pectolytic and cellulolytic enzymes *in vitro* and were compared. *In vitro* activities of pectin methylesterases (PME) and cellulase from filtrates of 14 day old developing hyphae growing on creeping bentgrass cell walls are reported in figures 3.04 and 3.05. Levels of PME and cellulase activity were adjusted with respect to dry weight of mycelia from cultures.

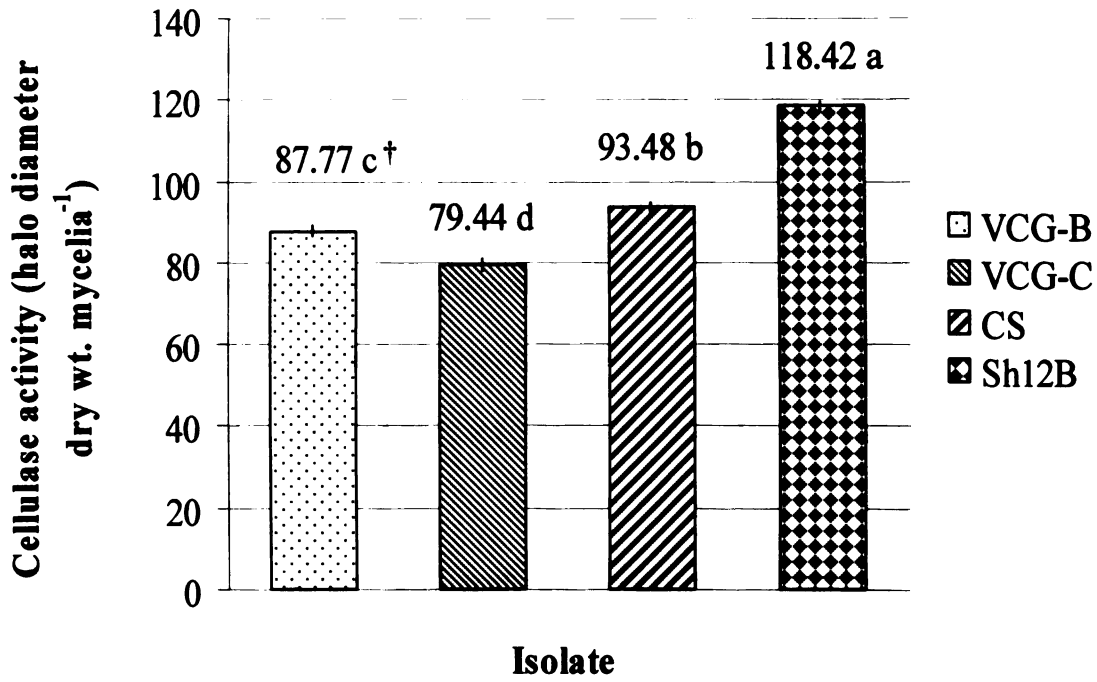
Figure 3.04. Pectin methylesterase activity* of four *R. floccosum* isolates grown in liquid culture with creeping bentgrass (*Agrostis palustris*) cell walls.



* Means are the average of 12 measurements from 4 replications.
† Means followed by different letters are significantly different according to Fisher's LSD ($P < 0.05$). Error bars represent the standard error (SE) of the mean.

When PME levels were adjusted with respect to mycelia dry weight, isolate Sh12B exhibited significantly greater activity than the other three isolates ($P < 0.05$). For cellulase activity, adjusted means showed Sh12B to have significantly higher enzyme activity than all other strains with respect to mycelium production ($P < 0.05$), while VCG-C had the lowest cellulase activity among all isolates.

Figure 3.05. Cellulase activity* of four *R. floccosum* isolates grown in liquid culture with creeping bentgrass (*Agrostis palustris*) cell walls.



* Means are the average of 12 measurements from 4 replications.
† Means followed by different letters are significantly different according to Fisher's LSD ($P < 0.05$). Error bars represent the standard error (SE) of the mean.

CONCLUSIONS

In order to investigate whether oxalic acid plays a crucial role in pathogen virulence, four isolates of *R. floccosum* were subjected to multiple assays related to OA production and extracellular enzyme activity. The isolate Sh12B, a known hypovirulent strain of *R. floccosum,* was evaluated and compared to three other known virulent isolates.

Virulence testing confirmed prior findings by Zhou and Boland (1997) that isolate Sh12B is indeed unable to maintain virulence on swards of creeping bentgrass. All other isolates displayed typical dollar spot disease symptoms on inoculated creeping bentgrass with bleached, circular spots, growing outward from the inoculation point, containing evident fluffy mycelium near the infected margins. Other studies involving hypovirulence and the production of OA have shown hypovirulent fungal strains exhibiting reduced or delayed accumulation of OA. They hypovirulence phenotype in isolate Sh12B *of R. floccosum* was previously associated with a reduced growth rate, atypical colony morphology, and reduced virulence, as well as the presence of double stranded RNA.

If pathogenesis is associated with the production of OA and/or extracellular enzymes as proposed by many authors in the past (Hancock, 1967; Lumsden, 1969, 1976, 1979; Hancock, 1967; Marciano et al., 1983; Noyes and Hancock, 1981), one could hypothesize that the hypovirulent isolate Sh12B should be deficient in both acid and cell wall degrading enzyme production.

Contrary to the initial hypothesis, isolate Sh12B displayed marked acid production when grown on BBPDA plates, displaying a high degree of color change in the indicator medium. Cultures of Sh12B tended to grow at about half the rate of the other virulent isolates, however, after 15 days of growth, the hypovirulent isolate Sh12B exhibited a higher degree of color change compared to VCG-B, and similar color change ratings to isolates VCG-C and CS. This initial examination of acid production in vitro indicated a possible delayed, yet sufficient production of acid by the hypovirulent strain when compared to the other *R. floccosum* isolates. The virulent isolate VCG-B displayed

significantly less color change ratings in the medium, indicating that overall acid production in vitro may not be directly associated with virulence on creeping bentgrass.

In order to confirm the production of OA, and make comparisons among fungal isolates culture filtrates OA levels were measured via HPLC/MS. The results confirmed the increased production of OA by virulent isolates compared to the hypovirulent counterpart. These results coincide with Callahan and Rowe (1991) and Zhou and Boland (1999) in that reduced or delayed accumulation of oxalic acid in hypovirulent isolates appears to be associated with reduced disease severity. However, the production of OA way relative to mycelial production was significantly higher in the hypovirulent isolate, indicating marked acid production even when fungal growth is at a minium. With these results, oxalic acid does not appear to be a sole pathogenic determinant in *R. floccosum*. *In vivo* studies should be done as well as the investigation into oxalic acid deficient mutants in *R. floccosum* to truly identify the importance of OA in pathogenesis.

Isolate Sh12B produced degradative enzymes (PME and cellulase) at levels equal to, or higher than the virulent strains evaluated. These hydrolytic enzymes have been associated with infection of tissue by *Sclerotinia* spp. (Hancock, 1966; Lumsden, 1979). However, these results coincide with Godoy et al. (1990), who found that non-pathogenic mutants of *Sclerotinia sclerotiorum* produced equal or greater amounts of pectolytic and cellulolytic enzymes when compared to wild type isolates. This could be an indication that factors other than pectolytic or cellulolytic enzymes are factors in the virulence of *R. floccosum*. *In vivo* studies involving extracellular cell wall degrading enzymes should be conducted in order to draw further conclusions regarding their importance in *R. floccosum* virulence and pathogenesis. This initial study set out to investigate differences

among virulent and non-virulent (hypovirulent) strains, and whether oxalic acid and/or degradative hydrolytic enzymes were essential in host/pathogen interactions. Isolate Sh12B may retain mechanisms necessary for the production of these enzymes for saprophytic survival purposes. If the isolate is to survive in nature by the acquisition of nutrients via other organisms, these degradative enzymes most likely are needed in the breakdown of detritus.

Oxalic acid is thought to play a major role in the pathogenesis of many plant pathogens, but the importance of OA production by the dollar spot pathogen *R. floccosum* has yet to be defined. Investigations into the importance of OA in pathogen virulence and infection could potentially lead to novel methods of disease control or reduction. The previously mentioned breakdown of OA via plant derived oxalate oxidase has been characterized as an option in transgenic plant production (Thompson et al. 1995; Liang et al., 2001; Hu et al., 2003). This same enzyme has been shown to be induced in certain grass species after wounding, and when subjected to heavy metal and temperature stress (Valentovicova et al., 2009; Le Deunff et al., 2004).

Prior chapters in this thesis pertained to the reduction of dollar spot disease through lightweight rolling of creeping bentgrass. A proposed means in disease suppression could be related to oxalate oxidase activity increasing in response to the rolling treatment. Initial research investigating this hypothesis proved sporadic and inconclusive (Appendix). However, without a basic knowledge of the role that oxalic acid plays in *R. floccosum* pathogenesis, it is difficult to make presumptions on whether OA degradation by oxalate oxidase could be having part in dollar spot disease suppression. The hypovirulent strain Sh12B which was unable to produce disease

symptoms on swards of creeping bentgrass, still exhibited marked acid and cell wall degrading enzyme production *in vitro*. These findings suggest that while oxalic acid and extracellular enzyme production may be contributing factors in *R. floccosum* pathogenesis, alone or in tandem, their production does not necessarily translate to disease development. It is likely that oxalic acid plays a diminutive role in the pathogenicity and disease development of *R. floccosum*, however, *in vivo* studies on the production of OA and potential cell wall degrading enzymes should be done.

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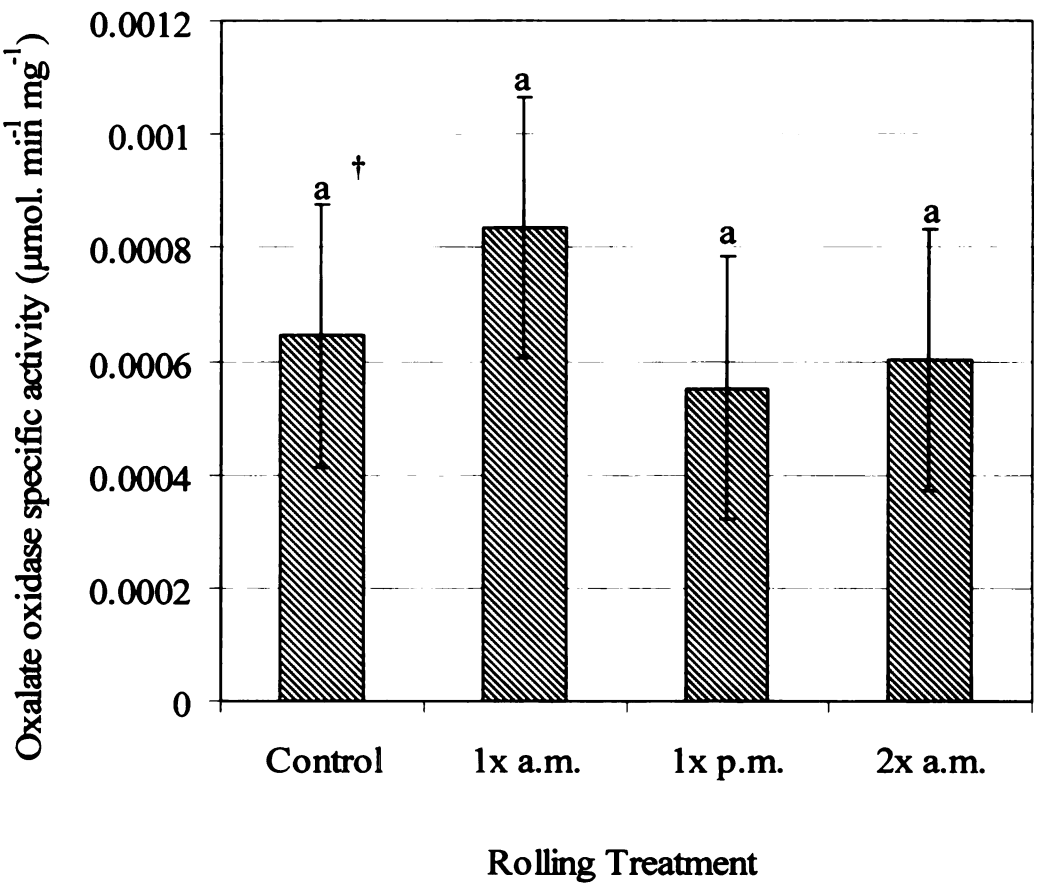
APPENDIX

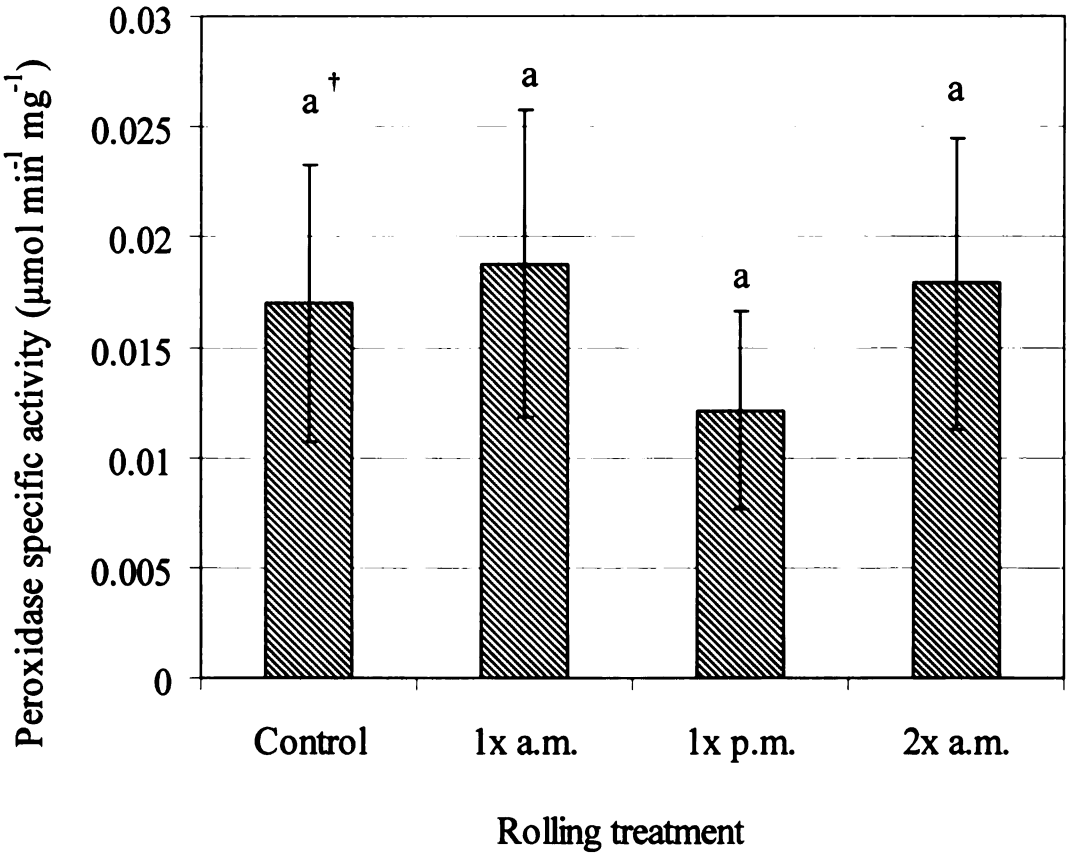
Figure A.1. Oxalate oxidase specific activity* in creeping bentgrass tissue from different rolling treatments.



*Means represent the average of 6 different assays performed on tissue throughout the growing seasons of 2008 and 2009

† Means followed by the same letter are statistically similar according to Fischer's LSD. Bars represent the standard error of the mean.

Figure A.2. Peroxidase specific activity* in creeping bentgrass tissue from different rolling treatments.



* Means represent the average of 4 different assays performed on tissue throughout the growing seasons of 2008 and 2009

† Means followed by the same letter are statistically similar according to Fischer's LSD. Bars represent the standard error of the mean.