IDENTIFICATION AND CHARACTERIZATION OF A NEW BACTERIAL DISEASE OF CREEPING BENTGRASS (*AGROSTIS STOLONIFERA* L.) CAUSED BY *ACIDOVORAX AVENEA* SUBSP. *AVENAE*

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF A NEW BACTERIAL DISEASE OF CREEPING BENTGRASS (*AGROSTIS STOLONIFERA* L.) CAUSED BY *ACIDOVORAX AVENAE* SUBSP. *AVENAE*

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A new bacterial disease was discovered in 2009 afflicting several creeping bentgrass (CBG) putting greens on a golf course in Charlotte, North Carolina. The reoccurring symptoms caused by this previously uncharacterized disease include chlorosis, etiolation, and a general thinning and decline of irregular shaped areas of turfgrass. Affected plants exhibited heavy bacterial streaming from vascular tissues when viewed microscopically. Initial isolation, pathogenicity tests and biochemical and molecular identification assays determined the causal agent to be the Gram-negative, aerobic, non-fluorescent member of the β-proteobacteria *Acidovorax avenae* subsp. *avenae* (*Aaa*). Hosts range inoculations showed the *Aaa* to be pathogenic on all cultivars of CBG with little to no pathogenicity on other hosts tested including several species of amenity turfgrass. Solicitation of symptomatic samples from golf courses between 2010-2013 confirmed *Aaa*-associated outbreaks of etiolation and decline on more than 30 different sites in 13 states. Inoculations of *Aaa* suspensions on CBG under a range of temperatures showed significant necrosis above 25°C and severe decline between 30°-35°C with high relative humidity. Root inoculation assays confirmed systemic movement of the turfgrass pathogen and the association of creeping bentgrass etiolation with *Aaa* infection. Controlled environment experiments with oxytetracycline and streptomycin sulfate significantly reduced symptoms of bacterial decline when pretreated onto plants as foliar spray suspensions prior to inoculation with *Aaa*. Field studies with inoculations of *Aaa* induced etiolation and showed the

growth regulator trinexapac-ethyl (TE) to significantly increase symptoms when compared to an untreated control. Additionally, ammonium sulfate and the combination of TE + ammonium sulfate consistently increased disease incidence in field plots. No treatment consistently suppressed symptoms of etiolation. Genetic investigations into *Aaa* using a previously designed Multilocus sequence analysis (MLSA) scheme show turfgrass isolates forming two distinct clades among other closely related *Acidovorax* spp. Whole genome sequencing of 12 representative isolates among the two turfgrass clades and three other *Acidovorax* isolates from different plant hosts allowed for the alignment-free design of discriminatory polymerase chain reaction (PCR) primer sets from draft genome assemblies. Predicted diagnostic primer sets were validated against a panel of genomic DNA from 21 target *Aaa* isolates and 16 non-target bacterial species via traditional PCR. Two primer sets, 0017 and 0019, showed the desired specificity to turfgrass isolates with 100% amplification accuracy. Probes were designed for the primer sets for use in real-time quantitative PCR assay development. For both primer sets, TaqMan and Zen probe assays were designed. Sensitive and specific detection of target organisms was accomplished from purified genomic DNA. Confirmation of detection specificity and sensitivity was accomplished from frozen samples of affected and unaffected turfgrass tissue. This assay allows for rapid and reliable diagnosis of *Acidovorax avenae* from turfgrass samples, and sets the stage for ecological and epidemiological research related to pathogen detection and disease proliferation.

To my wife Courtney; your grace, patience, love and support never cease to amaze me.

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CHAPTER ONE

ACIDOVORAX AVENAE AND BACTERIAL DISEASES OF AMENITY TURFGRASS

INTRODUCTION

The genus *Acidovorax* contains many important bacterial plant pathogens previously classified as members of the *Pseudomonas* genus. *Acidovorax* pathogens cause disease in a wide range of economically important monocotyledonous and dicotyledonous plants, including corn, rice, watermelon, anthurium, and orchids. The original pathogenic isolate was first described in 1909 by Manns (24), which caused leaf blight of oats (*Avenae sativa* L.) and was given the name *Pseudomonas avenae*. Subsequent research found the bacterium capable of causing disease on numerous species of the Graminaceae family (Syn. Poacea) including wheat (*Triticum aestivum* L.), finger millet (*Eleusine coracana* (L.) Gaertn.), Italian millet (*Setaria italic* (L.) Beauv.), pearl millet (*Pennisetum glaucum* (L.) R. Br.), and proso millet (*Panicum miliaceum* L.) (4, 9, 28). *Acidovorax avenae* subsp. *avenae* as it is known today has become an important pathogen of both corn (*Zea mays* L.) (37) and rice (*Oriza sativa*) (14), while the close relative *A. avenae* subsp. *citrulli* (synonymous with *P. pseudoalcaligenes* subsp. *citrulli*) has more recently been recognized as a serious pathogen of watermelon (*Citrullus lanatus* (Thunb.) Matsumura and Nakai) and other cucurbits around the world (38).

SYMPTOMS AND EPIDEMIOLOGY

Acidovorax avenae subsp. *avenae* can cause a range of symptoms on different monocotyledonous hosts including foliar blight, leaf stripe, stalk rot, brown stripe, leaf spots, leaf and stem streaks and others (4, 9, 14, 20, 24, 40). The hosts of economic importance primarily

include rice, maize and sugarcane, but many hosts of secondary importance exist (10). Heavy infections often cause significant yield reduction in rice nurseries, however, moderate foliar infections on both rice and corn do not typically decrease yield substantially enough to be considered a major pathogen (29).

The most typical symptoms produced on grass hosts by *A. avenae* subsp. *avenae* consist of leaf streaks and stripes, often extending into the leaf sheaths with occasional development of stalk rot (34). Symptoms tend to be more severe on young seedlings and immature plants than on developed, mature plants (41, 46). In rice, *A. avenae* subsp. *avenae* infects the primary leaves of seedlings in nurseries producing brown stripes on sheaths which frequently extend out to the leaf blade (47). Other symptoms on rice include the inhibition of seed germination, brown stripes on leaves, curving of the leaf sheath, and an abnormal elongation of the mesocotyl (21). Death and necrosis has been observed in severely affected seedlings, and often discoloration of both seeds and plants can take place leading to an overall decrease in germination rate and seed vigor (15, 36, 42).

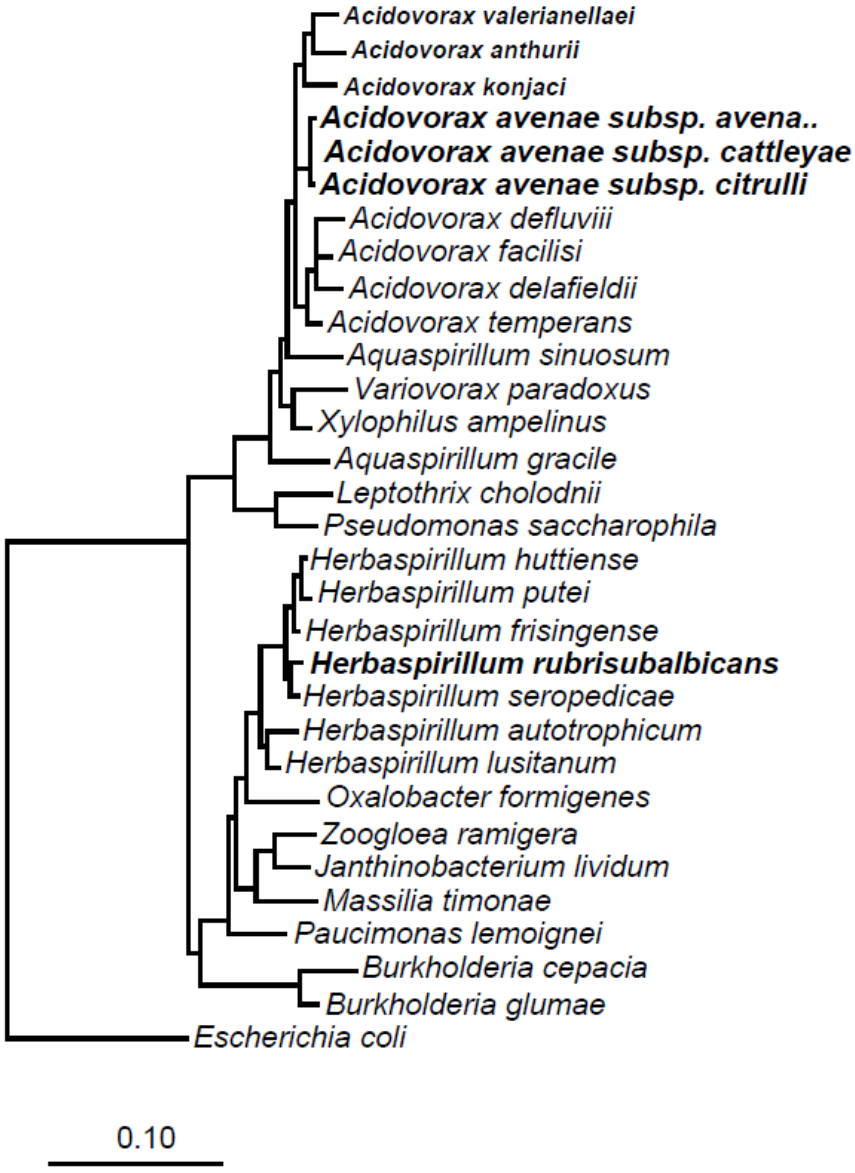
ACIDOVORAX TAXONOMY

Numerous studies over the years have resulted in the taxonomic reclassification of the *Acidovorax* genus while others have established many of its members as important, ecologically diverse members of the β-Proteobacteria. The classification of *A. avenae* previously included several subspecies, largely based on morphology and the host from which the pathogen was initially identified (14, 54, 55). More recently, Schaad et al. (2008) proposed new speciation based on the phylogenetic relatedness among plant pathogenic members of the *Acidovorax* genus. Specifically, the authors revealed "considerable" differences among subspecies of *A. avenae* that are plant pathogenic versus those that are not. The work suggested a speciation of *A.*

oryzae for rice pathogenic strains of *A. avenae* subsp. *avenae*, as well as elevating *A. avenae* subsp. *citrulli* and *A. avenae* subsp. *cattleyae* each to the species level, respectively (39). Phylogenetic analysis of the 16S rRNA sequences of *Acidovorax* members has shown the plant pathogenic strains to cluster together on one branch within the genus while other, non-plant pathogenic strains cluster together as a separate clade (Figure 1.1).

Much confusion has surrounded the taxonomic placement and proper nomenclature of *Acidovorax avenae* subsp. *avenae.* Heterogeneous strains of *A. avenae* subsp. *avenae* exist and have been confirmed by rep-PCR and DNA:DNA homology (30, 44), mostly attributed to plant hosts the individual strains were isolated from. For instance, strains of *A. avenae* subsp. *avenae* causing bacterial streak of maize have a mean overall DNA:DNA similarity of only 45% compared to strains that cause bacterial stripe of rice (30). Variation has also been found in the intergenic spacer (ITS) region of *A. avenae* subsp. *avenae* strains. The rice and maize pathogens group together based on their ITS sequences yet are sister clades to *A. avenae* subsp. *avenae* strains from rescuegrass and wheatgrass adding to the uncertainty of this group (10). Traditionally, pathogenicity has been an indicator of distinction among closely related pathovars or subspecies. Hu et al. (1997) found strains of *A. avenae* subsp. *avenae* that were virulent on only certain varieties of corn but weakly pathogenic on oats. Host specificity has also been shown in isolates of *A. avenae* subsp. *avenae* from rice, some of which have shown exclusive pathogenicity to rice in extensive inoculation experiments. Other studies have shown isolates of *A. avenae* subsp. *avenae* from alternative grass hosts unable to elicit disease in rice (3, 20).

Figure 1.1. 16S rRNA phylogenetic tree taken from Fegan (2006).



Tree inferred from 16SrRNA sequence information retrieved from the GenBank database. The tree shows phylogenetic positions of plant pathogenic *Acidovorax* spp. and *Herbaspirillum* spp. in bold text within the β-proteobacteria.

Most recently, *A. avenae* subsp. *avenae* has emerged as the cause of sterile panicles in rice in Italy (5). The condition was diagnosed initially as a physiological condition, but recent studies have shown the condition to be caused by *A. avenae* subsp. *avenae*.

Acidovorax avenae subsp. *avenae* is a known seed-borne pathogen with worldwide distribution (4, 42, 51). The bacterium has been found viable in the glumes and pericarp of rice seed and it is thought that seed may act as an important means of dissemination of the bacterium to a broad range of geographical regions (41, 42). Alternative hosts such as wild grasses have been reported as inoculum reservoirs within seed and leaf tissue for subsequent infection of the primary host maize (13), however, *Acidovorax avenae* subsp. *avenae* is not thought to survive well in soil or plant debris (46). The bacterium has been shown to invade rice though stomata in the coleoptiles, subsequently multiplying in substomatal chambers in the first leaf. The bacterium then invades intercellularly, reaching the lacunae which run along the full length of the leaves (41). In maize, infection has been demonstrated to occur through leaf stomata (12), while in pearl millet, it is proposed that hydathodes serve as the portal of entry for the bacterium (4). Mature rice plants that survive early seedling infection of *A. avenae* subsp. *avenae* have been shown to harbor latent infections (41, 42). Dissemination of this infection is thought to occur via farm equipment (13) as well as internal transmission from plant to seed in latently infected plants. The conditions that favor disease development caused by *A. avenae* subsp. *avenae* typically include high humidity and high temperatures (1, 19, 42, 47, 53).

BENTGRASS SUMMER DECLINE AND THE ETIOLATED TILLER SYNDROME

Creeping bentgrass (*Agrostis stolonifera* L.) is a cool-season turfgrass species most commonly used on golf course greens, tees and fairways. The species is best known for its fine texture and adaptation to mowing heights as low as 3 mm. Creeping bentgrass is a stoloniferous turfgrass, primarily adapted to cool, humid regions. Because of its performance on high-quality putting surfaces, *A. stolonifera* has been used in warmer climatic zones with increasing popularity over the last several decades (6). Although creeping bentgrass is susceptible to a wide range of diseases including dollar spot (*Sclerotinia homoeocarpa*), brown patch (*Rhizoctonia solani*), *Microdochium* patch (*Microdochium nivale*), and gray snow mold (*Typhula incarnata*/*T. ishikariensis*), many cultivars have been developed that show differential susceptibility to these important diseases (48, 50). Weather extremes have been known to damage creeping bentgrass turf, especially summer stress. Biotic and abiotic stresses can simultaneously result in symptoms of decline on creeping bentgrass which can be very difficult to diagnose or distinguish (6).

The cause of bentgrass summer decline has been attributed to numerous different factors including heat stress, drought stress, soil temperature, rooting depth and other sources of natural physiological plant breakdown (6, 8, 17, 18, 22, 23). Biological organisms, most typically fungal pathogens, have been proposed to be associated with the bentgrass summer decline "complex" as well (2, 6). Golf courses growing creeping bentgrass under stressful summer conditions are usually able to successfully control common fungal diseases with regular applications of fungicides (6, 43, 48). Although proper disease management measures may be in place, many putting greens, particularly those on high profile golf courses with adequate financial and agronomic resources continue to experience unique symptoms of bentgrass etiolation and decline.

The term etiolation is derived from the French *etioler*, meaning to grow pale and weak (35). The typical etiolated creeping bentgrass leaf blade has an abnormal appearance of yellow or light-green hue and is often accompanied by the abnormal growth of shoots and stems. By definition, etiolation is the growth of plant shoots in the absence of light or in limited light situations. The lack of chlorophyll production in low light conditions leads to stems and leaves becoming elongated and chlorotic. Etiolated tiller syndrome (ETS) was a name given to the widespread epidemic of etiolation observed on perennial ryegrass at Lebanon Country Club in North Cornwall, PA in 2004 and 2005 (11). The symptoms of etiolation have been observed on turfgrasses around the world (11, 45). In Northern Europe, including England and Scandinavia, the condition is called "ghost disease", with conditions described to appear during late spring to early summer, and re-appear in late summer to early fall (11). Others have coined the condition "mad tiller disease", and although no empirical studies have proven the causation, many have attributed the symptoms to fungi isolated from affected tillers including *Fusarium cumorum, Fusarium crookwellense* and *Rhizoctonia solani* (45).

Much like other plants, etiolation in turfgrass typically occurs under low light conditions, but the phenomenon is also a well characterized symptom of bacterial infection on the widely distributed invasive turfgrass species known as annual bluegrass (*Poa annua* L). Yellowing and chlorosis accompanied by stem elongation is commonly observed on annual bluegrass affected with the *Xanthomonas transulucens* pv. *poae* (7, 26). Bacterial wilt of *Poa annua* caused by *X. translucens* pv. *poae* is the only important bacterial disease of managed turfgrass, and etiolation is a key early diagnostic symptom of this largely cosmetic problem on golf course fairways and putting greens.

HISTORY OF BACTERIAL DISEASES ON TURFGRASS

Bacterial wilt caused by *Xanthomonas translucens* pv. *poae* occurs primarily on golf course putting greens throughout the United States. The disease most commonly causes pale elongated growth referred to as etiolation (bleaching and elongation of leaves), and yellowing and progresses to cause foliar necrosis and plant death under favorable conditions.

Xanthomonas campestris pv. *graminis* was first described in 1981 as causing bacterial wilt of Toronto C15 creeping bentgrass (*Agrostis stolonifera*). Toronto C15 is a vegetatively propagated cultivar of creeping bentgrass no longer widely used in the golf course industry due to its susceptibility to the devastating bacterial disease. Bacterial wilt of Toronto C15 did not affect other cultivars of bentgrass nor annual bluegrass (*Poa annua* var. *reptans* (Hauskins) Timm) (32). In 1985, Roberts and Vargas described a new pathogen, *Xanthomonas campestris* pv. *poa anua,* causing a wilt disease of annual bluegrass (33). As further confirmation of a bacterial disease, the antibiotic oxytetracycline was found to control symptoms when applied to putting greens at the excessive rate of 2.5 lbs per 1000 sq. feet (33). In 1995, Nishino et al. (27) described the occurrence of bacterial wilt caused by *Xanthomonas translucens* pv. *poae* in Japan. In 2005, Mitkowski et al (26) described the host range and virulence of several isolates of *Xanthomonas translucens* pv. *poae* from US golf courses. The taxonomy of the genus *Xanthomonas* has been reclassified and *Xanthomonas* isolates that infect grasses are now considered *Xanthomonas translucens.* Specifically, isolates infecting *Poa annua* are *Xanthomonas translucens* pv. *poae* and isolates infecting *Agrostis stononifera* are known as *Xanthomonas translucens* pv. *graminis* (31, 49).

There are very few options available for controlling bacterial wilt of *Poa annua*. Although applications of oxytetracycline have been shown to control bacterial wilt (33),

antibiotics are not labeled for use on turfgrass in the United States and therefore cannot be used. Copper containing pesticides such as copper hydroxide can be used to prevent bacterial wilt before disease symptoms develop, however these chemicals often convey a high risk of phytotoxicity, and are not likely to be effective against bacteria already occupying plant tissue (25). Most options for limiting damage from bacterial wilt revolve around reducing plant stress. Raising mowing height, increasing or providing adequate nitrogen fertility, reducing traffic and compaction, and increasing the overall vigor of the turfgrass stand is likely to discourage severe damage from the disease (7, 25). The major issue with these recommendations is that golfer expectations don't often relate to these changes in playing conditions, particularly considering the widespread trend in golf course management over the past several decades to increasingly lower mowing heights and create "faster" putting surfaces (52).

The rarity of bacterial pathogens and infrequent occurrences of prokaryotic diseases on high value turfgrass are reflected in most turfgrass disease management textbooks and compendiums. The focus on fungal pathogens in turfgrass systems may lead to misdiagnoses of maladies which are indeed caused by other biological organisms. One such example is the creeping bentgrass etiolation and decline problem that has plagued golf course putting greens for more than a decade. Anecdotally described as a stress related problem, the reoccurring symptoms were later found to be associated with a new bacterial disease caused by *Acidovorax avenae* subsp. *avenae.* The discovery, identification, characterization and progress toward specific molecular diagnostics of the new bacterial turfgrass pathogen are discussed herein.

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CHAPTER TWO

IDENTIFICATION, CHARACTERIZATION, AND DISTRIBUTION OF *ACIDOVORAX AVENAE SUBSP. AVENAE* ASSOCIATED WITH CREEPING BENTGRASS (*AGROSTIS STOLONIFERA L.)* ETIOLATION AND DECLINE

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ABSTRACT

Bacterial etiolation and decline caused by *Acidovorax avenae subsp. avenae* is an emerging disease of creeping bentgrass (*Agrostis stolonifera* L.) in the Mid-Atlantic and Transition Zone; a unique area of turfgrass culture between cool and warm regions of the United States. It is suspected that the disease has been present for many years, although diagnosis of the first occurrence was not reported until 2010. Solicitation of samples from golf courses in 2010-2011 was undertaken to investigate the prevalence and dissemination of *A. avenae* subsp. *avenae* on creeping bentgrass. At least 21 isolates from 13 states associated with these outbreaks on golf courses were confirmed as *A. avenae* subsp. *avenae* by pathogenicity assays and 16S rDNA sequence analysis at two independent locations. Pathogenicity testing of bacterial isolates from creeping bentgrass samples exhibiting heavy bacterial streaming confirmed *A. avenae* subsp. *avenae* as the only bacterium to cause significant disease symptoms and turfgrass decline. Host range inoculations revealed isolates of *A. avenae* subsp. *avenae* to be pathogenic on all *A. stolonifera* cultivars tested, with slight but significant differences in disease severity on particular cultivars. Other turfgrass hosts tested were only mildly susceptible to *A. avenae* subsp. *avenae* infection. This study initiated research on *Acidovorax avenae* subsp. *avenae* pathogenicity causing a previously uncharacterized disease of creeping bentgrass putting greens in the United States.

INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera* L.) is one of the most widely used turfgrass species on golf course putting greens in the United States. Most of the destructive diseases afflicting creeping bentgrass are caused by fungal pathogens, and are collectively managed by fungicide applications, host resistance, and integrated cultural practices. Bacterial diseases of turfgrasses however, are uncommon. To date, the only notable bacterial pathogens to cause significant damage to turfgrass are in the genus *Xanthomonas*, which have been shown to elicit disease in vegetatively propagated creeping bentgrass such as 'Toronto C-15' (23) and annual bluegrass (*Poa annua* L.) (2, 21, 25).

Summer stress and extreme weather conditions, particularly in the "transition zone" make managing creeping bentgrass a challenge in several regions of the U.S. This unique region of the U.S. ranging from the coastal states of Delaware, Maryland, and North Carolina, and stretching westward to Oklahoma and Kansas creates difficult growing conditions for cool season turfgrasses in the summer months. Recently, a bacterial pathogen within the genus *Acidovorax* has been detected in association with creeping bentgrass decline and etiolation in the United States (12). Symptoms of the disease, on golf greens begin as small, (15 – 30 cm) irregular-shaped areas of foliar discoloration progressing from green to a light-green /yellow in appearance. Often associated with discoloration, the affected creeping bentgrass rapidly etiolates, producing elongated, yellow blades of grass with stems that extend 0.75 - 4 cm above the turfgrass canopy. Although it is initially observed as a cosmetic problem, high summer temperatures (> 30^0 C) lead to thinning and necrosis of the foliar canopy; leaving irregularly shaped areas of dead grass in a creeping bentgrass sward. Initial symptoms may be confused with other diseases or conditions on creeping bentgrass, making diagnosis of the problem

difficult. Damage from the disease is most severe on highly maintained, intensively managed putting greens, and symptoms first appear on peripheral or outer edge areas of putting greens which tend to be the most stressed or trafficked due to frequent and repetitive mowing and rolling practices .

The genus *Acidovorax* contains many plant pathogenic species and subspecies that cause a wide range of economically important diseases on monocotyledonous and dicotyledonous plants including corn, watermelon, rice, anthurium, and orchids. *Acidovorax avenae* consists of three subspecies that differ mainly in their host range. *A. avenae* subsp. *avenae* is pathogenic on members of the *Gramineae* (3, 14, 15, 22, 29, 31), *A. avenae* subsp. *citrulli* is pathogenic on members of *Cucurbitaceae* (15, 31), and *A. avenae* subsp. *cattleyae* is pathogenic on *Cattleya* and *Phalaenopsis* only (1).

Aside from the initial isolation and identification of *Acidovorax avenae* subsp. *avenae* found in association with an emerging enigmatic syndrome plaguing golf course putting greens in the U.S. (12) and Japan (10), there has been limited research on *Acidovorax* spp. affecting amenity turfgrass. The research objective was to characterize bacteria isolated from symptomatic turfgrass, their pathogenicity on creeping bentgrass, and their association with the newly emerging etiolation and decline disease of creeping bentgrass on golf courses in the United States.

MATERIALS AND METHODS

Research sites

The isolation, identification, and inoculation of the bacterial specimens from creeping bentgrass took place at two sites during 2009-2011. Researchers at Michigan State University

(MSU) and the University of Rhode Island (URI) worked collaboratively using slightly different methodologies.

Isolation

During 2009-2011, samples of affected turfgrass from golf course putting greens were evaluated for common turfgrass fungal pathogens as well as bacteria by observing leaves, stems, and roots for common signs and symptoms of infection. Pathogen isolation from sample plugs at MSU was conducted at MSU after microscopic examination revealed bacterial streaming from the cut ends of individual leaves. Stems and leaves of affected tissues were cut into 1- to 4-mm long sections, surface disinfested with 5% sodium hypochlorite solution for 1 min, followed by a rinse in sterile distilled water for 1 min, and either plated onto nutrient agar (NA) (BD Difco, Sparks, MD), or put into Eppendorf tubes with 20 µl sterile phosphate buffer saline (PBS) solution (pH 7.0) and macerated with a sterile scalpel. In the latter case, serial dilutions up to 1 x 10^{-4} were performed in sterile PBS, with 10 µl of each suspension plated onto NA medium and incubated at room temperature for 3-5 days. At URI, symptomatic leaf blades were put onto a glass slide in 200 µl sterile water, and when bacterial streaming was observed, 1 µl of exudate was removed and plated onto yeast extract-dextrose-calcium carbonate agar medium (YDC) (26). Single colonies that developed were then streaked onto new NA (MSU) or YDC (URI) plates to obtain pure cultures for subsequent identification and inoculation experiments. All isolates were maintained on solid nutrient medium (King's B, NA, or YDC) for short-term use, or grown in 25 ml of trypticase soy broth (TSB) medium for 3-5 days, after which 1 ml of suspension was transferred to CryoTubes (Thermo-Fisher Scientific Inc., Rochester, NY) and stored at -80^{0} C in 30% glycerol for long-term storage.

Electron Microscopy

Naturally infected symptomatic leaves and stems of creeping bentgrass were cut into 2-mm long sections for observation via electron microscopy. Samples were fixed at 4^0 C for 2 h in 4% glutaraldehyde buffered with 0.1 M sodium phosphate at pH 7.4. Following a brief rinse (0.5 h) in the buffer, samples were dehydrated in an ethanol series (25%, 50%, 75%, 95%) for 30 min at each graduation with three 10-min changes in 100% ethanol. Samples were critical point dried in a critical point dryer (Model 010, Balzers Union Ltd., Balzers, Liechtenstein) using liquid carbon dioxide as the transitional fluid. Samples were mounted on aluminum stubs using high vacuum carbon tabs (SPI supplies, West Chester, PA), then coated with osmium (~10 nm thickness) in a NEOC-AT osmium coater (Meiwafosis Co., Ltd., Osaka, Japan). Samples were examined in a JEOL JSM-7500F (cold field emission electron emitter) scanning electron microscope (JEOL Ltd., Tokyo, Japan). Digital images were acquired using Analysis Pro software version 3.2 (Olympus Soft Imaging Solution Corp., Münster, Germany).

Fatty acid analysis

The organism isolated at MSU (QH-B1) was initially identified by fatty acid methyl ester analysis (FAME). The isolate was submitted for cellular fatty acid analysis for species identification. Microcheck (Northfield, VT), a commercial service, was used to perform the analysis and isolates were submitted per the preparation instructions of the company according to their published procedures.

ELISA identification

The URI bacterial isolates were identified in September 2010 with an indirect ELISA kit from Agdia (catalog number BRA 41900), using an alkaline phophatase label following the manufacturer's published protocol. Twenty-four-hour-old bacteria colonies were transferred

from YDC plates with a sterile toothpick into individual well plates for testing. A positive reading was indicated by a color change from clear to yellow. The positive control (*Acidovorax avenae*) included with the kit tested positive, and the negative control was negative.

16S rDNA sequencing and identification at MSU

Isolates of the most commonly recovered bacteria were grown for 3 to 5 days on King's B medium at room temperature. Single colonies were picked with sterile toothpicks and transferred into 100 µl of DNA lysis buffer (0.05 M KCl; 0.01 M Tris; and 1% Tween 20). Suspensions were incubated at 100^0 C for 10 min, supernatant was used directly as a template for PCR reactions. PCR reactions contained 50 to 100 ng of total DNA template. All PCR amplifications were performed in a 25 µl reaction mixture containing 1 µl of DNA template solution, and PCR reactants supplied by Invitrogen Co., (San Diego, CA) including: 18.1 ul of sterile distilled water, 2.5 µl of 10x PCR buffer, 0.2 µl of 10 mM dNTP mixture, 1 µl of 50 mM $MgCl_2$ solution , 1 µl of each primer 63f (5'- CAGGCCTAACACATGCAAGTC)/ 1387r (5'- GGGCGGWGTGTACAAGGC) (19) at a concentration of 10 µM, and 0.2 ul of *Taq* DNA polymerase at 5 U/ul. All reaction tubes were placed in an Applied Biosystems GeneAmp 2700 thermocycler96 programmed as follows: 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C, with a final extension of 5 min at 72°C. All PCR products were run on a 1% gel and stained with ethidium bromide to confirm amplification of 16S region. PCR products were purified with the QIA-quick PCR Purification Kit (QIAGen) according to the manufacturer's protocol. Purified DNA was recovered, standardized to 10 ng/µl via NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and sequenced at the Michigan State University Research Technology Support Facility using an ABI 3730 automated sequencer.

16S rDNA sequencing and identification at URI. Ribosomal DNA (16S) was amplified on a PTC-100 thermocycler (Waltham, MA), using the primers pA(=8F) (5'–AGAGTTTGATCMTGGCTCAG–3')/ pHr (5'–AAGGAGGTGATCCANCCRCA–3') (34). Single colonies of bacteria were placed into 100 µL of sterile water and frozen and thawed twice in order to obtain template DNA. All PCR amplifications were performed in a 25-µl reaction mixture containing 1 µl of template DNA with PCR reactants supplied by Promega Corp. (Madison, WI) including: 14 µl of sterile distilled water, 2.5 µl of 25mM $MgCl_2$, 2.5 µl of 10x magnesium free buffer, 1.3 µl of 200µM dNTPs and 0.5 µl of each 10 µM primer. All reaction tubes were placed into the thermal cycler at 94°C and 0.5 units of *Taq* DNA polymerase (Promega Corp.) were then added to each tube. The thermocycler program was: 1 min at 94°C, 1 min at 51°C and 2 min at 72°C repeated 25 times, followed by 5 min of extension at 72°C. PCR products were purified using a QUIAquick PCR Purification Kit (Qiagen). The DNA samples were sequenced at the URI Genomics and Sequencing Center using an Applied Biosystems 3130xl Genetic Analyzer.

Inoculation experiments (MSU)

Acidovorax avenae subsp. *avenae* [MSU1], *Stenotrophomonas maltophilia* [MSU7], *Herbaspirillum seropedicae* [MSU11], and *Ralstonia pickettii* [MSU12], initially isolated from serial dilutions of symptomatic samples, were used in inoculation experiments with seed-grown *A. stolonifera* cv. 'Penn-A4'. Seeds were sown in Styrofoam cups (8.5 by 10 cm) filled with 80:20 sand:peat at a rate of 0.0049 kg/m^2. Plants were allowed to grow for 8-10 weeks until pots were sufficiently filled with mature turfgrass. Fertilizer (20-20-20) was applied every 2-3 weeks as foliar sprays at a rate of 24.4 kg of nitrogen ha^{-1}. Plants were trimmed at regular intervals to a height of approximately 2-3 cm. Three replicate cups were included in all inoculations, and

experiments were repeated once unless otherwise noted. Inoculum preparation consisted of growing pure cultures of each bacterium from frozen seed stock in 25 ml trypticase soy broth (TSB) medium for 3-4 days in 100 ml Erlenmeyer flasks on a rotary shaker set to 100 rpm at room temperature (20-22°C). Before plant inoculations, cells were spun down in centrifuge tubes in a Sorvall Legend RT centrifuge at 3761xg for 3 min. Supernatant was decanted and cells were resuspended in 25 ml of sterile PBS. The bacterial cell suspension was vortexed, and turbidity measured at 640 nm. Concentrations were adjusted to range from 10^8 to 10^9 cells ml^{-1}. Prior to inoculation, turfgrass foliage was trimmed with sterile scissors to approximately 3 cm in length and misted with sterile distilled water. Inoculation was performed by dipping sterile scissors into a bacterial suspension, trimming plants to length of approximately 1-2 cm using the inoculated scissors, then submerging leaf tips in the same bacterial cell suspension for approximately 10 s by inverting the cup over liquid inoculums prep. Control plants were treated similarly, but scissors were sterile and leaf tips were dipped in sterile PBS. Cups were transferred to a Conviron HL-E8VH growth chamber set to 12-h day length and temperatures of 30-32°C (light) and 25-27°C (dark) at >70% relative humidity in order to simulate environmental conditions of origin. In inoculation experiment 1, plants were kept in the growth chamber for 15 days, watered daily or as needed with deionized tap water, and trimmed every 2-3 days with sterile scissors to simulate turfgrass mowing events. Plants were rated every 2-3 days for disease progression and symptomology. Foliar disease symptoms were rated on a 0-to-100% scale, where 0 = unaffected and 100 = every plant is completely necrotic or dead. Pooled experimental data from repeated experiments were analyzed by ANOVA using Proc GLM in SAS (v. 9.2; SAS Institute, Cary, NC). When a significant *F*-value was determined, means were separated by Tukey's multiple comparison test.

A second inoculation experiment at MSU used a single isolate (*Acidovorax avenae* subsp. *avenae* [MSU4]) to inoculate eight cultivars of *Agrostis stolonifera* (Table 2.2). All inoculations took place following the above-mentioned methods, all cups were replicated four times, and experiments conducted twice. Confirmation of bacterial streaming from plants was conducted 13-15 days post-inocualtion by excising four symptomatic plants per container from the base of the shoot, cutting into 2-to-3-mm-long sections, and observing at x200 under a compound microscope. Positive identification of streaming was tallied for each replicate and numbers compiled to form a percentage of plants with streaming identified. Isolation of inoculated bacterium was achieved via surface disinfestation of cut tissues and subsequent plating onto King's B medium. Disease ratings were taken every 2-3 days for 15 days. Data from repeated experiments were pooled among and analyzed by ANOVA using Proc GLM in SAS (v. 9.2; SAS Institute, Cary, NC). When a significant F-value was determined, means were separated by Tukey's multiple comparison test.

Inoculation experiments at URI. Approximately 20 seeds of each type of turfgrass listed in Table 2.2 were sown in 4-inch plastic pots in MetroMix 510 (Sun Gro Horticulture, Bellevue, WA) a peat, vermiculite, and pine bark based potting mix. Seedlings were grown under greenhouse conditions and turfgrass was not trimmed prior to inoculation. Six-to-8-week-old plants were used, each experiment consisted of three replications of plants, experiments were conducted twice. All inoculations included a negative control in every replicate of every experiment using sterile water. Glycerol stocks of bacteria were removed from the -80°C freezer and streaked onto YDC. Cultures were grown in 40 ml of a modified YSG broth medium (8) substituting 10 g of dextrose for glycerol and placed on a rotary shaker at 270 rpm in a 28°C incubator. All plants were inoculated by dipping scissors into the bacterial suspension and

clipping the leaves. Scissors were sterilized between isolate inoculations by spraying with 70% ethanol. Turf grasses were clipped to 2-4 cm in height during inoculation. For the larger non-turfgrass species, three plants were sown into each replicate pot including corn, barley, oats, wheat, and rice. For these larger plants, 2-4 cm of tissue was cut from the leaf tip during inoculation. The URI growth chambers ranged between 29-31°C during the daylight phase and 25-24°C during the night phase. Plants were misted once per day with a plastic spray bottle to increase the relative humidity inside the chamber. The plants were under continuous illumination, and were not clipped after inoculation. Initial symptoms were visible 3 days after inoculation, and plants were rated for disease symptoms 7 days after inoculation. When symptoms were visible on clipped leaf blades, visual ratings of disease on plants were taken as severe, moderate, or ambiguous/mild, (++, +, +\-, or -) respectively. Disease ratings were followed by microscopic observations of two symptomatic leaf blades per plant for bacterial streaming. When bacterial streaming was present, bacteria were re-isolated using the methods described above.

RESULTS

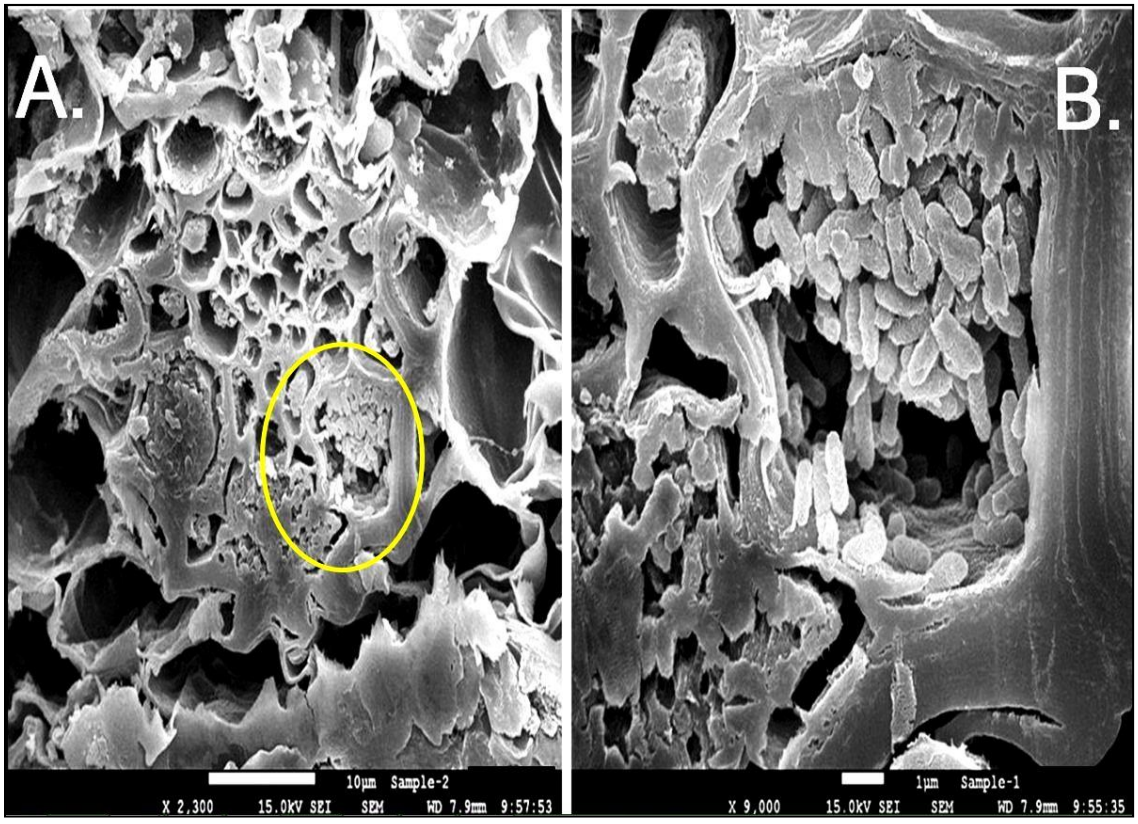
Isolation

Dilution plating and isolation from infected turfgrass leaves and stems consistently resulted in Gram-negative, rod-shaped, non-flourescent bacterial colonies on King's B medium. The colonies on YDC medium were white/cream with tan to brown centers, convex, smooth, 2-3 mm in diameter after 3 days growth.

Electron Microscopy

In the samples observed under scanning electron microscopy, the vascular cells had abundant rod-shaped bacterial colonization in the adaxial surface region. The vascular bundle showed abnormal impediment by bacteria (Figure 2.1) that were not present in tissues from asymptomatic plants. The scanning electron microscopy images revealed the presence of bacteria in all of the symptomatic plants studied. A large number of bacteria were observed in numerous adaxial and abaxial vascular spaces. Fungi were not detected in any of the electron microscopic analyses.

Figure 2.1. **Electron microscopy image of a cross section of naturally infected creeping bentgrass (*Agrostis stolonifera* L.) plant from a golf course putting green. A.** 2,300x view of a vascular bundle. Note the numerous rigid xylem vessels that are impeded. **B.** 9,000x view of impeded vascular tissue showing heavy colonization by rod-shaped bacteria.



Fatty acid analysis

The FAME identification system determined that the organism initially isolated from creeping bentgrass (MSU1) had similarity indices closest to *A. avenae subsp. cattleyae* (0.890) and *A. avenae subsp. avenae* (0.853) (data not shown).

16S rDNA sequencing and ELISA identification. From 2009-2011, 28 isolates of suspected causal bacterial agents were cultured from infected creeping bentgrass collected from more than 25 golf courses in 15 states by both MSU and URI researchers (Table 2.1). BLAST (NCBI BLAST Web Server) comparisons of the ribosomal DNA sequences from these isolates indicated high similarity indices (>95%) to *A. avenae* subsp. *avenae* in a majority of the isolated organisms. The most common genus that colonized the stem and leaf tissues of infected plants was *Acidovorax*. Other isolated bacterial genera were cultured and stored for further classification and pathogenicity testing.

Inoculation experiments at MSU

In the first experiment, percent necrosis of creeping bentgrass was significantly greater after 15 days incubation in MSU1-inoculated plants compared to all other bacterial inoculants and the sterile water control (Figure 2.2). None of the other bacteria isolated from creeping bentgrass (*Stenotrophomonas maltophilia*, *Ralstonia pickettii*, and *Herbaspirillium seropedicae*) produced significant turfgrass damage or necrosis when compared to the sterile water control (Figure 2.2), and post-inoculation bacterial streaming from lower tissues was observed only in plants inoculated with MSU1 (data not shown).

Table 2.1. Identification of bacterial isolates cultured from symptomatic turfgrass plants collected from various geographic regions in the United States. Information presented in Table 2.1 was derived from research at MSU and URI, using two different sets of universal bacterial 16S primers.

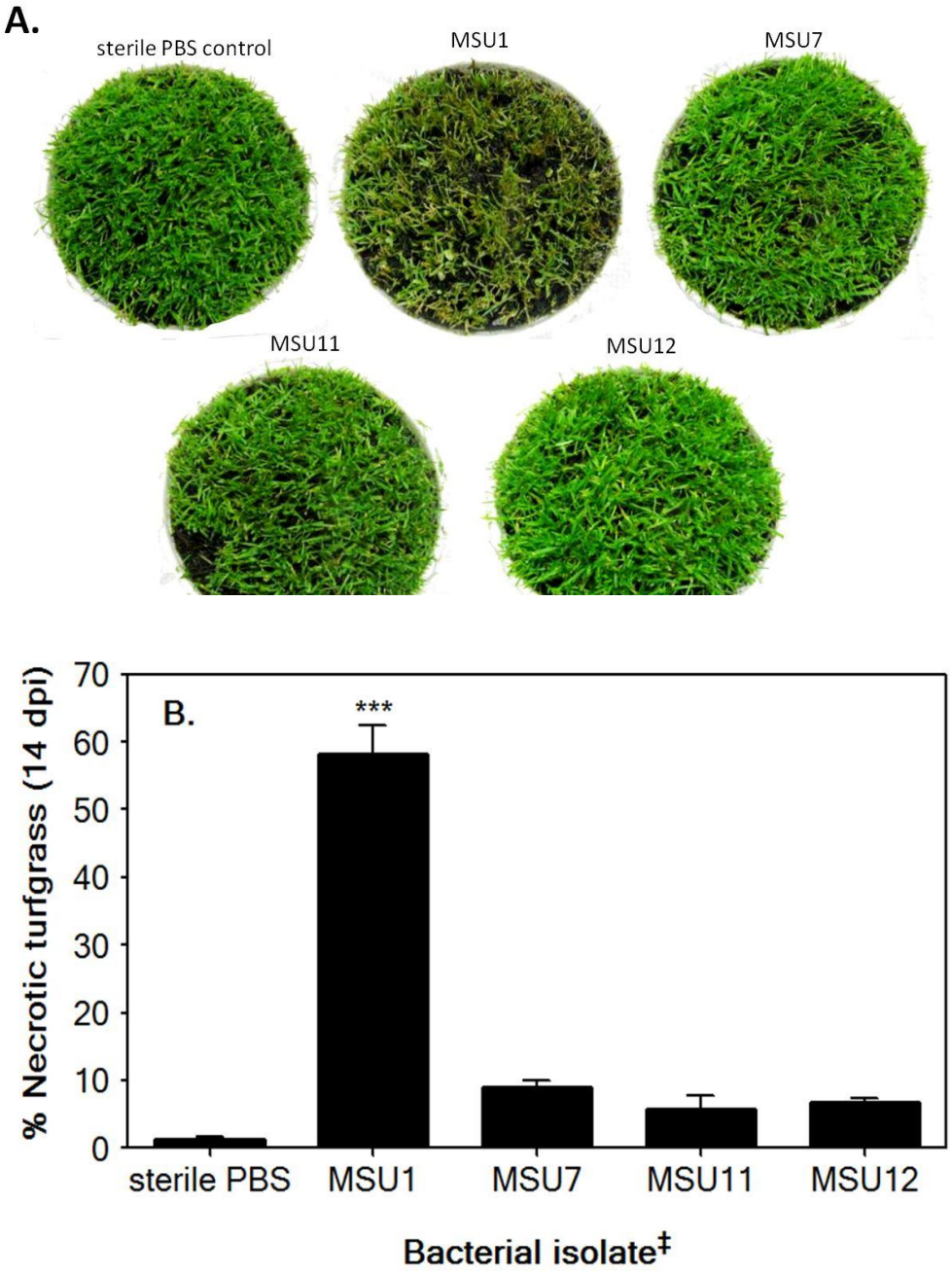
Isolate Name	Host	Geographic Location	BLAST results†	% I.D.‡	ELISA§
MSU1	*Agrostis stolonifera* cv. Penn G2	Charlotte, NC	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	99.24	NT
MSU2	*Agrostis stolonifera* cv. Penn A4	Irvine, TX	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	99.76	NT
MSU3	*Agrostis stolonifera* cv. unknown	Detroit, MI	*Ochrobactrum pseudogrignonense* strain: CCUG 30717; Accession # [NR_042589]	99.50	NT
MSU4	*Agrostis stolonifera* cv. Penn A4	Fort Worth, TX	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	99.92	NT
MSU5	*Agrostis stolonifera* cv. Penn A1	Athens, GA	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	99.59	NT
MSU6	*Agrostis stolonifera* cv. Penn-A1/A4	Athens, GA	*Microbacterium binotii* strain CIP 101303; Accession # [NR_044290]	100.0	NT
MSU7	*Agrostis stolonifera* cv. unknown	Silvis, IL	*Stenotrophomonas maltophilia* strain IAM 12423; Accession# [NR_041577]	99.44	NT
MSU8	*Agrostis stolonifera* cv. unknown	Newburgh, IN	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	99.92	NT
MSU9	*Agrostis stolonifera* cv. Penn G2	Toledo, OH	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	98.05	NT
MSU10	*Agrostis stolonifera* cv. unknown	Indianapolis, IN	*Stenotrophomonas maltophilia* strain IAM 12423; Accession# [NR_041577]	99.51	NT
MSU11	*Agrostis stolonifera* cv. unknown	South Hamilton, MA	*Herbaspirillum seropedicae* strain Z67; Accession# [NR_029329]	99.67	NT
MSU12	*Agrostis stolonifera* cv. unknown	St. Louis, MO	*Ralstonia pickettii* strain ATCC 27511; Accession # [NR_043152]	99.42	NT
MSU13	*Agrostis stolonifera* cv. Penn G2	Charlotte, NC	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	95.37	NT
MSU14	*Agrostis stolonifera* cv. Penn G2	Charlotte, NC	*Acidovorax avenae* subsp. *avenae* strain ICMP 3183; Accession # [NR_043752]	100.0	NT
URI1	*Agrostis stolonifera* cv. unknown	Greenwich, CT	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	98.86	+
URI2	*Agrostis stolonifera* cv. unknown	Walpole, MA	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+
URI3	*Agrostis stolonifera* cv. unknown	Midlothian, IL	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+
URI4	*Agrostis stolonifera* cv. unknown	Deal, NJ	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+
URI5	*Agrostis stolonifera* cv. unknown	Amagansett, NY	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+

Table 2.1 (Cont'd)

URI6	*Agrostis stolonifera* cv. L-93	Richmond, VA	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Accession # [CP002521]	99.77	+
URI7	*Agrostis stolonifera* cv. unknown	Willoughby, OH	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Accession # [CP002521]	99.93	+
URI8	*Agrostis stolonifera* cv. Alpha	Bethlehem, PA	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Accession # [CP002521]	99.93	+
URI9	*Agrostis stolonifera* cv. unknown	Lakeville, MA	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	99.93	+
URI10	*Agrostis stolonifera* cv. unknown	Charlotte, NC	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+
URI11	*Agrostis stolonifera* cv. unknown	Dayton, OH	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	99.86	+
URI12	*Agrostis canina L.* cv. unknown	Haverhill, MA	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+
URI13	*Agrostis canina L.* cv. Greenwich	Charlestown, RI	*Acidovorax avenae* subsp. *avenae* ATCC 19860; Acession # [CP002521]	100.0	+

† BLAST results indicate the top hits from each organism represented in the BLAST NCBI database.
‡ % I.D. represents the percentage of nucleotide matches from the queried organism 16S rDNA to the indicated organism identified as the top hit from the BLAST database.
§ Enzyme-linked immunosorbent assay (ELISA) identification of URI isolates was carried out using an indirect ELISA kit from Agida (catalog number BRA 41900) specific to A*cidovorax avenae*. (+) indicates a positive presence.

Figure 2.2. Percent (%) necrosis of creeping bentgrass (*Agrostis stolonifera* cv. 'Penn-A4') after inoculations with isolates of bacteria from infected golf course samples. A. Creeping bentgrass cups after inoculation with different bacterial isolates and 15 d incubation. **B.** Percent necrosis ratings after inoculation and 15 d incubation.



‡ Isolates were identified via 16S rDNA sequencing: MSU11 (*H. seropedicae*; [NR_029329]); MSU1 (*A. avenae* subsp. *avenae*; [NR_043752]), MSU12 (*R. pickettii*; [NR_043152]), and MSU7 (*S. maltophilia*; [NR_041577]). PBS is sterile phosphate buffered saline control. Bars represent ± standard error of mean. (***) mean is significantly different than all others according to Tukey's multiple comparison test ($P < 0.001$).

In the second inoculation experiment at MSU, the eight cultivars of *A. stolonifera* tested exhibited varying levels of susceptibility; however, all were susceptible to infection by *A. avenae* subsp. *avenae* isolate MSU4 (Table 2.2). Significantly less ($P \leq 0.05$) disease was detected on the cultivars 'Tyee' and 'Declaration' compared to the other cultivars tested after 15 days of incubation (Table 2.2). The cultivar '007' had significantly less disease ($P = 0.003$) than 'Penn-A4', but not 'Bengal', 'L-93', 'Penncross' or 'Penn G-2'. (Table 2.2). 'Declaration' and 'Tyee' showed a significant ($P \leq 0.05$) reduction in the percentage of plants with bacterial streaming compared to all other cultivars, while 'Penn-A4' had higher observed percentages of bacterial streaming from plant tissues than all other cultivars ($P \leq 0.05$) (Table 2.2).

Inoculation experiments at URI

Inoculation with *A. avenae* subsp. *avenae* (RI6) at URI confirmed the pathogenicity of the bacterium on creeping bentgrass. All cultivars of *A. stolonifera* tested displayed high levels of susceptibility with significant chlorosis and necrosis of inoculated plants (Table 2.2). Other *Agrostis* spp. (*A. canina, A. tenuis*) were also highly susceptible to *A. avenae* subsp. *avenae* infection (Table 2.2). Other turfgrass genera which were mildly susceptible to infection by the URI6 isolate include *Lolium multiflorum* and *Festuca arundinacea*; however, infection was limited to isolated areas of individual leaves, and progression of necrosis was not complete (Table 2.2). Disease severity was low on the tested stands of *P. annua, P. pratensis, F. rubra, L. perenne, Z. mays,* and *O. sativa*, with little to no necrosis progressing past the point of inoculation. Pathogenicity on the tested cultivars of *H. vulgare, A. sativa,* and *T. aestivum* was significant, with extensive chlorosis, heavy bacterial streaming and eventual necrosis of inoculated tissues. In asymptomatic hosts, weak bacterial streaming was detected only from tissues inoculated directly with a bacterial suspension (Table 2.2).

Table 2.2. Host range inoculations conducted at Michigan State University (MSU) and the University of Rhode Island (URI) with *Acidovorax avenae* subsp. *avenae* isolated from *Agrostis stolonifera* L.

Host tested	Percent disease[u]	Streaming from plants (%)[v]	Re-isolation of *A. avenea* subsp. *avenae*[w]
	MSU Inoculation (MSU4)[x]		
Agrostis stolonifera cv. 007	47.5 b[y]	68.7 b[y]	+
Agrostis stolonifera cv. Bengal	50.0 ab	64.7 b	+
Agrostis stolonifera cv. Declaration	32.1 c	48.5 c	+
Agrostis stolonifera cv. Tyee	28.1 c	44.5 c	+
Agrostis stolonifera cv. L-93	56.5 ab	64.7 b	+
Agrostis stolonifera cv. Penncross	55.0 ab	68.7 b	+
Agrostis stolonifera cv. Penn G-2	59.1 ab	60.7 b	+
Agrostis stolonifera cv. Penn A-4	66.8 a	80.8 a	+
	URI inoculations (URI6)[z]		
Agrostis stolonifera cv. Southshore	++	91.7	+
Agrostis stolonifera cv. Crenshaw	++	100.0	+
Agrostis stolonifera cv. Providence	++	100.0	+
Agrostis stolonifera cv. Mackenzie	++	100.0	+
Agrostis stolonifera cv. Cato	++	100.0	+
Agrostis stolonifera cv. SR-1150	++	100.0	+
Agrostis stolonifera cv. PennLinks	++	100.0	+
Agrostis stolonifera cv. PennEagle	++	91.7	+
Agrostis canina cv. SR7200	++	100.0	+
Agrostis tenuis cv. Exeter	++	100.0	+
Agrostis tenuis cv. Alistar	++	100.0	+
Lolium multiflorum cv. Unknown	+	100.0	+
Poa annua var. *annua*	+/-	100.0	+
Poa pratensis cv. Nuglade	+/-	100.0	+
Festuca rubra var. *rubra* cv. Cindy Lou	-	50.0	+
Lolium perenne cv. Turf Star	-	100.0	+
Festuca arundinacea cv. Pixie	+	100.0	+
Zea mays cv. Sweet Perfection	+/-	41.7	+
Zea mays cv. Early Choice	+/-	100.0	+
Zea mays cv. Jubilee	+/-	16.7	-
Zea mays cv. Bi-licious Hybrid	+/-	100.0	+
Hordeum vulgare cv. Conlon	++	50.0	+
Avena sativa cv. Marion	++	100.0	+
Avena sativa cv. Montezuma	++	100.0	+
Avena sativa cv. Richland	++	100.0	+
Triticum aestivum cv. Anza	++	100.0	+
Oryza sativa cv. Nortai	+/-	66.7	+
Oryza sativa cv. Koshikari	+/-	58.3	+

[u]Percent disease was the mean percentage of total turf necrosis in each container. Data were pooled from initial and repeated trials of the experiment.

Table 2.2. (Cont'd)

[v]Bacterial streaming (MSU) was the percentage of plants with confirmed bacterial streaming from four randomly selected shoots examined from each container (pooled n = 32).
[w]Confirmation (+/-) of *A. avenae* subsp. *avenae* isolation was achieved by dilution plating and subsequent 16S rDNA sequencing.
[x]Disease ratings (MSU) were recorded 15 days post-inoculation with *Acidovorax avenae* subsp. *avenae* (isolate MSU4).
[y]Means followed by the same letter are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).
[z]Disease ratings recorded 7 days post inoculation with *Acidovorax avenae* subsp. *avenae* (isolate URI6). Disease ratings (pooled n = 6) were as follows: Limited infection approximately 1 mm wide at the end of the cut blade = (-); Limited yellowing present with weak bacterial streaming = (+/-); Yellowing present in leaves, early necrosis = (+); Severe necrosis and yellowing with heavy bacterial streaming = (++). Bacterial streaming (URI) is from two chosen symptomatic leaves per plant (pooled n = 12). Data were pooled from initial and repeated trial of the experiment.

DISCUSSION

The infection of creeping bentgrass by *Acidovorax avenae* subsp. *avenae* in golf course putting green settings has not been characterized in the United States until this point. Symptoms of etiolation, necrosis, and thinning of the turfgrass stand in irregular areas become noticeable in many locations under high temperatures, accompanied by severe physical and/or mechanical stress. Consistent isolation of high populations of *A. avenae* subsp. *avenae* from diseased creeping bentgrass from several locations in the United States, and results of subsequent inoculation studies have shown that the bacterium is capable of causing symptoms of discoloration, thinning, and necrosis on creeping bentgrass. In addition, the bacterium caused minor disease symptoms on other turfgrass hosts such as *A. tenuis*, *P. annua*, and *F. arundinacea* in controlled environment studies.

Symptoms on host plants (e.g. *A. Sativa*, *Z. mays*, *S. officianarum*, and *O. sativa*) infected by *A. avenae* subsp. *avenae* typically consist of leaf streaking, brown stripes, and occasional rots; however, in rice, *A. avenae* subsp. *avenae* infection can cause curving of the leaf sheath, and an abnormal elongation of the mesocotyl (16). The similar symptomology associated with infected creeping bentgrass etiolation, must be further explored. Creeping bentgrass

etiolation and subsequent decline has been hypothesized to be caused by numerous biotic and abiotic effects (9, 18); however, a pathogenic agent has never been thoroughly characterized. While this study demonstrated the ability of *A. avenae* subsp. *avenae* to infect and cause disease on creeping bentgrass, the phenomenon of early stage etiolation is still relatively uncharacterized. We hypothesize that the natural infection of the bacterium at low-to-moderate levels during cooler temperatures causes a physiological or hormonal response in the plant by which elongation of the newest shoot and leaf occurs. Observations of this atypical growth habit can also be observed in annual bluegrass (*Poa annua* var. *annua*) infected with the bacterial pathogen *Xanthomonas translucens* pv. *poae* (6).

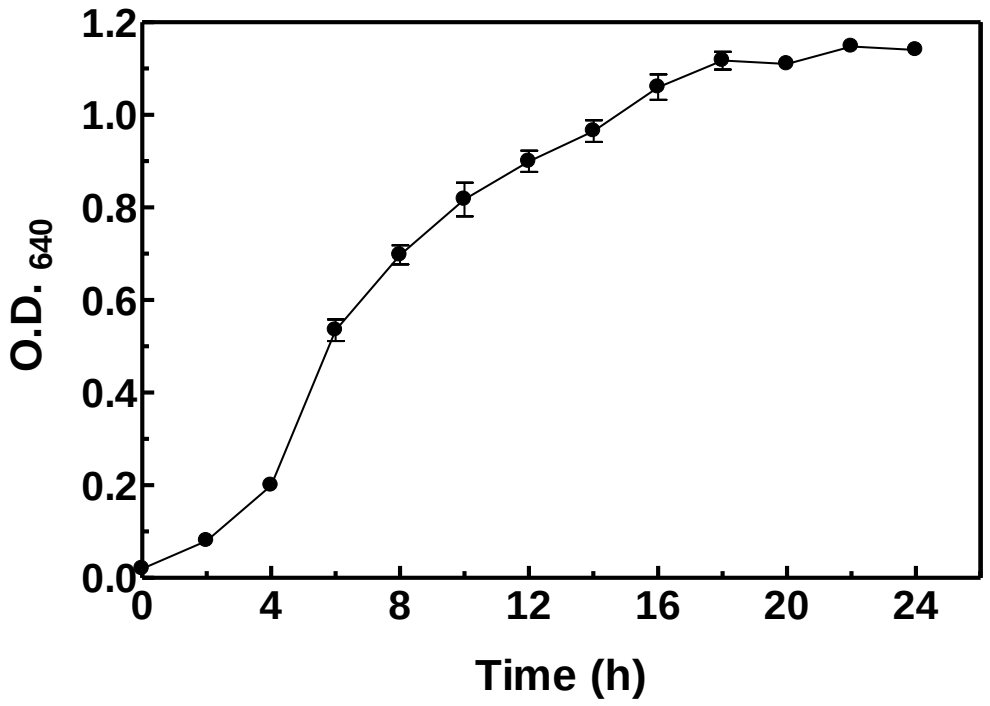
The bacterium *Acidovorax avenae* subsp. *avenae* is a common seed-borne pathogen (3, 7, 13, 32, 33) in graminaceous species, and has long been considered a weak pathogen of most hosts, having only minor economic impact in corn, oats, rice, and millet (5, 4). More recently however, *A. avenae* strains have caused severe losses in rice throughout the world (17), and have emerged as the cause of numerous diseases of importance on other hosts as well (19, 30, 11, 28). The emergence of *A. avenae* subsp. *avenae* as a pathogen on turfgrass is consistent with observations in other cropping and planting systems. Extreme environmental conditions, particularly high temperatures, sustained humidity, and periods of heavy rainfall in many regions of the United States have made an already intensively managed species such as creeping bentgrass increasingly difficult to manage in summer months. Infection of creeping bentgrass by *A. avenae* subsp. *avenae* and the rapid population of tissues during high heat and humidity results in necrosis and plant death. Latent infection of *A. avenae* subsp. *avenae* has been observed in rice (32, 33), in which asymptomatic plants harbor the bacterium and are able to transmit infection from plant to seed, and symptom development is favored by high temperature

and humidity. Until a robust diagnostic assay for the turfgrass pathogen is developed, the existence of latent infection among turfgrass stands is still uncertain. It is also unclear based on 16S rDNA sequencing how closely related the turfgrass pathogen is to other plant pathogenic *A. avenae* subsp. *avenae*. Genetic and genomic analyses related to host specificity and phylogenetic divergence are underway.

'Bacterial brown stripe' is a name given to the disease caused by *Acidovorax avenae* subsp. *avenae* on creeping bentgrass in Japan (10). While this original name was likely adopted to match nomenclature to diseases caused by *A. avenae* subsp. *avenae* on other hosts (i.e. corn, rice, sugarcane etc.), the symptoms on creeping bentgrass in the United States tend to be those of chlorosis, etiolation, necrosis, and decline rather than brown striping. Therefore, we refrain from using this terminology and recommend the disease be referred to as bacterial etiolation or bacterial decline. Bacterial infection of creeping bentgrass has been considered a stress-related condition on golf course putting greens, and is thought to be exacerbated by low heights of cut and aggressive cultural practices such as sand top-dressing and grooming. The emerging problem associated with *Acidovorax avenae* subsp. *avenae* infection on turfgrass is likely stress induced. Daily mowing, frequent aggressive cultural practices, and heavy traffic are all effective means by which inoculum can spread to neighboring plants throughout a turfgrass stand. Symptoms are not often observed on turfgrass that is kept at higher heights of cut, or subjected to less rigorous maintenance regimes. Control options for bacterial diseases in turfgrass are extremely limited. More work remains in characterizing appropriate cultural and potential chemical control options for this emerging disease on golf course putting greens.

APPENDIX

Figure A-2.1. **Growth curve of *Acidovorax avenae* subsp. *avenae* isolates (MSU4, MSU1, MSU13 and URI1) in trypticase soy broth (TSB) at 21°C.** Standard error bars represent error among different isolates. O.D. is logatithmic scale of absorbance units.



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CHAPTER THREE

INFLUENCE OF TEMPERATURE AND INOCULATION METHOD ON *ACIDOVORAX AVENAE* SUBSP. *AVENAE* PATHOGENICITY AND SYMPTOMOLOGY IN CREEPING BENTGRASS

ABSTRACT

The emerging disease known as bacterial etiolation and decline on creeping bentgrass (*Agrostis stolonifera* L.) caused by *Acidovorax avenae* subsp. *avenae* is a major concern on golf courses around the United States. Little is known about the epidemiology of the pathogen on turfgrass, and initial studies have only characterized partial aspects of the disease. Experiments using six day/night temperature regimens and inoculations with a pathogenic isolate of *A. avenae* subsp. *avenae* (MSU4) were conducted to determine the influence of temperature on disease development and severity on creeping bentgrass. The experiments demonstrated that disease was most pronounced at temperatures above 25° C, with stand necrosis being significantly greater, and chlorophyll index significantly lower in inoculated pots when compared to the controls after five days and nine days, respectively. A separate set of experiments was executed aimed at inducing etiolation symptoms on creeping bentgrass through an alternative method of inoculation. Root dip assays with suspensions of the MSU4 isolate of *A. avenae* subsp. *avenae* caused symptoms of stem elongation, chlorosis, and heavy bacterial streaming compared to the maize *A. avenae* subsp. *avenae* isolate (AAA 99-2) and a sterile water control. Together, these results provide valuable introductory principles on the etiology of an emerging disease on an important species of amenity turfgrass.

INTRODUCTION

Bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae* has been responsible for significant damage to infected creeping bentgrass (*Agrostis stolonifera* L.) putting greens in a range of states around the U.S. including areas in the Southeast, Mid-Atlantic, and Midwest. The disease was initially identified in 2009 (6), however, anecdotal reports suggest it has been present for longer. Typical symptoms initially appear as chlorosis on individual plants in small (15-30 cm) irregular-shaped or diffuse areas on putting greens. Plants with foliar discoloration often exhibit etiolation and rapid stem elongation, causing infected plants to protrude up to 4 cm above the surrounding turfgrass canopy. Etiolated turf plants start out as a cosmetic nuisance that can affect the playability of the putting green by disrupting ball roll and surface uniformity. The disease becomes more destructive when warm temperatures persist in conjunction with high humidity. At this point, the chlorotic, etiolated plants begin to show symptoms of water-soaking, necrosis, and thinning in irregular areas of the turfgrass stand, resulting in bare areas on the putting green.

This enigmatic turfgrass pathogen is one of few bacterial agents capable of causing significant damage on amenity turfgrass (7, 15, 17). With little known about the disease, diagnoses are often based on visual assessments of symptomology in the field, and supported by with microscopic observations of heavy bacterial streaming from infected plant stems. Diagnostic confirmation shows occurrence of *A. avenae* subsp. *avenae* in creeping bentgrass to be relatively widespread among different regions of the country including the Southeast, Mid-Atlantic, and Midwest (7). Disease severity however, is variable, with many putting greens in cooler climates experiencing only etiolation, and areas further south seeing both etiolation and

decline. Additionally, the infection does not appear to be limited to any particular *A. stolonifera* cultivars (7).

The mechanism of natural infection and optimal temperature for disease progression on *A. stolonifera* is uncharacterized. Experiments utilizing a dipped scissor inoculation method with liquid suspensions of isolates of *A. avenae* subsp. *avenae* have produced leaf necrosis, discoloration, and thinning of the stand under controlled environmental conditions (7). These experiments have demonstrated the general pathogenicity of *A. avenae* subsp. *avenae* isolates on creeping bentgrass through inundation of leaf tissues with high concentrations of bacteria; however, the inoculation strategy likely fails to mimic natural plant invasion, and subsequently fails to induce the symptom of etiolation that is observed in natural environments. Thus, quantifiable etiolation has not been reliably reproduced on creeping bentgrass through experimental inoculation with *A. avenae* subsp. *avenae*.

The objectives of this study were to 1) Elucidate the temperature(s) at which *A. avenae* subsp. *avenae* is most virulent on creeping bentgrass through foliar inoculations, 2) Employ an alternative inoculation strategy to encourage the early stage etiolation that is commonly observed in naturally infected creeping bentgrass plants.

MATERIALS AND METHODS

Bacterial isolates and culture conditions

The bacterial organisms used in inoculation studies herein include the MSU4 isolate of *Acidovorax avenae* subsp. *avenae* isolated from naturally infected creeping bentgrass (*Agrostis stolonifera* L.) from a golf course putting green sample originating in Fort Worth, Texas in 2010, and the AAA 99-2 isolate of *Acidovorax avenae* subsp. *avenae*, originally isolated from a

symptomatic sweet corn (*Zea maize*) plant, and provided by Dr. R. Walcott at the University of Georgia.

For isolation of the turfgrass strain, MSU4; plants were cut into 1- to 4-mm-long sections, surface disinfested with 5% sodium hypochlorite for 1 min followed by a rinse in sterile distilled water for 1 min, and placed into Eppendorf tubes with 20 µl of sterile phosphate-buffered saline (PBS) solution (pH 7.0) and macerated with a sterile scalpel. Serial dilutions were performed up to 1 x 10^{-4} in sterile PBS, with 10 µl of each suspension plated onto nutrient agar (NA) medium and incubated at room temperature. Once the bacteria were in pure culture, preliminary pathogenicity tests were performed, and the bacterial isolate was later identified via 16s rDNA sequencing as described in Giordano et al., (2012). All bacteria used in inoculation, including *Acidovorax aveane* subsp. *avenae* AAA 99-2, were maintained on solid NA medium for short-term use, or grown in 25 ml of trypticase soy broth (TSB) (BD Difco) medium for 72 hours, after which 1 ml of suspension was transferred to CryoTubes (Thermo-Fisher Scientific Inc.) and stored at -80°C in 30% glycerol for long-term storage.

Temperature range foliar inoculation assays

The MSU4 isolate of *Acidovorax avenae* subsp. *avenae* was used in inoculation experiments on seed-grown *Agrostis stolonifera* 'Penn-G2'All plants were grown under standard greenhouse conditions (20 to 25° C) in polystyrene cups (8.5 by 10 cm) filled with 80:20 sand/peat at a rate of 0.005 kg/m^2. Plants were watered daily and fertilized every 2-3 weeks with foliar sprays of 20-20-20 complete fertilizer at rate of 24.4 kg nitrogen ha^{-1}. Eight replicate cups for each temperature treatment were established and allowed to mature for 12 weeks until pots were filled with complete turfgrass coverage. For inoculation, 'MSU4' was grown in 25 ml TSB

media for 48 h at room temperature on a rotary shaker set to 100 rpm. Cells were spun down in centrifuge tubes in a Sorvall Legen RT centrifuge at 3,760 x g for 3 min. Supernatant was decanted and cells were resuspended in 50 ml of sterile PBS. After vortexing, suspension turbidity was measured at 640 nm on a Spectronic 20 spectrophotometer (Milton Roy Co.) and adjusted to 10^8 cells ml^{-1}. Sterile scissors were soaked with bacterial suspension by pipetting 1 ml of inoculum onto the scissor blades and trimming plants immediately after. A total of 5 ml of bacterial suspension was used for each inoculated replicate cup and plants were trimmed to a final length of about 3 cm. Control plants were treated similarly by trimming with sterile scissors soaked with a suspension of sterile PBS. After inoculation, cups were transferred to a Conviron HL-E8VH growth chamber set to 12-h day length and one of six temperature treatments administered in this study; 15°C/11°C, 20°C/15°C, 25°C/21°C, 30°C/23°C, 35°C/28°C, or 40°C/32°C (day/night). Temperatures were monitored using a WS-9133TWC-IT Wireless Weather Station (La Crosse Technology, USA), and relative humidity was kept between 75-85% by misting the chamber with deionized tap water three times per day. Pots were watered daily with 25 ml of deionized tap water by pouring the liquid over each pot so that water was evenly distributed into the underlying soil. Pots of creeping bentgrass were trimmed after 5 days in the growth chamber to a height of approximately 3 cm in order to simulate a mowing event.

Foliar disease symptoms were rated every two days on a 0-to-100% visual scale, where 0 = unaffected, 50 = approximately half of the pot exhibited chlorotic or necrotic symptoms, and 100 = entire pot was completely necrotic or dead. Chlorophyll ratings of the turfgrass canopy for each cup were taken at the same time as symptom assessments using a FieldScout CM 1000 ‘Point-and-Shoot’ Chlorophyll Meter (Spectrum Technologies, Aurora, IL) held at a consistent

measurement distance of 60 cm under constant fluorescent indoor lighting. Ten replicate measurements were assessed for each cup rating and mean measurement recorded.

Root dip inoculation assays

A second inoculation assay was developed to deduce the effects of alternative infection and acropetal movement of *A. avenae* subsp. *avenae* in turfgrass plants. Inoculation preparation for bacterial isolates took place by growing cultures from single colonies in 50 ml TSB media for 36 h at room temperature on a rotary shaker set to 100 rpm. Cells were spun down in centrifuge tubes in a Sorvall Legen RT centrifuge at 3,760 x g for 3 min. Supernatant was decanted and cells were resuspended in 50 ml of sterile distilled water. After vortexing, turbidity was measured for each bacterial isolate at 640 nm on a Spectronic 20 spectrophotometer (Milton Roy Co.) and adjusted to 10^6 cells ml^{-1}. Inoculations took place in a 48 well, flat bottomed, polystyrene, Costar cell culture plate (Corning Inc., Corning, NY) with 1 ml of bacterial suspension in each well (i.e. approximately 10^6 bacterial cells); control wells contained 1 ml of sterile distilled water. Plates were covered with AlumaSeal II pierceable adhesive sealing foil (RPI corp.) and punctured with a sterile disposable wooden toothpick in the center of each well to accommodate plant insertion.

Creeping bentgrass (cv. 'Penn G-2') was first established from seed in a potting tray (53.3 x 25.4 x 6.4 cm) filled with horticultural grade perlite under standard greenhouse conditions. Seedlings were watered every other day with 500 ml of half-strength Hoagland's solution (8) distributed evenly over the potting tray with a watering can. Six-week-old plants were removed from perlite trays and transported to the laboratory for preparation. Roots were washed with sterile distilled water until free of perlite debris. Prior to inoculation, whole plants were disinfested in 10% sodium hypochlorite for 1 min followed immediately by a rinse in

sterile distilled water for 1 min. Top growth from individual plants was trimmed with a sterile razor blade so that each plant measured 2 cm from the crown. Lower roots were sliced with a sterile razor blade so that all roots measured 10 mm in length from the longest root tip to the crown, and immediately placed into designated wells in cell culture plates to encourage bacterial infection. Sixteen replicates of each suspension of bacterial isolate and sterile water control were included on each plate and the experiment was repeated twice. Plates were placed in the greenhouse under standard conditions (25°/17° C day/night) and monitored daily.

Individual plants were removed from designated wells with forceps and measurements of plant etiolation were taken 2, 4, 6, 8, and 10 days post inoculation with a 15 cm acrylic office ruler by measuring from the crown to the growing tip of the youngest leaf. Individual plant leaf and stem chlorophyll index was measured using a CCM-300 chlorophyll content meter (Opti-Sciences, Hudson, NH). The meter is a modulated ratio fluorescence chlorophyll flourometer that measures chlorophyll content with a source detector that does not require the aperture to be completely covered and allows for accurate, non-destructive fluorescence measurements on smaller plants like turf grasses. Three measurements per plant were taken on each rating occasion; lower stem, mid stem, and leaf. Measurements were averaged and recorded for each rating date of 2, 4, 6, 8, and 10 days post inoculation. Data from repeated experiments were pooled before statistical analysis.

Bacterial streaming from root dip inoculated tissues was determined 10 days post inoculation by separating individual plant root tissue from stem tissue, and stem tissue from leaf tissue. Root, stem and leaf tissues were sliced with a sterile razor into 2-3 mm segments and plant components were viewed at 40x - 100x for bacterial streaming using a compound microscope. Streaming was assessed using a 0 - 3 rating scale where 0 = no streaming detected, 1

= minor streaming observed from roots, 2 = Streaming observed in roots and lower stems, and 3 = heavy streaming from roots, lower, and upper stems/leaves. Streaming data is derived from two repeated experiments with results pooled for statistical analysis.

Re-isolation of bacteria from individual plants was accomplished by surface sterilizing three replicate plants from each inoculation treatment (MSU4, AAA 99-2 and control). Bacteria were isolated by following the above mentioned serial dilution procedure and plated onto King's B medium. Single colonies were picked with sterile toothpicks and transferred into 100 µl of DNA lysis buffer (0.05 M KCl; 0.01 M Tris; and 1% Tween 20). Suspensions were incubated at 100° C for 10 min, supernatant was used directly as a template for PCR reactions. PCR reactions contained 50 to 100 ng of total DNA template. All PCR amplifications were performed in a 25 µl reaction mixture containing 1 µl of DNA template solution, and PCR reactants supplied by Invitrogen Co., (San Diego, CA) including: 18.1 ul of sterile distilled water, 2.5 µl of 10x PCR buffer, 0.2 µl of 10 mM dNTP mixture, 1 µl of 50 mM $MgCl_2$ solution , 1 µl of each primer 63f (5'- CAGGCCTAACACATGCAAGTC)/ 1387r (5'-GGGCGGWGTGTACAAGGC) taken from (19), at a concentration of 10 µM and 0.2 ul of *Taq* DNA Polymerase at 5 U/ul. All reaction tubes were placed in an Applied Biosystems GeneAmp 2700 thermocycler96 programmed as follows: 5 min at 95° C, followed by 35 cycles of 1 min at 95° C, 1 min at 55°C, and 1.5 min at 72°C, with a final extension of 5 min at 72° C. All PCR products were resolved on a 1% gel and stained with ethidium bromide to confirm amplification of 16S region. PCR products were purified with the QIA-quick PCR Purification Kit (QIAGen) according to the manufacturer's protocol. Purified DNA was recovered, standardized to 10 ng/µl via NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and sequenced at the Michigan State University Research Technology Support Facility using an ABI 3730 automated sequencer.

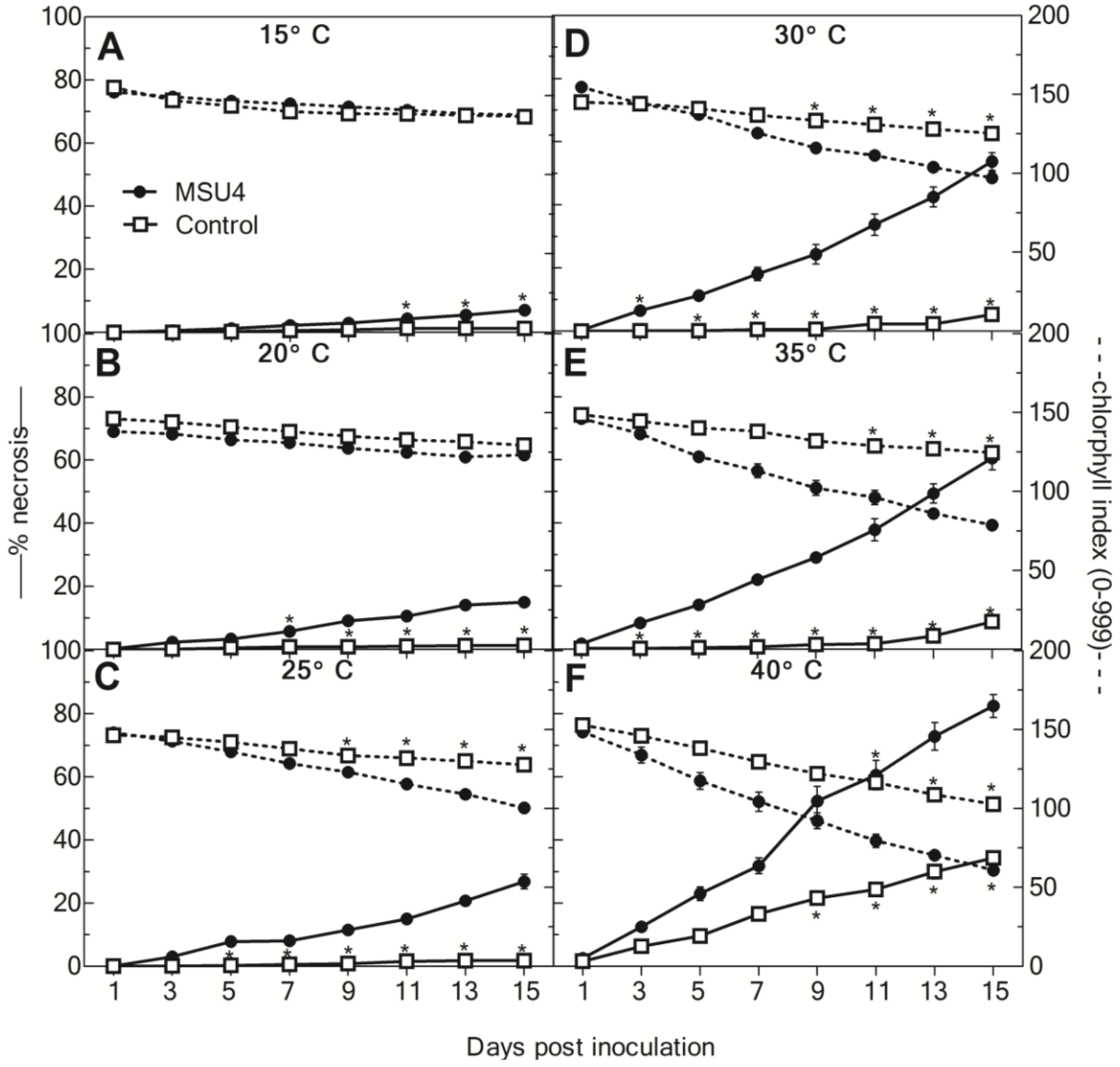
All data including disease ratings, etiolation measurements, chlorophyll content and bacterial streaming in all studies was subjected to an analysis of variance (ANOVA) using PROC GLM in SAS (11). When a significant F value ($P < 0.05$) was determined, means were separated by Tukey's multiple comparison test.

RESULTS

Temperature range foliar inoculation assays

As temperature increased, disease severity (percent stand necrosis) increased on swards of creeping bentgrass inoculated with MSU4 (Figure 3.3.1). Significant disease was not present in inoculated pots at 15° C until 11, 13 and 15 days post inoculation (dpi) when compared to the sterile PBS control (P = 0.003, 0.004, and 0.003, respectively). At 20° C, significant disease incidence was visually observed on inoculated cups 9, 11, 13, and 15 dpi (Figure 3.3.1B) (P = <0.001, 0.003, <0.001, <0.001, and <0.001, respectively). At 25° C, significant necrosis was observed on inoculated cups 7, 9, 11, 13, and 15 dpi (Figure 3.3.1C) (P = 0.005, <0.001, <0.001, <0.001 and <0.001, respectively). When plants were incubated at 30° C, inoculated pots displayed significantly more stand necrosis than sterile PBS on all rating dates except 1 dpi (Figure 3.3.1D) (P = 0.003, 0.010, 0.004, 0.004, 0.001, and 0.004, respectively). Similarly, at 35° C, inoculated pots had significantly more disease than control pots on all rating dates beyond 1 dpi (Figure 3.3.1E) (P = 0.002, 0.006, 0.002, <0.001, and <0.001, respectively). At 40° C, a significant increase in plant necrosis was observed on inoculated pots at 9, 11, 13, and 15 dpi (Figure 3.3.1F) (P = 0.007, 0.003, 0.002, and <0.001, respectively).

Figure 3.1. Percent necrosis and chlorophyll index of creeping bentgrass pots (*Agrostis stolonifera* cv. 'Penn-G2') after inoculation with *Acidovorax avenae* subsp. *avenae* as influenced by temperature.



Necrosis symptoms were assessed on a 0-100 scale, where 0 = unaffected, 50 = half of the turfgrass stand necrotic or symptomatic, and 100 = entire stand dead. Chlorophyll index was assessed with a FieldScout CM 1000 'Point-and-Shoot' Chlorophyll Meter.

Solid lines and dashed lines correspond to percent necrosis (left y axis) and chlorophyll index values (right y axis), respectively. Error bars represent standard error of mean (n = 8).

Within rating day and assessment, * represents a significant difference between treatments according to Tukey's multiple comparison test ($P \leq 0.05$).

Chlorophyll measurements were taken on replicate pots of MSU4-inoculated and sterile PBS control swards of creeping bentgrass to obtain an objective measurement of overall stand color and turfgrass quality. Significant differences in chlorophyll measurements were not detected at any measurement date when plants were incubated at 15° C and 20° C (Figures 1A and 1B respectively). At 25° C, significant decreases in chlorophyll index were detected in the MSU4 inoculated treatment 9, 11, 13, and 15 dpi (Figure 3.3.1C) (P = 0.032, 0.032, 0.009, and <0.001, respectively). At 30° C, significant differences in chlorophyll content were observed among treatments at 9, 11, 13, and 15 dpi (Figure 3.3.1D) (P = 0.011, 0.013, 0.004, and 0.010, respectively). Significant differences existed among treatments incubated at 35° C 11, 13, and 15 dpi (Figure 3.3.1E) (P = 0.048, 0.005, and 0.001, respectively). At 40° C, significant decreases in chlorophyll index were detected in the inoculated pots at 11, 13, and 15 dpi (Figure 3.3.1F) (P = 0.034, 0.017, and 0.008, respectively).

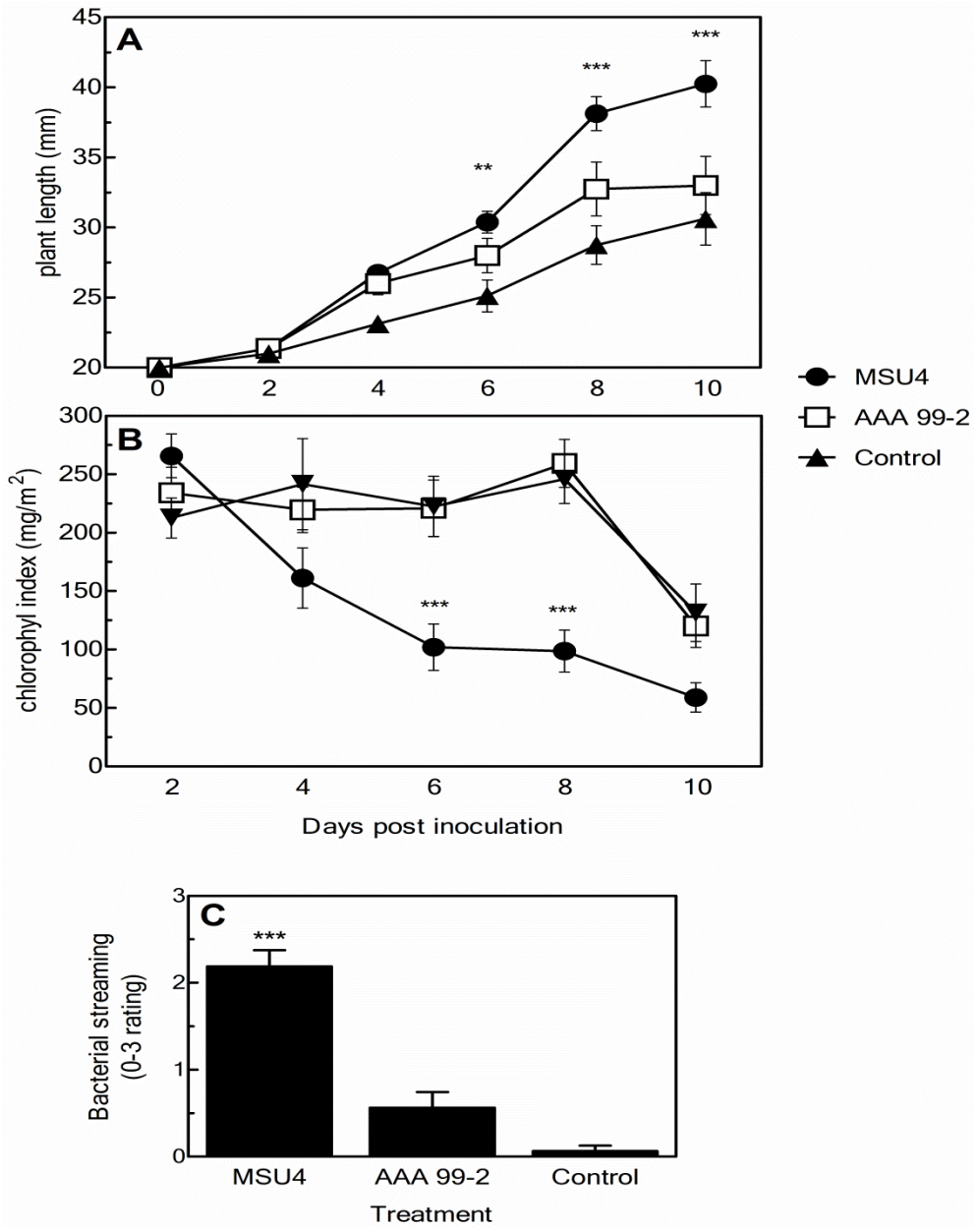
Root dip inoculation assays

An alternative inoculation method using a root dip technique was employed to assess plant etiolation, and subsequently, detect minor differences in plant length and chlorophyll content among two *A. avenae* subsp. *avneae* isolates originating from different hosts and a sterile water control. As time progressed, plant growth in the bacterial suspension or sterile water control could be sufficiently measured and recorded. No differences in plant growth were observed among treatments until 6 dpi. At this time, a significant increase in plant length was detected in the MSU4 inoculated plants when compared to the maize pathogen AAA 99-2 and the sterile water control (P = <0.010 and <0.001, respectively) (Figure 3.3.2A). Beyond 6 dpi,

the MSU4 treatment displayed significantly increased plant length when compared to the AAA 99-2 and the sterile water control on each measurement date ($P < 0.001$) (Figure 3.3.2A).

Similar to plant length, chlorophyll index remained relatively consistent among treatments until 6 dpi. At this rating point, chlorophyll measurements were significantly lower in the MSU4 inoculated treatment when compared to the AAA 99-2 isolate and the sterile water control ($P < 0.001$) (Figure 3.3.2B). Chlorophyll content was significantly lower in the MSU4 inoculated treatment at 8 dpi when compared to the AAA 99-2 and the non-inoculated control ($P < 0.001$) (Figure 3.3.2B). At 10 dpi, chlorophyll index was not statistically different among treatments; however, mean indices for AAA 99-2 and the sterile water control were more than two times those of the MSU4 treatment (131.4, 120.3 and 58.8, respectively). Additionally, at 10 dpi, mean bacterial streaming rating was significantly higher in the MSU4 inoculated plants compared to the AAA 99-2 and the sterile water control (Figure 3.3.2C), indicating heavy colonization in roots, lower stem, and upper stem of the MSU4 isolate in root dip inoculated creeping bentgrass plants. *Acidovorax avenae* subsp. *avenae* was re-isolated and identified via 16S rDNA sequencing from symptomatic roots, stems, and leaves of the MSU4 inoculated plants, however *A. avenae* subsp. *avenae* could only be reliably re-isolated and identified from roots from the AAA 99-2 inoculated plants (data not shown). These results indicate a systemic movement of the MSU4 isolate within creeping bentgrass, as well as putative host specificity of the AAA 99-2 to maize.

Figure 3.2. Etiolation of creeping bentgrass (*Agrostis stolonifera* cv. 'Penn G-2') after root dip inoculation with *Acidovorax avenae* subsp. *avenae* isolates from turfgrass (MSU4) or maize (AAA 99-2).



A. Mean plant length measurements (stem and leaf) (n = 32). **B.** Mean chlorophyll index (n = 32). **C.** Mean bacterial streaming ratings from plants 10 days post inoculation (n = 16); where 0 = no streaming detected, 1 = minor streaming observed from roots, 2 = Streaming observed in roots and lower stems, and 3 = heavy streaming from roots, lower, and upper stems/leaves.

*, **, *** indicates significant difference between MSU4 and the untreated control according to Tukey's multiple comparison test ($P \leq 0.05$, 0.01, and 0.001, respectively). Error bars represent standard error of mean.

Figure 3.3. Etiolation symptoms of root inoculated creeping bentgrass plants and control plants.

A. Plants were subjected to inoculation with a pathogenic strain of *A. avenae* subsp. *avenae* (MSU4) originating from a naturally infected creeping bentgrass stand. **B.** Plants subjected to sterile water only and displayed no significant difference from the AAA 99-2 isolate of *A. avenae* subsp. *avenae* originating from *Z. mays* (not shown).

DISCUSSION

Effective inoculation methods and optimal environmental conditions are critical components of accurate disease assessments when characterizing an enigmatic disease such as bacterial etiolation and decline of creeping bentgrass. The optimum temperature for growth on agar medium of *Acidovorax* spp. typically ranges from 30 to 36° C (4, 18), and previous studies have indicated various optimal temperatures for disease occurrence ranging from 18 to 30° C depending on host plant and bacterial isolate (9, 19). Very little is known about the nature and development of *A. avenae* subsp. *avenae* infection on creeping bentgrass. Field and anecdotal observations have attributed initial etiolation symptom onset to mild days and cool nights, and

severe disease outbreaks to warmer, humid temperatures. Inoculations and disease characterizations have established pathogenicity of *A. avenae* subsp. *avenae* (MSU4) on creeping bentgrass at high (~30° C) temperatures, which mimic initial environment of origin (7). However, given the breadth of incidence and variability in geographic location, conditions that most favor disease development have not been established in a turfgrass system.

Based on this study, as temperature increases, damage to creeping bentgrass caused by MSU4 becomes more severe. Minor damage occurred at lower temperatures of 15° and 20° C, however, significant disease symptoms were not evident until more than 10 days after inoculation. Our results indicate disease onset and progression to be most severe at temperatures between 25° and 35° C. It is at these temperatures plant necrosis was significantly apparent in as little as 3 days post inoculation, and progressed throughout the duration of the incubation period with minimal damage to the sterile water control pots. Additionally, inoculation with the MSU4 isolate had the most pronounced effect on chlorophyll index ratings at temperatures between 25° and 35° C when compared to the sterile water control. Beyond 35° C, non-inoculated pots treated with only sterile PBS, succumbed to high temperature-induced physiological decline.

Necrosis and plant health ratings were confounded by heat stress and natural plant desiccation at this extremely high temperature. Several studies have shown the detrimental biochemical and physiological effects of high air and soil temperatures on creeping bentgrass (1, 2, 10, 13, 14, 22), and it is likely that factors other than pathogen-induced necrosis and decline were responsible for elevated disease ratings and decreased chlorophyll indices at the 40° C incubation temperature in this study. The optimal temperatures for disease development and creeping bentgrass decline established in this study allow for a better understanding of *A. avenae* subsp. *avneae* pathogenicity by turfgrass researchers and golf course superintendents.

Etiolation on turfgrass remains an enigmatic problem, often associated with high internal populations of bacterial cells in affected creeping bentgrass plants. Abnormal elongation of the terminal shoot and evident chlorosis in plant stems are initial symptoms linked with *A. avenae* subsp. *aveane* isolation from *A. stolonifera*. To date, quantifiable etiolation has only been observed in naturally affected stands of turfgrass, while necrosis and general decline has been reproduced through pathogenicity assays conducted in controlled environments (7). Root dip inoculations implemented in this study produced considerable etiolation symptoms on healthy creeping bentgrass; evident by significant increases in plant length and reductions in chlorophyll content several days after inoculation. Confirmation of bacterial colonization in roots, lower stems, and upper stems of MSU4 inoculated plants also indicates acropetal movement of bacteria within creeping bentgrass subjected to a root dip inoculation. Minimal observations of bacterial colonization in plants inoculated with the maize pathogen AAA 99-2 indicates a potential pathogenic specificity among isolates on various graminacious hosts. Host specificity among *A. avenae* subsp. *avenae* has been documented, and separation among strains based on sequence variation in the intergenic spacer (ITS) region also suggests distinction among isolates from different hosts (3, 9, 12).

This study demonstrates the influence of temperature and entry method of *A. avenae* subsp. *avenae* on creeping bentgrass plants as it pertains to disease severity and symptomology. On rice, *A. avenae* subsp. *avneae* is thought to be seed borne (20, 21); the same goes for *A. citrulli* transmission in melon (16). Little is known however, about transmission and natural infection mechanisms in creeping bentgrass. Based on this study and field observations, we speculate initial colonization of creeping bentgrass plants occurs through latent growth of existing internal bacterial populations, or by uptake from the thatch/soil microbial reservoir.

Further research is required to determine if seed transmission is solely responsible for pathogen prevalence in creeping bentgrass stands. Other possibilities include alternative hosts, insect vectors, contaminated equipment or water sources, and survival of the pathogen in turfgrass thatch or soil debris. It is likely that daily mowing of infected stands contributes significantly to the spread of inoculum, however the origination of the infection on creeping bentgrass remains uncertain.

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LITERATURE CITED

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CHAPTER FOUR

INVESTIGATIONS INTO PREVENTIVE AND REMEDIAL TREATMENT OF BACTERIAL ETIOLATION AND DECLINE CAUSED BY *ACIDOVORAX AVENAE SUBSP. AVENAE*

ABSTRACT

The recent characterization of bacterial etiolation and decline on creeping bentgrass caused by *Acidovorax avenae* subsp. *avenae* has caused widespread concern in the golf and turfgrass industries. Symptoms range from aesthetic etiolation, to necrotic death and decline of large areas, particularly on intensively managed putting greens. Bacterial diseases are notoriously difficult to control, and currently, no antibiotic products are labeled for use on turfgrass. Controlled environment experiments showed two agricultural antibiotics; oxytetracycline and streptomycin sulfate significantly reduced symptoms of bacterial decline when pretreated onto creeping bentgrass prior to inoculation with a virulent isolate of *A. avenae* subsp. *avenae*; however, only oxytetracycline kept plants at an acceptable level of health compared to the untreated control. Field studies showed widespread etiolation during peak disease pressure and significant differences among treatments in 2013. The growth regulator trinexapac-ethyl (TE) was found to significantly increase etiolation when compared to the untreated control. Additionally, ammonium sulfate and the combination of TE + ammonium sulfate consistently increased disease incidence in field plots. These results suggest limited availability of remedial treatments for bacterial etiolation and decline. Additionally, this research helps to confirm the anecdotal association of TE and ammonium sulfate applications in disease perturbation.

INTRODUCTION

Turfgrass is subject to many different diseases caused by soil and environmental microorganisms. Fungal pathogens make up a majority of the disease-causing agents of amenity turf species (13). For many years, bacterial pathogens were not considered primary disease causing agents on high value turfgrass (11, 12). One exception includes bacterial wilt of annual bluegrass (*Poa annua* L.) caused by *Xanthomonas translucens* pv. *poae,* a vascular disease that is widely prevalent and can cause a variety of symptoms under different environmental conditions (11). Control of *X. translucens* has been relatively unsuccessful on annual bluegrass, and currently, no antibiotics are labeled for commercial use on turfgrass. Previous studies with the closely related pathogen *X. campestris* pv. *graminis* of Toronto C-15 creeping bentgrass (*Agrostis palustris* Huds.) showed successful control of bacterial wilt symptoms with extremely high (10x the highest labeled rate) concentrations of the antibiotic oxytetracycline (12). Considering the rarity of bacterial diseases in turfgrass systems, it is no surprise there is an overall lack of research pertaining to products and methods related to their control.

Acidovorax avenae subsp. *avenae* was diagnosed as a major bacterial threat of established creeping bentgrass (*Agrostis stolonifera* L.) putting greens in 2009 (4). The pathogen causes widespread chlorosis of individual leaf blades, sometimes occurring in patches, or exhibiting a more diffuse pattern spread throughout the playing surface. The chlorosis of tissue is typically accompanied by an abnormal elongation of the plant stems. Subsequent to this etiolation symptom, a general decline of the turfgrass is observed when temperatures reach 25^0 – 30^0 C for a sustained period of time, resulting in necrosis and death of the affected plants, leaving areas on the putting green devoid of turfgrass cover. The disease is characterized by the initial symptoms of etiolation, and diagnosed traditionally by microscopic confirmation of heavy

bacterial streaming from cut ends of symptomatic creeping bentgrass stems (5). Bacterial etiolation and decline has been confirmed on dozens of golf courses around the country, and is considered a major limiting factor for creeping bentgrass summer health and development during the warm summer months. Regions like the Mid-Atlantic and the Transition Zone, a unique region of the U.S. ranging from the coastal states of Delaware, Maryland, and North Carolina, and stretching westward to Oklahoma and Kansas, are typically where damage is most severe, likely due to the sustained humidity and high day and night time temperatures during much of the growing season.

Effective control measures for bacterial etiolation and decline caused by *A. avenae* are largely unresolved. Most recommendations for symptom alleviation involve limiting unnecessary stress to the plant during times of optimal disease pressure. Putting green turfgrass is subjected to intensive management practices that can cause extreme physiological stress on the plants (10). Daily mowing to a very low height (< 0.31 cm), mechanical traffic, compaction, sand topdressing, and frequent applications of plant growth regulators like trinexapac-ethyl are thought to have negative implications on plant health with regard to *A. avenae* infection. Cultural methods that reduce plant stress including raising the height of cut, increasing overall plant nutrition with fertilization, and reducing traffic may decrease the incidence of bacterial etiolation and decline. Vigorously growing healthy grass is thought to be less likely to suffer severe damage from bacterial pathogens (2). Several problems exist with this particular strategy of symptom avoidance. First, modern advancements in technology and superior germplasm in turfgrass cultivars has allowed for a dramatic reduction in the allowable height of cut. This reduction has significantly increased the ball roll distance otherwise known as green speed, resulting in a current standard of expectations that is unattainable by raising the height of cut on

greens for disease management. Thus the practice is not widely adopted by members of the golf industry (15).

Early reports of this new disease on golf courses gave rise to widespread speculation and hyper-vigilant investigations into potential causes or contributors. Anecdotal effects of the growth regulator trinexapac-ethyl on etiolation symptoms in creeping bentgrass have since spurred research efforts into the investigation of this commonly used product. Trinexapac-ethyl, (Primo, Syngenta) inhibits late gibberellin biosynthesis from GA1 to GA20 and is widely utilized in turfgrass management for clipping reduction, annual bluegrass seed head suppression, germination enhancement, increased ball roll distance, and improvement of overall turf quality, color, and density (8). Additionally, since the early reports of this disease on golf course putting greens, applications of ammonium-based nitrogen fertilizers have also been under scrutiny as a potential factor related to *Acidovorax* infection and symptom severity. These observations have been made by golf course superintendents, professional consultants, and turfgrass diagnosticians attempting to make sense of this emerging and enigmatic disease on creeping bentgrass. Very few control options have been reported or anecdotally observed that show any potential for reducing the severity of bacterial etiolation and decline.

The objective of this study was to investigate the role of different plant health products including, fungicides, antibiotics, fertilizers, biological controls, and plant growth regulators for their control and/or exacerbation of etiolation and decline symptoms caused by *A. avenae* subsp. *avenae* on creeping bentgrass putting greens. Studies included a controlled environment growth chamber setting with pre-treatment of several products before inoculations with *A. avenae* subsp. *avenae.* Additionally, separate field studies were conducted on putting greens with both

naturally occurring *Acidovorax* symptoms, as well as a field site where frequent inoculations of virulent *A. avenae* subsp. *avenae* took place.

MATERIALS AND METHODS

Growth Chamber Study

Acidovorax avenae subsp. *avenae* (MSU4), initially isolated from serial dilutions of symptomatic tissue from a golf course in Fort Worth, TX, was used in the inoculation with seed-grown *A. stolonifera* cv. 'Penn-A4'. Seeds were sown in Styrofoam cups (8.5 by 10 cm) filled with 80:20 sand:peat at a rate of 0.0049 kg/m^2. Plants were allowed to grow for 10 weeks until pots were sufficiently filled with mature turfgrass. Fertilizer (20-20-20) was applied every 2 weeks as foliar sprays at a rate of 24.4 kg of nitrogen ha^{-1}. Plants were trimmed at regular intervals to a height off approximately 3 cm. Four replicate cups were included in all inoculations, and experiments were repeated twice.

Prior to inoculation, replicate cups of creeping bentgrass were treated with different agrochemical products listed in Figure 4.1. All products were applied to turfgrass foliage as spray applications 48 hours prior to inoculation with *Acidovorax avenae* subsp. *avenae*. Replicate cups of creeping bentgrass were placed along the center transect of a pre measured 0.6 m by 1.8 m square area on the greenhouse floor. Treatments were applied using a CO_2 backpack sprayer at 38 PSI and 448.9 L ha^{-1} (48 gallons per acre) application volume using a single 8002E Tee Jet flat fan nozzle. All treatments were applied at 897.9 L ha^{-1} (96 GPA) by spraying over the pre-measured plot area in two directions at the 48 GPA application rate. Cups were immediately removed from the area and placed on the greenhouse bench in a randomized

fashion. Initial baseline ratings of pots took place immediately following application of treatments.

Table 4.1. Product name, active ingredient, and rate of agrochemicals investigated under controlled environmental conditions for the suppression of bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae* on creeping bentgrass (*Agrostis stolonifera* L.).

Product	Active Ingredient	Rate
Firewall	Streptomycin sulfate	200 ppm
Mycoshield	oxytetracycline	200 ppm
Actigard	acibenzolar-S-methyl	52.5 g/ha
Primo	trinexapac-ethyl	0.79 l/ha
Daconil Action	chlorothalonil + acibenzolar-S-methyl	11.45 L/ha
Sonnet	QST 713 strain of *B. subtillus*	2.28 kg/ha
Negative control	-	Untreated, not inoculated
Positive control	-	Untreated, inoculated

Inoculum preparation consisted of growing pure cultures of the *A. avenae* subsp. *avenae* (MSU4) from frozen seed stock in 25 ml trypticase soy broth (TSB) medium for 3-4 days in 100 ml Erlenmeyer flasks on a rotary shaker set to 100 rpm at room temperature (20-22^0 C). Cells were spun down in centrifuge tubes in a Sorvall Legend RT centrifuge at 3761 xg for 3 min prior to plant inoculations. Supernatant was decanted and cells were resuspended in 25 ml of sterile PBS. Bacterial cell suspension was vortexed and turbidity was measured at 640 nm. Concentrations were adjusted to approximately 10^8 CFU ml^{-1}. Prior to inoculation onto cups of established, pre-treated creeping bentgrass, foliage was misted with sterile distilled water. Inoculation of each replicate cup was performed by dipping sterile scissors into a the bacterial suspension, trimming plants to a length of approximately 1-2 cm, using the inoculated scissors,

then submerging the leaf tips in the same bacterial suspension for approximately 10 s by inverting the cup over liquid inoculums preps. Control plants were treated similarly, but scissors were sterile and leaf tips were dipped in sterile PBS. After inoculations, cups were transferred to a Conviron HL-E8VH growth chamber set to 12-h day length and temperatures of 30^0 C (light) and 25^0 C (dark). Relative humidity was kept high by misting the chamber three times per day for the duration of the experiment with distilled water in a spray bottle. Plants were watered daily with 25 ml deionized tap water by pouring the liquid over each pot so that water was evenly distributed into the underlying soil. Pots were trimmed after 5 days in the growth chamber to a height of approximately 3 cm in order to simulate a mowing event.

Foliar disease symptoms were rated every two days on a 0-100% visual scale, where 0 = unaffected, 50 = approximately half of the pot exhibited chlorotic or necrotic symptoms, and 100 = entire pot was completely necrotic or dead. Pooled experimental data from repeated experiments were analyzed by ANOVA using Proc GLM in SAS (v. 9.2; SAS Institute, Cary, NC). When a significant F-value was determined, means were separated by Tukey's multiple comparison test.

Field Study – Inverness C.C.

In 2013, a field study was conducted on a mixed stand of annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis stolonifera* cv. 'Penn G-2') putting green at Inverness Country Club in Toledo, Ohio. Natural infection of *Acidovorax avenae* subsp. *avenae* was confirmed in the summers of 2011 and 2012 by isolation and identification of the pathogen via 16S rDNA sequencing (see chapter 1). Reoccurring symptoms of etiolation and subsequent decline were present on the particular area of the putting green at Inverness C.C., thus the selection of this area warranted the research undertaken.

The study was conducted on a 15-year old, sand-based, creeping bentgrass putting green established to USGA specification (14). The area was mowed daily at a 0.254 cm height of cut during the growing season. General, maintenance of the putting green from April 2, 2013 until the completion of the study on July 25, 2013 was carried out by the golf course superintendent and turfgrass maintenance crew with the exception of fertilization and chemical pesticide application which was under strict control of the research program herein. In general, the putting green received weekly applications of sand topdressing, was subjected to rolling treatments at least three times weekly, and was irrigated or hand watered as needed in order to keep turf healthy and free from drought stress. The study area was set up in a randomized complete block design with four replicate 0.6 m by 1.83 m plots. Nitrogen fertility was incorporated into each spray treatment as urea (46-0-0) at a rate of 12.2 kg N ha^{-1} every two weeks unless nitrogen was a constituent of the individual treatment (see Figure 4.2). Treatment applications began 4/29, prior to visual symptoms of etiolation. Subsequent applications of the 14 day interval treatments were made on 5/13, 5/27, 6/10, 6/24, 7/8, and 7/22. A background spray of Boscalid (Emerald, BASF) was applied at a rate of 0.45 kg ha^{-1} over the entire study area on 7/5 to selectively prevent dollar spot caused by the fungal pathogen *Sclerotinia homoeocarpa*.

Table 4.2. Active ingredients and application parameters of chemical treatments investigated at Inverness C.C. in Toledo, OH for the control of bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae* on a creeping bentgrass (*Agrostis stolonifera* L.) putting green.

Product	Active Ingredient	Application Rate	Application Interval	Spray Volume
Untreated	-	-	-	
Mycoshield	Oxytetracycline	3.36 kg/ha	14 days	96 GPA
Primo	trinexapac-ethyl	0.79 l/ha	14 days	96 GPA
Signature	aluminum tris	24.4 kg/ha	14 days	96 GPA
Daconil Action	chlorothalonil+acibenzolar-S-methyl	11.46 l/ha	14 days	96 GPA
Ammonium Sulfate	$(NH_4)_2SO_4$	6.1 kg/ha	14 days	96 GPA
Primo + Ammonium sulfate	trinexapac-ethyl $(NH_4)_2SO_4$	0.79 l/ha 6.1 kg/ha	14 days	96 GPA
Trimmit	Paclobutrazol	0.47 l/ha	14 days	96 GPA

Field Study - Hancock Turfgrass Research Center 2012

In 2012, a field study was undertaken at The Hancock Turfgrass Research Center (HTRC) on the campus of Michigan State University in East Lansing, Michigan. Research was conducted from May to August on a research putting green consisting of a mixed sward of creeping bentgrass ('Independence' *Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.). The sand-based soil mix (~90:10 sand:peat v/v) was constructed in 2005 using USGA specifications (14). Sand topdressing was applied on a light, frequent (weekly) basis throughout the growing season to simulate typical golf course putting green maintenance practices in the area. No vertical mowing or core cultivation occurred on the research plots during the study to minimize turfgrass and soil disruption. Nitrogen fertility was applied using foliar applications of water soluble urea at a rate of 24.4 kg N ha^{-1} mo^{-1} during the growing season from May to September of each year except on 26 Apr 2012, where nitrogen was applied as a granular application (18-9-18) at a rate of 48.8 kg N ha^{-1}. Irrigation was applied via four Rain Bird Eagle

irrigation heads, model 750 (Rain Bird Distribution Co., Azusa, CA), located at the corners of the research area. Irrigation was applied as needed to keep the turf healthy and free of wilting symptoms. Plots were mowed at a height of 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.

The research plots were set up in a randomized complete block design and consisted of four replicate 0.91 m by 1.82 m plots for each treatment with 0.30 m alleys between plots. The area was inoculated with *Acidovorax avenae* subsp. *avenae* (MSU4 isolate) after growing populations from seed stock in 2 L flasks of TSB culture media (8 L total) on a rotary shaker for 72 hours, and evenly applying inoculum (average = 2.3 x 10^7 CFU/ml) over the study area twice per week for the duration of the study. Inoculations were applied using a nitrogen powered backpack sprayer calibrated to 38 PSI and 448.9 L ha^{-1} (48 gallons per acre) application volume using six 8002E Tee Jet flat fan nozzles. In order to promote bacterial entry and infection, the study area was mowed immediately after application of bacterial inoculum. Initial treatment applications were made on 5/24, prior to symptom development. Subsequent applications of the 14-day interval treatments were made on 6/7, 6/21 and 7/5, 2012. Background sprays of boscalid (Emerald, BASF) were applied at a rate of 0.45 kg ha^{-1} over the entire study area on 5/15, 6/12, and 7/17, 2012 to selectively prevent dollar spot. Additionally, background sprays of azoxystrobin (Heritage, Syngenta) were applied at a rate of 0.92 kg ha^{-1} for the control of brown patch caused by the fungal pathogen *Rhizoctonia solani* on 7/10 and 7/25, 2012.

Table 4.3. Active ingredients and application parameters of chemical treatments investigated at the Hancock Turfgrass Research Center in East Lansing, MI for the control of bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae* on a creeping bentgrass (*Agrostis stolonifera* L.) putting green in 2012.

Product	Active Ingredient	Application Rate	Application Interval	Spray Volume
Untreated	-	-	-	
Mycoshield	Oxytetracycline	3.36 kg/ha	14 days	96 GPA
Ammonium Sulfate	$(NH_4)_2SO_4$	12.2 kg N/ha	14 days	96 GPA
Firewall	Streptomycin sulfate	4.57 kg/ha	14 days	96 GPA
Primo	trinexapac-ethyl	0.75 l/ha	14 days	96 GPA
Primo + Ammonium Sulfate	trinexapac-ethyl $(NH_4)_2SO_4$	0.75 l/ha 12.2 kg N/ha	14 days 14 days	96 GPA 96 GPA
Signature	aluminum tris	24.4 kg/ha	14 days	96 GPA
Daconil Action	chlorothalonil+acibenzolar-S-methyl	11.46 l/ha	14 days	96 GPA
Kasumin	Kasugamycin			96 GPA

Field Study - Hancock Turfgrass Research Center 2013

In 2013, research was conducted on the same site as 2012 (indicated above). In April 2013, the research area was stripped of the existing turfgrass using a sod cutter. Established top growth, thatch, and a three-inch layer of soil were removed from the study area and replaced with a three-inch layer of subsoil consisting of a sand:peat (90:10 v/v) mixture spread evenly over the area to prepare for new sod establishment. Creeping bentgrass (cv. 'Independence) sod (Great Lakes Bentgrass, Ann Arbor, MI) was laid onto the study area on 5/7/2013. The area was fertilized with granular applications of 18-9-18 Contec DG fertilizer (Andersons Inc.) on 5/9, 5/19/ and 5/29 at a rate of 24.4 kg N ha^{-1} to ensure adequate growth and root establishment. Sand topdressing was performed using a rotary fertilizer spreader, evenly distributing sand across the study every other day for 4 weeks until sod establishment was adequate. Sod was hand watered daily as needed in order to keep the study area alive and free of wilt symptoms.

The study area was set up in a randomized complete block design with four replicate 0.6 m by 1.83 m plots. Inoculations were initiated on 5/31 with foliar sprays of *Acidovorax avenae* subsp. *avenae* (MSU4 isolate). Similar to the study conducted in 2012, the area was inoculated twice weekly with suspensions of bacterial inoculum grown from seed stock in 8 liters of TSB liquid medium using a nitrogen powered backpack sprayer calibrated to 38 PSI and 448.9 L ha^{-1} (48 gallons per acre) application volume using six 8002E Tee Jet flat fan nozzles. Immediately following spray inoculations, the study area was mowed at a height of 3.96 mm with a walk-behind Toro Greensmaster 1000 greens mower (Toro Co., Bloomington, MN). After study initiation, sand topdressing was performed weekly with light applications, typically following an inoculation event to encourage wounding and abrasion for subsequent bacterial infection. The study was rolled with a lightweight greens roller three times per week to simulate typical management practices on golf courses in the region. After study initiation, nitrogen fertility was incorporated into each spray treatment as urea (46-0-0) at a rate of 12.2 kg N ha^{-1} every two weeks unless nitrogen was a constituent of the individual treatment (see Figure 4.4). Initial treatments were applied on 6/19 before visual symptoms of etiolation were present. Subsequent applications of the 14-day interval treatments were applied on 7/3, 7/17, and 7/31.

Boscalid (Emerald. BASF) was sprayed over the entire study area on 7/5 at a rate of 0.46 kg ha^{-1} as a background spray to selectively control dollar spot caused by the fungal pathogen *Sclerotinia homoeocarpa*. Mefenoxam (Subdue Maxx, Syngenta) was sprayed over the entire area on 7/10/2013 at a rate of 3.18 l ha^{-1} to prevent the encroachment of yellow tuft caused by the oomycete pathogen *Scleropthora macrospora* into the study.

Table 4.4. Active ingredients and application parameters of chemical treatments investigated at the Hancock Turfgrass Research Center in East Lansing, MI for the control of bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae* on a creeping bentgrass (*Agrostis stolonifera* L.) putting green in 2013.

Product	Active Ingredient	Application Rate	Application Interval	Spray Volume
Untreated	-	-	-	-
Mycoshield	Oxytetracycline	3.36 kg/ha	14 days	96 GPA
Primo	trinexapac-ethyl	1.5 l/ha	14 days	96 GPA
Ammonium Sulfate	$(NH_4)_2SO_4$ (low)	6.1 kg N/ha	14 days	96 GPA
Primo + Ammonium sulfate	trinexapac-ethyl + $(NH_4)_2SO_4$	1.51 l/ha 6.1 kg/ha	14 days	96 GPA
Ammonium Sulfate	$(NH_4)_2SO_4$ (high)	12.2 kg N/ha	14 days	96 GPA
Urea	CH_4N_2O	12.2 kg N/ha	14 days	96 GPA
Signature	aluminum tris	24.4 kg/ha	14 days	96 GPA
Daconil Action	chlorothalonil+acibenzolar-S-methyl	11.46 l/ha	14 days	96 GPA

In all three field studies, treatments were applied using a CO_2 powered backpack sprayer at 38 PSI and 448.9 L ha^{-1} (48 gallons per acre) application volume using a single 8002E Tee Jet flat fan nozzle. All treatments applied at 897.9 L ha^{-1} (96 GPA) were administered by spraying over the pre-measured plot area in two directions at the 48 GPA application rate. All ratings were taken in the morning before mowing, when conditions were conducive to observing etiolation symptoms. Disease ratings were taken with regard to typical field symptoms of bacterial etiolation (chlorosis and stem elongation) with etiolation ratings being visually estimated on a percent scale of 0-100 where 0=no etiolation present in plot, 50= half of the plants in the plot are symptomatic, and 100= all plants in the plot are etiolated. If decline was present, turfgrass quality ratings were taken using a 1-9 scale where 7 = acceptable turfgrass quality, and 1 = completely dead turf. When etiolation symptoms were present, ransom samples of stem and leaf tissue were selected from symptomatic plots and investigated for bacterial streaming from

cut ends of stems and leaves. Data from all three field studies were analyzed using ANOVA in Proc MIXED of SAS (6). When significant F values existed, means were separated by Tukey's HSD ($P \leq 0.05$).

Identification and isolation of *Acidovorax avenae* from inoculated field plots was achieved using stems and leaves of affected tissues. Stems and leaves were cut into 1- to 4-mm long sections, surface disinfested with 5% sodium hypochlorite solution for 1 min, followed by a rinse in sterile distilled water for 1 min, and either plated onto nutrient agar (NA) (BD Difco, Sparks, MD), or put into Eppendorf tubes with 20 µl sterile phosphate buffer saline (PBS) solution (pH 7.0) and macerated with a sterile scalpel. In the latter case, serial dilutions up to 1 x 10^{-4} were performed in sterile PBS, with 10 µl of each suspension plated onto *A. avenae* SMART media and incubated at room temperature for 3-5 days. The SMART media was made following the protocol from Kawanishi et al. (7) and is based on selective carbon sources and antibiotics for the growth of *Acidovorax avenae*. Ingredients for SMART media used in this study are listed in Figure 4.1.

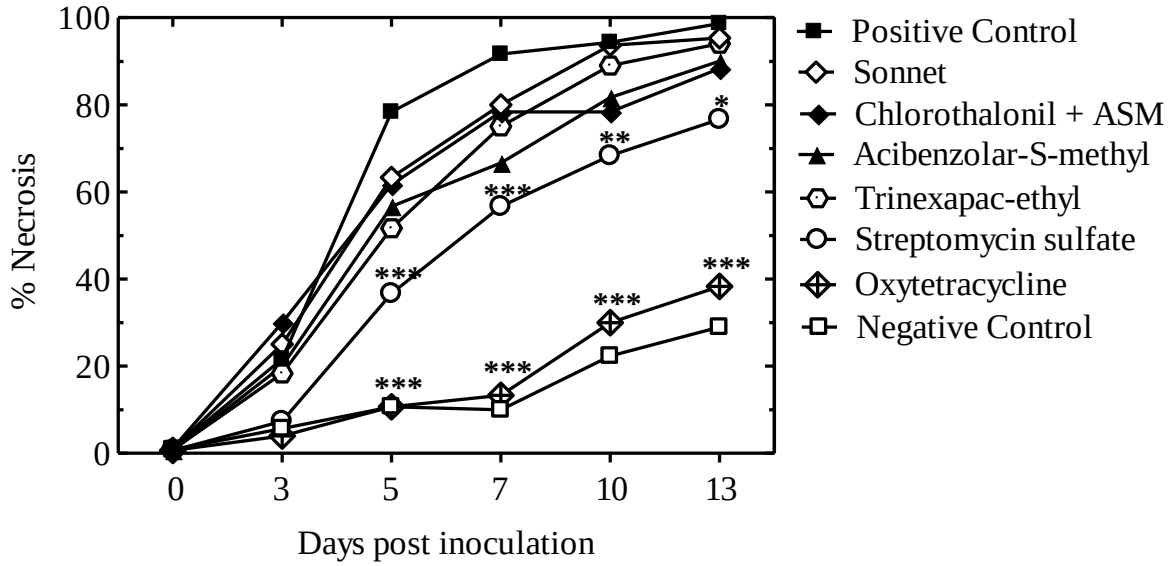
RESULTS

Growth Chamber Study

Pretreatment of cups prior to inoculation with *A. avenae* subsp. *avenae* resulted in significant treatment effects on percent turfgrass stand necrosis (Figure 4.1). Symptoms of leaf blight, yellowing and decline were evident 3 days post inoculation (dpi). A significant reduction in necrosis was observed in both the oxytetracycline and the non-inoculated untreated control (negative control) compared to the inoculated untreated control (positive control) 5 dpi ($P < 0.0001$). Both the oxytetracycline and the negative control treatments were lower in percent

necrosis compared to the positive control at 7, 10, and 13 dpi with a high degree of statistical separation ($P < 0.0001$) (Figure 4.1). Treatment with streptomycin sulfate also showed a slight but significant reduction in necrosis after inoculation with *A. avenae* subsp. *avenae* when compared to the positive control 5, 7, 10, and 13 dpi ($P < 0.0001$, 0.01, 0.01, and 0.05 respectively) (Figure 4.1). All other treatments tested in this study showed no significant difference with regard to symptom development compared to the inoculated control when pre-treated onto creeping bentgrass prior to inoculations with the MSU4 isolate of *A. avenae* subsp. *avenae* ($P > 0.05$).

Figure 4.1. Percent necrosis of creeping bentgrass (*Agrostis stolonifera* cv. 'Penn-A4') after inoculation with *Acidovorax avenae* subsp. *avenae* after and incubation at 30° C. All treatments were applied 48 hours prior to inoculation with the MSU4 isolate.



* Mean is significantly different than the inoculated untreated positive control according to Tukey's HSD (*, **, *** P < 0.05, < 0.01 and < 0.001 respectively).

ANOVA

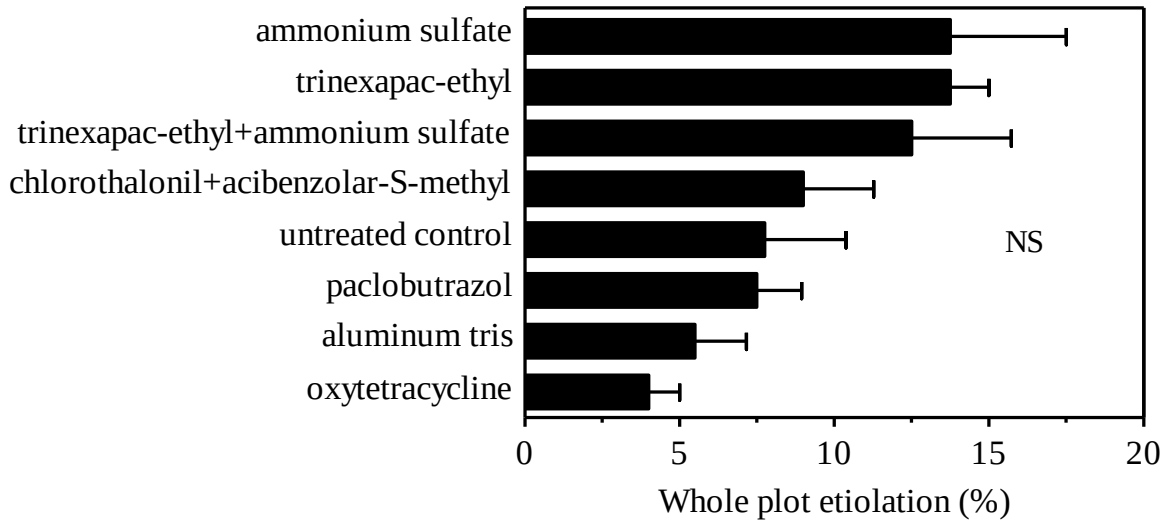
Source of variation	*df*	P > F
Treatment	14	< 0.0001
Day post inoculation (dpi)	5	< 0.0001
Treatment x dpi	70	< 0.0001

Field Study – Inverness C.C.

Naturally occurring symptoms of bacterial etiolation were first noticed in the study area on 7/22/2013. Disease symptoms were prevalent for a 10-day period in July when temperatures were most conducive. Symptoms consisted of typical chlorosis and stem elongation observed across the research plots, exclusively affecting the creeping bentgrass. Ratings were taken as whole plot etiolation percentages based on the amount of creeping bentgrass within each individual plot.

No significant differences in etiolation symptoms were observed among any of the treatments at Inverness C.C. when disease pressure was highest (Figure 4.2). Etiolation was visibly higher in several of the plots, particularly those treated with trinexapac-ethyl and ammonium sulfate. However, statistical separation among means did not exist. Stem elongation and chlorosis of individual plants within plots were the only observed symptoms. When symptoms of etiolation were present within the study, representative samples of affected turfgrass plants were selected from individual plots and confirmation of bacterial streaming as well as isolation of *Acidovorax* was achieved on SMART media (data not shown). Quality ratings showed no significant differences among treatments in overall turfgrass quality during peak disease pressure on 7/22/13 (Figure 4.3). Chlorosis and stem elongation were evident in many of the plots, however no decline or thinning of the creeping bentgrass was present in 2013 at the Inverness C.C. site. Turfgrass quality remained similar among all treatments for the duration of the study due to overall low disease pressure for the season (data not shown).

Figure 4.2. Percent plot etiolation on a mixed stand of annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis stolonifera* cv. 'Penn-G2') naturally infected with *Acidovorax avenae* subsp. *avenae* at Inverness C.C. in Toledo, Ohio. Ratings were taken on July 22, 2013 during peak disease pressure. Treatment applications began April 29, 2013 with subsequent 14-day repeat applications as described in "Materials and Methods".

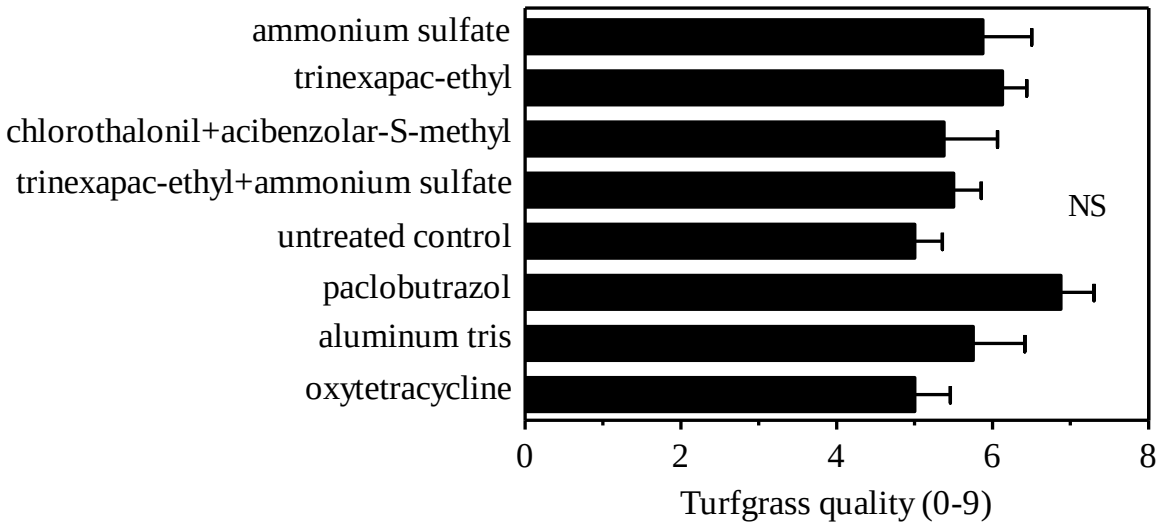


NS = no significant differences among treatment means according to Tukey's HSD (P > 0.05).
Error bars represent standard error of mean (n = 4).

ANOVA

Source of variation	df	P > F
Treatment	7	0.0410
Residual	24	

Figure 4.3. Turfgrass quality ratings on a mixed stand of annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis stolonifera* cv. 'Penn-G2') naturally infected with *Acidovorax avenae* subsp. *avenae* at Inverness C.C. in Toledo, Ohio. Ratings were taken on July 22, 2013 during peak disease pressure. Treatment applications began April 29, 2013 with subsequent 14-day repeat applications as described in "Materials and Methods".



NS = no significant differences among treatment means according to Tukey's HSD (P > 0.05).
Error bars represent standard error of mean (n = 4).

ANOVA

Source of variation	df	P > F
Treatment	7	0.2074
Residual	24	

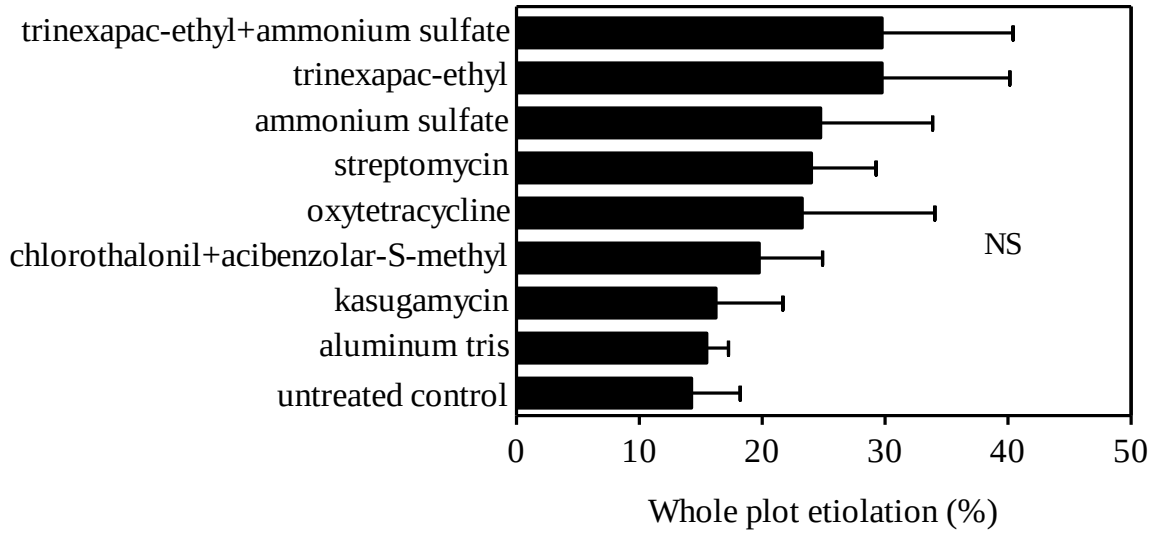
Field Study - Hancock Turfgrass Research Center 2012

In 2012, symptoms of etiolation were reproduced in field plots at the Hancock Turfgrass Research Center in East Lansing, Michigan with weekly inoculations of *Acidovorax avenae* subsp. *avenae (*MSU4). Stem elongation, abnormal growth, and chlorosis were first noticed across the study on 7/5/2012. Symptoms persisted during a 2-week span of unusually warm and humid weather in Michigan (See weather data, Figure 4.2). The inoculation and subsequent symptom development at the HTRC marked the first known reproduction of bacterial etiolation symptoms caused by *Acidovorax avenae* subsp. *avenae* in the field. Data taken during peak disease pressure are shown.

No significant differences in bacterial etiolation symptoms were observed among treatments at the HTRC site in 2012 ($P > 0.05$) (Figure 4.4). All plots including the untreated control showed relatively uniform symptoms of yellowing and abnormal elongation on the creeping bentgrass. The untreated control resulted in the lowest mean etiolation among any of the treatments, while other chemical products exacerbated symptoms. Treatments of trinexapac-ethyl, alone, or in combination with ammonium sulfate resulted in noticeably higher, yet statistically similar etiolation when compared to the untreated control and several of the other treatments (Figure 4.4).

During peak disease pressure, turfgrass quality was not significantly different among plots ($P > 0.05$) (Figure 4.5). All plots showed some level of etiolation with chlorosis and stem elongation; however, none of the treatments progressed to more detrimental advanced stages of decline.

Figure 4.4. Percent plot etiolation on a creeping bentgrass (*Agrostis stolonifera* cv. 'Independence') research area inoculated twice weekly with *Acidovorax avenae* subsp. *avenae* (MSU4) at the Hancock Turfgrass Research Center in East Lansing, Michigan. Ratings were taken on July 5, 2012 during peak disease pressure. Treatment applications began May 24, 2012 with subsequent 14-day repeat applications as described in "Materials and Methods".

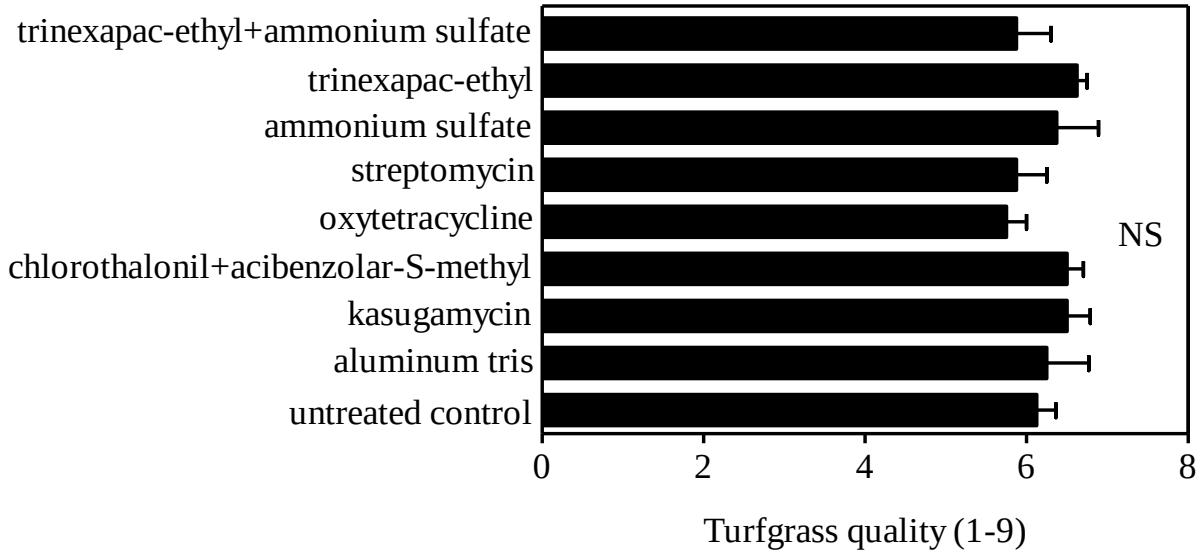


NS = no significant differences among treatment means according to Tukey's HSD (P > 0.05).
Error bars represent standard error of mean (n = 4).

ANOVA

Source of variation	df	P > F
Treatment	8	0.780
Residual	27	

Figure 4.5. Turfgrass quality on a creeping bentgrass (*Agrostis stolonifera* cv. 'Independence') research area inoculated twice weekly with *Acidovorax avenae* subsp. *avenae* (MSU4) at the Hancock Turfgrass Research Center in East Lansing, Michigan. Ratings were taken on July 17, 2012 after peak disease pressure. Treatment applications began May 24, 2012 with subsequent 14-day repeat applications as described in "Materials and Methods".



NS = no significant differences among treatment means according to Tukey's HSD (P > 0.05).
Error bars represent standard error of mean (n = 4).

ANOVA

Source of variation	*df*	P > F
Treatment	8	0.406
Residual	27	

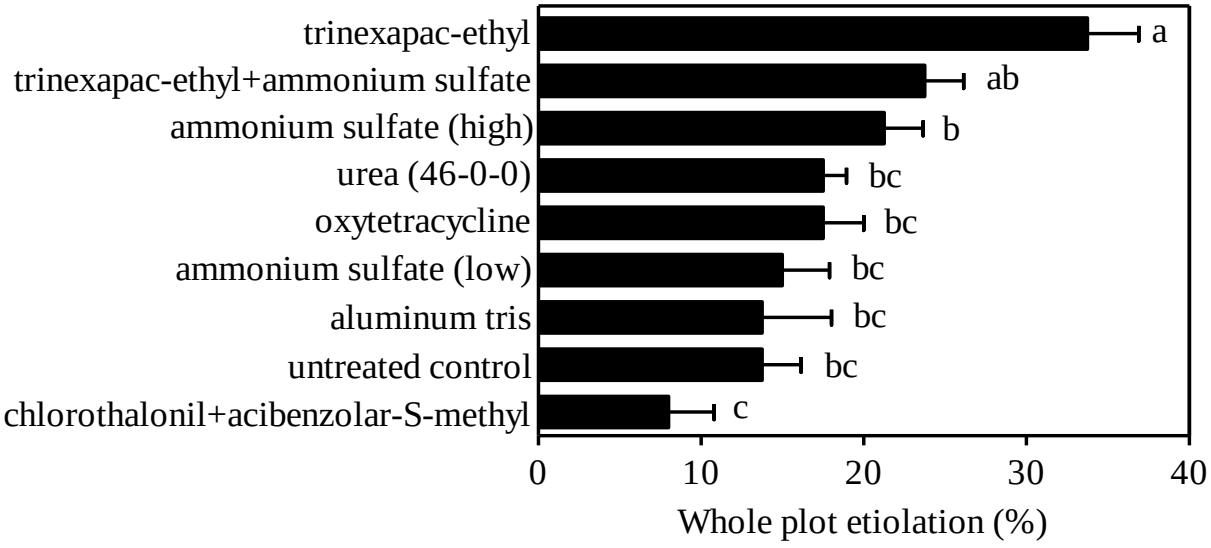
Field Study - Hancock Turfgrass Research Center 2013

Inoculations of *Acidovorax avenae* subsp. *avenae* (MSU4) onto newly established creeping bentgrass sod at the HTRC in 2013 resulted in a widespread outbreak of bacterial etiolation in late June and early July. Disease symptoms peaked on 6/26/2013 when weather conditions were conducive in the region, and subsided when temperatures cooled. Data shown were taken during peak disease pressure.

Significant differences in bacterial etiolation symptoms were observed among treatments in 2013 ($P < 0.05$) (Figure 4.6). The treatment of trinexapac-ethyl resulted in significantly higher incidence of chlorosis and abnormal stem elongation than all other treatments except the trinexapac-ethyl and ammonium sulfate combination treatment (Figure 4.6). The treatments that contained either trinexapac-ethyl or ammonium sulfate (high rate) resulted in significantly more bacterial etiolation than the chlorothalonil+acibenzolar-S-methyl treatment ($P < 0.05$) (Figure 4.6).

Significant differences existed with regard to symptoms of etiolation among treatments, however, no decline or necrosis was observed on the inoculated study at the HTRC in 2013. Turfgrass quality was not significantly different among any treatment for the duration of the study, and all treatments resulted in statistically similar ($P > 0.05$) turfgrass quality ratings during peak disease pressure in late June, 3013 (Figure 4.7).

Figure 4.6. Percent plot etiolation on a creeping bentgrass (*Agrostis stolonifera* cv. 'Independence') research area inoculated twice weekly with *Acidovorax avenae* subsp. *avenae* (MSU4) at the Hancock Turfgrass Research Center in East Lansing, Michigan. Ratings were taken on June 26, 2013 during peak disease pressure. Treatment applications began June 19, 2013 with subsequent 14-day repeat applications as described in "Materials and Methods".

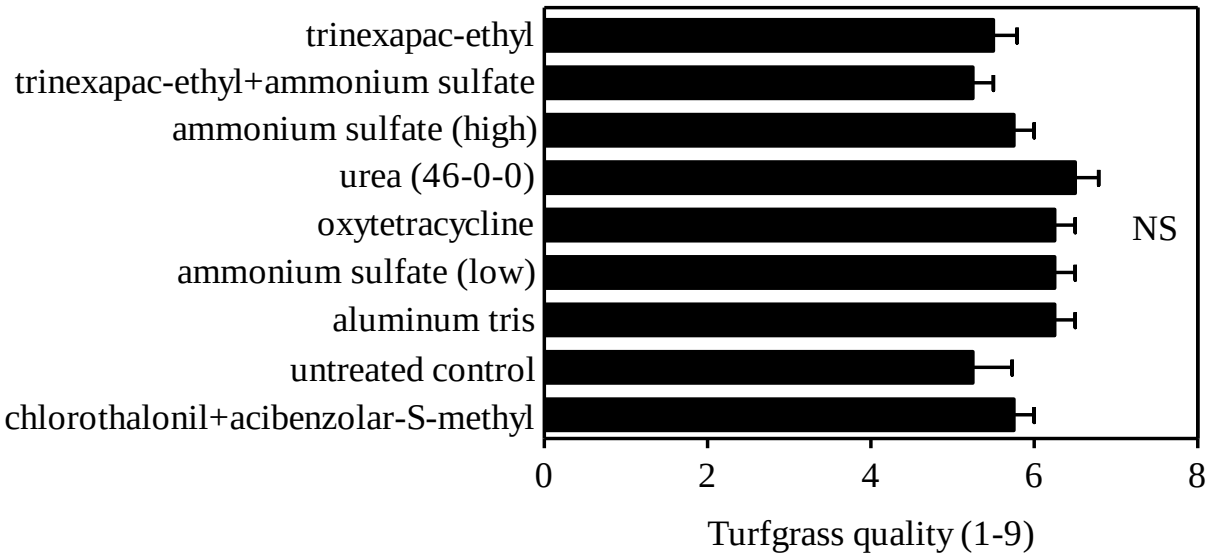


Means followed by different letters are significantly different according to Tukey's HSD ($P < 0.05$).
Error bars represent standard error of mean (n = 4).

ANOVA

Source of variation	*df*	P > F
Treatment	8	< 0.0001
Residual	27	

Figure 4.7. Turfgrass quality on a creeping bentgrass (*Agrostis stolonifera* cv. 'Independence') research area inoculated twice weekly with *Acidovorax avenae* subsp. *avenae* (MSU4) at the Hancock Turfgrass Research Center in East Lansing, Michigan. Ratings were taken on June 26, 2013 during peak disease pressure. Treatment applications began June 19, 2013 with subsequent 14-day repeat applications as described in "Materials and Methods".



NS = no significant differences among treatment means according to Tukey's HSD (P > 0.05).
Error bars represent standard error of mean (n = 4).

ANOVA

Source of variation	*df*	P > F
Treatment	8	0.019
Residual	27	

DISCUSSION

Acidovorax avenae subsp. *avenae* poses a serious problem to creeping bentgrass putting greens given the aesthetic and functional detriment associated with its infection. The current lack of knowledge related to treatment of bacterial etiolation and decline along with the overall misunderstanding of management practices that contribute to symptom development warrant further investigation into this novel disease system (9).

Inoculations with a virulent strain of *A. avenae* (MSU4) under conditions similar to the extreme summer environment in the "Transition Zone" of the United States have shown the pathogen to cause extensive necrosis and decline of creeping bentgrass (5). Pretreatment of creeping bentgrass with an array of chemicals in this study resulted in significantly reduced symptoms of necrosis and decline. Most notably the treatment of cups with a high labeled rate (200 ppm) of the agricultural antibiotic oxytetracycline protected plants from pathogen infection and severe disease onset. The antibiotic streptomycin sulfate also reduced symptoms significantly when compared to the untreated control, although necrosis ratings were still upwards of 40-70%. Only the oxytetracycline treatment provided acceptable levels of turfgrass quality after 14 days incubation at 30° C in both of the replicate growth chamber studies. Previous bacterial turfgrass disease research with *X. campestris* found high rates of oxytetracycline effective in preventing infection on *A. stolonifera* (12). Additionally, these results correlate with *in vitro* assays which found significant reductions of *A. avenae* growth on poison media amended with oxytetracycline but not streptomycin sulfate (data not shown). Although preventative treatment of turfgrass with antibiotics shows promise in stifling bacterial disease incidence, it is unlikely these products will ever be labeled for commercial use in amenity turfgrass systems. Additionally, the pretreatment of cups of creeping bentgrass with

high labeled rates of antibiotics such as oxytetracycline may show promise in thwarting subsequent infection from leaf inundation with *A. avenae* inoculum in a controlled environment, but translating and reproducing these results *in situ* with natural infection and field inoculation is imperative in order to make inferences toward potential remedial treatments for bacterial etiolation and decline.

The field studies undertaken mark the first reported reproduction of symptoms of etiolation through inoculation with *Acidovorax avenae* subsp. *avenae* onto a managed creeping bentgrass putting green. The study methodology and empirical confirmation of disease causation is significant for future research aimed at measuring control mechanisms for bacterial etiolation and decline. Field studies conducted herein rendered significant findings related to disease aggravation through repeated applications of commonly used turfgrass products. Although no treatment consistently controlled disease symptoms in any of the three reported studies, a common trend existed among the research that clearly supports anecdotal accounts from golf course superintendents battling this enigmatic disease.

Several turfgrass managers have indicated increased etiolation after applications of the growth regulator trinexapac-ethyl (Jeff Kent, personal communication). Additionally, acidifying nitrogen fertilizers, particularly ammonium sulfate, have been speculated to stimulate *Acidovorax*-mediated etiolation as well. In the three studies reported, symptoms of etiolation were markedly greater in plots treated with trinexapac-ethyl within the labeled rate every two weeks. However, other plant growth regulators such as paclobutrazol, did not show the same adverse effects on disease symptoms when used according to the manufacturer's recommendations. Similarly, ammonium sulfate-based nitrogen fertilizer exacerbated symptoms of etiolation when compared to most other treatments. Compared to a urea-based nitrogen source

standard, the ammonium sulfate treatment, with the same amount of total nitrogen, consistently resulted in higher mean etiolation ratings.

The exact role of plant growth regulators, particularly trinexapac-ethyl in bacterial etiolation and decline is not well understood (1). Trinexapac-ethyl is a late gibberellic acid (GA) synthesis inhibitor that is frequently applied to creeping bentgrass to reduce uneven shoot growth and improve ball roll distance (3). Whether the reduction of GA alters plant responses to *A. avenae* infection is not yet known. A potential problem with reducing GA could be the significant interaction between GA and pathogen response pathways in the plant which utilize salicylic acid (SA) and/or jasmonic acid (JA). The induced systemic resistance pathway, mediated by JA, protects plants from biotrophic pathogens (16). Since *Acidovorax* is mostly considered a biotroph, the inhibition of GA could ultimately result in a reduction of the JA signal, thus limiting the induced systemic response and increasing plant susceptibility to *Acidovorax* following PGR application. Similarly, the effects of ammonium sulfate alone or in synergy with GA inhibiting plant growth regulators on turfgrass *Acidovorax*-associated etiolation must be further investigated. Many potential mechanisms may be responsible for stimulating bacterial growth within the plant microenvironment. Whether a reduction in pH or the presence and utilization of the particular ammoniacal nitrogen source is responsible for disease perturbation remains unclear.

The results presented herein provide a foundation for future management and recommendations of bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae.* Although investigations into pathogen detection and etiology are discussed in previous chapters, the true mechanism and cause of disease severity remains unclear. Future research on bacterial pathogens of turfgrass including *Acidovorax avenae* subsp. *avenae* must focus on

integrated management of the disease. Most results have indicated a general increase in symptoms when attempting to treat with common plant health products such as fungicides and fertilizers.

With no reliable treatment available, current recommendations for managing bacterial etiolation and decline are to cease unnecessary chemical inputs, raise the height of cut, and abstain from stress-inducing mechanical practices particularly during hot, humid weather conditions. Additionally, based on the data collected from several field studies, as well as observations from consultations and visits to numerous golf courses, use of trinexapac-ethyl is not advised on creeping bentgrass putting greens when disease is active, or during the hot weather of summer. Moreover, there appears to be potential for ammonium sulfate to exacerbate disease symptoms during peak pathogen activity. Therefore, urea-based nitrogen sources for foliar feeding applications may serve as a better choice on at-risk putting greens, particularly during stressful

APPENDIX

Table A-4.1. SMART *Acidovorax* media used for isolation from infected creeping bentgrass tissue. Recipe adopted exactly from Kawanishi et al., 2011.

SMART-Aac medium plate
For *Acidovorax avenae*

L-methionine	1 g
Na2HPO4	3 g
KH2PO4	3 g
NH4Cl	1 g
MgSO4	0.25 g
FeSO4	5 mg
Crystal violet	3 mg
Agar	15 g
Deionized water	1 L
Cycloheximide	50 mg
Ampicillin	10 mg
Cetrimonium	10 mg
Polymixin	10 mg

Figure A-4.1. Maximum air temperatures between May 15 and September 15 for 2012 and 2013 growing seasons. Data was collected from the weather station located at the Hancock Turfgrass Research Center in East Lansing, Michigan and retrieved from the Michigan State University Enviro-Weather website (http://www.agweather.geo.msu.edu).

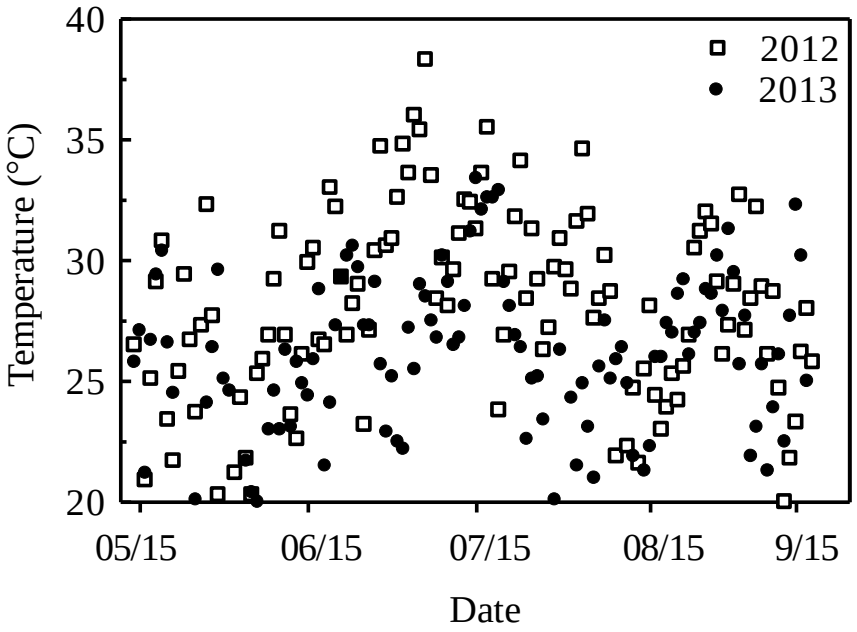
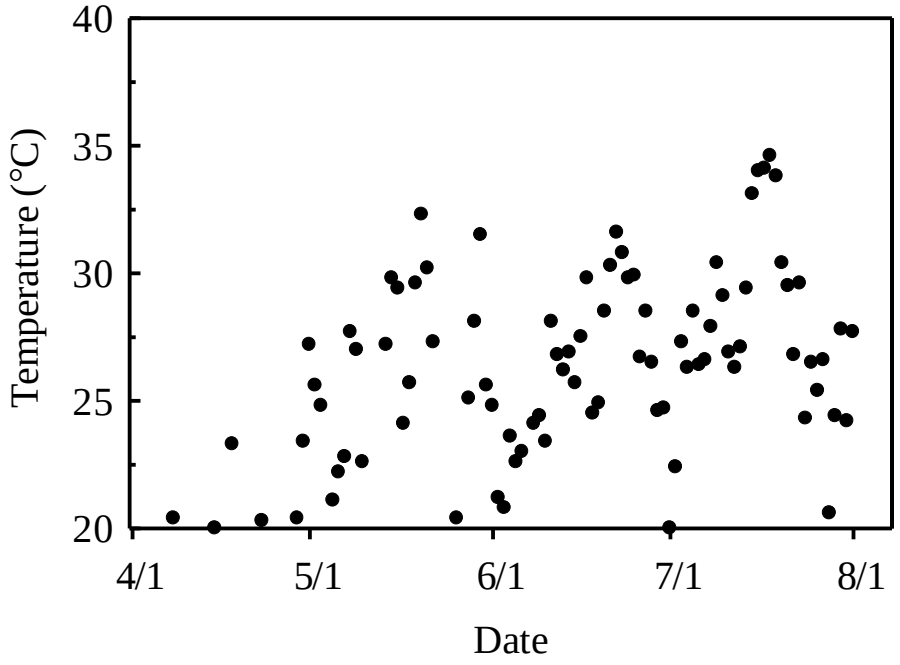


Figure A-4.2. Maximum air temperatures between April 1 and August 1, 2013 at Inverness C.C. in Toledo, OH. Data was collected from the weather station located at Cliley Farms in Dundee, Michigan, and retrieved from the Michigan State University Enviro-Weather website (http://www.agweather.geo.msu.edu).



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CHAPTER FIVE

PYLOGGENETIC RELATIONSHIPS AND HOST SPECIFICITY OF *ACIDOVORAX AVENAE* SUBSP. *AVENAE*: TOWARDS SPECIFIC MOLECULAR DIAGNOSIS OF THE TURFGRASS PATHOGEN

ABSTRACT

Acidovorax avenae subsp. *avenae* (*Aaa*) is the causal agent of bacterial etiolation and decline, causing considerable damage to creeping bentgrass putting greens across the United States. The emergence and spread of this pathogen is not well understood, and reliable identification currently is based on laborious and antiquated culture methods. In order to gain a better understanding of *Aaa* from turfgrass, 28 isolates collected from infected samples around the U.S. were evaluated in a seven-gene multilocus sequence analysis (MLSA) and compared to 17 other *Acidovorax* species and subspecies. Maximum Likelihood and Bayesian analyses rendered two well-supported clades of *Aaa* strains originating from diseased turfgrass distinct from other *Acidovorax* spp. from grass family hosts. Multi-gene phylogenetic relationships were used to select 15 representative isolates for whole-genome sequencing. Draft genome assemblies were used as input into a novel, alignment-free, primer design pipeline. Predicted primer set specificity to turfgrass pathogenic *Aaa* was validated via traditional PCR and a real-time PCR assay was developed. Primer sets 0017 and 0019 coupled with an internal oligo probe showed optimal sensitivity and specificity when evaluated with the target pathogen, closely related bacterial species and a number of other microorganisms that inhabit the same host and soil environment. Utilization of this assay in diagnostic laboratories will enable turfgrass managers to more quickly and effectively detect and potentially reduce unneeded fungicide usage through early and accurate identification of the pathogen.

INTRODUCTION

Since its initial identification in 2009, *Acidovorax avenae* subsp. *avenae* has emerged as a serious pathogen of creeping bentgrass (*Agrostis stolonifera* L.) (3). The disease caused by *A. avenae* subsp. *avenae* (*Aaa*) known as bacterial etiolation and decline, has been diagnosed on dozens of golf courses around the U.S. (4). Diagnoses of the disease have been confounded by several factors; first, bacterial diseases on turfgrass are very rare. Only one other major disease of turfgrass is caused by a bacterial pathogen which affects annual bluegrass (*Poa annua* L.) known as bacterial wilt caused by *Xanthomonas translucens* pv. *poae,* thus, turfgrass diagnosticians are typically not adequately equipped for bacterial identification. Second, *Acidovorax* pathogens are often seed-borne (5, 32, 41), and while this has not been confirmed in creeping bentgrass, the pathogen does not always produce distinct symptoms under field conditions. The disease is often very difficult to diagnose, and in such cases diagnosis must rely on time-consuming isolation and pathogenicity testing. Finally, the turfgrass pathogenic *Acidovorax* is closely related genetically to other plant pathogenic members of the *Acidovorax* genus including *A. citrulli*, *A. cattleyae*, *A. facilis, A. konjaci,* and other *Acidovorax avenae* subsp. *avenae* pathogens which are known to infect many different graminaceous hosts, but are often heterogeneous, displaying a range of host specificity (20, 36).

The ability to distinguish accurately among bacterial strains is important in determining the emergence and evolution of pathogens. DNA fingerprinting using techniques such as pulse-field gel electrophoresis and repetitive extragenic palindromic polymerase chain reaction (REP-PCR) have been previously employed with different members of the *Acidovorax* genus (35, 40, 42). Unfortunately these methods have several drawbacks, including poor reproducibility between and within labs, and an inherent inability to quantify genetic relationships (35).

Although amplified fragment length polymorphism (AFLP) and PFGE can be highly discriminating, they are unable to determine genetic relationships among and between strains at deeper phylogenetic levels (19).

Multilocus sequence analysis (MLSA) is a powerful tool for identifying and fingerprinting strains of bacteria based on the principles of multilocus enzyme electrophoresis (8, 16, 27). Nucleotide sequences from 5 to 10 protein coding genes are used as a conserved, reproducible model for bacterial profiling. An existing MLSA gene set was developed by Feng et al., (2009) to characterize the relationship among *Acidovorax citrulli* clonal complexes. The genes chosen were based on an MLSA scheme developed for *Burkholderia pseudomallei* (18), and were initially evaluated based on their divergence among two available *Acidovorax* genomes (*A. citrulli* AAC00-1 and *Acidovorax* sp. JS42). Feng el al., (2009) provides an informative analysis of relationships among *A. citrulli* species from different geographic regions; however, the study only resolves phylogenetic relationships among the two clades of *A. citrulli*, using other plant pathogenic members of the *Acidovorax* genus as outgroups.

The ability of diagnostic techniques such as real-time quantitative PCR (qPCR) to deliver sensitive and quantifiable results is dependent on the availability of primer sets that distinguish a target organism or organisms from non-target organisms (21). The design of discriminatory primer sets is typically guided by common sequences in the target organisms, but divergent, or absent, in non-target organisms. The most commonly used sequences include intergenic transcribed spacer regions, ribosomal DNA, 'housekeeping' genes and virulence genes (6, 28, 29, 39). Diagnostic amplification of DNA from members of the *Acidovorax* genus by conventional PCR has been largely based on the sequence of the 16S-23S ribosomal DNA intergenic transcribed spacer region (ITS) (33), or unique DNA fragments from profiles using

rep-PCR (1). Primers have been described for many plant pathogenic bacteria including *Acidovorax* at the genus level (34), however, the *Acidovorax*-specific primers react with all species and subspecies of *Acidovorax* and are not specific to strains pathogenic only to grass species, particularly turfgrass. An important advantage of a real-time PCR assay is its rapidity (31). The time necessary to obtain definitive results with molecular methods is less than one working day. Conversely, isolation on agar media requires at least several days.

This study was undertaken to investigate the genetic relationships among newly identified pathogenic *Acidovorax avenae* subsp. *avenae* on turfgrass to closely related plant pathogenic members of the genus *Acidovorax*. Employing MLSA using a subset of seven genes from Feng et al., (2009), and a representative set of taxa within the *Acidovorax* genus including 28 isolates originating from naturally infected stands of creeping bentgrass, the aim is to develop a robust phylogenetic topology that can be exploited for further genomic investigations. Using this information, whole genome sequencing was implemented on select divergent isolates within the genus to identify candidate primer sets that discriminate between arbitrary subgroups of the sequenced bacteria. The sensitively, specificity, and rapidity of the molecular methods refined in this study will not only allow for a tool to detect and diagnose infection by the pathogen, but will be useful in ecological studies of the bacterium i.e. to determine when and where the pathogen proliferates under certain conditions.

MATERIALS AND METHODS

Strains used and DNA extraction

A total of 45 strains of *Acidovorax* spp. were studied in the MLSA. This includes 11 strains of *A. avenae* subsp. *avenae* from five different Poaceae hosts (rice, millet, sorghum, vasseygrass, and maize), and 28 strains of *A. avenae* subsp. *avenae* isolated from naturally

infected creeping bentgrass (*Agrostis stolonifera* L.). Outgroups chosen for the phylogenetic analysis consisted of four strains of *A. citrulli* from melon, and one strain each of *A. konjaci* and *A. cattleyae* originally isolated from konjac and orchid respectively; all of which are now classified as individual species, and infect hosts outside of the Poaceae family (30) (Table 5.1). All isolates from creeping bentgrass were obtained from diseased turfgrass samples sent to the MSU turfgrass diagnostic laboratory between 2009 and 2012. All other *Acidovorax* isolates were obtained from Dr. Ron Walcott at the University of Georgia except for isolates 1-8 in Table 5.1, in which nucleotide sequences for individual genes were obtained from an *Acidovorax* MLST database at (http://genome.ppws.vt.edu/cgi-bin/MLST/search_alleles.pl).

Prior to all experiments, bacteria were grown on King's B medium (13) and DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN Inc., San Diego, CA) according to manufacturer's protocol. All 28 strains of *A. avenae* subsp. *avenae* from turfgrass were determined to be pathogenic by inoculating creeping bentgrass cups as described (4).

Multilocus sequence analysis

Table 5.2 indicates the set of genes and associated primers adopted in this study from the original MLSA by Feng et al., (2009). Genes from bacterial isolates originating in this study were PCR amplified in 25 µl reaction mixtures containing DNA template at 5 to 20 ng/µl, 0.2 µM each primer, and 12.5 µl of a 2x MeanGreen PCR Master Mix (Syzygy Biotech, Grand Rapids, MI). All reaction tubes were placed in an Applied Biosystems GeneAmp 2700 thermocycler96 programmed as follows: 35 cycles of 95°C for 30 s for denaturing, 60°C for 30 s for primer annealing, and an extension period at 72°C for 30 s. The final step was an extension period at 72°C for 5 min. Purification of the PCR products was done with Exonuclease I (Fermentas Life Sciences, Vilnius, Lithuania) and FastAP Thermosensitive Alkaline Phosphatase

(Fermentas Life Sciences, Vilnius, Lithuania) according to the manufacturer's protocol. DNA was recovered, standardized to 10 ng/µl via NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and sequenced at the Michigan State University Research Technology Support Facility using an ABI 3730 automated sequencer.

Table 5.1. Isolate, 16S rDNA identification, geographic origin and host of plant pathogenic *Acidovorax* species and subspecies investigated in this study.

Taxon name	I.D. (16S rDNA)	Geographic Origin	Host of Origin
AA _30003	*Acidovorax avenae* subsp. *avenae*	Japan	Rice
AA _30015	*Acidovorax avenae* subsp. *avenae*	Nigeria	Rice
AA _30044	*Acidovorax avenae* subsp. *avenae*	Nigeria	Millet
AA _30179	*Acidovorax avenae* subsp. *avenae*	Brazil	Sorghum
AA _30296	*Acidovorax avenae* subsp. *avenae*	Japan	Rice
AA _30297	*Acidovorax avenae* subsp. *avenae*	Japan	Rice
AA _30298	*Acidovorax avenae* subsp. *avenae*	Japan	Rice
AA _30305	*Acidovorax avenae* subsp. *avenae*	United States	Vassey grass
AA78_5	*Acidovorax avenae* subsp. *avenae*	Georgia, USA	Maize
AA81_1	*Acidovorax avenae* subsp. *avenae*	Georgia, USA	Maize
AA99_2	*Acidovorax avenae* subsp. *avenae*	Georgia, USA	Maize
CAT98_1	*Acidovorax avenae subsp. cattleyae*	California, USA	Orchid
CIT00_1	*Acidovorax citrulli*	Georgia, USA	Melon
CIT92_17	*Acidovorax citrulli*	Georgia, USA	Melon
CIT92_300	*Acidovorax citrulli*	Georgia, USA	Melon
CIT96_9	*Acidovorax citrulli*	Georgia, USA	Melon
KONJ	*Acidovorax konjaci*	Japan	Konjac
MSU_1	*Acidovorax avenae* subsp. *avenae*	North Carolina, USA	Creeping bentgrass
MSU_13	*Acidovorax avenae* subsp. *avenae*	North Carolina, USA	Creeping bentgrass
MSU_14	*Acidovorax avenae* subsp. *avenae*	North Carolina, USA	Creeping bentgrass
MSU_15	*Acidovorax avenae* subsp. *avenae*	Idaho, USA	Creeping bentgrass
MSU_16	*Acidovorax avenae* subsp. *avenae*	Indiana, USA	Creeping bentgrass
MSU_17	*Acidovorax avenae* subsp. *avenae*	Maryland, USA	Creeping bentgrass
MSU_18	*Acidovorax avenae* subsp. *avenae*	Ohio, USA	Creeping bentgrass
MSU_19	*Acidovorax avenae* subsp. *avenae*	Michigan, USA	Creeping bentgrass
MSU_2	*Acidovorax avenae* subsp. *avenae*	Texas, USA	Creeping bentgrass
MSU_4	*Acidovorax avenae* subsp. *avenae*	Texas, USA	Creeping bentgrass
MSU_8	*Acidovorax avenae* subsp. *avenae*	Indiana, USA	Creeping bentgrass
MSU_9	*Acidovorax avenae* subsp. *avenae*	Ohio, USA	Creeping bentgrass
URI_1	*Acidovorax avenae* subsp. *avenae*	Connecticut, USA	Creeping bentgrass
URI_10	*Acidovorax avenae* subsp. *avenae*	North Carolina, USA	Creeping bentgrass
URI_11	*Acidovorax avenae* subsp. *avenae*	North Carolina, USA	Creeping bentgrass
URI_14	*Acidovorax avenae* subsp. *avenae*	Rhode Island, USA	Colonial Bentgrass
URI_15	*Acidovorax avenae* subsp. *avenae*	North Carolina, USA	Creeping bentgrass
URI_16	*Acidovorax avenae* subsp. *avenae*	Michigan, USA	Creeping bentgrass
URI_17	*Acidovorax avenae* subsp. *avenae*	Rhode Island, USA	Creeping bentgrass
URI_18	*Acidovorax avenae* subsp. *avenae*	New York, USA	Creeping bentgrass
URI_2	*Acidovorax avenae* subsp. *avenae*	Massachusetts, USA	Creeping bentgrass
URI_3	*Acidovorax avenae* subsp. *avenae*	Illinois, USA	Creeping bentgrass
URI_4	*Acidovorax avenae* subsp. *avenae*	New Jersey, USA	Creeping bentgrass
URI_5	*Acidovorax avenae* subsp. *avenae*	New York, USA	Creeping bentgrass
URI_6	*Acidovorax avenae* subsp. *avenae*	Virginia, USA	Creeping bentgrass
URI_7	*Acidovorax avenae* subsp. *avenae*	Ohio, USA	Creeping bentgrass
URI_8	*Acidovorax avenae* subsp. *avenae*	Pennsylvania, USA	Creeping bentgrass
URI_9	*Acidovorax avenae* subsp. *avenae*	Massachusetts, USA	Creeping bentgrass

Sequences were visualized, manually edited, and trimmed in the CLC Workbench package (v. 4.0, CLC bio). Subsequent multiple sequence alignments for all individual genes were performed with ClustalW in MEGA5 (14) using the default settings. The first step of the phylogenetic analysis was to investigate the potential incongruence among three randomly chosen genes from the seven-gene MLSA scheme developed by Feng et al. (2009). Single-gene phylogenies were constructed for the *gltA*, *gmc*, and *lepA* loci using optimality criteria of maximum likelihood in MEGA5. The model of sequence evolution was determined using the likelihood ratio test and selecting the model with the lowest Bayesian Information Criterion (BIC) for each gene. Tree space was searched heuristically, and reliability was assessed using bootstrap re-sampling of 100 replicates. Congruence tests among the three loci (gltA, gmc, and lepA) were carried out in PAUP (v. 4.0) (37) using the Hompart function with 1000 replications and seed value of 123. A heuristic search for homogeneity of sequences was conducted employing the TBR swapping algorithm.

Concatenated 7-gene phylogenetic analysis.

Maximum likelihood analysis resulted in ML trees for the three individual genes (*gltA*, *gmc*, *lepA*), the concatenated 3-gene sequence, and the 7-gene concatenated sequence as a comparison (Appendix 5). In each case, the optimality criterion for ML was determined using MEGA5 to find the model of sequence evolution through likelihood ratio tests. The model with the lowest Bayesian inference criterion (BIC) in each of the five different analyses was chosen and used as a parameter in ML analysis. Treespace was searched using a Close-Neighbor-Interchange heuristic method and all codons (1^{st}, 2^{nd}, 3^{rd}, non-coding) were included.

Bayesian method of phylogenetic inference was also employed (17, 22) and implemented in MrBayes 3.0 (26). For the full 7-gene concatenated set, a GTR model was assumed with estimated parameter values for base frequencies, proportion of invariant sites, and for the shape of the gamma distribution with six categories. Ten million generations of two simultaneous Markov chain Monte Carlo chains were run, and trees were sampled every 500 generations with heated chains at a temperature of 0.10 and a swap frequency of one. For burn-in, the first 250,000 generations were discarded (likelihood values stabilized after the first 100,000 generations). The remaining trees were computed into a majority-rule consensus with the percent of times a clade occurred among the sampled trees as the posterior probability of that clade existing (11).

Whole genome sequencing

Genomic DNA was extracted from 15 strains of *Acidovorax* spp. (Table 5.3) using the QIAGEN protocol described above. Isolates were selected based on their phylogenetic relationships in the previous MLSA, host specificity, and availability of genomic sequence information in public databases. Genomic DNA was submitted to the Michigan State University Research Technology Support Facility for sequencing and assembly. Sequencing libraries were prepared using standard Illumina TruSeq DNA Sample Prep Kit v2. Paired end (PE) 2 x 250 bp sequencing was performed on the Illumina MiSeq (MiSeq Control Software v 2.2.0) with MiSeq Reagent Kit v2. Image analysis and base calling were performed on-board with Real Time Analysis (RTA) software v1.17.28. BCL to FASTQ conversion and de-multiplexing were carried out offline with CASAVA v1.8.2.

Adapter and quality trimming was done with Trimmomatic (v0.30). Read pairs with overlapping 3' ends were identified and stitched together into pseudo long reads with

FLASH (v1.2.6). Non overlapping read pairs and these pseudo long reads were aligned to the *Acidovorax avenae* reference genome (subsp. *avenae* ATCC 19860, NCBI accession # NC_015138.1) using bwa (v0.7.5a). Variant calling and filtering was performed with Genome Analysis Toolkit (GATK v2.4-9). The UnifiedGenotyper tool of GATK was used for variant calling with ploidy set to one. *De novo* assembly was performed using velvet (v1.2.7). For each strain a range of kmers were scanned from 23 to 93 in steps of 10. From the set of assemblies for each strain the optimal kmer assembly was chosen based on maximization of the length of long scaffolds while minimizing the total number of scaffolds making up the majority of the assembly. All sequenced strains and their identifiers for this study are indicated in Table 5.3, additional information pertaining to sequence and assembly statistics can be found in Appendix 5.

Table 5.2. Names, functions, lengths, and polymerase chain reaction primers for the seven protein coding genes adopted for analysis of isolates in this study from Feng et al., (2009) in a multi-locus phylogenetic analysis of *Acidovorax* spp.

Gene name	Function	Length (bp)	Primer sequences
gmc	Glucose-methanol-choline oxidoreductase	484	5'-TGGTTGACCTC*AAA*TAGCC- 3' / 5'-TTTCGACTTCATCGTCATCG-3'
ugpB	Extracellular solute-binding protein, family I	452	5'-TGAAGG*AAA*TCTCGGTCGTC-3' / 5'-CTTGACGTCGTTGCTGAAGA-3'
pilT	Twitching motility protein	404	5'-GAGTACATCTGCGCCACCTT-3' / 5'-AATACGGGCACATCCTGAC-3'
lepA	GTP-binding protein	489	5'-GATCGACACGCCCGGACAC-3' / 5'-TGATGTAGCCCACCTCGCC-3'
trpB	Tryptophan synthase subunit beta	439	5'-GCCACTTCGGCCGCTATG-3' / 5'-CCTCGTTGAGCGCATCCTT-3'
gltA	Type II citrate synthase	484	5'-GAAGTCCACGTTCGGGTAGA-3' / 5'-ACATGTACCCGCAGAACCA-3'
phaC	Poly(R)-hydroxyalkanoic acid synthase, class I	431	5'-ATCGCCAACCTGCTGCAC -3' / 5'-GAACGTGGTGAGGAAGGTGG-3'

Table 5.3. Bacterial strains selected for whole genome sequencing, 16S DNA identification, host of origin, and source of isolates.

Strain name	16S rDNA Identification	Host of Origin	Source
AA78-5	*Acidovorax avenae* subsp. *avenae*	Maize	R. Walcott (9)
AA99-2	*Acidovorax avenae* subsp. *avenae*	Maize	R. Walcott (9)
CAT98-1	*Acidovorax avenae* subsp. *cattleyae*	Orchid	R. Walcott (9)
MSU4	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
MSU16	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
MSU9	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
URI7	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
MSU17	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
MSU18	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
URI15	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
URI11	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
MSU1	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
URI5	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
URI14	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study
URI8	*Acidovorax avenae* subsp. *avenae*	Creeping bentgrass	This study

Primer prediction pipeline

The primer design pipeline was adopted from Pritchard et al., (2012) with minor adjustments for the purposes of this study. A comprehensive description of the methodology can be found in the supplementary materials of Pritchard et al., (2012). Briefly, a configuration file specifying the location of input sequences of each isolate in FASTA format, and the species classification of that isolate, was provided to the primer design script, (available for download at https://github.com/widdowquinn/find_differential_primers). A single pseudochromosome was compiled for the set of draft contigs belonging to each isolate. Prediction of coding sequences was carried out on the pseudochromosome using PRODIGAL (12) where necessary. 20,000 primer sets were predicted for each isolate pseudochromosome using EPRIMER3 (23), keeping only the primer sets that exist entirely within a predicted CDS. Functional annotation of the CDS to which primers were designed was not carried out. PRIMERSEARCH (23) was used to identify cross-amplification of each isolate. A filter for off-target amplification was implemented by BLASTING primer sets against sequenced Enterobacteriaceae and other Beta Proteobacteria (but not *Acidovorax*) genomes. Primer sets were classified according to their ability to amplify host (turfgrass) specific *Acidovorax* species. The results were written to a summary output file. Thermodynamics specified for amplification primers during the prediction phase included a T_m between 58-60°C (59°C optimal), a GC content between 30-80% with no more than two G+C in last five nt at 3' end. Runs of identical nucleotides were avoided, and sequence constraints were applied during the prediction process to design sets of amplicons of length of 150-250 bp. Primer sets contained flanking primer sequences only, or flanking primer sequences and an internal oligomer for post-amplification hybridization. Hybridization probes were specified to have a length of 13-30 bp with a T_m between 68-70°C. Non-redundant,

putative turfgrass pathogenic *A. avenae* subsp. *avenae* specific primer sets are indicated in Table 5.4.

Primer evaluation

Seven bacterial isolates were first screened with each of the 20 putative specific primer sets; two target isolates of *Acidovorax avenae* subsp. *avenae* MSU4 and URI1, and five non-target isolates including *Acidovorax avenae* subsp. *avenae* AA81-1 and AA99-2 (non-target *A. a. avenae*), *Acidovorax cattleyae* CAT98-1 (non-target *Acidovorax* sp.), *Acidovorax citrulli* CIT00-1 (non-target *Acidovorax* sp.), *Acidovorax konjaci* KON (non target *Acidovorax* sp.), and *Xanthomonas translucens* pv. *poae* PAR5 (non-target turf pathogen). Once screened, primers with specificity to target turfgrass isolates were further tested against a broader range of target and non-target bacterial isolates listed in Table 5.1 as well as several other bacterial organisms isolated from turfgrass soil and debris. Reactions of 25 µl contained DNA template at 5 to 20 ng/µl, 0.2 µM each primer, and 12.5 µl of a 2x MeanGreen PCR Master Mix (Syzygy Biotech, Grand Rapids, MI). Annealing temperature was set to 60°C due to the proposed optimal annealing temperature for each primer being predicted near 60°C by the pipeline. Standard cycling conditions included one cycle of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute; one cycle of 72°C for five minutes. Products were resolved on a 1% agarose gel in tris-EDTA (TE) buffer at 0.5x concentration, alongside a 1 kb+ ladder (Life Technologies). The images were captured under standard conditions in a gel documentation system at 300 dpi and saved in TIFF format. Image manipulations (combination of multiple gel results into single figure were carried out using Adobe Illustrator.

Further assay optimization was carried out with chosen primer sets (0003, 0010, 0017, 0019) utilizing a gradient range of annealing temperatures. Briefly, 10 ng of genomic DNA from

target (MSU1, MSU4, MSU14, MSU18) and non-target (AA78-5, CAT98-1, CIT00-1, FAC96-2) isolates was used in PCR preparations according to the aforementioned protocol. The PCR was run using a gradient of four annealing temperatures including 58°C, 60°C, 64°C and 66°C using the following cycling conditions on an Eppendorf Mastercycler nexus thermal cycler: initial denaturing at 94°C for 90 sec; followed by 35 cycles of 94°C denaturing for 15 sec, gradient temperature (°C) annealing for 20 sec, and 72°C extension for 30 sec with a final cycle of 72°C for 7 min. Gel electrophoresis and band imaging were performed as mentioned above. Further testing of all target and non-target isolates on hand (listed in Figure 5.2) was carried out with the primer sets 0017 and 0019 and performed under the protocol described above with the selected annealing temperature of 64°C during PCR thermal cycling.

Real-time qPCR evaluation

Two primer sets (0017 and 0019) with specificity to turfgrass pathogenic *A. avenae* subsp. *avenae* via conventional PCR were selected for further evaluation in real-time qPCR assay development. The Primer Express 3.0 software package (Applied Biosystems, Foster City, California) was used for the TaqMan probe design. Candidate probe sequences were BLAST-queried against the GenBank database for Enterobacteriaceae as well as the genomic sequences derived in this study, and *A. avenae* subsp. *avenae* - specific oligonucleotides were selected. The TaqMan probe: 6FAM-TACGGGTACGCCCACAA-MGBNFQ, was synthesized by Life Technologies (http://www.lifetechnologies.com) and consists of a minor DNA groove binding quencher. The ZEN probe: 6FAM-ACCGTCGCTTACCATCACCGTAGATTCG-/3IABkFQ/ was synthesized by Integrated DNA Technologies (http://www.idtdna.com) and incorporates an additional internal ZEN quencher. Real-time PCR assays for detecting *Aaa* were performed in 25 µl reaction mixtures containing 2 µl template DNA, 20 µM each primer, 10 µM TaqMan

probe and 2.5 uL TaqMan Universal PCR Master Mix (Applied Biosystems, California). Each sample was run as three technical replicates. Amplifications were carried out on an ABI Step One Plus real–time PCR system (Applied Biosystems, California) using the program of an initial denaturing step at 95° C for 10 min followed by 40 cycles, each consisting of denaturing at 95° C for 15 s and extension at 60° C for 60 s.

Standard regression curves were plotted for tenfold serial dilutions of *A. avenae* DNA against cycle threshold (Ct) values. Every set of reactions also included a water control to verify no contamination of reagents with template DNA.

DNA extraction from turfgrass samples

To further examine assay detection specificity and sensitivity *in vivo*, eight samples of turfgrass from various sources listed in Table 5.6 were prepared for DNA extractions. Briefly, 15-20 mg of stem and leaf tissue was macerated in bead mill homogenizing tubes on a mini bead beater for 1 min in 1.5 ml of AP1 buffer. DNA was prepared using the Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions. Concentrations of DNA were measured using a ND-1000 spectrophotometer (ThermoFisher Scientific) and DNA sample preps were used directly in real-time PCR assays. Real-time assays were conducted according to the specifications indicated above.

Table 5.4. *Acidovorax* PCR primers used in validation studies. Primer sequences were predicted by the pipeline and used in validation assays for the detection of turfgrass pathogenic strains of *Acidovorax avenae* subsp. *avenae*. Predicted primers in the table were non-redundant pairs selected from the complete set of predicted primers with desired specificity.

Set name	Forward primer	Reverse primer
001	5'-GCATGGAAATGCACGTAGAG-3'	5'-GCTGAAGCAGTACCCTTTCC-3'
002	5'-ATCGATGTGATCACCTGTGG-3'	5'-TTCTTCGTCGCATAACGAAC-3'
003	5'-TCACCTACACTCTGGCGAAG-3'	5'-GTGGAAGGGTGACGAGACTT-3'
004	5'-GGTAGCACGACTCCACCTCT-3'	5'-ATGGAACTCTTCAGGGCAGT-3'
005	5'-GCCATATCGCAAGAATTGTG-3'	5'-TTAGCCCAAATCAGCAACTG-3'
006	5'-TTTGACGCTTTGTTCGAGTC-3'	5'-CCTCGACTTCCAAGTTCACA-3'
007	5'-AGATTAGGCGACGGTTGAGT-3'	5'-GGTGCCTCTTATGGGTGATT-3'
008	5'-TTTGACGCTTTGTTCGAGTC-3'	5'-CGACTTCCAAGTTCACATGC-3'
009	5'-CATGGAAATGCACGTAGAGC-3'	5'-GCTGAAGCAGTACCCTTTCC-3'
0010	5'-TAGAGCGCTTGCTGGTAGTG-3'	5'-ATCCTATGCCAACAGCACAG-3'
0011	5'-ACCGAAGAGAAGGTCGTCAC-3'	5'-CGACGATCCAGATCACGTAG-3'
0012	5'-AGAGAAGGTCGTCACCATCC-3'	5'-CGACGATCCAGATCACGTAG-3'
0013	5'-CCAAGTTCACATGCTCCAAT-3'	5'-CCGGTACTTTGACCCTTTGT-3'
0014	5'-CTGGAACGCTCTGATGAAGA-3'	5'-AGAGCTTCACACGATTCACG-3'
0015	5'-CCTTCGTTGTGTTTCTGGTG-3'	5'-GCCATGAGCATGTAGCTGTT-3'
0016	5'-TTTGACGCTTTGTTCGAGTC-3'	5'-ACCTCGACTTCCAGGTTCAC-3'
0017	5'-GCGCAAAGATGTAGGTTTGA-3'	5'-AGTCCGCCCAATACGATTAC-3'
0018	5'-TTGGAGCAACGAATGATTTC-3'	5'-TTCAACGGTGTATTGCCATT-3'
0019	5'-GGAGCGCAAAGATGTAGGTT-3'	5'-AGTCCGCCCAATACGATTAC-3'
0020	5'-CCGGTACTTTGACCCTTTGT-3'	5'-GCTTCCAAGTTCACATGCTC-3'

RESULTS

Multilocus sequence analysis

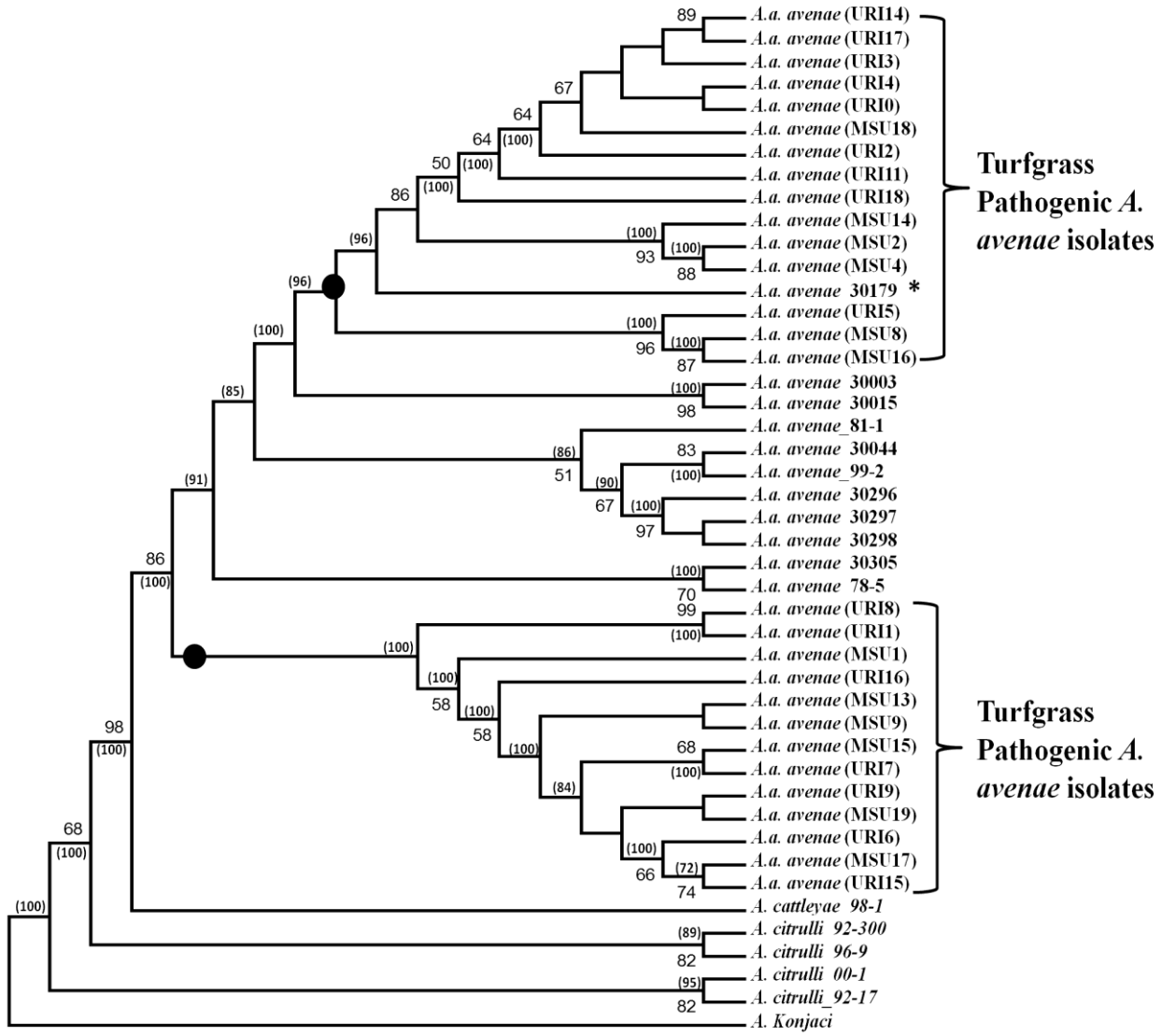
Analysis of three individual genes (*gltA, gmc,* and *lepA*) rendered three unique ML trees with different topologies (Appendix 5). To assess the degree of support for the partitions in each of the trees, 100 bootstrap replicates were generated and summarized as 50% majority-rule consensus trees. Partition homogeneity tests among the three genes demonstrated a high degree of difference in phylogenetic signal based on the P-value in each comparison being 0.001. This significance indicates a rejection of the null hypothesis with a high degree of confidence that there phylogenetic signal among the individual genes used is significantly different. However, combining and expanding the phylogenetic analysis by concatenating the three-and seven-gene sets respectively resulted in a progressively more robust resolution of *Acidovorax* isolates into distinct clades with clearly defined relationships traced to host specificity (Appendix 5).

When all seven genes are included in the analysis, the maximum likelihood bootstrap consensus tree showed strong support for the a group of *Aaa* turfgrass isolates forming a clade near the top of the tree (86%), as well as strong support for the larger clade consisting of all *Aaa* isolates (86%) (Figure 5.1). Strains of *Acidovorax* isolated from turfgrass grouped together in two distinct clades, both unique from other *A. avenae* subsp. *avenae* isolates from other grass hosts such as corn and rice with the exception of *A.a. avenae*_30179 from sorghum which was placed among the top turfgrass clade although with less than 50% ML BS support (Figure 5.1). The rest of the *A. avenae* subsp. *avenae* isolates were placed between the two turfgrass clades with strong support for inner nodes, but weak-to-no ML BS support for the outer nodes of these groupings. Additionally, the outgroups of *A. cattleyae*, *A. citrulli*, and *A. konjaci* are distinct from the *A. avenae* isolates with 98% ML BS support.

Bayesian analysis rendered a similar tree to ML analysis for the 7-gene MLSA (not shown) with two distinct clades of *A. avenae* subsp. *avenae* from turfgrass. Other *Acidovorax avenae* taxa from monocot hosts similarly formed unique clades of their own, sister to the turfgrass strains. Bayesian analysis provided much stronger posterior probability support for many of the inner and outer nodes of the ML tree. Posterior probability from Bayesian analysis is shown in parentheses on nodes in Figure 5.1. The sorghum strain, *A.a*. *avenae*_30179 placed among the upper clade of turfgrass isolates which was not well supported by ML BS analysis, is supported by a 96% posterior probability after Bayesian analysis (Figure 5.1). Strong (> 70%) support in the entire concatenated 7-gene tree indicates a high degree of confidence and a robust representation of the phylogenetic relationships rendered in the resulting topology. No trends or groupings appear to exist based on geographic location or virulence among the turfgrass isolates of *Aaa* from this analysis. The information gained from the multi-gene phylogenetic analysis however allowed for the informed selection of a representative group of isolates for whole-genome sequencing and subsequent investigation into unique and potentially discriminatory regions for molecular diagnostics.

Twelve isolates of turfgrass pathogenic *Aaa* were selected for whole genome sequencing. Six isolates were selected from one of the clades containing turfgrass pathogenic *Aaa* and six chosen from the other clade based on the results of the 7-gene MLSA. The other three isolates sequenced were selected based on their phylogenetic and taxonomic relationship to the turfgrass pathogenic isolates and their availability for this project.

Figure 5.1. Maximum likelihood tree for the 7-gene concatenated MLSA set.



Tree was inferred using the Maximum Likelihood method and the best fit DNA model as determined by the Model Selection tool in MEGA5 set to default parameters. The best fit model was the Tamura 3-parameter (T92 + G+ I) model. All codon positions were included. Tree is a bootstrap consensus tree inferred from 500 replicates with numbers corresponding to partitions reproduced in more than 50% of the bootstrap replications are shown. Numbers in parentheses indicate posterior probabilities derived from Bayesian analysis of two independent MCMCMC runs with 10,000,000 generations in MrBayes 3.0. Tree with the highest log likelihood is shown (-12662.14 ML and -9644.245 Bayes). Black circles indicate groupings of isolates pathogenic to turfgrass with * indicating the exception within the turf clade of the sorghum isolate of *A. avenae* subsp. *avenae*.

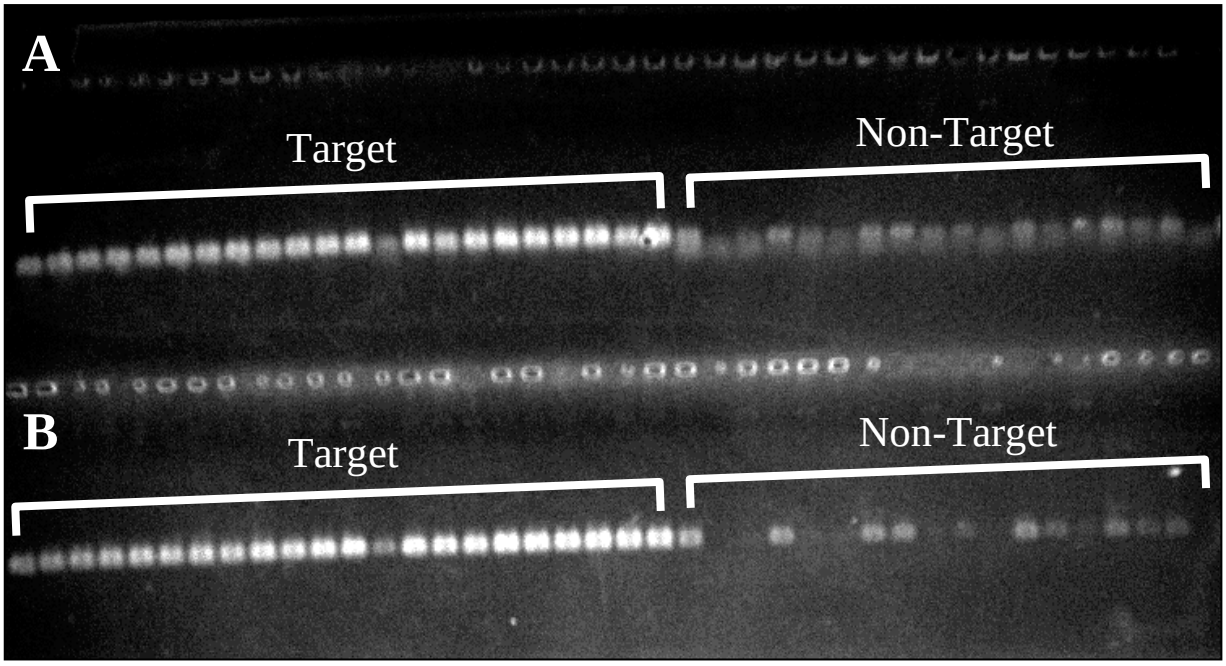
Whole genome sequencing, primer prediction, and PCR validation

The 15 draft genome assemblies of *Acidovorax* spp. were used as input to the primer prediction pipeline with host of origin (i.e. target classification) as indicated in Table 5.1. Two runs of primer design were performed, both generating primers with hybridization oligos screening against a BLAST database comprising all chromosome and plasmid sequences from all Enterobacteriaceae in the September 2013 GenBank database at ftp://ftp.ncbi.nih.gov/genomes/bacteria. A total of 68 primer pairings were proposed as putative discriminatory diagnostic primers for turfgrass pathogenic isolates. After discarding redundant primer sets, 20 pairs remained for further testing.

Primers that amplified each of the initial 2 target turfgrass strains with adequate intensity and little-to-no amplification of the 6 non-target bacterial DNA were subjected to a broader range of isolate testing via traditional PCR. Primer sets 0017 and 0019 produced positive PCR results with each of the 22 evaluated target strains giving a single band of the expected product size of 200 bp (Figure 5.2). Under the pre-determined annealing temperature of 60°C, both sets produced faint positive results with other *Acidovorax* spp. and subspecies, as well as other bacterial organisms assessed in this study (Figure 5.2). Gradient thermal cycling with annealing temperatures ranging from 58° – 66°C determined specific binding to only target DNA in turfgrass isolates to take place at the annealing temperature of 64°C. At 66°C, sensitivity of the PCR assay was lost in several of the target isolates (data not shown). Further confirmation of specificity of both the 0017 and 0019 primer sets at 64°C showed only amplification of DNA from target isolates with complete absence of banding from non-target bacterial isolate DNA (Figure 5.3). Adjustment of the annealing temperature provided the desired specificity of the

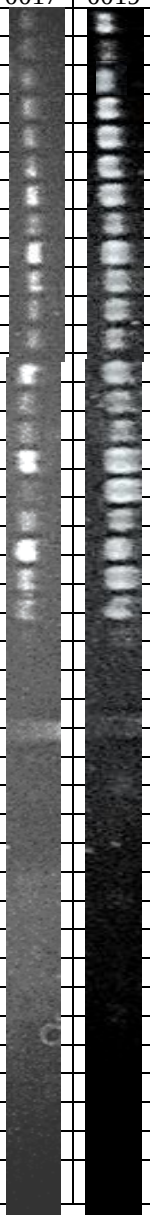
primers to turfgrass pathogenic strains of *Acidovorax avenae* subsp. *avenae* while limiting non-specific, or phantom banding from the non-target bacterial hosts (Figure 5.3).

Figure 5.2. PCR amplification of target and non-target DNA from bacterial isolates using select primer sets designed from whole genome sequences of different *Acidovorax* spp.



All reactions took place using the annealing temperature specified by the primer design pipeline (60°C). **A.** Amplification of target isolates (left) and non-target isolates (right) using the primer set 0017. **B.** Amplification of target isolates (left) and non-target isolates (right) using the primer set 0019. Note the intensified banding on target isolates and subtle banding on non-target isolates.

Table 5.5. PCR amplification from target and non-target bacterial isolates grouped by primer set with optimized primer annealing temperature.

Isolate	Primer Set		Isolate I.D.	Host	Target
	0017	0019			
URI_15			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_17			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_17			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
UR_I7			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_9			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_5			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_13			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_18			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_19			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_16			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_1			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_4			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_6			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_10			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_14			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_9			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_18			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_3			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_1			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_8			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_5			*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_3			*Ochrobactrum pseudogrignonense*	Creeping bentgrass	-
negative			n/a	n/a	-
negative			n/a	n/a	-
1 Kb+ ladder			n/a	n/a	-
AA99-2			*Acidovorax avenae subsp. avenae*	Maize	-
AA81-1			*Acidovorax avenae subsp. avenae*	Maize	-
AA78-5			*Acidovorax avenae subsp. avenae*	Vasseygrass	-
FAC96-2			*Acidovorax facilis*	Soil environment	-
FAC94-9			*Acidovorax facilis*	Soil environment	-
CAT98-1			*Acidovorax cattleyae*	Orchid	-
CIT00-1			*Acidovorax citrulli*	Melon	-
CIT96-9			*Acidovorax citrulli*	Melon	-
CIT92-17			*Acidovorax citrulli*	Melon	-
KON			*Acidovorax konjaci*	Konjac	-
MSU_6			*Microbacterium binotii*	Creeping bentgrass	-
MSU_7			*Stenotrophomonas maltophilia*	Creeping bentgrass	-
MSU_11			*Herbaspirillum seropedicae*	Creeping bentgrass	-
XAN-1			*Xanthomonas translucens pv. poae*	Annual bluegrass	-
XAN-2			*Xanthomonas campestris pv. translucens*	Perennial ryegrass	-
negative			n/a	n/a	-

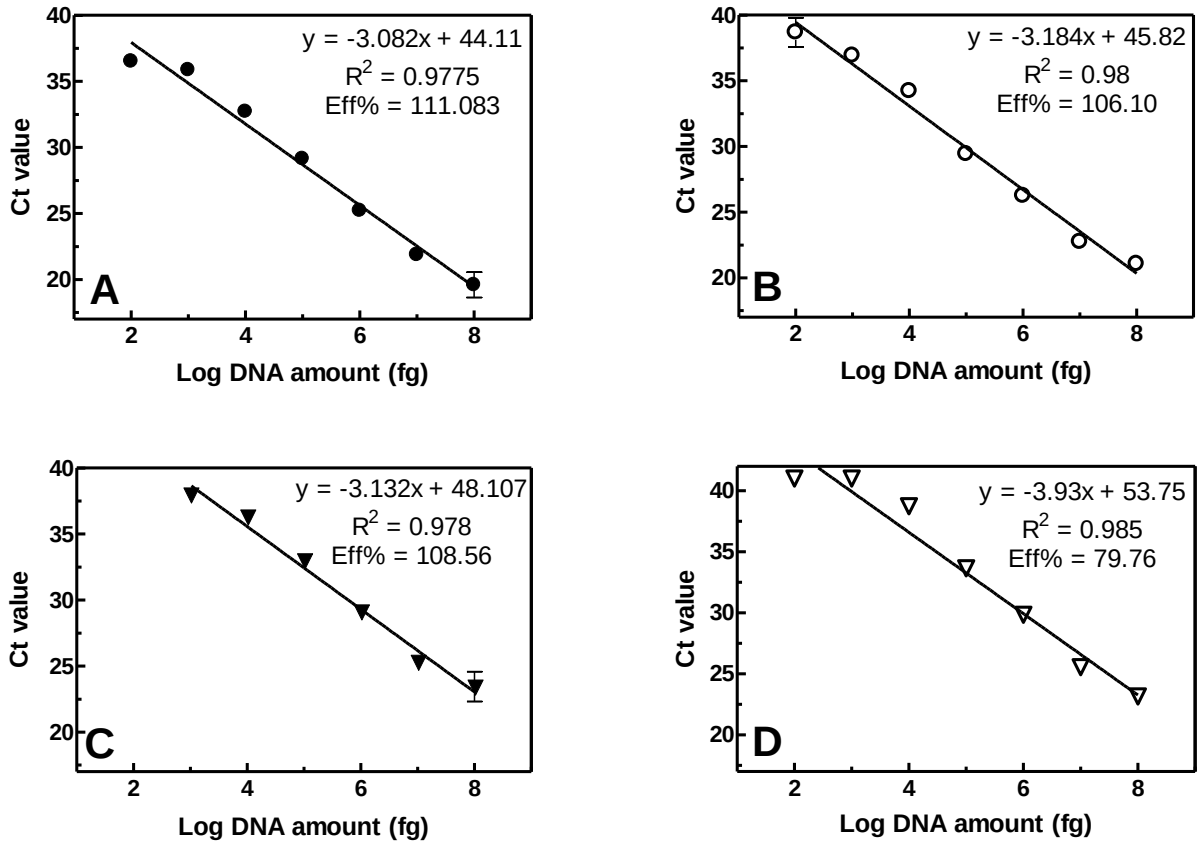
Images are from agarose gels that have been aligned against the matching isolate designation (leftmost column) and below the primer names (top row). The image is split so that target isolates of pathogenic *Acidovrax avenae* subsp. *avenae* originating from creeping bentgrass are located in the top section. The bottom section includes several different non-target *Acidovorax* spp. as bacterial organisms isolated from the turfgrass phyllosphere and rhizosphere including two pathogenic *Xanthomonas* spp. The two sections are bound by a 1 kb+ ladder. Both primer sets show results after optimization of annealing temperature at 64°C during thermal cycling.

Real-time PCR

Detection limits for real-time PCR were determined with 10-fold serial dilutions of 100 ng initial *A. avenae* subsp. *avenae* genomic DNA under conditions described above. Both primer sets and the two different probes (TaqMan and ZEN) were used in real-time PCR assays. The regression curves between the DNA amounts and the Ct values were linear: for 0017/TaqMan, $y = -3.18x + 45.82$ ($R^2 = 0.980$) with a PCR efficiency of 111.08, and for 0017/ZEN, $y = -3.082x + 44.11$ ($R^2 = 0.9775$) with a PCR efficiency of 106.10 (Figure 5.3). For 0019/TaqMan, $y = 3.93x + 53.75$ ($R^2 = 0.985$) with a PCR efficiency of 208.56 and for 0019/ZEN $y = -3.132x + 48.11$ ($R^2 = 0.978$) with a PCR efficiency of 79.76 (Figure 5.3). Although the real-time PCR assays could detect concentrations as low as 0.1 pg pure *A. avenae* subsp. a*venae* DNA at Ct values of 35 or higher, we considered samples with Ct values greater than 31 as *Aaa*-negative based on our preliminary tests to ensure detection specificity. Therefore, the actual detection limit for the real-time PCR assay is between 10 -100 pg *Aaa* DNA, based on formula of efficiency.

The bacterial isolates listed in Table 5.6 were used to assess the specificity of the real-time PCR assay. Both assays (0017 and 0019 primer sets) conducted using the ZEN probe produced positive results with all eight target *A. avenae* subsp. *avenae* from turfgrass ($Ct < 31$), and negative results with all other bacterial species tested ($Ct > 31$) (Table 5.6). The assays conducted with the TaqMan probe, however, showed reduced specificity to some of the target isolates, particularly for isolate URI_1. Nonetheless, overall Ct values in all of the assays remained below the determined limit of detection ($Ct < 31$) for target isolates, and above the limit of detection for non-target organisms.

Figure 5.3. Real-time PCR standard curves of the log of *Acidovorax avenae* subsp. *avenae* (MSU4) input genomic DNA concentration versus the corresponding cycle threshold (Ct) values. The DNA concentration was 0.1 pg – 100 ng. Standard errors are labeled for each data point.



A. Standard curve for primer set 0017 with the ZEN probe. **B.** Standard curve for the primer set 0017 with the TaqMan probe. **C.** Standard curve for the primer set 0019 with the ZEN probe. **D.** Standard curve for the primer set 0019 with the TaqMan probe. Note: The values of the standard errors are small and may be difficult to view.

Table 5.6. Isolate name, sensitivity and specificity of select qPCR primers and probes on DNA preparations from target and non-target bacterial organisms.

Isolate	Real-time PCR results[a]				Species[d]	Host	Target
	primer set						
	0017		0019				
	probe type						
	TaqMan[b]	ZEN[c]	TaqMan	ZEN			
MSU_9	22.8	22.1	23.6	22.7	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_1	34.1	29.7	30.3	26.4	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_18	27.9	23.7	29.8	25.5	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_16	28.6	24.3	30.0	25.6	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_4	28.6	24.7	28.8	25.0	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
MSU_14	28.0	23.9	30.8	26.5	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_18	20.9	20.4	20.8	20.2	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
URI_5	28.6	24.8	29.6	25.9	*Acidovorax avenae subsp. avenae*	Creeping bentgrass	+
AA99-2	ND	38.7	ND	ND	*Acidovorax avenae subsp. avenae*	Maize	-
AA81-1	ND	38.7	ND	ND	*Acidovorax avenae subsp. avenae*	Maize	-
AA78-5	35.8	34.6	36.4	37.8	*Acidovorax avenae subsp. avenae*	Vasseygrass	-
CAT98-1	39.5	36.5	ND	ND	*Acidovorax cattleyae*	Orchid	-
CIT00-1	ND	39.0	ND	ND	*Acidovorax citrulli*	Melon	-
CIT96-9	36.7	35.3	39.2	35.6	*Acidovorax citrulli*	Melon	-
CIT92-17	36.7	36.5	ND	39.5	*Acidovorax citrulli*	Melon	-
FAC94-9	37.1	35.2	39.2	36.3	*Acidovorax facilis*	Soil	-
FAC96-2	34.1	32.8	34.6	33.5	*Acidovorax facilis*	Soil	-
KONJ	ND	ND	ND	ND	*Acidovorax konjaci*	Konjac	-
MSU_3	33.9	32.0	34.2	33.0	*Ochrobactrum pseudogrignonense*	Creeping bentgrass	-
MSU_6	38.6	37.1	39.8	38.4	*Microbacterium binotii*	Creeping bentgrass	-
MSU_7	32.1	31.1	32.9	31.4	*Stenotrophomonas maltophilia*	Creeping bentgrass	-
XAN-1	39.4	37.9	ND	ND	*Xanthomonas translucens pv. poae*	Annual bluegrass	-
XAN-2	34.9	32.9	35.2	33.6	*Xanthomonas campestris pv. translucens*	Perennial ryegrass	-

[a] Mean real time PCR Ct value of two technical replicates.

[b] TaqMan probe was standard hydrolysis probe with an MGB quencher to inhibit fluorescence

[c] Zen probe was a double quenched TaqMan probe with additional internal ZEN quencher.

[d] Species were originally identified via 16S rDNA sequencing (see: Giordano et al., 2012).

ND = Not determined due to lack of amplification

In vivo detection of *Aaa* in symptomatic turfgrass tissues was assessed using the above real-time qPCR assays. *Acidovorax avenae* was positively detected (Ct < 31) from 4 of 4 suspected infected samples (HTRC_2012, IL_HIN, HTRC_2013, LVL12) using all four primer/probe assay combinations (Figure 5.7). In positive detection experiments, Ct values correlated with visual disease symptoms, intensity of microscopic bacterial streaming and confirmation of *A. avenae* subsp. *avenae* via selective media plating (i.e. lower Ct values from heavily infected samples). Negative results (Ct > 31) were obtained from four samples (GLET10, BNTSD1, GLNRM, BCCX) which were included based on their symptomology and origin. The GLET10 sample displayed symptoms of etiolation and chlorosis, however failed to produce signs of bacterial streaming or result in isolation of *A. avenae* onto selective media. The BNTSD1 sample was from newly established creeping bentgrass seedlings and showed no symptoms or signs of bacterial infection. The GLNRM sample was from an adjacent, asymptomatic area of a golf course putting green displaying etiolation symptoms. This sample showed no signs of disease. The BCCX sample originated from a perennial ryegrass golf course fairway displaying symptoms of etiolation and decline. Heavy bacterial streaming was detected and isolations of *Xanthomoans campestris* pv. *translucens* were achieved however, no *Acidovorax avenae* was isolated from this sample.

Table 5.7. Specific detection of *Acidovorax avenae* subsp. *avenae (Aaa)* with designed primers and probes from frozen and fresh turfgrass samples.

Sample	Real-time PCR results[a] primer set 0017 probe type TaqMan[b]	0017 ZEN[c]	0019 TaqMan	0019 ZEN	Description	Host	Isolation of *A. avenae* subsp. *avenae* (*Aaa*)[d]
HTRC_2012	25.7 (+)	24.6 (+)	23.9 (+)	23.5 (+)	Research plot, putting green turf, inoculated with *Aaa*, etiolation, streaming (+), frozen	Creeping bentgrass	+
HTRC_2013	19.6 (+)	19.2 (+)	18.5 (+)	20.6 (+)	Research plot, putting green turf, inoculated with *Aaa*, etiolation, streaming (+), frozen	Creeping bentgrass	+
LVL12	20.4 (+)	19.8 (+)	18.9 (+)	21.7 (+)	Golf course, putting green turf, etiolation, decline, streaming (+), frozen	Creeping bentgrass	+
IL_HIN	22.0 (+)	21.4 (+)	20.8 (+)	22.4 (+)	Golf course, putting green turf, etiolation, decline, streaming (+), frozen	Creeping bentgrass	+
GLET10	37.4 (-)	37.1 (-)	38.8 (-)	ND (-)	Golf course, putting green turf, etiolation, streaming (-), frozen	Creeping bentgrass	-
BNTSD1	37.5 (-)	37.2 (-)	37.6 (-)	ND (-)	5-week-old seedlings, greenhouse grown, no symptoms, streaming (-), fresh	Creeping bentgrass	-
GLNRM	37.0 (-)	36.9 (-)	38.6 (-)	ND (-)	Golf course, putting green turf, adjacent to etiolated area, no symptoms, streaming (-), frozen	Creeping bentgrass	-
BCCX	31.8 (-)	31.0 (-)	31.1 (-)	31.3 (-)	Golf course, fairway turf, etiolation, streaming (+), isolated and confirmed *Xanthomonas campestris* pv. *translucens*, frozen	Perennial ryegrass	-

[a] Mean real time PCR Ct value of two technical replicates.

[b] TaqMan probe was standard hydrolysis probe with an MGB quencher to inhibit fluorescence

[c] Zen probe was a double quenched TaqMan probe with additional internal ZEN quencher.

[d] If bacterial infection was suspected, isolation attempts took place using serial dilutions of macerated tissue onto selective media for *A. avneae* spp. + = isolation and identification of *A. avenae* subsp. *avenae* via 16S rDNA sequencing.

ND = Not detected due to lack of amplification

Samples consisted of 15-20 mg of fresh or frozen plant material. All Ct values were derived from two technical replicates of DNA preparations.

DISCUSSION

Very little information exists pertaining to the newly identified pathogenic strains of *Aaa* isolated from infected golf course samples around the U.S. Proper identification and diagnosis of *Aaa* in turfgrass systems is imperative for proper management recommendations and foundational epidemiological research.

This study was undertaken to first investigate the relationship among different *Aaa* strains isolated from different plant hosts. In particular, the phylogenetic placement of the newly identified pathogenic strains attacking creeping bentgrass in the United States. The previously employed 7-gene MLST scheme was used as a template to explore genetic relationships among these bacterial organisms. The adoption of the pre-existing scheme lead to initial investigations into the appropriateness of the gene set chosen by Feng et al., (2009) for phylogenetic analysis.

The individual gene trees indicate a disparity in topology among the isolates. The lack of congruence among the genes indicates a strong probability of supporting incorrect relationships among the taxa when using a small number of genes. The source of incongruence is unknown at this point; however, there are several putative explanations including genetic drift, different evolutionary histories, hybridization, and horizontal gene transfer among others. Rokas et al. (25) explained that two genes with different evolutionary histories for a particular taxonomic group, will by definition be incongruent while still depicting true histories. Here, we validated that theory by using three incongruent genes, concatenating them, and conducting phylogenetic analyses that supported (relatively strongly) a single phylogeny.

Concatenation of the three individual genes rendered a majority of the taxa grouped in or near their respective host of origin clades in both ML and Bayesian analyses. When all seven genes were used as a comparison, the ML and Bayesian trees both showed strong bootstrap

support for a majority of the taxa groupings. Additional to the strong bootstrap and posterior support, many of the resulting groupings make biological sense with regard to host of origin and pathogenic specificity. One point of contention which requires further investigation is the clear separation among the two clades of *Aaa* from turfgrass. Even though these isolates are grouped within the larger *A. avenae* clade, there are well supported phylogenetic differences among them, indicating that either pathogenicity on turfgrass is polyphyletic, or the determinants of pathogenicity within this group of isolates cannot be parsed out with the sampling of seven genes used in this analysis.

Much like the study conducted by Rokas et al. (2003), it appears that the more genes added to an analysis the more robust the sequence analysis becomes for predicting the phylogenetic relationships among the taxa. The unreliability of single-gene data sets (or sets composed of linked genes, such as genes from the mitochondria or other organelles) stems from the fact that each gene is shaped by a unique set of functional constraints through evolution. Such problems can be avoided by genome-wide sampling of independently evolving genes. The results of this study have aided in our understanding of the emergent turfgrass pathogenic strains of *Aaa*, and have allowed for a phylogenetically directed selection of particular isolates for further analysis with whole-genome sequencing. The ultimate goal of this project was to develop specific and sensitive primer to be used in a rapid and reliable molecular diagnostic assay.

The techniques employed herein exemplify the utility of high throughput sequencing in the development of discriminatory diagnostic primer sets. The sets of primers in the output from the Python script pipeline all showed some level of discriminatory ability and specificity to the predefined group of input sequences. Validation of select primers against a wide array of target and non-target bacterial isolates confirmed specificity of the primer sets which can separate very

closely related strains. Isolates of *A. avenae* subsp. *avenae* from corn, rice, sorghum, and other grass family hosts as well as other *Acidovorax* species were not amplified during PCR assays under optimal primer annealing conditions. Some differences in the intensity of the PCR bands were evident among target turfgrass pathogenic *Aaa* isolates tested, although the vast majority were strongly positive, indicative of high concentration of amplified product.

An essential aspect of this project was the diagnostic capability of the designed discriminatory primers, and the subsequent development of a reproducible assay to be used among the turfgrass industry for specific detection of pathogenic *Aaa*. Real-time qPCR has become a popular diagnostic tool for rapid and sensitive detection and quantification of pathogens in various host samples (7, 10, 15, 38, 43). The primers and probes designed in this study allowed us to detect *Acidovorax avenae* subsp. *avenae*, the causal agent of bacterial etiolation and decline, in stems and leaves of creeping bentgrass plants. The real-time PCR assays described here were all relatively sensitive. The best performing assay in terms of specificity and sensitivity utilized primer set 0019 and the double quenched ZEN probe. It can detect as little as 10 pg of pathogenic *A. avenae* genomic DNA in turfgrass samples, equivalent to 1956 genomes.

The 0019/ZEN assay developed here is also highly specific. All samples of *Aaa* tested from various creeping bentgrass sources yielded unambiguous positive detection with Ct values < 27. The other *Acidovorax* species and turfgrass bacterial inhabitants used in this study produced no fluorescence signal or Ct values > 31 and were considered negative.

Further validation of the real-time PCR assay involved testing DNA from samples of live and frozen turfgrass stems and leaves collected from symptomatic and asymptomatic turfgrass between 2012 and 2013. Results were compared with visual estimates of disease severity at the

time of collection, microscopic observations of bacterial streaming, and isolation onto selective media and molecular identification via 16S rDNA sequencing. The real-time PCR assay detected *Aaa* in 4 of the 8 samples. These results agreed not only with the symptoms of etiolation and decline observed among the samples, but with presence of bacterial streaming from tissues determined by microscopic detection and isolation of *Aaa* on selective media which detected the pathogen in 4 of 8 samples as well. The one exception is the BCCX sample from perennial ryegrass with Ct values around 31. This sample showed heavy bacterial streaming from stems and leaves and advanced symptoms of etiolation and decline. However, when isolation measures were taken, only a *Xanthomonas* pathogen could be recovered and characterized out of the tissue. It is likely that *Aaa* is capable of co-occupying turfgrass plants with *Xanthomonas* species and other pathogenic bacterial organisms. Trace amounts of *Aaa* within that sample could be resulting in reduced Ct values and elevated amplification. Roberts et al. (2013) recently reported the isolation and characterization of a *Xanthomonas* species associated with creeping bentgrass etiolation in tandem with *Aaa* in the southeastern United States. It is therefore likely that this sample of perennial ryegrass could harbor both the suspected primary *Xanthomonas* causal agent as well as the target organism *Aaa*.

In summary, we present here a TaqMan real-time PCR assay that is sensitive and specific for turfgrass pathogenic *Acidovorax avenae* subsp. *avenae* detection. This assay allows for the detection of *Aaa* directly from infected turfgrass plants within 5 hours and therefore represents a rapid and simple method to identify bacterial etiolation and decline in the field and evaluate the effectiveness of treatments used for its control. This assay also has many other potentially useful applications in plant breeding, diseases quarantine, and a wide array of epidemiological studies.

FINAL CONCLUSIONS AND FUTURE PROSPECTIVE

An emergent disease of creeping bentgrass caused by the bacterial plant pathogen *Acidovorax avenae* subsp. *avenae* is described. This unique problem to intensively managed turf stands such as golf course putting greens poses an imminent threat to managers and superintendents due to the lack of reliable control tactics available for bacterial diseases. The research herein provides inaugural descriptions of the diagnosis, general pathogenicity, and epidemiology of *Acidovorax avenae* on creeping bentgrass.

Based on diagnostic sampling and anecdotal discussions, it appears that the pathogen is relatively ubiquitous within North America. The high temperatures and humidity that favor disease development are not observed for long periods of time in more temperate regions of the United States. Therefore, this disease should be considered of marginal concern on creeping bentgrass in northern climates. Based on this assessment and studies conducted aimed at chemical treatment of etiolation and decline symptoms, minimal agitation of the affected site is suggested during intermittent events of disease occurrence. On sites with a history of bacterial etiolation, limiting the use of trinexapac-ethyl and ammonium sulfate prior to and during peak disease pressure is also recommended.

In regions such as the Southeastern United States, where temperatures exceed 30° C for extended periods of time with high humidity, management strategies should be aimed at limiting turfgrass stress during summer months to manage disease severity. Under such conditions, bacterial etiolation and decline caused by *Acidovorax avenae* subsp. *avenae* can be the limiting factor for healthy stands of intensely managed creeping bentgrass. Based on observations and previous literature describing symptoms of etiolation and summer decline on creeping bentgrass, it is likely that the pathogen has been prevalent in turfgrass systems for a many years, yet

overlooked by managers and pathologists as an abiotic stress response during extenuating weather conditions. Recent technological and mechanical advances and drastic changes in management practices within the turfgrass industry have likely resulted in unique pressures on pathogen populations within the system. Diseases that were once prevailing on turfgrass stands are now discouraged by persistent broad spectrum fungicide applications and effective management strategies which limit their proliferation. Perhaps this selective pressure on normally predominant fungal pathogens coupled with aggressive cultural management of creeping bentgrass has favored the increased pervasiveness of *Acidovorax avenae* in the turfgrass system.

One question that remains unanswered is the origin of the pathogen in turfgrass stands. This research has shown both foliar and root initiated inoculations to successfully convey diseases symptoms of etiolation and decline respectively. It is unclear however, where the inoculum comes from in a natural environment such as a golf course or sod farm, and to what concentrations are bacterial populations needed to incite particular disease symptoms. Speculation aside, the development of the specific real-time PCR assay in this study will undoubtedly help to answer these questions. Not only can an unlimited number of turfgrass and environmental samples be queried for *Acidovorax avenae* detection and quantification, but experimentation aimed at further characterizing disease etiology will be much easier with a reliable and rapid real-time PCR assay available.

APPENDIX

Figure A-5.1. Comparison of three maximum likelihood trees from individual protein coding genes.

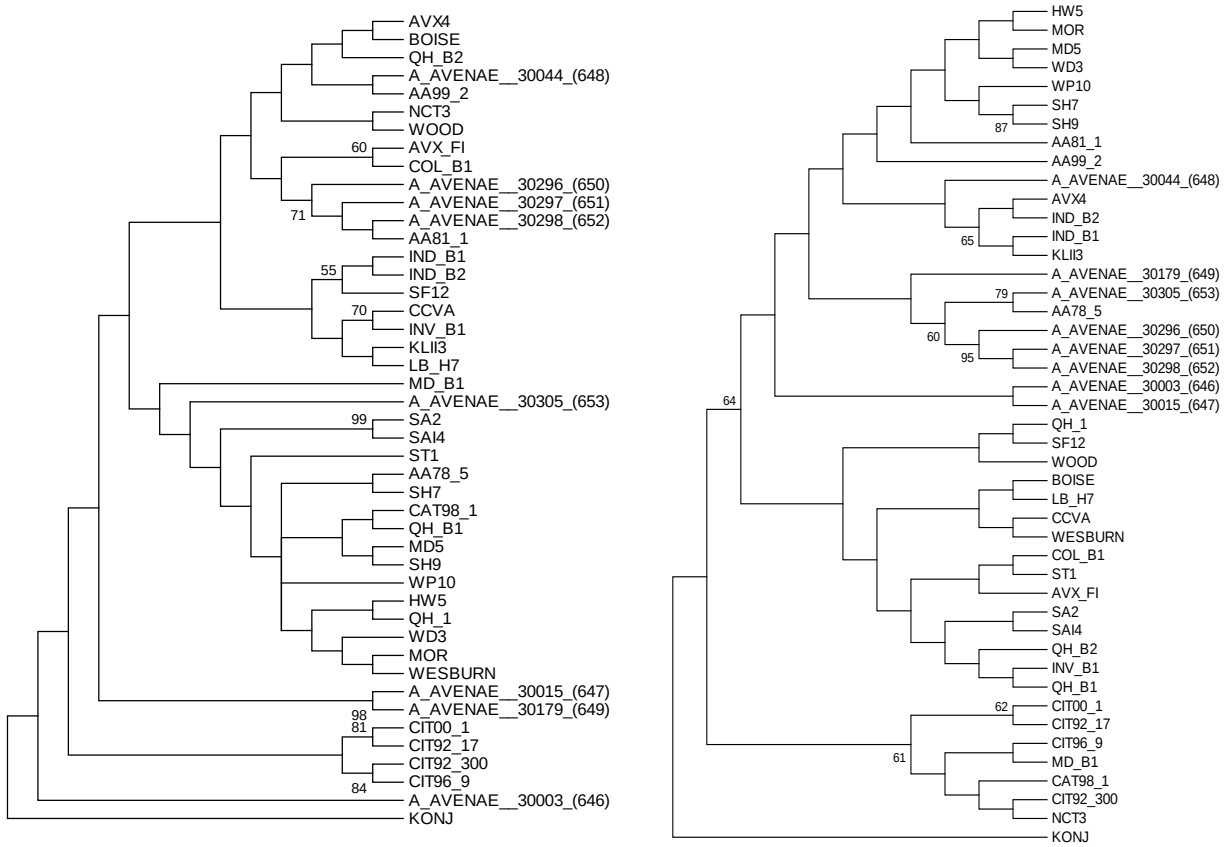
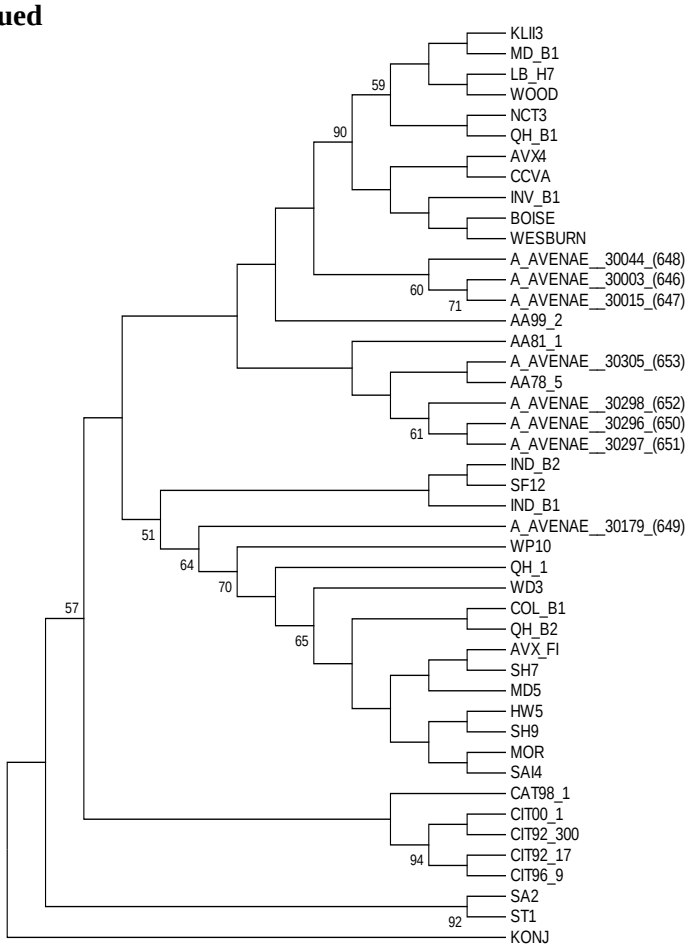
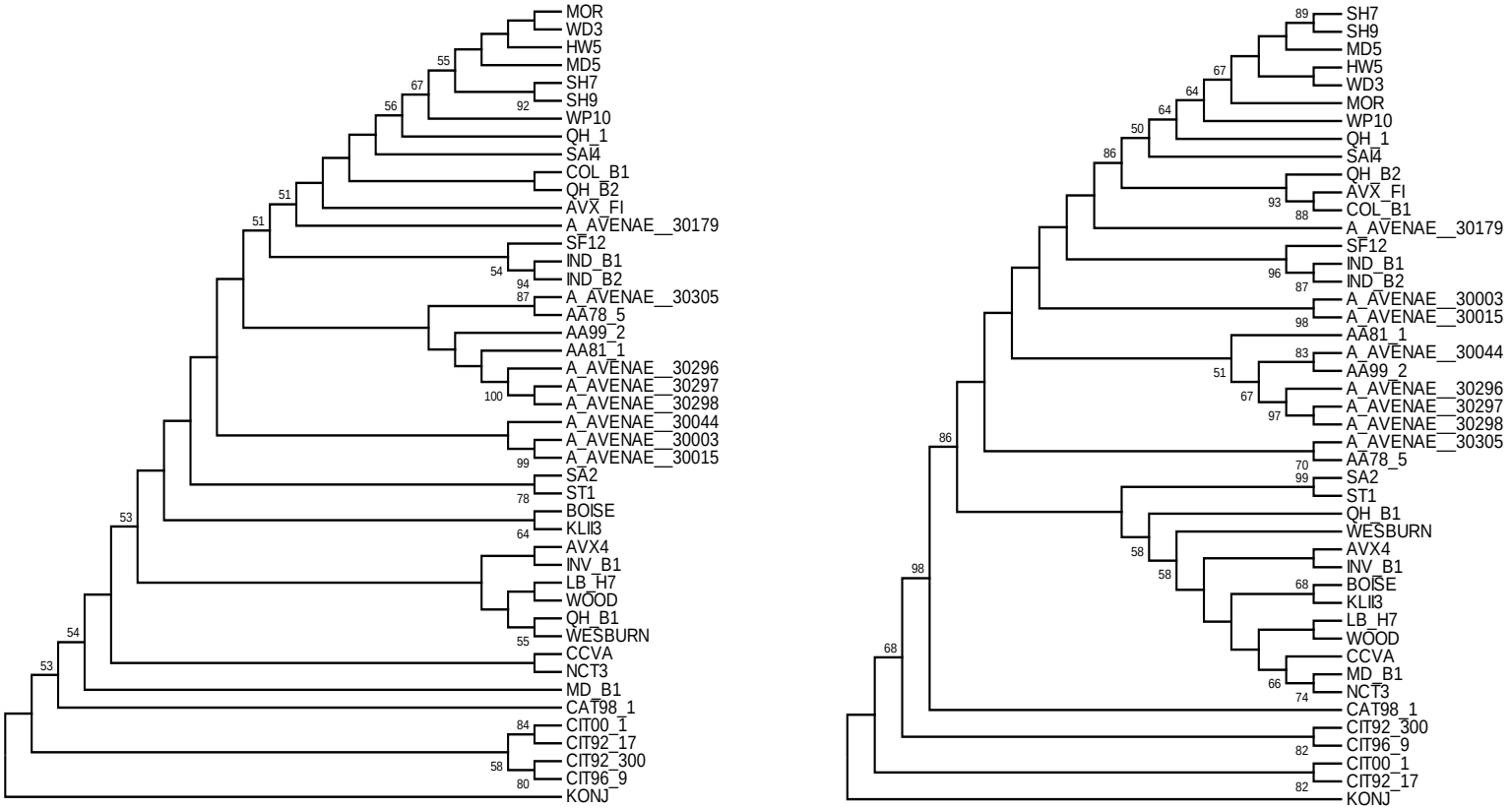


Figure A-5.1 continued



All trees were inferred using the Maximum Likelihood method and the best fit DNA model as determined by the Model Selection tool in MEGA5 set to default parameters. For all three trees, the best fit model was the Tamura 3-parameter (T92 + G+ I) model. All codon positions were included. Numbers corresponding to partitions reproduced in more than 50% of the bootstrap replications are shown. All trees are bootstrap consensus trees inferred from 100 replicates. **A.** ML tree for the gltA locus. Tree with the highest log likelihood was -988.02. **B.** ML tree for the gmc locus. Tree with the highest log likelihood was 12662.14. **C.** ML tree for the lepA locus. Tree with the highest log likelihood was -1405.56.

Figure A-5.2. **Maximum likelihood trees for the 3-gene (A) and 7-gene (B) concatenated sequence sets**.



All trees were inferred using the Maximum Likelihood method and the best fit DNA model as determined by the Model Selection tool in MEGA5 set to default parameters. For both trees, the best fit model was the Tamura 3-parameter (T92 + G+ I) model. All codon positions were included. Numbers corresponding to partitions reproduced in more than 50% of the bootstrap replications are shown. Trees are bootstrap consensus trees inferred from 500 replicates.
A. ML BS consensus tree for the 3-gene sequence set (gltA, gmc, lepA). Tree with the highest log likelihood is The tree with the highest log likelihood was -4347.527 **B.** ML BS consensus tree for the entire 7-gene sequence set (gltA, gmc, lepA, phaC, pilT, trpB, and ugpB) locus.
Tree with the highest log likelihood is shown (-12662.14). Green circles indicate groupings of isolates pathogenic to turfgrass. The blue circle indicates the one isolate of *A. avenae* among the turfgrass isolates that is not from turfgrass but from sorghum.

Table A-5.1. Assembly statistics for sequenced bacterial genomes.

Strain	kmer	Total reads	Reads Assembled	% Assembled	Median Coverage	Total length (bp)	# of Scaffolds	Largest scaffold (bp)	N50 size (bp)	N50 #
AA78-5	43	727580	711709	97.8%	23.8	4884148	849	120416	11467	115
Aa99-2	33	736366	727003	98.7%	26.0	4949784	836	86210	14775	89
COLB1	43	720217	711988	98.9%	22.9	5306692	723	98761	18825	78
Cat98-1	43	686632	672352	97.9%	20.8	5173556	781	63435	14020	107
INDB2	53	698219	685557	98.2%	21.5	5321978	889	80273	13954	108
INV	33	717235	707916	98.7%	23.8	5411630	1000	108620	14055	102
KL3	53	774472	751253	97.0%	22.1	4951859	917	61577	11799	119
MDB1	33	635802	633414	99.6%	23.9	5583247	717	131134	30534	51
MOR	43	735838	711054	96.6%	22.1	5260015	914	81858	15245	91
NCT3	43	670610	662267	98.8%	23.8	5249730	732	119219	18606	76
QH1	43	700303	690273	98.6%	23.9	5263646	674	83012	20439	73
QHB1	43	702121	692834	98.7%	22.8	5483924	649	138551	25205	59
SF12	43	771406	755157	97.9%	22.5	5226612	795	101561	16927	87
SH7	43	763397	751255	98.4%	22.6	5243968	841	89423	15053	98
Sa2	43	729052	718401	98.5%	22.8	5321833	778	92377	18042	79

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