ABSTRACT

Title of Dissertation: INVESTIGATING CULTURAL PRACTICES TO IMPROVE THE EFFICACY AND RELIABILITY OF BIOLOGICAL CONTROL ORGANISMS IN TURFGRASS SYSTEMS

Joseph Ryan Doherty, Doctor of Philosophy, 2021

Dissertation Directed by: Dr. Angus Murphy, Professor
Plant Science and Landscape Architecture

Biological control, or suppressing plant pathogens through natural predators or competitors, has been an area of scientific intrigue for many decades. However, inconsistent efficacy remains the chief reason for a lack of widespread adoption by growers. This dissertation was developed to address the inconsistencies of biological control through three practices. First, reducing competition from resident foliar microbial communities, or niche clearing, was explored for brown patch suppression and biological control organism establishment. Second, biological control organisms were applied to the rhizosphere and evaluated for suppression of root infecting pathogens. Finally, combinations of monthly biochar topdressing and biweekly or weekly biological control organism applications were evaluated for foliar pathogen suppression and biological control organism establishment. In each study, biological control organisms were observed to suppress pathogens and reduce disease severity. However, neither niche clearing nor biochar topdressing increased disease control compared to the biological control organism applied alone. While biochar applications did not improve the efficacy of biological control organisms, they did reduce the severity of disease unaffected by

biological control organisms and increased turfgrass quality. Furthermore, biological control organisms were most successful under low to moderate levels of disease pressure, as control was lost when disease pressure peaked each year. Once again, neither niche clearing nor biochar topdressing increased populations of biological control organisms compared to the organisms applied alone. Weekly applications of biological control organisms did result in higher population levels compared to biweekly applications, and rhizosphere targeted applications appeared to have resulted in an establishment of the biological control organism into the rhizosphere community. Findings from these research projects suggest that biological control organisms should be applied weekly to increase odds of successful disease reduction. Given the lack of control under high disease pressure in all studies, successful implementation of biological controls should be targeted to the shoulders of the season when disease pressure is lower, or biological control organisms should be implemented into a season long program to supplement a traditional fungicide program.

INVESTIGATING CULTURAL PRACTICES TO IMPROVE THE EFFICACY AND RELIABILITY OF INTRODUCED BIOLOGICAL CONTROL ORGANISMS IN TURFGRASS SYSTEMS

by

Joseph Ryan Doherty

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Advisory Committee:

Professor Angus Murphy, Chair
Dr. Jo Anne Crouch
Professor Mengjun Hu
Professor James Kerns
Professor Joseph Roberts
Professor Stephanie Yarwood

Dedication

To my incredible parents, Chris and Jen, whose words of encouragement and emotional support have been a cornerstone throughout my life. To my brother Connor, who has always been there to provide laughter during tough times. Sara, my partner and best friend, your care and encouragement through this endeavor has been my saving grace more times than you know. I dedicate this work to you all, and the rest of the family. I would not be where I am today without all of you.

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List of Abbreviations

BCO	biological control organism
qPCR	quantitative real-time polymerase chain reaction
ZT	hydrogen peroxide + peroxyacetic acid
QST713	*Bacillus subtilis* QST713
AFS009	*Pseudomonas chlororaphis* AFS009
W9	*Bacillus subtilis* EXP-W9
CFU	colony forming unit
LN_2	liquid nitrogen
PRR	Pythium root rot
ASV	amplicon sequence variant
PERMANOVA	permutational analysis of variance
ANOSIM	analysis of similarity

Chapter 1: Review of Literature

1.i Biological Control of Pathogens

Plant pathogens have been infecting and causing plant loss for as long as plants and microorganisms have been interacting. Humans have put forth considerable efforts to mitigate plant pathogens and prevent significant crop losses that would impact human survival. In 1885, Pierre Alexis Millardet found the right combination of copper sulfate and hydrated lime to control downy mildew on grapevines (Agrios, 2005). Thus, the Bordeaux mixture was born, and the era of fungicidal control of plant pathogens began. Research continued into fungicides for controlling plant pathogens, and to date, a multitude of different modes of action and fungicide classes have been discovered.

Dr. Carl Freiherr von Tubeuf was the first plant pathologist to introduce the concept of biological control of pathogens (1914). Freiherr's concept of biological control, or biocontrol, used a broad interpretation of "control of one organism by another, excluding man" (Baker, 1987). Scientific advancements since this initial definition have resulted in more nuance being woven into defining biological control. The US Department of Agriculture has defined biocontrol as "reducing pest populations through the use of natural enemies such as parasitoids, predators, pathogens, antagonists, or competitors to suppress pest populations" (US Department of Agriculture, 2020). Some groups go further and include genes and gene products as forms of biocontrol (US Congress Office of Technology Assessment, 1995). Despite variations in technical definitions of biocontrol, stemming from different scientific fields, the underlying principle of "using one organism to reduce the damaging effects of another organism" remains. For this review, and dissertation at large, biocontrol will focus specifically on

controlling plant pathogens, and their associated damages, through the deliberate utilization of introduced microorganisms or stimulation of resident microbial communities.

Biocontrol of a pathogen can involve either specific or generalized suppression mechanisms (Schlatter, et al., 2017). General suppression occurs through the activity of a consortium of microorganisms and is not transferrable to conducive soils, whereas specific suppression occurs through the activity of a singular microorganism and is transferrable to susceptible soils (Baker, 1987). Disease suppressive soils, where stimulation of native rhizosphere microorganisms through plant rhizodeposition leads to a reduction in pathogen load or disease severity, are primary examples of general suppression (Schlatter, et al., 2017). These disease suppressive soils can occur naturally, like some soils from the Châteaurenard region of France that are highly suppressive to Fusarium wilts (Alabouvette, 1986), or they can occur through stimulation of native microbiota, like the soils of an avocado grove in Queensland, Australia that were supplemented with organic material to keep the soil organic matter near 12% which lead to suppression of Phytophthora root rot in a soil infested with *Phytophthora cinnamomi* (Baker and Cook, 1974). The specific mechanisms behind general suppression are shrouded by the web of interactions between microbes that results in pathogen reduction, but with emerging technologies, such as high-throughput sequencing and advances in metabolomics, proteomics, and stable-isotope probing scientists are primed to begin elucidating these mechanisms.

In specific suppression, the population of a single microorganism, the biological control organism (BCO), is responsible for pathogen suppression. Researchers have

elucidated four modes of action by which a BCO suppresses a pathogen. These four main modes of action are antibiosis, competition, parasitism/predation, and plant defense induction (Whipps, 2001). Some assert there are more modes of action, such as lytic enzymes and unregulated waste products, by which biocontrols inhibit pathogens (Köhl, et al., 2019; Pal and Gardener, 2006). However, these proposed modes of action can be sorted into one of the aforementioned modes of action.

Antibiosis of plant pathogens can involve growth suppression via antibiotics, membrane disruptors, metabolic inhibitors, or siderophores in the form of peptides, specialized metabolites, or volatiles (Haggag and Mohamed, 2007). Antibiotic production by a BCO has been observed to be quite effective in reducing a pathogen and its associated damages, though most successes come from *in vitro* and *in situ* studies. Lytic enzymes have been attributed to biocontrol of pathogens, such as chitinase production in *Serratia marcescens* mediating control of *Sclerotium rolfsii* (Ordentlich, et al., 1988). It should be noted that lytic enzymes are also produced to degrade organic materials for nutrient acquisition; therefore, in natural systems, it would be difficult to discern what percent of the present lytic enzymes were produced solely due to microbe-microbe interactions. Volatile compounds produced by several strains of *Pseudomonas* were shown to completely inhibit *Sclerotinia sclerotiorum* sclerotia formation and mycelial growth, indicating these compounds have a role in biocontrol (Fernando, et al., 2005). An entire literature review could be dedicated to the compounds underlying antibiosis, and indeed some have been (Fravel, 1988; Raaijmakers, et al., 2002), which underscores the importance of this biocontrol mode of action. However, the production and role of these compounds in nature remain entangled in a complex web of interactions that hinder our

ability to discern the necessary qualifications to reliably achieve disease suppression in the field.

Competition between pathogens and BCOs can be separated into two types. First, there is competition for limited nutrients. In the rhizosphere, iron availability is too low to freely meet plant and microbe demands (Colombo, et al., 2014). Siderophores are widely produced by various microbes (Neilands, 1984), and are key molecules in iron acquisition. Siderophore production, and in turn increased competitive ability for iron, has been linked to disease suppression by several microbes, especially amongst fluorescent pseudomonads (Arya, et al., 2018; Duijff, et al., 1993; Loper and Buyer, 1991; Naureen, et al., 2015). The second type of competition is competition for space. Colonizing infection courts before a pathogen arrives allows a BCO to protect the host by blocking pathogens from accessing the infection court (Di Francesco, et al., 2017). It is reasonable to think these two types of competition are not mutually exclusive, as a BCO that can more effectively compete for nutritional resources can better colonize a host as it is less starved for resources.

Parasitism/predation occurs when a BCO directly attacks a pathogen or destroys pathogen propagules (Pal and Gardener, 2006). Hyperparasites, or BCOs that parasitize pathogens, use cell wall degrading enzymes, such as glucanases or chitinases, to attack a pathogen (Chet, et al., 1981; Geraldine, et al., 2013). Also, within this mode of action is hypovirulence, wherein a fungal virus reduces the pathogenicity of a plant pathogen (Nuss, 2005). Hypoviruses that reduce the pathogenicity of *Cryphonectria parasitica* are the best example of hypovirulence in plant pathology (Milgroom and Cortesi, 2004). This

mode of action is the smallest of the four in pathology, although it is more common amongst biocontrol of insect pests (Rosenheim, et al., 1995).

Finally, a BCO can reduce disease severity through plant defense activation. A plant's innate defenses can be activated through two mechanisms, which can be differentiated based on the elicitor and regulatory pathways involved. First, there is systemic acquired resistance (SAR), which can be triggered through exposure to pathogens, non-pathogenic microbes, or through chemicals that activates salicylate pathways resulting in salicylic acid and pathogenesis-related (PR) protein accumulation (Sticher, et al., 1997). Conversely, induced systemic resistance (ISR) is triggered by plant-growth-promoting rhizobacteria (PGPR) and utilizes metabolic pathways regulated by jasmonate and ethylene (van Loon, et al., 1998; Yan, et al., 2002). Additionally, there is no accumulation of PR proteins in ISR (Pieterse, et al., 1996).

The lines between the different biocontrol modes of action may not be as discrete as the lines between synthetic fungicide modes of action. Several *Trichoderma* strains were found to use NAGase and β-1,3-glucanase to weaken the cell walls and parasitize *S. sclerotiorum* (Geraldine, et al., 2013). Other research has shown β-1,3-glucanase produced by *Pseudomonas cepacia* to be involved in damaging cell walls of *R. solani*, but no parasitic activity was observed in microscopic imagery (Fridlender, et al., 1993). Siderophores, which have been implicated in *Pseudomonas* competitive biocontrol (Arya, et al., 2018; Naureen, et al., 2015), can also be elicitors of ISR in various systems (Leeman, et al., 1996; Meziane, et al., 2005). Overlaps such as these blur the lines between the different biocontrol modes of action. The truth of the matter may be that biocontrols simply do not act through single modes of action like synthetic fungicides do.

Continuing research efforts are needed to further decipher these modes of action, as a thorough understanding of them is needed to develop reliable biocontrol methods.

1.ii *Bacillus* as a Biological Control Organism

Bacillus is one of the most widely studied bacterial genera for biocontrol potential and the most formulated BCO. A search of the U.S. Environmental Protection Agency's database of registered biopesticides, which includes biochemical and microbial active ingredients, shows that 51 of the 390 registered products are *Bacillus* spp. strains, more than 4 times higher than the next microbial active ingredient *Pseudomonas* spp. with 13 registrations (https://bit.ly/3bvMrEH; accessed 9 Mar 2021). Researchers have consistently identified multiple species of *Bacillus* that are capable of producing antibiotic compounds, producing endospores, exhibiting rhizosphere competence, forming biofilms, and inducing plant defenses. All of these characteristics are highly valued in a candidate BCO.

Production of antibiotic compounds is the most important feature of *Bacillus* species for implementation as a BCO. More than a dozen antibiotics produced within the *Bacillus* genus have been documented (Fira, et al., 2018; Shafi, et al., 2017; Stein, 2005). Cyclic lipopeptides, which include iturins, surfactins, and fengycins, are key factors of plant pathogen biocontrol (Ongena and Jacques, 2008; Yánez-Mendizábal, et al., 2012). Several strains of *B. subtilis* and *B. amyloliquefaciens* produce all three of the aforementioned cyclic lipopeptides and can modulate production in response to pathogen presence. When *B. subtilis* 98S was grown in the presence of *Pythium* or *Fusarium*, larger quantities of iturins and fengycins were present in the zone of inhibition compared to the same strain grown in the presence of *Botrytis* where no increase of any cyclic

lipopeptides were observed (Cawoy, et al., 2015). The cyclic lipopeptides can also impact pathogens through multiple means. The surfactin WH1fungin, produced by *B. amyloliquefaciens* WH1, can induce apoptosis of *Rhizoctonia solani* cell walls and bind to mitochondrial membranes causing reduced ATPase activity (Qi, et al., 2010). The importance of antibiotic production in *Bacillus-mediated* biocontrol of plant pathogens cannot be understated. Extensive reviews have been conducted entirely on the phenomenon (Fira, et al., 2018; Stein, 2005).

If a BCO is to be effective, it must be possible to formulate, store, and establish well in a new environment. Members of the *Bacillus* genus are capable of producing endospores that allow them to overwinter and survive unfavorable conditions (Nicholson, 2002). This would help a potential BCO survive through an offseason, and the BCO population would return naturally when endospores germinate. BCOs that could be stored as endospore suspensions would thus exhibit the shelf life expected from commercial products. Rhizosphere competence, or the ability of an organism to establish, function, and reproduce in the rhizosphere, is a commonly documented characteristic within the *Bacillus* genus (Abdallah, et al., 2018; Chowdhury, et al., 2013; Singh, et al., 2008). Without rhizosphere competence an introduced BCO would be outcompeted and fail to establish a population in the rhizosphere, thus pathogen suppression would never occur.

The ability to produce a biofilm can be beneficial for a potential BCO to attach and persist on a plant host, which may contribute to rhizosphere competence. Biofilms are formed by excretions of extracellular polymeric substances, which immobilize cells on a surface, and once a biofilm is formed intracellular interactions, such as horizontal gene transfer and quorum sensing, occur (Flemming and Wingender, 2010). An extensive

study of *Bacillus cereus* ATCC14579 revealed significant shifts in cellular metabolism that facilitates defense in biofilm-associated cells (Caro-Astorga, et al., 2020). These changes include thickened cell walls, thiocillin production, scavenging of reactive oxygen species, and triggering of sporulation. The importance of biofilm formation to biocontrol success clearly goes beyond advantages in establishment and are likely ubiquitous across similar species.

1.iii Hurdles to Biological Control Adoption

Research has shown biocontrol to be an effective method of reducing disease severity in greenhouse and field evaluations, yet adoption by practitioners remains minimal. Many practitioners fail to see reliable disease control when implementing biocontrol products, but applications of synthetic fungicides reliably prevent and reduce disease severity. For there to be widespread adoption of biocontrol practices, the reliability of pathogen biocontrol needs to be improved.

First and foremost, there needs to be an understanding that BCO populations are crucial to success. Secondary metabolites, which include antibiotic compounds, are produced through metabolic pathways regulated via quorum sensing, or the regulation of gene expression in response to fluctuations in population density (Miller and Bassler, 2001). Quorum sensing is involved in the production of surfactin by *B. subtilis* (Hamoen, et al., 2003), pyrrolnitrin by *Serratia plymuthica* (Liu, et al., 2007), the efficacy of *Pseudomonas fluorescens* 2P24 (Liang, et al., 2020), and regulation of multiple genes affecting biocontrol traits of *P. chlororaphis* PA23 (Shah, et al., 2020). Sporulation can be triggered in endospore-forming bacteria through quorum sensing recognizing low cell density (Miller and Bassler, 2001). Interestingly, disruption of quorum sensing signals in

P. *fluorescens* 2P24 decreased biofilm formation and biocontrol efficacy, but antagonistic secondary metabolites were not impacted (Wei and Zhang, 2006). Failure to establish BCO populations to a high enough concentration will result in genes crucial to biocontrol not being activated through quorum sensing. Conversely, failure to maintain BCO populations above a certain threshold will lead to sporulation. Improving the reliability of biocontrol practices will rely on our ability to develop reliable methods of introducing and establishing sufficient populations of a BCO.

The environments where BCOs are applied are characterized by a diverse resident microbial community. The bacterial component of microbiomes associated with turfgrass and similar systems comprise species from a wide range of taxa (Doherty, et al., 2020; Gomes, et al., 2018; Roquigny, et al., 2018). Given this taxonomic diversity, it is likely that the available ecological niches have been filled. Multiple species competing for the same ecological niche will result in one species outcompeting the other (Gause, 1934). Since the resident microbes are already competing or occupying their niche, any introduced BCO will have a significant hurdle to overcome.

In addition to concerns regarding the technical practicalities of BCO application and use, any biocontrol implementation effort must include programs to manage practitioner expectations. Within the United States, farmers, landscapers, golf course superintendents, and other plant growers have access to many synthetic fungicides that are highly efficacious and reliable. When applied at the right time intervals, these products can provide near-complete control. However, with the link between BCO populations and efficacy, biocontrol products require more frequent applications. Furthermore, the dependence of populations relinquishes biocontrol to preventative

applications. In its current state, biocontrol is unlikely to see widespread adoption any time soon.

1.iv Summary and Dissertation Overview

Biocontrol of plant pathogens has been a topic of interest for the past 100 years. However, over that same period, there have been incredible advances in synthetic fungicides. Resulting in fungicides that are capable of being applied at fractions of an ounce per acre, while still providing near-total control of the target pathogen. As reliance on synthetic fungicides increased, there is a similarly growing concern about their environmental impact and overall sustainability. This has led to increased interest in biocontrol as a more environmentally sustainable alternative to synthetic fungicides. Research efforts have resulted in the discovery of BCOs that have successfully reduced disease severity in greenhouse and field settings. Unfortunately, their adoption has been slow. Turfgrass managers will attribute the cost of biological products and their unreliable disease control are the leading reasons why industry adoption is lacking. There is a disconnect between research trials, including field trials, and real-world implementation that can explain the unreliable control observed by practitioners.

Our current understanding of the underlying mechanisms of biocontrol would lead one to believe that BCO populations are key to success. Maintaining a critical population level under research conditions is likely much easier than under real-world conditions. Although researchers attempt to replicate real-world conditions for field trials, it is impossible to completely replicate everything. For example, despite researchers mimicking the cultural and fertility practices of their region when conducting experiments on a research putting green, it is impossible for researchers to adequately

mimic the variables introduced by play from golfers. Additionally, product applications to turfgrass are commonly made as tank mixes of several different chemicals and fertilizers.

There is an abundance of information on BCOs being tested *in vitro, in situ,* and *in vitro,* although disease suppression varies. Researchers are continuing to decipher the complex metabolic and molecular pathways underlying the biocontrol of pathogens. Given that quorum sensing, and therefore populations, play a significant factor in biocontrol, research efforts should be focused on monitoring BCO populations after application. The objectives of this dissertation were to 1) evaluate niche-clearing practices to increase biological control organism establishment and efficacy against brown patch in home lawn turfgrass, 2) appraise effects of soil directed biological control organism applications on the rhizosphere microbiome and control of root-infecting pathogens, and 3) assess effects of compost topdressing on biological control phyllosphere establishment and control of foliar pathogens. Results from this research will elucidate BCO population dynamics following application and may provide improved recommendations to turfgrass managers for implementing biocontrol practices.

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Chapter 2: Effects of niche clearing on establishment and efficacy of introduced biological control organisms on *Rhizoctonia solani* in home lawn turfgrass

2.i Abstract

Biological control organisms (BCOs) remain poorly utilized in the turfgrass industry given their history of being unreliable and less effective than synthetic fungicides. Resident turfgrass microbial populations may be hindering the ability of BCOs to establish into the microbiome and suppress plant pathogens. Reducing resident communities via a surface sterilant (i.e., niche clearing) may be a method of improving the establishment and efficacy of BCOs. A greenhouse study was initiated using pots of *Lolium perenne* L. cv. "Express II" to evaluate combinations of niche clearing (H_2O_2+$C_2H_4O_3$) and BCOs (*Bacillus subtilis* QST713, *Pseudomonas chlororaphis* AFS009, and *B. subtilis* EXP-W9). These same combinations were evaluated under field conditions using a stand of *L. perenne* cv. "Salinas II". Treatments were applied on a 14 d interval, and niche clearing occurred 3 h prior to BCO application. Foliage samples were collected in the greenhouse trial at 0, 2, 4, 7, 10, and 13 days after treatment, and were used to monitor culturable bacterial communities in addition to BCO populations via qPCR. Brown patch severity and turfgrass quality were measured every 2 days in the greenhouse and 7 to 14 d in the field study. Niche clearing did not increase BCO populations compared to the BCO being applied alone, nor did it increase brown patch control. Treatment combinations did not significantly impact culturable bacterial communities either. Under field conditions BCO applications did reduce brown patch severity compared to the non-treated control, but when disease pressure increased control was lost. These findings indicate niche clearing alone will not improve BCO establishment or efficacy, and future research efforts should investigate other avenues.

2.ii Introduction

Biological control of plant pathogens has been an area of interest for many decades (Baker, 1987; Baker and Cook, 1974). The main driver of interest in biological control is to reduce the use of synthetic fungicides and deleterious off-target effects of mitigating plant pathogens. Numerous biological control organisms (BCOs) have been identified and have been effective in reducing disease in research settings (Pal and Gardener, 2006). However, grower adoption of BCOs remains sporadic at best. BCOs may have been shown to be effective under research settings, but most research projects happen either under controlled conditions (i.e., a greenhouse) or on small scale research plots. Scaled up to whole field applications, biological control has proven to be too unreliable and not effective enough for growers to rely solely on them for disease suppression.

Failure of an applied BCO to establish into the microbiome to which it is introduced to is likely a considerable driver of inconsistent efficacy. Population levels are undoubtedly crucial to biological control success regardless of the BCO's mode of action. Quorum sensing plays a role in the success of BCOs through gene regulation. Production of antibiotics in multiple BCOs has been demonstrated to be regulated by quorum sensing (Hamoen, et al., 2003; Liu, et al., 2007; Shah, et al., 2020). Furthermore, quorum sensing can trigger entire metabolic shifts, such as in *B. cereus* ATCC14579 where cells within a biofilm underwent extensive metabolic shifts to protect themselves including the production of thickened cells walls, ROS scavenging, and antibiotic production (Caro-Astorga, et al., 2020). Quorum sensing can also trigger sporulation in some endospore forming bacteria, enhancing survival potential through unfavorable conditions (Miller

and Bassler, 2001). While the exact mechanisms behind the importance of quorum sensing to biological control success may not be completely understood, it is clear that it plays a significant role. As such, populations of BCOs need to be maintained at a high titer to obtain reliable control of pathogens.

Establishing into a host's microbiome is immediately challenged by the microbes already inhabiting it. The plant microbiome is diverse; inhabited by thousands of taxa from all microbial groups (Doherty, et al., 2021; Gomes, et al., 2018; Ma, et al., 2021). Even newly emerged seedlings have diverse microbial communities associated with them (Doherty, et al., 2020; Tannenbaum, et al., 2020). With these diverse microbial communities, ecological niches are already being competed for and, quite possibly, are already fulfilled. This poses a problem as two species with identical niche requirements cannot exist simultaneously (Gause, 1934). In other words, one species will outcompete the other to occupy the ecological niche and meet their needs. However, temporarily disrupting the resident microbial communities through a surface disinfestant may provide an opportunity for an introduced BCO to gain a competitive advantage.

Synthetic fungicides provide excellent and reliable control of plant pathogens when used properly. With the risk of fungicide resistant pathogens developing and growing concerns regarding the impact of repeated fungicide applications on the environment, there is a need to improve alternative disease control methods. Improving the ability of introduced BCOs to establish and persist in a new environment should in turn increase the reliability of their pathogen suppression. To that end, a greenhouse and a multi-year field trial were developed to evaluate niche clearing practices for their ability to improve BCO establishment and efficacy against foliar pathogens. We hypothesized

that 1) BCO populations would be higher in turfgrass pre-treated with a surface disinfestant than in turfgrass where the BCO was applied alone and 2) disease control would be greater in turfgrass receiving BCO and surface disinfestant pre-treatment than in turfgrass only receiving BCO applications.

2.iii Materials and Methods

Two separate experiments were developed to evaluate if pre-treating turfgrass with a surface disinfestant, to reduce resident microbial populations, can increase the establishment and efficacy of introduced biological control organisms (BCOs). The first experiment was conducted in a greenhouse with a benchtop humidity chamber, while the second experiment was conducted under field conditions to evaluate real-world implementation of treatment combinations.

Greenhouse Growth Conditions

A growth medium was created using a 50:50 mix of Fafard 3B potting mix (Sungro Horticulture; Agawam, MA) and Scotts Lawn Soil (The Scotts Company, LLC; Marysville, OH). During mixing the growth medium was amended with Shaw's 44-0-0 100% SurfCote-4 (Knox Fertilizer Company; Knox, IN) at a 0.59 kg N m^{-3} rate to provide fertility for the duration of the experiment. This growth medium was used to fill 10.1 cm square by 12.7 cm deep pots (Belden Plastics; St. Paul, MN). Pots were then seeded with "Express II" perennial ryegrass (*Lolium perenne* L. cv. "Express II") at a rate equivalent to 9.07 kg 92.9 m^{-2}. Turfgrass plants were grown for one month following germination under ambient greenhouse conditions before being relocated into a benchtop humidity chamber in the greenhouse. Turfgrass was maintained at a height of 5 cm by

mowing 2x wk^{-1} with flame sterilized scissors, to prevent cross contamination once treatments were initiated.

A 160 cm x 106.7 cm x 60.9 cm benchtop humidity chamber was constructed from angle aluminum and 6 mm polyethylene greenhouse film (Grower's Solution, LLC; Cookeville, TN), to create a controlled environment where conditions favorable for disease development could be maintained. Air temperature and relative humidity within the humidity chamber were monitored with a WatchDog B102 button logger (Spectrum Technologies, Inc., Aurora, IL). Heat mats and two overhead mister arrays inside the humidity chamber were used to maintain daily high/low temperatures at 33° C/28° C and relative humidity constantly between 85-90%. Overhead misters ran from 8 am to 5 pm for 30 s every 15 min, delivering 165 mL water from each array. No supplemental irrigation was provided as this misting cycle provided sufficient water.

Greenhouse Experimental Design

The greenhouse experiment utilized a split-split plot experimental design with a 2 x 2 x 4 factorial treatment arrangement. The main plot factor was pathogen inoculation with half of the pots being inoculated with *Rhizoctonia solani* Kühn infested rye grain while the other half of the pots were not inoculated. Sub plot treatments consisted of niche clearing with hydrogen peroxide + peroxyacetic acid [ZT (ZeroTol 2.0; BioSafe Systems LLC; East Hartford, CT)] and no niche clearing. Sub sub plot treatments were BCO applications which consisted of *Bacillus subtilis* QST713 [QST713 (Rhapsody®; Bayer Crop Science; Research Triangle Park, NC)], *Pseudomonas chlororaphis* AFS009 [AFS009 (Zio™; SePRO Corporation; Carmel, IN)], *B. subtilis* EXP-W9 (W9), and a non-treated control. Each treatment combination was replicated 4 times. Niche clearing

and BCO treatment combinations were applied every 14 d. Three experimental runs were completed.

Niche clearing treatments were applied at 5.18 L hydrogen peroxide + 0.38 L peroxyacetic acid ha^{-1} (19.1 L ZeroTol® 2.0 ha^{-1}). This was delivered through a compressed air single nozzle sprayer (R&D Sprayers; St. Landry Parish, LA) using an air-induction nozzle calibrated to deliver 1,222.4 L ha^{-1} over two passes. Three hours following niche clearing treatments, BCO treatments were applied to sub sub plots. QST713 was applied at $8.54x10^9$ colony forming units (CFUs) *B. subtilis* QST713 ha^{-1} (31.8 L Rhapsody® ha^{-1}), AFS009 was applied at $9.13x10^9$ CFUs *P. chlororaphis* AFS009 ha^{-1} (9.1 kg Zio™ ha^{-1}), and W9 was applied as a cellular suspension with a titer of 10^9 CFUs ml^{-1}. BCO treatments were applied using a compressed air single nozzle sprayer (R&D Sprayers) with an air-induction nozzle calibrated to deliver 814.9 L ha^{-1} evenly across the pots.

The suspension of W9 was created by growing the W9 isolate on plates of general nutrient agar (Beckton, Dickinson, and Co.; Franklin Lakes, NJ) for 24 hours. Plates were then flooded with sterile 0.1% peptone water and the bacteria were dislodged from the agar with a flame sterilized rubber policeman. Suspended W9 cells were titrated to 10^9 CFUs ml^{-1} (OD ~1) using additional sterilized 0.1% peptone water, and the resulting suspension was used for treatment application. A fresh suspension of W9 cells was created for each treatment application.

Pathogen infested rye grain was prepared by combining 250 cc rye grain, 6.25 g calcium carbonate, and 220 ml water in a 1 L Erlenmeyer flask. Flasks were capped with cotton plugs and aluminum foil before autoclaving at 121º C for 45 min. Autoclaved rye

grain was allowed to cool for 48 h. Ten 3 mm diameter plugs were pulled from potato dextrose agar plates colonized by *R. solani* Kühn AG2-2IIIB and added to the cooled rye grain. Inoculum was incubated at room temperature for 7 d. Every 48 hours during incubation the inoculum was shaken to disperse pathogen mycelium and ensure thorough infestation. Inoculation occurred 21 d after trial initiation. A single infested rye grain was placed in the center and 1.25 cm from each corner of inoculated pots.

Pots were monitored every 48 h following inoculation. Brown patch severity was evaluated on a 1-9 scale, where 1 = non-symptomatic turfgrass and 9 = completely necrotic pots. Additionally, images of pots were collected for digital image analysis in TurfAnalyzer (Green Research Services, LLC; Fayetteville, AR) as a secondary measure of disease severity.

Monitoring Microbial Populations

Following trial initiation, samples were collected from each experimental unit at 0, 2, 4, 7, 10, and 13 days after treatment. For the samples collected on the day of treatment application, 4 h elapsed between ZT application and sample collection. Samples consisted of 3 ryegrass plants, removed from the soil surface using flame sterilized forceps and scissors, placed into pre-weighed sterile 15 ml centrifuge tubes. All samples were kept on ice to limit microbial activity prior to analysis. In the lab, centrifuge tubes were weighed again to obtain sample weights for data normalization. To dislodge epiphytic microbiota, 15 ml sterile 0.1% peptone water was added to each sample and tubes were placed in a CPX3800 Ultrasonic Bath (Thermo Fisher Scientific; Waltham, MA) for 1 min. Using an Autoplate 5000 (Advanced Instruments Inc.; Norwood, MA), leaf washings were plated onto *Bacillus* spp. selective media [MYP

agar+ polymyxin B (Beckton, Dickinson, and Co.)], *Pseudomonas* spp. selective media [Pseudomonas isolation agar (Beckton, Dickinson, and Co.)], and a non-selective general nutrient agar (Beckton, Dickinson, and Co.). Plates were incubated at room temperature. CFUs were enumerated using a QCount Color (Advanced Instruments Inc.) 48 h after plating.

Monitoring Biological Control Organisms

In the second and third experimental runs a second identical sample was taken from experimental units and placed into pre-weighed sterile 2 ml microcentrifuge tubes (Fisher Scientific). Samples were immediately placed on ice to limit microbial activity during transportation from the greenhouse to the laboratory. Once in the lab, tubes were re-weighed to obtain sample weights and subsequently placed in a -20º C freezer to maintain populations present at the time of sampling. Total DNA was extracted from samples by adding a sterile 3 mm diameter tungsten carbide bead (Qiagen) to the sample tubes, freezing them in LN_2, and running them on a TissueLyser II (Qiagen; Gaithersburg, MD) at 30 Hz for 2 min. Disrupted samples were then processed with the Qiagen DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Extracted DNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

Populations of *B. subtilis* QST713 and *P. chlororaphis* AFS009 were quantified via qPCR. Primers, probe, and qPCR protocol for *B. subtilis* QST713 were followed as previously described (Mendis, et al., 2018). Briefly, reactions were performed in the StepOnePlus Real-Time PCR System (Applied Biosystems; Foster City, CA) at a final volume of 20 µl, containing 2 µl of sample DNA, PerfeCTa qPCR ToughMix ROX

(Quantabio; Beverly, MA), and 150 nM each of forward primer, reverse primer, TaqMan® probe (Integrated DNA Technologies; Coralville, IA). Thermal conditions were 95ºC for 15 min followed by 39 cycles of 95ºC for 15 s and 58ºC for 1 min. Aliquots of extracted DNA were sent to collaborators in the turfgrass pathology lab at University of Connecticut for quantification of *P. chlororaphis* AFS009 populations with strain specific primers and probe.

Field Trial

A multi-year field trial was initiated 6 June 2018 on a stand of "Salinas II" perennial ryegrass, maintained as a home lawn, at the Paint Branch Turfgrass Research Facility in College Park, MD. Turfgrass was mowed weekly to a height of 6.35 cm with a Toro Z-Master (The Toro Company; Bloomington, MN). Turfgrass was irrigated to prevent drought stress. Annually 144.17 kg N ha^{-1} was applied to the trial area as 73.16 kg water insoluble N ha^{-1} (Shaw's 44-0-0 Surfcote; Knox Fertilizer Company; Knox, IN) each April and as 23.67 kg water soluble N ha^{-1} (46-0-0) each month. Annual broadleaf weeds and sedges were controlled with applications of mesotrione at 7.61 kg a.i. ha^{-1} and halosulfuron at 0.069 kg a.i. ha^{-1}, respectively.

The trial utilized a split plot experimental design with a 2 x 5 factorial treatment arrangement. Main plots measured 1.8 m x 10.2 m with a 0.3 m buffer between plots, and sub plots measured 1.8 m x 1.8 m with a 0.3 m buffer between plots. The main plot factor was niche clearing applied as 5.18 L hydrogen peroxide + 0.38 L peroxyacetic acid ha^{-1} (19.1 L ZeroTol® 2.0 ha^{-1}) or no niche clearing. Niche clearing treatments were applied to plots in two passes through a M4 battery powered backpack sprayer (MY4SONS; Santa Rosa, CA) using an air-induction nozzle calibrated to apply 1,222.4 L ha^{-1} evenly

across the plots over the two passes. Three hours after niche clearing treatments were applied, sub plot treatments were applied. Sub plot treatments consisted of QST713 applied at $8.54x10^9$ colony forming units (CFUs) *B. subtilis* QST713 ha^{-1} (31.8 L Rhapsody® ha^{-1}), AFS009 applied at $9.13x10^9$ CFUs *P. chlororaphis* AFS009 ha^{-1} (9.1 kg Zio™ ha^{-1}), W9 applied as a cellular suspension with a titer of 10^9 CFUs ml^{-1}, azoxystrobin applied at 0.306 kg a.i. ha^{-1} (3.14 L Heritage® TL ha^{-1}; Syngenta Crop Protection; Greensboro, NC) and a non-treated control. Sub plot treatments were applied through a compressed air pressurized system using an air-induction nozzle calibrated to apply 814.9 L ha^{-1} evenly across the plots. Treatments were applied biweekly from 6 June to 2 August 2018 and from 7 June to 31 July 2019. Data were collected every 7-14 d during the trial periods. Brown patch severity was measured as percent plot area symptomatic. Turfgrass quality was measured on a 1-9 scale, where 1 = completely dead turfgrass, 6= minimal acceptable quality, and 9 = completely healthy turfgrass.

Statistical Analysis

Data collected from both projects were analyzed using PROC MIXED in SAS 9.4 (SAS; Cary, NC). Means were separated using Tukey's HSD. Unless specified, a p-value ≤ 0.05 was considered significant. Figures were generated in the R environment (R Core Team, 2020) with the package 'ggplot2' v. 3.3.2 (Wickham, 2016).

2.iv Results

Brown Patch Suppression

Niche clearing practices did not affect brown patch severity in the greenhouse experiment nor in the field trial. Brown patch progressed rapidly in the greenhouse. In all experimental runs, turfgrass in inoculated pots was completely dead by 7 – 10 d after

inoculation. There were no significant effects of treatments on brown patch severity (Fig 2.1). Statistical analysis of percent green cover from digital image analysis also did not show any significant treatment effects (Appendix A; Fig. S2.1). Under field conditions, BCO treatments reduced brown patch severity over the entire season (AUDPC) compared to the non-treated control, but only in 2019 (Fig 2.2A). Azoxystrobin reduced brown patch severity over the entire season in both years of the field trial (Fig 2.2A). In 2018, QST713 did reduce brown patch severity compared to the non-treated control before disease pressure increased in mid-July (Appendix A; Fig. S2.2). Turfgrass quality over the entire season was only improved by azoxystrobin applications in 2018 and 2019 (Fig 2.2B).

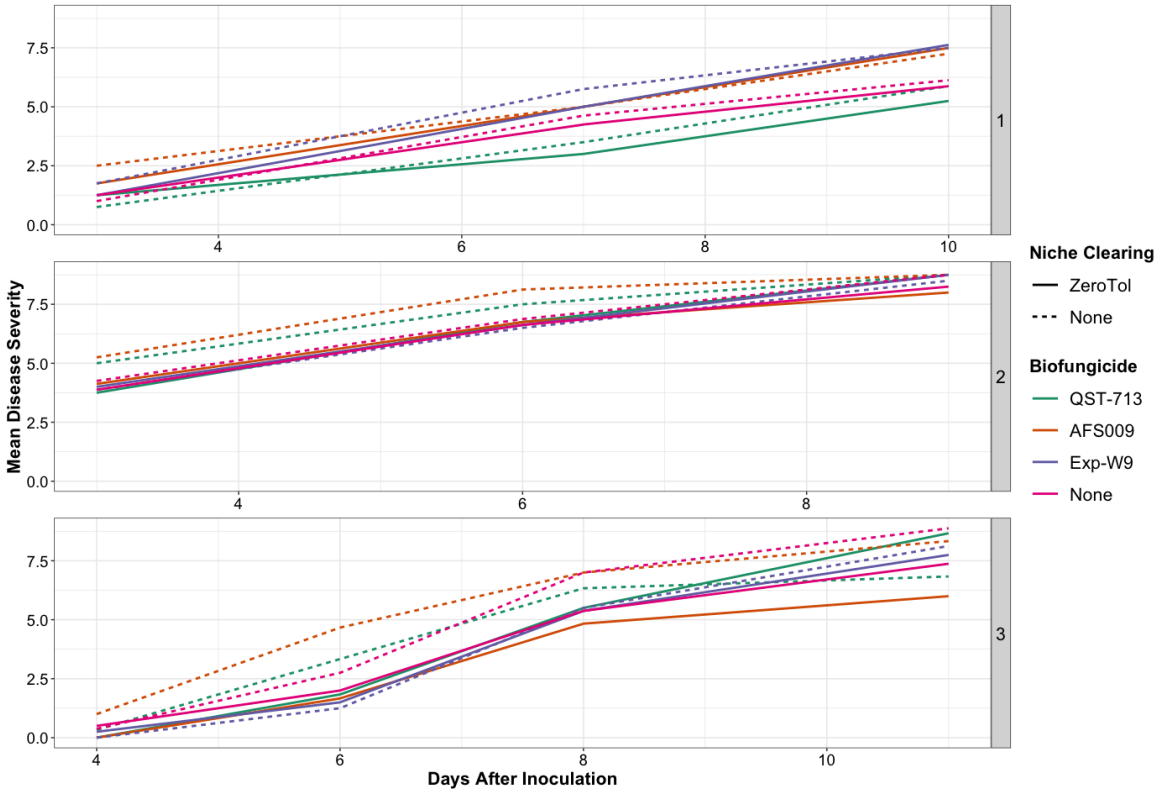

Fig 2.1. Mean brown patch severity over time in response to applications of niche clearing and biofungicide combinations to pots of "Express II" perennial ryegrass inside a greenhouse benchtop humidity chamber. Disease severity was rated on a 0-9 scale, where 0 = no disease and 9 = completely diseased pot. Facets 1, 2, and 3 represent each experimental run. QST713, AFS009, Exp-W9, and none refer to the biofungicide treatments *Bacillus subtilis* QST713, *Pseudomonas chlororaphis* AFS009, *B. subtilis* Exp-W9, and non-treated, respectively.

Foliar Bacterial Communities

Overall, there was no clear pattern in the effects of treatment combinations on culturable *Bacillus*, *Psuedomonas*, or bacterial communities (Appendix A; Fig. S2.3, S2.4, S2.5). When significant effects were observed on a given sample date, the only separation was between the highest and lowest values. Samples from 0 days after treatment did not show a consistent decrease in any of the culturable communities from ZT applications (Appendix A; Fig. Fig. S2.3, S2.4, S2.5).

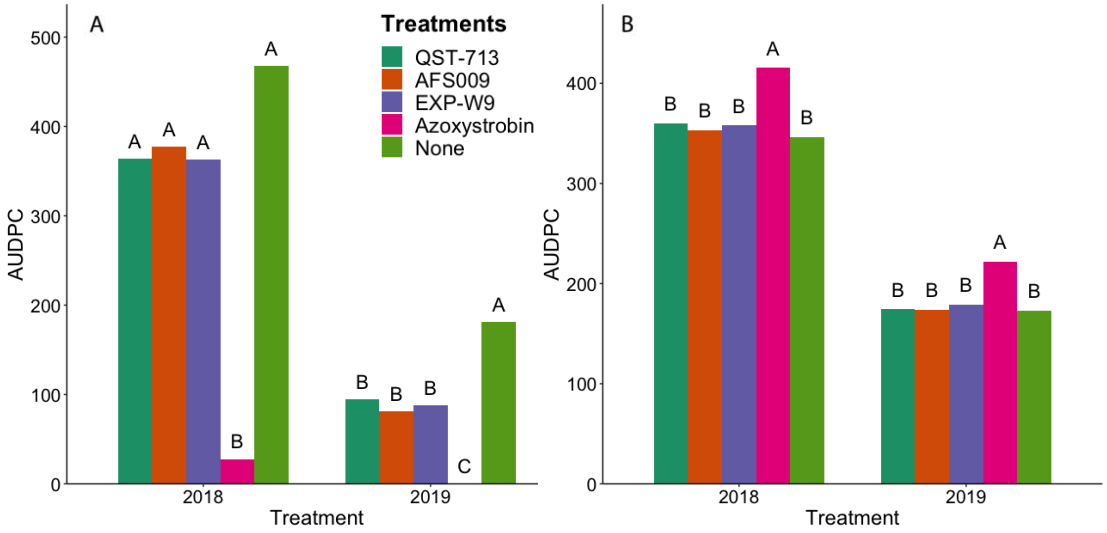

Fig 2.2. Area under the progress curve values for (A) disease severity and (B) turfgrass quality in response to (bio)fungicide applications to "Salinas II" perennial ryegrass maintained as a home lawn. Treatments sharing a letter within a given year are not statistically different (Tukey's HSD; $p \leq 0.05$).

Biological Control Organism Populations

Niche clearing practices did not consistently impact populations of either QST713 or AFS009 (Fig 2.3). In one experimental run, QST713 populations were lower in niche cleared pots (Fig. 2.3B). However, there was no niche clearing effects on QST713 in the other experimental run and AFS009 populations were never significantly impacted by niche clearing (Fig 2.3). Populations of *B.* subtilis QST713 and *P. chlororaphis* AFS009

decreased rapidly following application. Populations stopped declining by 7 d after application but did not completely diminish (Fig 2.3).

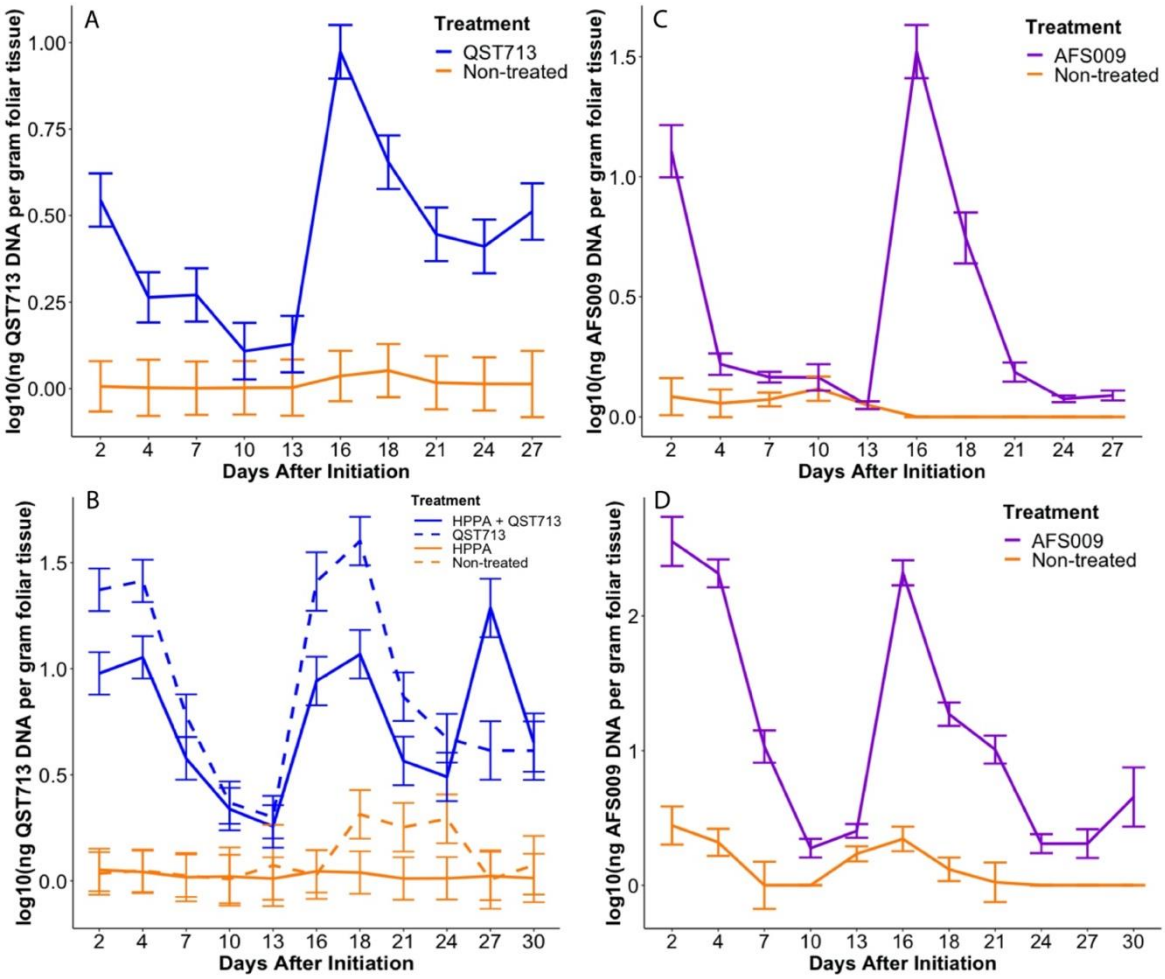

Fig 2.3. Foliar concentration of (A &B) *Bacillus subtilis* QST713 and (C & D) *Pseudomonas chlororaphis* AFS009 DNA, measured through qPCR assays, in response to treatment applications to pots of "Express II" perennial ryegrass in a greenhouse benchtop humidity chamber. Treatment applications were made at trial initiation and every 14d thereafter. Two experimental runs were conducted. Experimental run 1 presented in A & C and experimental run 2 presented in B & D. QST713, AFS009, and HPPA refer to *B. subtilis* QST713, *P. chlororaphis* AFS009, and hydrogen peroxide + peroxyacetic acid. Error bars represent standard errors of the means.

2.v Discussion

The goals of these projects were to evaluate niche clearing practices for their impact on the establishment of applied BCOs and on their preventative efficacy against the brown patch pathogen *Rhizoctonia solani*. Niche clearing practices did not result in

increased populations of introduced BCOs. Similarly, a recent study in apples did not see enhanced *Aureobasidium pullulans* colonization of apple flowers after treating them with a surface sterilant (Slack, et al., 2019). These results suggest applications of a surface sterilant will not provide an introduced BCO enough of a competitive advantage to colonize the host before native populations rebound.

Preventative efficacy of the tested BCOs was not improved by niche clearing practices. Likewise, biological control of fire blight in apple orchards was not improved through pre-treating apple blossoms with a surface sterilant before introducing *A. pullulans* (Slack, et al., 2019). We postulated that increased populations of a biological control organism would lead to increased efficacy against *R. solani*. However, as mentioned earlier, when measuring QST713 populations via qPCR we observed that niche clearing practices did not have an impact. Therefore, not observing an effect of niche clearing practices on preventative efficacy is logical. Combining H_2O_2 with a biological control organism can reduce disease more than the biological control organism alone. Soaking fava bean seeds for 6 h in a suspension of *B. megaterium* and H_2O_2 reduced incidence of root rot and damping off more than soaking the seeds in a suspension of *B. megaterium* alone (Abdel-Monaim, 2013). Although, in this study H_2O_2 was utilized as a chemical plant defense inducer not as a niche clearing product specifically. Perhaps utilizing a tank mixture of ZT and QST713 could prove more effective than sequentially applying them, but future research would need to be conducted to test this.

Interestingly, we did not observe decreases in culturable foliar microbial populations in sample collected on the day of application. Conversely, the

aforementioned similar study in apples did observe decreased culturable microbial populations within 4 h of surface sterilant application (Slack, et al., 2019). This difference might pertain to the difference in plant tissue, i.e., turfgrass foliage and apple flowers, or the growth habit of turfgrass. The foliage of turfgrass plants is in close proximity to the soil surface, where a large reservoir of microbiota resides. Furthermore, the active growing point of turfgrass is at or just below the soil surface constantly pushing new foliar tissue through the soil microbial reservoir. Microbial load is undoubtedly higher for turfgrass foliage compared to apple flowers, and as such microbial communities may have rebounded faster or been minimally impacted. Regardless of these differences, the apple flower microbial communities rebounded to pre-sterilant levels within 24 h (Slack, et al., 2019). Indicating that in both systems niche clearing practices were unable to reduce microbial competition long enough for the introduced BCO to gain a competitive advantage.

It is important to consider two possibilities that may help explain the complete lack of disease suppression observed in the humidity chamber trials. First, is that due to the highly favorable brown patch conditions within the humidity chamber, *R. solani* was able to easily overwhelm the applied BCOs. Secondly, it is also probable that the misting used to maintain favorable humidity levels for disease development was impacting BCO survival or dislodging the BCO from the foliage surface, thus preventing the BCO from providing disease suppression.

In conclusion, tested BCOs were able to reduce brown patch severity compared to the non-treated control, but they were not as effective or reliable as azoxystrobin. Niche clearing practices did not improve the establishment of applied BCOs, nor did they

improve the efficacy against the brown patch pathogen *R. solani*. While disease control was not improved through niche clearing practices, it was not decreased either. Suggesting that niche clearing practices did not give a colonization or infection advantage to *R. solani* either. In their current form, biological control organisms cannot provide the reliable control that turfgrass managers require from disease management products. However, given their efficacy under low to moderate disease pressure, future research efforts should focus on using BCOs as an integrated management option to reduce fungicide rates or extend intervals.

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Chapter 3: Investigating chemical and biological control applications for pythium root rot prevention and impacts on creeping bentgrass putting green rhizosphere bacterial communities

Joseph R. Doherty[1] and Joseph A. Roberts[1*]

[1]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742, U.S.A.

[2]Plant and Environmental Sciences Department, Clemson University, Florence, SC 29506, U.S.A.

[*]Current address: Plant and Environmental Sciences Department, Clemson University, Florence, SC 29506 U.S.A.

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3.i Abstract

Pythium root rot (PRR) is a disease that can rapidly devastate large swaths of golf course putting greens, with little recourse once symptoms appear. Golf courses routinely apply preventative fungicides for root diseases, which may be altering the rhizosphere microbiome leading to unintended impacts to plant health. A multi-year field trial was initiated on a 'T-1' creeping bentgrass (*Agrostis stolonifera* L. cv. 'T-1') putting green in College Park, Maryland to evaluate preventative PRR management for disease suppression and impacts to rhizosphere bacterial communities. Fungicides commonly used to prevent PRR and a biological fungicide were repeatedly applied to experimental plots throughout the growing season. Rhizosphere samples were collected twice annually from each plot to evaluate rhizosphere bacterial communities through amplicon sequencing and monitor biological control organism populations via qPCR. Cyazofamid was the only treatment to suppress PRR in both years compared to the control. Fosetyl-Al on a 14 d interval and *Bacillus subtilis* QST713 also reduced PRR severity in 2019 compared to the non-treated control. Treatments did not significantly affect bacterial diversity or relative abundances of bacterial classes, however seasonal environmental changes did. Repeated rhizosphere targeted applications of *B. subtilis* QST713 appear to have established the bacterium into the rhizosphere, as populations increased between samples, even after applications stopped. These findings suggest that QST713 may reduce pathogen pressure when repeatedly applied and can reduce fungicide usage during periods of low PRR pressure.

3.ii Introduction

Pythium root rot (PRR), caused by multiple *Pythium* spp., can cause rapid decline and death of large areas of turfgrass. While all turfgrasses are susceptible to PRR, this disease is most problematic on golf course putting greens. A multitude of *Pythium* spp. have been associated with PRR (Abad et al. 1994; Hampy et al. 2021; Hendrik et al. 1970; Nelson and Craft 1991), with differing aggressiveness at a given soil temperature. For example, *P. volutum* is more aggressive at 28ºC than 16ºC, *P. torulosum* aggressiveness was highest at 32ºC and quickly declined with temperature, and *P. ultimum* var. *ultimum* was most aggressive at 16 ºC (Abad et al. 1994; Nelson and Craft 1991). Excessive soil moisture has been correlated with increased *Pythium* spp. infection (Martin and Loper 1999; Schlub and Lockwood 1981; Stanghellini and Burr 1973). At a given soil temperature, higher soil water potential resulted in more severe PRR of snap beans compared to lower soil water potential (Pieczarka and Abawi 1978). In cool season putting greens like creeping bentgrass (*Agrostis stolonifera* L.), PRR is most common during the summer months, where symptoms manifest from the combination of root systems compromised by PRR, heat stress, and moisture stress. However, as evident from the varying soil temperatures conducive to *Pythium* spp. infection, PRR may manifest any time of year if the soil is sufficiently moist.

Multiple fungicide active ingredients are effective at controlling *Pythium* spp. in turfgrass systems, including mefenoxam, fluopicolide, cyazofamid, etridiazole, propamocarb, fosetyl-Al, azoxystrobin, fluoxastrobin, and pyraclostrobin (Cohen and Coffey 1986; Cook et al. 2009; Kerns et al. 2009). Recent research has shown preventative programs provide better control of PRR on golf course putting greens,

though curative programs can still be utilized to reduce symptoms (Hampy et al. 2021). Treatments including cyazofamid provided the best preventative control of PRR, while cyazofamid was the only treatment to curatively reduce PRR symptoms (Hampy et al. 2021). Disease severity was reduced with preventative applications of pyraclostrobin, fluoxastrobin, azoxystrobin, mefenoxam, and etridiazole compared to the non-treated control; however, none were as effective as cyazofamid (Hampy et al. 2021).

Fungicides can provide excellent suppression of PRR; however, biological based control has also been documented to reduce the severity of multiple Pythium diseases. In sand-based putting greens applications of organic amendments have reduced PRR severity (Craft and Nelson 1996). The authors attribute this suppression to the microbial activity of the organic amendments, which may indicate suppression through competition or antibiosis. In addition to general suppression via organic amendments, *Bacillus* spp. have been successfully utilized to reduce PRR symptoms in other cropping systems. *Bacillus subtilis* reduced PRR of cauliflower, caused by *P. ultimum* var. *ultimum*, when the bacterium was mixed directly into the soil (Abdelzaher 2003). *Bacillus* spp. have also reduced severity of Pythium diseases in tomato (Jayaraj et al. 2005; Kipngeno et al. 2015), hot pepper (Nakkeeran et al. 2006), and tobacco (Shang et al. 1999). *Bacillus* spp. strains that are utilized in formulated biological control products are commonly isolated from the soil. Therefore, successful establishment and control may be more reliable by using these products to challenge root-infecting pathogens.

Mitigating plant pathogens through the application of fungicides holds the potential to significantly alter the plant microbiome, especially with broad spectrum fungicides. Considering the plant microbiome plays a key role in the health status of a

plant (Berendsen et al. 2012; Lambers et al. 2009), it is crucial to understand the impacts of management practices on these communities. Repeated applications of propiconazole, chlorothalonil, triadimefon, iprodione, flutolanil, mefenoxam, and cyproconazole were found to not alter the rhizosphere microbial communities of a creeping bentgrass putting greens, as measured through culture-based assays, BIOLOG metabolic profiles, and fatty acid profiles (Harman et al. 2006). Conversely, phyllosphere fungal communities contained higher proportions of yeasts than filamentous fungi following applications of chlorothalonil, iprodione, or propiconazole (Harman et al. 2006). Foliar populations of actinomycetes, bacteria, fungi, and fluorescent pseudomonads were reduced by multiple fungicides, but effects were transient and no clear pattern was observed over the course of two growing seasons (Doherty et al. 2017). However, fungicides utilized to mitigate *Pythium* spp. tend to be specific to oomycetes, and there is little known regarding their impact on microbial communities in the soil.

Turfgrass managers regularly use timely fungicide applications (e.g., cyazofamid, propamocarb, and fosetyl-Al) to control PRR. However, there is a lack of data on the impacts of preventative PRR management on the turfgrass rhizosphere microbiome. Furthermore, the formulated product containing *B. subtilis* QST713 has activity against *Pythium* spp. diseases in ornamentals, but efficacy against *Pythium* spp. diseases in turfgrasses is not well understood. Therefore, a two-year field study was developed to 1) evaluate *B. subtilis* QST713 for its ability to suppress PRR development compared to commonly used fungicides and 2) elucidate the impact of these control measures on the rhizosphere bacterial communities. We hypothesized that 1) *B. subtilis* QST713 applications would reduce PRR severity compared to the non-treated control and 2)

treatment applications would cause shifts in the composition of rhizosphere bacterial communities.

3.iii Materials & Methods

Field Sites

A two-year field study was established 29 May 2019 on a "T-1" creeping bentgrass (*Agrostis stolonifera* L. cv. "T-1") modified push-up style putting green (60% sand/20% soil/20% peat) to evaluate preventative disease control applications on Pythium root rot development and impacts on the rhizosphere bacterial communities. The trial area was mowed five times per week with a Jacobsen Greens King IV Plus triplex reel mower (Jacobsen Manufacturing; Ipswich, England, UK) with a bench height of cut setting of 3.5 mm. Annually, 61 kg N ha^{-1} was applied to the trial area. Using a complete source (i.e., 20-20-20) 6.1 kg N ha^{-1} was applied to the trial area on 18 and 29 April 2019 and in 2020 on 17 and 29 April. Additional N was applied biweekly as soluble urea (46-0-0) at 6.1 kg ha^{-1} from 8 May to 23 Aug 2019 and from 5 May to 22 Aug 2020. Irrigation was provided to prevent drought stress. The fungal diseases dollar spot (*Clarireedia* spp. Salgado-Salazar), brown patch (*Rhizoctonia solani* Kühn), and take-all patch (*Gaeumannomyces gramminis* (Sacc.) Arx & D. Olivier var. *graminis*) were controlled with the fungicides chlorothalonil (Daconil Ultrex; Syngenta Crop Protection, Greensboro, NC), boscalid (Emerald; BASF Corp., Research Triangle Park, NC), and thiophanate-methyl (3336; NuFarm, Alsip, IL).

Plots were inoculated with *Pythium aphanidermatum* infested creeping bentgrass leaves on 25 June 2019 adapting methods described previously (Kerns and Tredway 2008). Briefly, four 3 mm mycelial plugs were placed into sterile water containing ten

sterilized creeping bentgrass leaves. Inoculum was grown for 3 days under constant lighting at 23ºC. A 7 cm deep core was taken at the center of each plot. Cores were pruned to a depth of 5 cm and fresh sand was placed into the hole in each plot to replace the discarded rhizosphere. Inoculum was then poured on top of the fresh sand and the core was returned. The trial area was irrigated thrice daily with 3.175 mm irrigation at 6 pm, 12 am, and 6 am for 2 weeks following inoculation in 2019. To encourage disease in 2020, the trial area was irrigated daily at 6 pm, 12 am, and 6 am with 3.175 mm of water for the entire month of May.

Experimental Design

The study was a randomized complete block design with four replications. Plots measured 0.9 m x 1.8 m. The same plots were used in both years. Treatments consisted of fosetyl-Al (Signature™ XTRA Stressgard®; Bayer Crop Science, Research Triangle Park, NC) applied at 7.31 kg AI ha^{-1} (12.18 kg Signature™ XTRA Stressgard® ha^{-1}) every 14 d, cyazofamid (Segway®; PBI-Gordon Corp., Shawnee, KS) applied at 0.572 kg AI ha^{-1} (1.433 L Segway® ha^{-1}) every 14 d, *B. subtilis* QST713 (Rhapsody®; Bayer Crop Science) applied at 0.308 kg *B. subtilis* QST713 ha^{-1} (31.8 L Rhapsody® ha^{-1}) every 14 d, fosetyl-Al (Signature™ XTRA Stressgard®; Bayer Crop Science) applied at 3.66 kg AI ha^{-1} (6.09 kg Signature™ XTRA Stressgard® ha^{-1}) every 7 d, propamocarb (Banol®; Bayer Crop Science) applied at 0.985 kg AI ha^{-1} (1.37 L Banol® ha^{-1}) every 14 d, and a non-treated control. Treatments were applied through a CO_2-pressurized system using an air-induction nozzle calibrated to apply 814.9 L ha^{-1} evenly across the plot. Treatments were applied from 29 May to 1 August 2019 and from 11 June to 5 August 2020. Immediately following application, plots were watered with 5 mm of irrigation.

Field Measurements and Analysis

Pythium root rot severity was measured, as percent symptomatic plot area, every 7 - 14 d. Turfgrass quality, an aggregate rating of color, density, and uniformity, was measured on a 1 - 9 scale, where 1 = completely dead turf, 9 = completely healthy, and 6 = minimal acceptable quality. Data were analyzed using PROC MIXED in SAS 9.4 (SAS, Cary, NC). Means were separated using Tukey's honest significant difference. A p-value of ≤ 0.05 was considered significant, unless specified otherwise. Figures were created using the R package 'ggplot2' v. 3.3.2 (Wickham 2016).

Rhizosphere Sampling and Processing

Rhizosphere samples were collected prior to trial initiation and 7 d after the final application each year. From each plot, four 2 cm soil cores were collected to a depth of 7.6 cm. Random locations within each plot were chosen using a random number generator and a 0.9 m x 1.3 m grid containing 171 0.05 m x 0.05 m sampling locations. The soil corer was sterilized with 75% EtOH between each plot, and holes were backfilled with autoclaved topdressing sand. The top 2 mm of each core was removed, to exclude thatch (i.e., layer of decaying organic material) and verdure, and the four cores for each plot were homogenized to form a representative sample. Homogenized samples were kept at -20℃ to prevent degradation of microbial communities. Environmental DNA (eDNA) was extracted from rhizosphere samples using the Qiagen PowerSoils kit (Qiagen, Gaithersburg, MD) with a modified protocol for low biomass soils as described by Beirn et al. (2016).

Amplicon Sequencing

Quality and dsDNA yields of extractions were measured using a Nanodrop 1000

(Thermo Fisher Scientific, Waltham, MA) and a Qubit fluorometer (Life Technologies, Grand Island, NY), respectively. Samples were diluted to 1.5 ng ul^{-1} based off Qubit quantification. Amplicons were generated using a two-step PCR process. First, bacterial 16S rDNA amplicons were PCR generated using the 515F-806R primer pair (Caporaso et al. 2011). Illumina adapters were appended to the 5' end of the primers for attachment of indices and Illumina sequencing adapters. Reverse primers were synthesized in four versions with 0-3 mixed sequence bases and combined into an equimolar mixture to increase diversity within the sequencing run, as low diversity libraries can result in low quality base calls without proper controls (Fadrosh et al. 2014). All PCR reactions and Illumina index incorporations were performed as previously described (Beirn et al. 2016). Before Illumina indices were incorporated, amplicons were purified using HighPrep PCR magnetic beads (MagBio Genomics, Gaithersburg, MD) following the manufacturer's protocol. Libraries were purified a second time with HighPrep PCR (MagBio Genomics) after index incorporation. Purified libraries were shipped for quality checking and sequencing at the Genomics Core at George Washington University. Amplicons were sequenced as paired-end reads (2 x 300) on an Illumina MiSeq with a 600-cycle MiSeq v.3 Reagent Cartridge (Illumina, San Diego, CA).

Sequence Analysis

Demultiplexed sequence data were parsed with cutadapt 1.9.1 (Martin 2011) for removal of non-biological sequences. Processed sequence data were imported into the R environment (R Core Team 2020) where the package 'DADA2' v. 1.18 (Callahan et al. 2016) was used for denoising, error correction, read joining, amplicon sequence variance (ASV) inference, chimera removal, and taxonomy assignment. Taxonomy was assigned

using the SILVA 138 SSU database (Quast et al. 2012; Yilmaz et al. 2013). A basic local alignment search tool (BLAST) query was used to check ASVs that were not assigned taxonomy from the SILVA database. BLAST results were only used to assign taxonomy if identity was > 98%, E-values were low, and the taxonomy was repeatedly returned. Any ASVs identified as Protista, chloroplasts, or mitochondria were removed from the analysis.

Diversity and statistical analyses of ASVs were conducted using the R packages 'phyloseq' v. 1.34.0 (McMurdie and Holmes 2013). Differences in alpha diversity estimates, calculated as Shannon and Simpson indices, were determined using nonparametric Kruskal-Wallis tests. Beta diversity was measured using Bray-Curtis dissimilarity matrices, with non-metric multidimensional scaling (NMDS) for ordination. Permutational analysis of variance (PERMANOVA) was used to compare bacterial community centroids. Pairwise permutation tests for homogeneity of multivariate dispersions were used, as PERMANOVA assumes equal beta dispersion. When beta dispersions were significantly different between groups, analysis of similarity (ANOSIM) was used to determine significance, as ANOSIM does not assume equal beta dispersions. Raw sequence data is available in the NCBI Sequence Read Archive as project PRJNA745161.

qPCR Analysis

Extracted eDNA was additionally subjected to qPCR analysis to assess populations of QST713 over the two-year study. Populations were monitored using qPCR primers and TaqMan® probes as previously described (Mendis et al. 2018). Reactions were performed in the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster

City, California). Final reaction volume was 20 µl, containing 2 µl of extracted DNA, 150 nM each of forward primer, reverse primer, TaqMan® probe (Integrated DNA Technologies, Coralville, IA), and PerfeCTa qPCR ToughMix ROX (Quantabio, Beverly, MA). Thermal conditions were as follows: 95º C for 15 min followed by 39 cycles of 95º C for 15 s and 58º C for 1 min. Data were subjected to statistical analysis in SAS 9.4 (SAS) using PROC MIXED and Tukey's HSD for means separation.

3.iv Results

Pythium Root Rot Control

Pythium root rot symptoms developed in early August each year. Disease severity peaked in the non-treated controls at 9.5% and 29% in 2019 and 2020, respectively. Symptoms completely resolved by 10 days following the final rating date. Cyazofamid was the only treatment to reduce PRR severity in both years (Fig. 3.1A). *B. subtilis* QST713 and fosetyl-Al on a 14d interval were the only other treatments to reduce PRR severity compared to the non-treated control in 2019 (Fig. 3.1A). Under low disease pressure (i.e., 2019), QST713 reduced PRR severity compared to the non-treated control; however, under high disease pressure (i.e., 2020), QST713 did not provide any suppression of PRR (Fig. 3.1). In both years turfgrass quality was highest in cyazofamid treated plots once PRR symptoms developed. Plots treated with fosetyl-Al weekly had higher turfgrass quality than propamocarb and non-treated plots in 2019 (Fig. 3.1B). PRR development drove turfgrass quality in 2020, with cyazofamid treated plots having higher turfgrass quality than *B. subtilis* QST713 and non-treated plots (Fig. 3.1B).

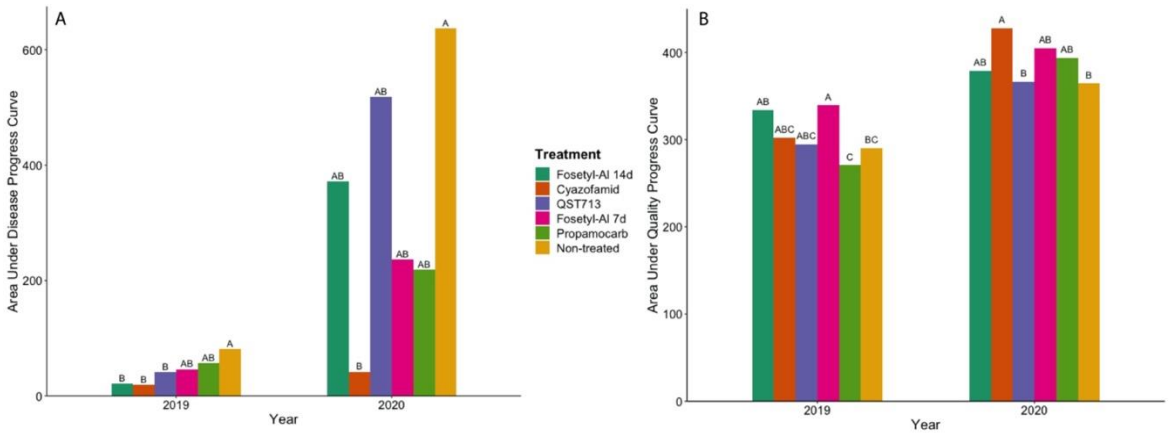

Fig 3.1. Area under the progress curves for (A) disease severity and (B) turfgrass quality in response to treatment applications in 2019 and 2020. Values were calculated from (A) disease severity and (B) turfgrass quality values collected from late May to late August 2019 and early June to late August 2020 at the Paint Branch Turfgrass Research Facility in College Park, MD. For each graph, bars sharing a letter within a year are not statistically different according to Tukey's HSD ($p \leq 0.05$).

QST713 Populations

Populations of QST713 increased at each sampling point throughout the trial (Fig. 3.2). Between August 2019 and June 2020 (i.e., when no treatments were being applied), QST713 populations continued to increase.

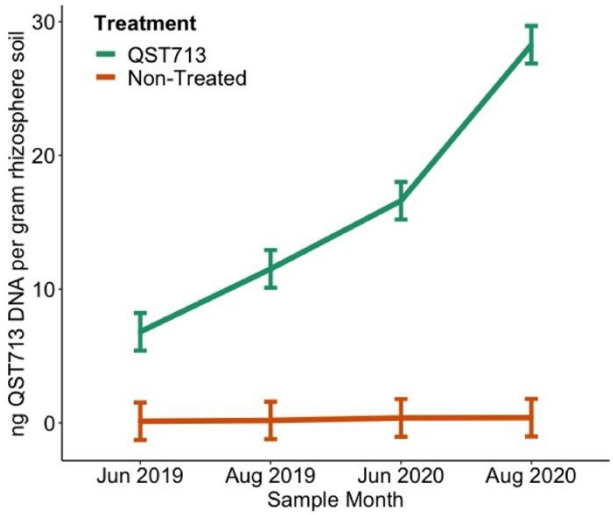

Fig 3.2. Rhizosphere populations of *Bacillus subtilis* QST713, measured through qPCR assays, in response to biweekly applications from 29 May to 1 Aug 2019 and from 11 June to 5 Aug 2020. Applications were watered into the rhizosphere with 5 mm post-application irrigation. Error bars represent standard error of the means.

Amplicon Sequence Variants

Processing of raw reads through the DADA2 pipeline resulted in 6.47 x 10^6 high quality sequences usable in downstream analyses. From these sequences, 19,050 amplicon sequence variants (ASVs) were inferred. There were 12,567 ASVs remaining following the removal of contaminant and unassignable ASVs from the data set.

Alpha and Beta Diversity Metrics

Shannon and Simpson indices were higher in 2020 than in 2019 (Fig. 3.3). In 2019, alpha diversity was higher in August than June (Fig. 3.3A). However, there was no significant difference between treatments within each month (Fig. 3.3A). There was no significant difference in alpha diversity between months or treatments in 2020 (Fig. 3B). Ordination of Bray-Curtis dissimilarity matrices showed samples clustering together by year, and by month within each year (Fig. 3.4). Community centroids were significantly different between 2019 and 2020 (Table 3.1). Within each year, community centroids for June and August were significantly different (Table 3.1). Treatments did not impact bacterial community centroids (Table 3.1).

Table 3.1. Centroid tests of beta diversity metrics for rhizosphere bacterial communities of a creeping bentgrass putting green receiving preventative treatments for Pythium Root Rot

	PERMANOVA	Dispersion	ANOSIM			
Year	0.001 ***	0.001***	0.001 ***			
	2019			**2020**		
	PERMANOVA	Dispersion	ANOSIM	PERMANOVA	Dispersion	ANOSIM
Month	0.001 ***	0.268	0.001 ***	0.001 ***	0.858	0.001 ***
Treatment	0.985	0.384	0.928	0.991	0.926	0.993

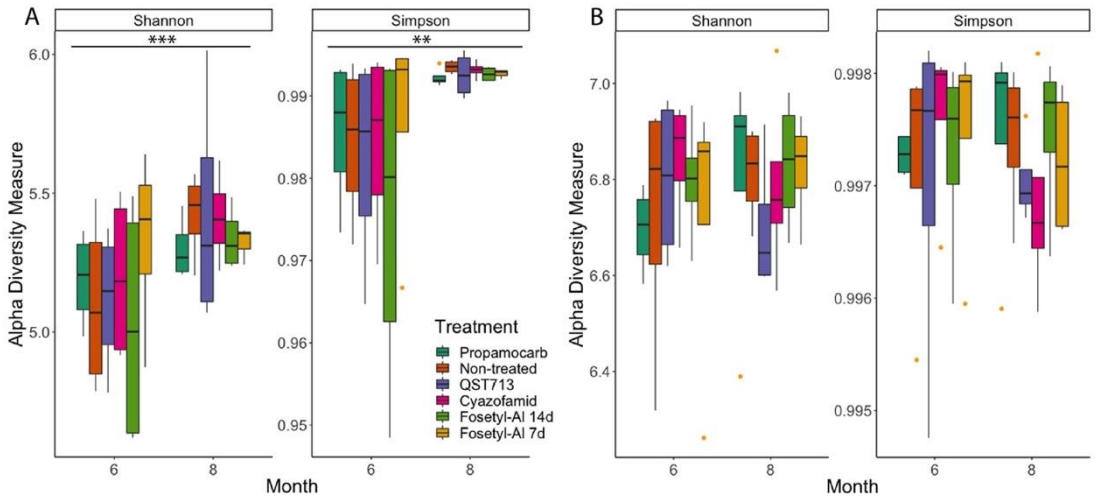

Fig 3.3. Alpha diversity estimates for rhizosphere samples collected in (A) 2019 and (B) 2020. Labels "6" and "8" refer to samples collected in "June" and "August" each year, respectively. Estimates were calculated in R using the 'phyloseq' package. Orange points represent outliers. *** denotes significance at the 0.01 level. ** denotes significance at the 0.05 level. Significance determined using non-parametric Kruskal-Wallis tests.

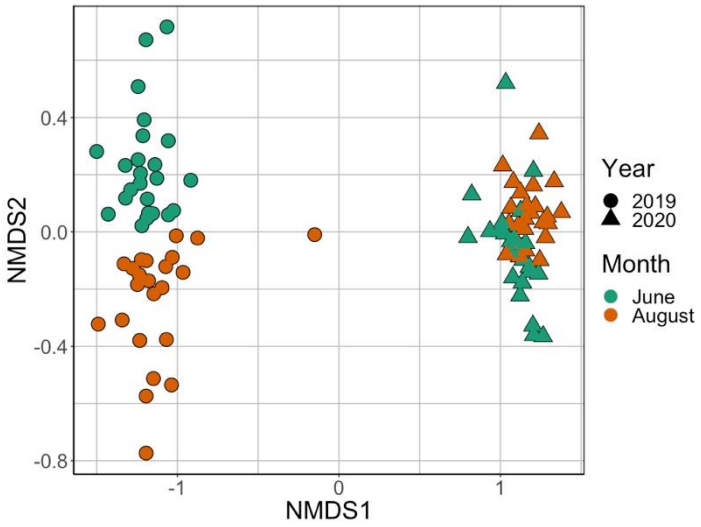

Fig 3.4. Non-metric multidimensional scaling of Bray-Curtis distance matrices. Shape indicates year and color indicates month sampled. Distance matrices and ordinations calculated in R using the 'phyloseq' package. Supporting statistics available in Table 1.

Taxonomic Composition of Bacterial Communities

Relative abundances of bacterial taxa were similar throughout the study. Alphaproteobacteria and Gammaproteobacteria were the two taxa present at the highest

relative abundances at each sampling point (Fig.3.5). Alphaproteobacteria relative abundances were slightly lower in 2020 than 2019, while Gammaproteobacteria relative abundances were consistent between each sampling point. Actinobacteria relative abundances decreased from June to August 2019 and remained at the lower relative abundance throughout 2020. Desulfuromonadia was not detected above 2% relative abundance in June 2019 but was detected at all subsequent sampling points (Fig. 3.5). Bacilli were not detected above 2% relative abundance until August 2020 (Fig. 3.5). Firmicutes were also not detected above 2% relative abundance until the final sample (data not shown).

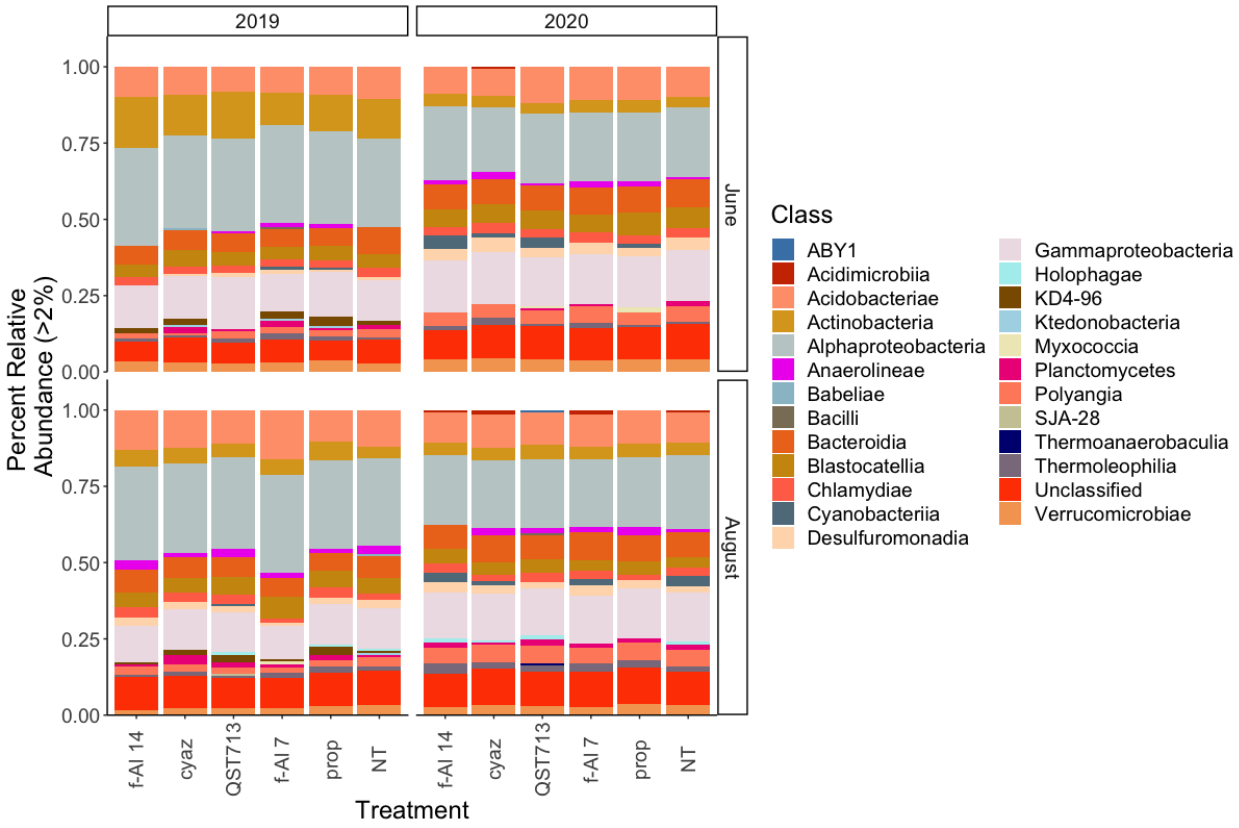

Figure 5. Relative abundances of bacterial classes present in rhizosphere samples collected from putting green plots treated with products to prevent Pythium root rot development. “f-Al 14”, “cyaz”, “QST713”, “f-Al 7”, “prop”, and “NT” refer to “biweekly fosetyl-Al”, “cyazofamid”, “*Bacillus subtilis* QST713”, “weekly fosetyl-Al”, “propamocarb”, and “non-treated”, respectively.

3.v Discussion

Pythium root rot is a perennial problem on golf course putting greens and preventative fungicide programs are commonly utilized to control this disease. The biological control agent *B. subtilis* QST713 and multiple fungicides were evaluated for preventative control of Pythium root rot, in addition to their off-target effects on rhizosphere bacterial communities. To our knowledge, this is the first study to utilize amplicon sequencing to evaluate off-target effects of preventative fungicide applications on turfgrass rhizosphere communities. Findings from this study indicate repeated fungicide applications for PRR have no measurable impact on rhizosphere bacterial communities, and under low disease pressure *B. subtilis* QST713 can reduce PRR severity.

Cyazofamid was found to be the most effective chemical option for preventing PRR development, keeping symptomatic turfgrass to less than 2.5% and 4% of the plot area in 2019 and 2020, respectively. Similarly in field studies conducted in North Carolina, treatments containing cyazofamid provided the best preventative control of PRR in multiple years and was the only active ingredient that provided curative control of PRR (Hampy et al. 2021). In the same study, pyraclostrobin, fluoxastrobin, azoxystrobin, mefenoxam, and etridiazole also preventatively controlled PRR compared to the non-treated check; however, they were not as effective as cyazofamid (Hampy et al. 2021). Preventative applications of cyazofamid are the best option for controlling PRR, but its site-specific mode of action underlies a pressing need to identify alternative or supplemental practices to control PRR before resistance develops.

Applications of *B. subtilis* QST713 reduced disease severity about 50% compared to the non-treated control in a low disease pressure year (2019) but were not effective under high disease pressure observed in 2020. However, in both years turfgrass quality in QST713 treated plots was similar to the non-treated control, albeit from different causes. Disease severity in 2019 was low enough that quality did not diverge, while in 2020 both treatments were similarly symptomatic. Under high disease pressure, biological control organisms have been successful though. On a creeping bentgrass putting green, where brown patch severity in the non-treated control reached 73.8% of the plot area, weekly applications of *B. subtilis* QST713 did reduce brown patch severity to 22% of the plot area (Roberts et al. 2019). *B. subtilis* QST713 and a *Trichoderma* based biological fungicide both reduced microdochium patch on an annual bluegrass putting green by 50% and 66%, respectively (Mattox et al. 2018). It is important to note that the biological control organisms in both studies were not able to completely suppress disease comparable to many synthetic fungicides, which underscores the importance of continuing to research methods of implementing biological products into management regimes to maximize disease reduction in the absence of synthetic fungicides.

Interestingly, rhizosphere populations of *B. subtilis* QST713 in this study were higher in 2020 than 2019. Given the role quorum sensing plays in the regulation of secondary metabolite production (Miller and Bassler 2001) or that outcompeting another organism for space and/or resources would be better facilitated at higher populations, one would expect more success with biological control organisms at higher populations. Though we did not measure *Pythium* spp. populations, so the pathogen may have simply outnumbered QST713 in 2020. Environmental conditions in 2020 may have been more

conducive for the *Pythium* spp. present. There was significantly more precipitation during the trial period in 2020 (55.52 cm) than 2019 (24.87 cm), in addition to the additional irrigation in May 2020 to encourage disease development. While most of the extra rainfall of 2020 occurred in August (28.65 cm), June 2020 (11.66 cm) precipitation totals were nearly double the precipitation in June 2019 (7.09 cm). Soil temperatures were colder in May 2020 (17.31ºC) than 2019 (20.29ºC) and warmer in July 2020 (27.50ºC) than July 2019 (26.88ºC). Previous research has observed *P. aphanidermatum* oospore germination and germ tube growth rates being 3 and 4 times faster, respectively, in 27ºC soil compared to 20ºC (Tedia and Stanghellini 1992). Yet bacterial communities in the same study had a generation time of 8 h at both soil temperatures. This suggests that at warmer soil temperatures, *P. aphanidermatum* can readily outcompete soil bacteria and rapidly infect turfgrass roots. Regardless of which reason explains the lack of control in 2020, these data highlight the inconsistencies inherent in utilizing biological controls, which is a major limitation to widespread adoption of biological control organisms in their current state.

Populations of *B. subtilis* QST713 increased over the course of the study. Interestingly, QST713 populations even increased between August 2019 and June 2020 (i.e., when no treatment applications were made), suggesting that QST713 was able to establish and overwinter in the rhizosphere. Similar results were observed with *Pseudomonas aureofaciens* TX-1, a biological control organism applied to turfgrass through an irrigation system (Sigler et al. 2001). In both cases, the biological control agent was repeatedly applied. Repeated application of a biological control organism appears to be crucial to establishing the organism into the rhizosphere, as *B. subtilis*

GB03 applied once to the soil at potato planting, the FAME profile of GB03 was not detectable by harvest (Larkin 2016). This is logical, as the soil is flush with microbial life and introducing a new organism requires overcoming significant competition from those resident microbes.

Amplicon sequencing of rhizosphere bacterial communities did not detect *Bacillus* spp. or Firmicutes above 2% relative abundance until the final samples in August 2020, and they were only detected in plots treated with *B. subtilis* QST713. This coincides with the highest measured QST713 populations in qPCR data. However, the levels measured through qPCR are not necessarily the critical levels to break 2% relative abundance in all rhizosphere communities. During amplicon generation, some more prevalent community members may have been amplified more than less abundant members, such as *Bacillus* spp. Furthermore, relative abundance is not a quantitative measurement of taxa prevalence and is data set dependent, but is one way to visualize differences in community structure that analyses like PERMANOVA and ordination of beta diversity measures detect.

Rhizosphere bacterial communities were changing between sampling dates, highlighting seasonality effects on the communities. This is not a unique observation, as others have observed this phenomenon in managed and natural systems (Beirn et al. 2016; Cho et al. 2008; Dunfield and Germida 2003). However, the data do not show lasting alterations to bacterial communities from repeated fungicide or biological control organism applications. Culturable soil bacterial communities were not increased or reduced by chemical seed treatment or applications of biological control organisms at potato planting (Larkin 2016). However, substrate use and fatty acid methyl ester profiles

revealed treatments did alter soil microbial populations (Larkin 2016). Fungicide impacts on microbial communities can vary. Iprodione has been observed to reduce soil bacterial communities until the chemical has dissipated from the soil (Wang et al. 2004), and tebuconazole transiently altered bacterial community beta diversity (Storck et al. 2018). However, these studies were evaluating more broad-spectrum fungicides while our study utilized fungicides that were specific to oomycetes. Perhaps this specificity of the active ingredients resulted in undetectable or transient changes in bacterial communities. Conversely, it is possible that selected primers or sequencing technology was insufficient to detect changes. In either case, the presented data alongside prior findings suggest that seasonal variations express greater influence over rhizosphere bacterial communities than fungicide treatments.

Research presented here demonstrates that Pythium root rot can be preventatively managed with minimal alterations to rhizosphere bacterial community structure. Furthermore, rhizosphere populations of beneficial bacteria can be established and sustained without causing significant alterations to the rhizosphere bacterial community. Considering *B. subtilis* QST713 appears to be able to establish and overwinter in a putting green rhizosphere, it may be a valuable tool to integrate into season long management programs to suppress disease under periods of low pressure. Which could lead to reductions in overall fungicide inputs and allowing more effective products to be utilized when increased disease pressure necessitates an application. Additional research is needed, however, to determine how full fungicide programs and cultural practices would impact establishment and survival of applied biological control organisms.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Chapter 4: Combining compost topdressing and biological control applications for season long control of foliar pathogens in a creeping bentgrass fairway

4.i Abstract

Compost and biological control agents can suppress pathogens alone; however, their reliability and efficacy are not as acceptable as synthetic fungicides commonly used to suppress pathogens. A multi-year field study was initiated to evaluate combinations of monthly compost and weekly or biweekly *Bacillus subtilis* QST713 (QST713) applications for their ability to suppress foliar pathogens on a creeping bentgrass (*Agrostis stolonifera* L.) fairway and to measure their impact on QST713 establishment. Disease severity and turfgrass quality were measured every 14 d throughout the growing season. Populations of QST713 were quantified by qPCR analysis on DNA extracted from foliage samples collected throughout the trial. Compost applications increased turfgrass quality in both years of the study and reduced dollar spot severity in 2021. Weekly QST713 applications reduced copper spot severity compared to biweekly applications and the non-treated control in 2020, yet monthly compost and weekly QST713 applications completely suppressed copper spot in 2021. Populations of QST713 were highest in weekly treated plots, and monthly compost applications did not affect QST713 establishment. While there was not an interaction between compost and QST713 applications, implementing both in a season long program will benefit turfgrass health and reduce disease severity.

4.ii Introduction

Synthetic fungicides are commonly used to reduce damages from plant pathogens in many agrosystems. While these products are highly effective, reliance on them needs to be reduced to minimize environmental impacts or fungicide resistance development. Evolution of fungicide resistance is a common phenomenon, that renders repeatedly used fungicides either less effective or completely ineffective (Lucas et al. 2015). While this remains a minimal issue in turfgrass systems, the potential for resistance development still remains with the use of site specific fungicides. Developing and improving alternative disease management practices can facilitate a reduction in synthetic fungicide use. For example, anthracnose on annual bluegrass [*Poa annua* L.f. reptans (Hausskn) T. Koyama] putting greens is dramatically reduced without fungicide applications by increasing mowing heights, increasing sand topdressing rates, and increasing rates of nitrogen (Hempfling et al. 2017). Dollar spot (*Clarireedia* spp.) severity is reduced through cultural practices like lightweight rolling (Nikolai et al. 2001), nitrogen fertilizer (Golembiewski and Danneberger 1998; Townsend et al. 2021), and plant growth regulators (Golembiewski and Danneberger 1998; Putman and Kaminski 2011). However, individual cultural practices are typically not enough to control diseases as well as synthetic fungicides do. As such, investigating all possible alternative practices to quell disease development is key to reducing the use of synthetic fungicides.

Biochar (i.e., pyrolyzed biomass) is an organic amendment that has multiple qualities that suggest incorporation into alternative and/or wholistic disease management programs. A meta-analysis of 371 studies utilizing biochar as a soil amendment revealed increased soil N, P, K, and C, crop yields, soil microbial biomass, and rhizobia

nodulation (Biederman and Harpole 2013). Amending the sand-based root zone of a golf course putting green increased water retention and creeping bentgrass growth compared to the non-amended root zone (Brockhoff et al. 2010; Vaughn et al. 2018). However, it is important to note that there is significant variation in the degree to which many of these soil physiochemical properties are impacted. Furthermore, biochar may be beneficial in reducing damage from plant pathogens. Beckley and Roberts (2021) recently observed reductions in dollar spot from biochar incorporation on a creeping bentgrass fairway in both years of their study. Vermicompost and synthetic fertilizer both reduced dollar spot in both years as well. Potting growth substrates amended with biochar suppressed gray mold and powdery mildew of peppers and tomatoes (Elad et al. 2010) and early blight of tomatoes (Rasool et al. 2021). The mechanism behind biochar mediated disease suppression in strawberries was an increased expression of five plant defense related genes (Harel et al. 2012). Suggesting, that biochar may provide broad base level benefits to plant health. Even if disease is not completely suppressed, a boost to plant defenses from biochar would reduce the pressure on other pathogen suppression practices. Caution should be used as having plants constitutively express defense mechanisms will result in costs to fitness and growth (Denancé et al. 2013; Vos et al. 2013)

Biological control organisms (BCOs) are another alternative that can have shown some degree of success in suppressing plant pathogens in the field (Pal and Gardener 2006). However, the adoption of BCOs by growers remains limited. Reliability of BCOs remains one of the chief limitations to their adoption. In a summary of 24 Plant Disease Management Reports spanning multiple turfgrass diseases, biofungicides (i.e., commercially formulated biological control organism products) only reduced disease in

33% of trials (Latin 2011). Lack of reliability may stem from resident microbiota outcompeting introduced BCOs. Research shows that turfgrass hosts have diverse microbial communities (Beirn et al. 2016; Crouch et al. 2017; Doherty et al. 2021). As such, overcoming competition from resident communities will be key in improving reliability of BCO disease control.

The objectives of this study were to 1) assess combinations of monthly biochar topdressing applications with weekly or biweekly biofungicide applications for their ability to suppress foliar diseases and 2) monitor BCO populations in response to treatment combinations. We hypothesized that combinations would suppress foliar diseases more than biochar or biofungicides alone, and that BCO populations would be higher in combination treatments than in biofungicide only treatments.

4.iii Materials & Methods

Site Description

A 2-year field trial was initiated in May 2020 on a "007" creeping bentgrass (*Agrostis stolonifera* L. cv. "007") fairway at the Paint Branch Turfgrass Research Facility in College Park, MD. The trial area was mowed three times per week at a bench height of 1.25 cm with a Jacobsen Greens King IV Plus triplex reel mower (Jacobson Manufacturing; Ipswich, England, UK). The trial area was irrigated to prevent drought stress. Annual weeds were controlled preemergence with dithiopyr (Dimension® 2EW; Dow AgroSciences; Indianapolis, IN) at a rate of 0.56 kg ai ha^{-1} on 28 April 2020 and 12 April 2021. Annual bluegrass [*Poa annua* L.f. reptans (Hausskn) T. Koyama] was controlled postemergence with amicarbazone (Xonerate® 2SC; FMC Corp.; Philadelphia, PA) at a rate of 0.17 kg ai ha^{-1} on 28 April 2020, 13 May 2020, 27 April

2021, and 10 May 2021. Weed control applications were made using a Toro Multi Pro 1750 (The Toro Company; Bloomington, MN) equipped with TeeJet AIC11008 nozzles calibrated to deliver applications in 814.9 L H_2O ha^{-1}.

Experimental Design

This trial was arranged as a randomized complete block split plot design with a 2 x 3 factorial treatment arrangement. Treatment combinations were replicated 4 times. Dimensions of the main plots measured 1.8 m by 6.1 m and were split into 3 sub plots measuring 1.8 m by 1.8 m with a 0.3 m buffer between sub plots. Main plot treatments were a modified sub-set of two main plot treatments from a previous study on the site by Beckley and Roberts (2021). The first main plot treatment was monthly topdressing applications of biochar (2-2-2 Mirimichi Green™ Pro Soil Enhancer; Mirimichi Green Express; Castle Hayne, NC) while the second main plot treatment was a non-treated control. Biochar applications were made monthly from May through August each year at a rate of 20.4 kg N ha^{-1}. Topdressing was performed using a shaker jar and a hard landscape rake to evenly distribute the application across the plot area.

Sub plot treatments consisted of weekly and biweekly *Bacillus subtilis* QST713 (Rhapsody®; Bayer Crop Science; Research Triangle Park, NC) and a non-treated control. Both intervals of *B. subtilis* QST713 were applied at a rate of 0.308 kg *B. subtilis* QST713 ha^{-1} (31.8 L Rhapsody® ha^{-1}). Treatments were applied through a CO_2-pressurized system using an air-induction nozzle calibrated to apply 814.9 L ha^{-1} evenly across the plot. Sub plot treatments were applied at their respective intervals from 11 June to 19 Aug 2020 and from 2 June to 11 Aug 2021.

Dollar spot and copper spot severity were evaluated by counting infection centers within each plot. Brown patch severity was evaluated through visual estimation of percent area within plots exhibiting brown patch symptoms. Turfgrass quality (i.e., color, density, and uniformity) was evaluated visually on a 1 to 9 scale where 6 represented minimal acceptable quality and 9 represented the highest quality possible. Disease severity and turfgrass quality were evaluated every 7 to 14 d throughout the trial period.

Monitoring QST713 Populations

To evaluate the establishment of QST713 in response to treatment combinations, samples were collected in 2020 on 22 May and 19 August and in 2021 on 25 May, 22 June, 21 July, and 18 August. For each sub plot, samples of five turfgrass plants were collected, using flame sterilized forceps, from four randomized locations within the plot. Senescent foliage and root tissue were removed, and samples were placed into pre-weighed 1.5 ml microcentrifuge tubes. Tubes were kept on ice in the field and during transit to the lab, whereupon they were re-weighed and placed into a -20º C freezer to limit community degradation. Samples were lyophilized for 48 h prior to DNA extraction to optimize yields. DNA was extracted from samples using the Qiagen DNeasy Plant Mini Kit (Qiagen; Gaithersburg, MD), following the manufacturer's protocol.

Pure cultures of QST713 were isolated through dilution plating of the commercial product Rhapsody (Bayer Crop Science). Once a pure culture was obtained, QST713 cells were suspended in sterile DI H_2O and diluted to an optical density of 1.0 (~10^9 CFUs ml^{-1}). DNA was extracted from cellular suspensions using the Zymo Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research Corporation; Irvine, CA) following the manufacturer's instructions. Extracted DNA was quantified using a

NanoDrop One spectrophotometer (Thermo Fisher Scientific; Waltham, MA). DNA was then diluted to a concentration of 10 ng QST713 DNA μl^{-1}. A serial dilution was performed to obtain additional concentrations of 1, 0.1, 0.01, and 0.001 ng QST713 DNA μl^{-1}, forming a standard curve with 5 steps.

Using QST713 primers, probe, and protocol as previously described (Mendis et al. 2018), qPCR reactions were completed in the StepOnePlus Real-Time PCR System (Applied Biosystems; Foster City, CA) with a final volume of 20 µl, containing 2 µl of sample DNA, PerfeCTa qPCR ToughMix ROX (Quantabio; Beverly, MA), and 150 nM each of forward primer, reverse primer, TaqMan® probe (Integrated DNA Technologies; Coralville, IA). Thermal conditions for qPCR reactions were as follows: 95º C for 15 min followed by 39 cycles of 95º C for 15 s and 58º C for 1 min.

Statistical Analysis

All data were analyzed using a two-way repeated measures analysis of variance (ANOVA) in SAS 9.4 (SAS Institute; Cary, NC). Logarithmic transformations were applied to qPCR data to correct patterns in the residuals. For statistically significant factors, Tukey's HSD post hoc test was used to compare treatment means. Graphs were generated using 'ggplot2' in R (Wickham 2016).

4.iv Results

Field Data

Dollar spot developed naturally in June each year, brown patch developed naturally in July 2020, and copper spot developed naturally in August 2020 and late-July 2021. Brown patch did not develop in 2021. In 2020, dollar spot severity was unaffected by treatments, in combination or alone. Compost topdressing applications reduced brown

patch and dollar spot severity in 2020 and 2021, respectively (Fig. 4.1 A&B). Weekly QST713 applications reduced copper spot severity by 50% compared to other treatments in 2020, and by 95% compared to the non-treated control in 2021 (Fig. 4.1 C). In both years, plots receiving monthly compost topdressings had significantly higher quality than plots not receiving compost topdressing (Fig. 4.2).

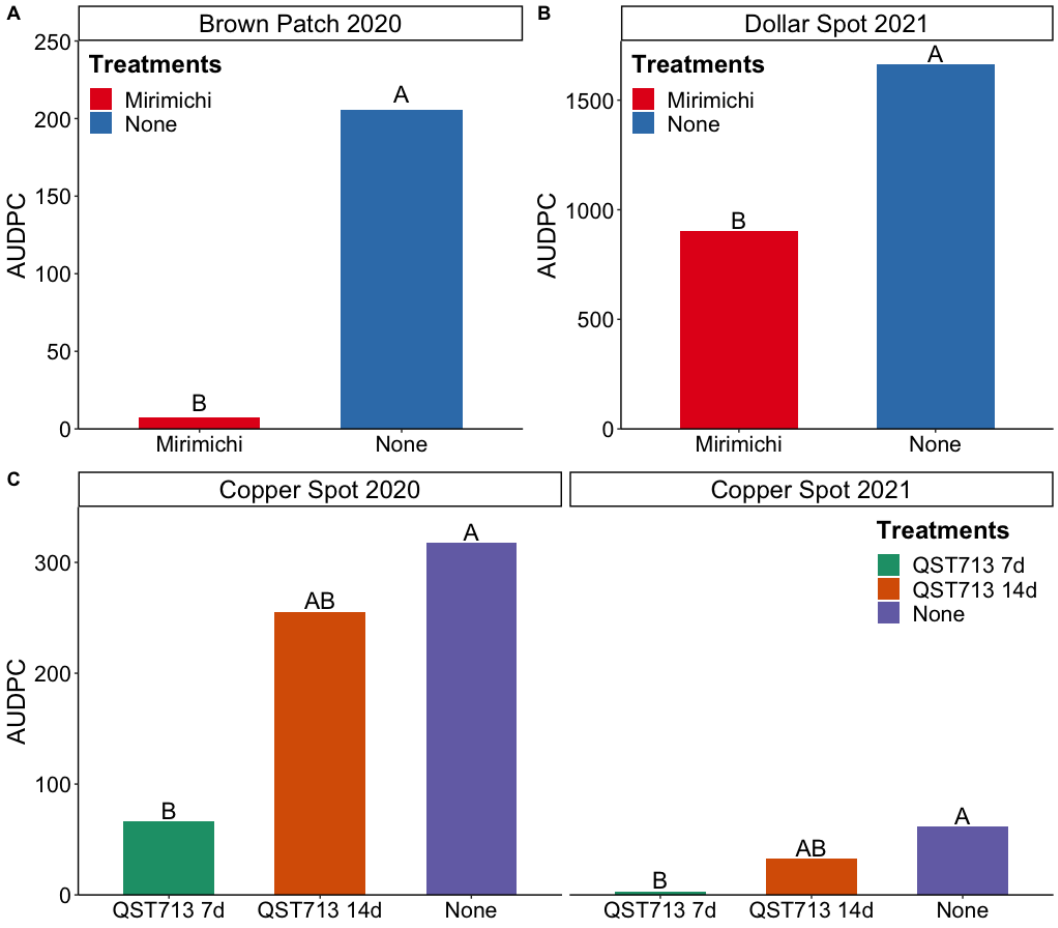

Fig. 4.1. Area under the disease progress curve values in response to monthly biochar topdressing (A & B) and weekly or biweekly *Bacillus subtilis* QST713 applications (C & D). Values calculated from A) 2020 brown patch severity (% plot area symptomatic), B) 2021 dollar spot (infection centers), C) 2020 and 2021 copper spot (infection centers). Treatments, within each quadrant, sharing a letter are not significantly different according to Tukey's HSD ($p \leq 0.05$)

QST713 Populations

In 2020, populations of QST713 were highest in plots receiving weekly QST713 applications, and there was no difference between the non-treated control and biweekly QST713 applications (Fig. 4.3). By May 2021, QST713 was undetectable in all plots. Similar to 2020, QST713 populations were highest in plots receiving weekly QST713 applications regardless of compost topdressing (Fig. 4.3).

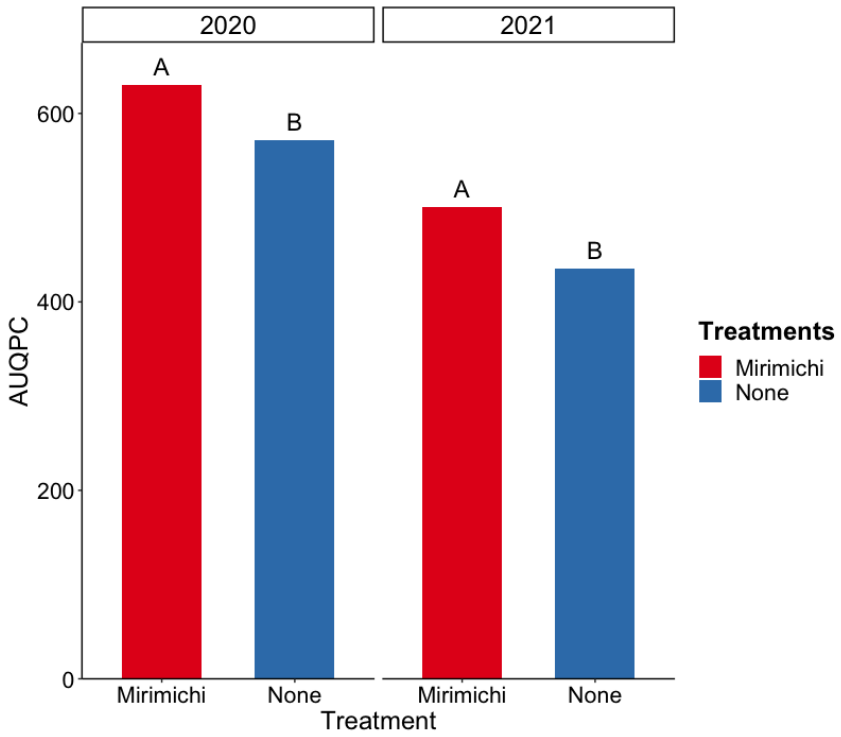

Fig. 4.2. Area under the quality progress curve values in response to monthly biochar topdressing. Values calculated from visual turfgrass quality ratings collected in 2020 and 2021. Treatments, within each year, sharing a letter are not significantly different according to Tukey's HSD ($p \leq 0.05$)

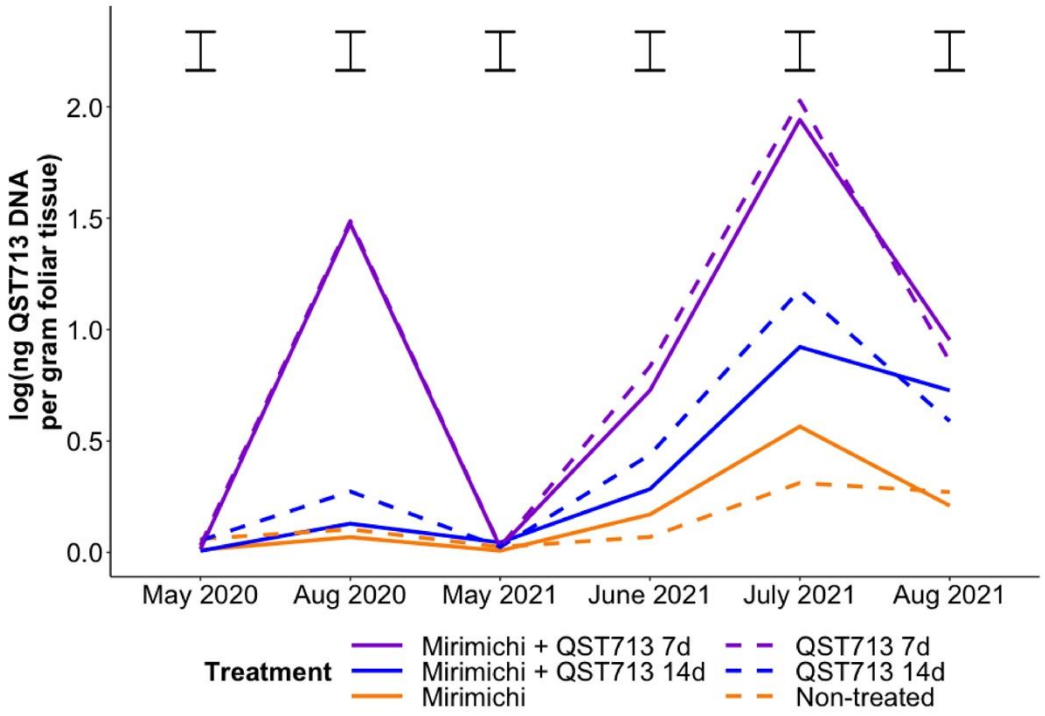

Fig. 4.3. Foliar concentrations of *Bacillus subtilis* QST713 DNA, measured through qPCR assays, in response to combinations of monthly biochar topdressing and weekly or biweekly *B. subtilis* QST713 applications made to a "007" creeping bentgrass fairway. Error bars represent standard errors of the means for each sampling date.

4.v Discussion

Applications of biochar material and biological control organisms can each provide plant health benefits. This research set out to evaluate combinations of these two materials in season-long programs for their effects on disease severity, turfgrass quality, and establishment of applied biological control organisms. No synergistic treatment effects on turfgrass quality or disease severity were observed. However, each product provided benefits that the other did not.

Applications of biochar proved to be more beneficial than QST713 in reducing disease and improving turfgrass quality. The observed benefits from biochar applications may stem from alterations to microbial communities, increased N fertility, or a combination of the two. Microbiota associated with N cycling have shown to be

stimulated by biochar applications (Clough et al. 2013; Xu et al. 2014), which may lead to increased N available to the plants. However, some biochar material may decrease plant N concentration (Lentz and Ippolito 2012; O'Toole et al. 2013), while some increase it (Jones et al. 2012; Uzoma et al. 2011), and still others had no effect (Jones et al. 2012; Prendergast-Miller et al. 2011). The biochar material used in this study also contained compost, so perhaps stimulated N cycling resulted in more plant available N. Further research would be needed to test this hypothesis. If this is true though, it would explain the decreased dollar spot severity, as increased N fertility has been documented to reduce severity of diseases like dollar spot (Beckley and Roberts 2021; Golembiewski and Danneberger 1998), and the increased turfgrass quality.

Brown patch is relatively unaffected by N fertility though, with rates of 293.1 kg N ha^{-1} yr^{-1} or higher for 2 years needed to intensify brown patch severity (Butler et al. 2019). Perhaps the suppression of brown patch stems from alterations to the plant microbiome in response to biochar applications. Kolton and others (2017) observed biochar increasing microbial and functional diversity of tomato rhizosphere bacterial communities and decreasing gray mold severity. Additionally, biochar did not result in changes to plant physiological characteristics, leading the authors to believe the alterations to microbial communities to be a significant factor in the reduced gray mold severity. Plant defense mechanisms have also been observed to be elicited by biochar applications (Harel et al. 2012). Biochar applications upregulated plant defense pathways in tomato and led to a reduction in Fusarium crown rot severity (Jaiswal et al. 2020). It is important to note that in most studies of biochar it is incorporated into the soil or potting growth medium, whereas in our study the material was applied monthly over the top of

the turfgrass. Our findings suggest that topdressing biochar may still provide disease suppression benefits observed in soil incorporated biochar; although, the underlying mechanisms remain to be elucidated in biochar topdressing.

In the absence of a regular fungicide program, uncommon diseases may become more problematic. We observed an outbreak of copper spot (*Gloeocercospora sorghi* Bain & Edgerton ex Deighton) in both years of this study. Although copper spot has been described for some time (Smith et al. 1989), little information is available regarding fungicide efficacies (Clarke et al. 2019). It is likely that fungicide programs for managing more persistent or problematic turfgrass diseases also control copper spot completely. Therefore, little data exists for fungicide efficacy. Regardless, copper spot is often considered a nuisance disease that is of low priority, as turf readily recovers from *G. sorghi* damage. It is quite possible that by switching away from conventional management regimes, with synthetic fungicides, we may see an increase in epidemics of less common or nuisance diseases, like copper spot. Which underscores the importance of future research on hybrid and organic management regimes now, so unforeseeable problems can be addressed before turfgrass managers are saddled with restrictions that necessitate a switch away from synthetic fungicides.

Findings from this research suggests that implementing both products in a season-long program is advisable for improved turfgrass quality in the absence of synthetic chemicals or fertilizers. Biochar reduced disease severity that QST713 did not, and vice-versa, in addition to providing increased turfgrass quality. Additionally, biochar did not significantly impact QST713 populations. Further research is needed to continue

exploring alternative disease control measures to reduce the turfgrass industry's reliance on synthetic fungicides.

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Dissertation Conclusions

Research designed for this dissertation set out to monitor biological control organisms (BCOs) in turfgrass systems and examine cultural practices and application techniques to improve efficacy and reliability. Our research was successful in understanding survival of introduced BCOs across a variety of experimental conditions. While cultural practices examined showed limited improvement to the efficacy of the BCOs, results from this dissertation highlight several important caveats to consider when implementing BCOs.

First, repeated applications of a BCO need to be made no more than 7 d apart. This practice will help maintain higher populations of the applied BCO. Efficacy isn't guaranteed by using a tighter application interval. But it will provide a higher chance for success, as evident in the QST713 application interval dose-response in copper spot suppression observed in chapter 4.

Second, biological control of turfgrass pathogens is not feasible under periods of high disease pressure. In every chapter of this dissertation, apart from copper spot, BCOs did not suppress disease during periods of high disease pressure. Perhaps during these periods of high disease pressure BCOs are overwhelmed by rapidly growing pathogens. If this is the case, making extra applications of a BCO prior to favorable disease development conditions could bolster BCO populations and improve disease suppression. Future research is needed to test this hypothesis, or other means to suppress pathogens during favorable conditions in the absence of synthetic fungicides.

Finally, disease control in the absence of synthetic fungicides will require a multifaceted approach. Synthetic fungicides can provide superb disease control at low use

rates, especially new chemistries. Conversely, biofungicides (i.e., commercial products with a BCO as the active ingredient) have much higher use rates and do not control disease as well. Using integrated pest management practices (i.e., the combined use of cultural, biological, and chemical means of disease control) can be beneficial in extending intervals or reducing use rates of synthetic fungicides. Integrated pest management tools will be essential in improving the efficacy of BCOs, as it is clear from this dissertation that BCOs are not effective or reliable as a sole means of disease control.

There is clearly more research that needs to be conducted on BCOs. One potential avenue of future research is investigating BCO and pathogen population dynamics to identify when control is achieved and when the pathogen breaks through that control. Alternatively, it may be beneficial to "flip the script" and instead of identifying why BCOs are not working in the environments they are applied to, focus on identifying the mechanisms behind their success in natural environments where the BCO was identified. Turfgrass managers rely on our research endeavors to continue controlling pathogens and it is our imperative to continue to innovate and investigate all methods of controlling pathogens, including biological control.

Appendix A. Supplementary information for Chapter 2

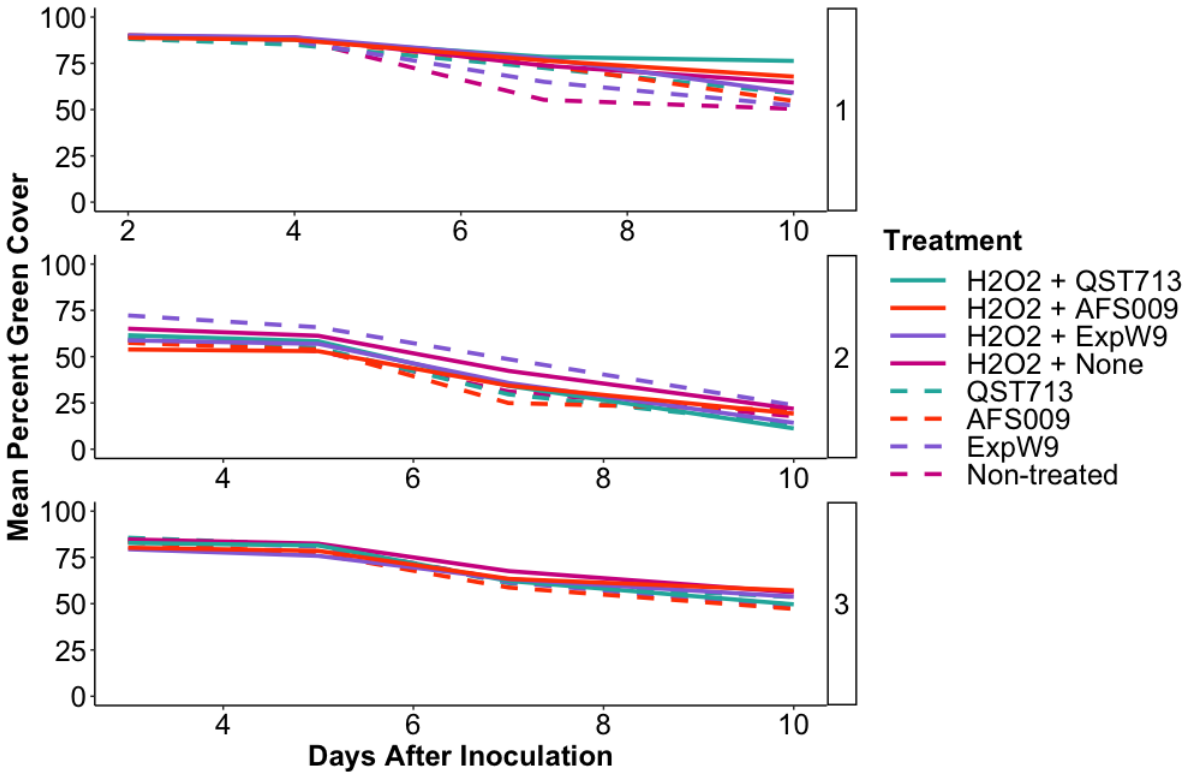

Figure S2.1. Mean percent green cover of perennial ryegrass pots grown in a benchtop humidity chamber over time after inoculation with *Rhizoctonia solani* Kühn infested rye grain. Lower percent green cover indicates more brown patch disease. Percent green cover values obtain through digital image analysis in the program TurfAnalyzer. Facets 1, 2, and 3 represent each experimental run. H2O2, QST713, AFS009, and ExpW9 refer to the treatments hydrogen peroxide, *Bacillus subtilis* QST713, *Pseudomonas chlororaphis* AFS009, and *B. subtilis* ExpW9, respectively.

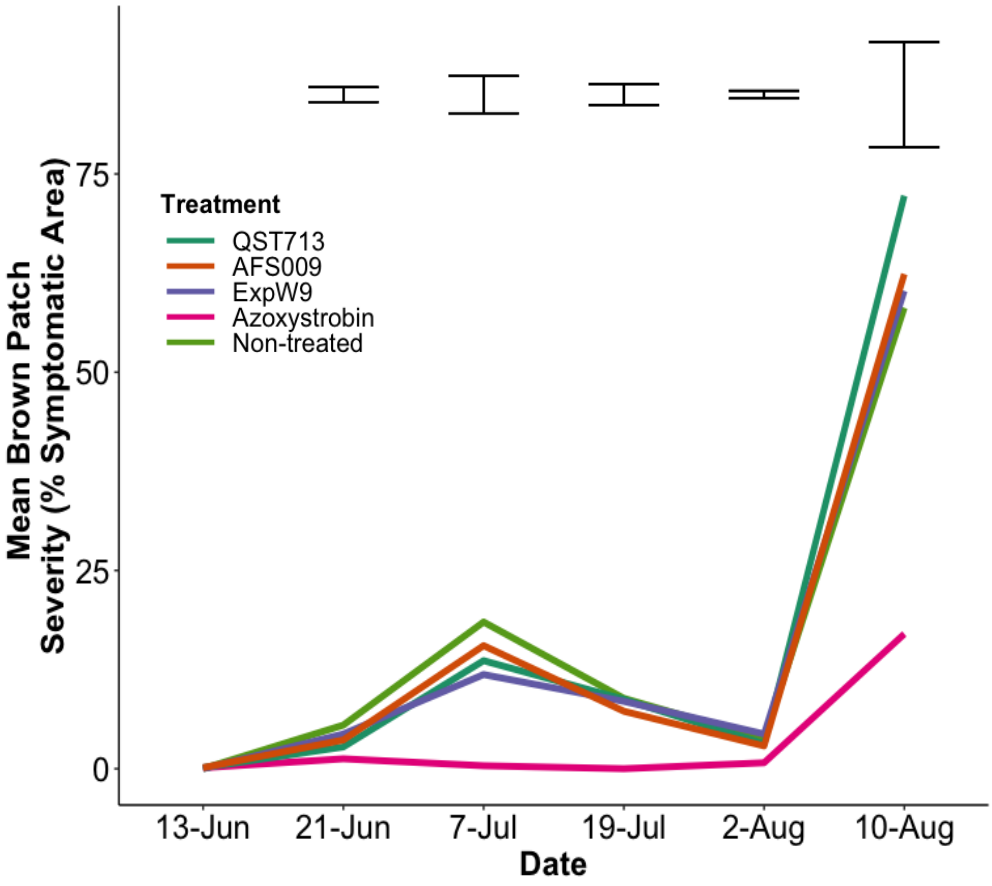

Figure S2.2. Brown patch severity, measured as percent symptomatic plot area, in response to biweekly biofungicide applications to "Salinas II" perennial ryegrass maintained as a home lawn. Error bars represent standard errors of the means for each rating date.

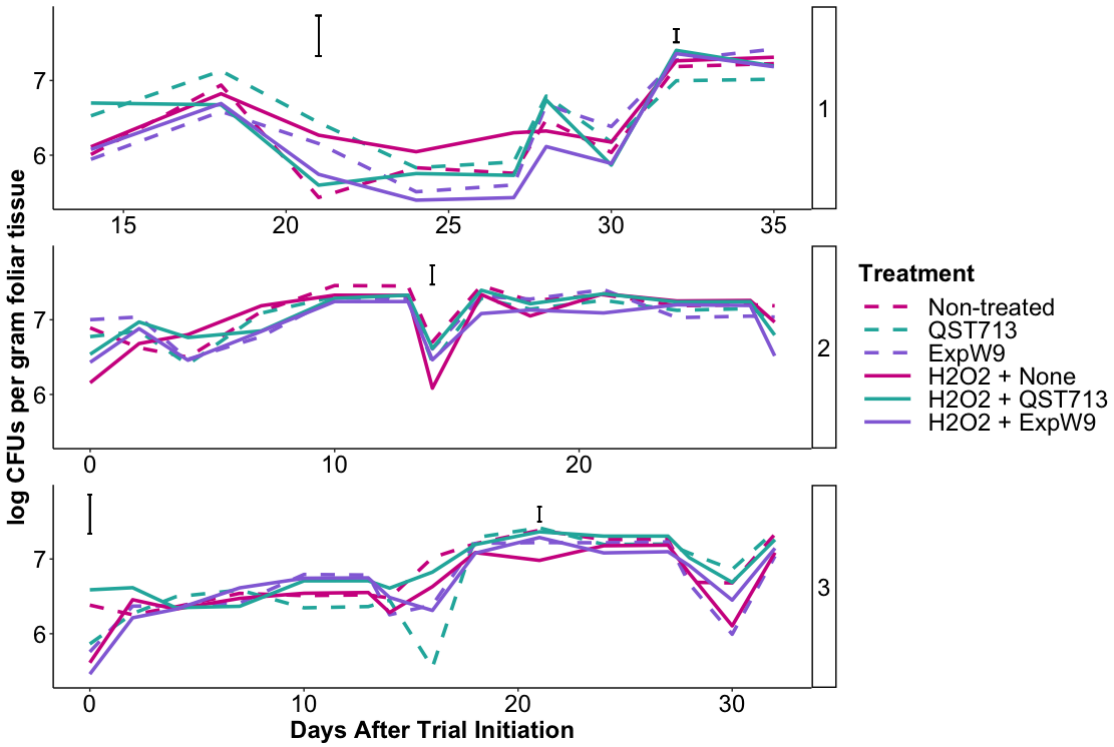

Figure S2.3. Colony forming units (CFUs) of *Bacillus* spp., grown on MYP + polymyxin B agar, in response to combinations of niche clearing and biological control organism applications. Facets 1, 2, and 3 represent each experimental run. H2O2, QST713, and ExpW9 refer to the treatments hydrogen peroxide, *Bacillus subtilis* QST713, and *B. subtilis* ExpW9, respectively. Error bars represent standard errors of the means for each rating date where statistically significant differences were present ($p \leq 0.05$).

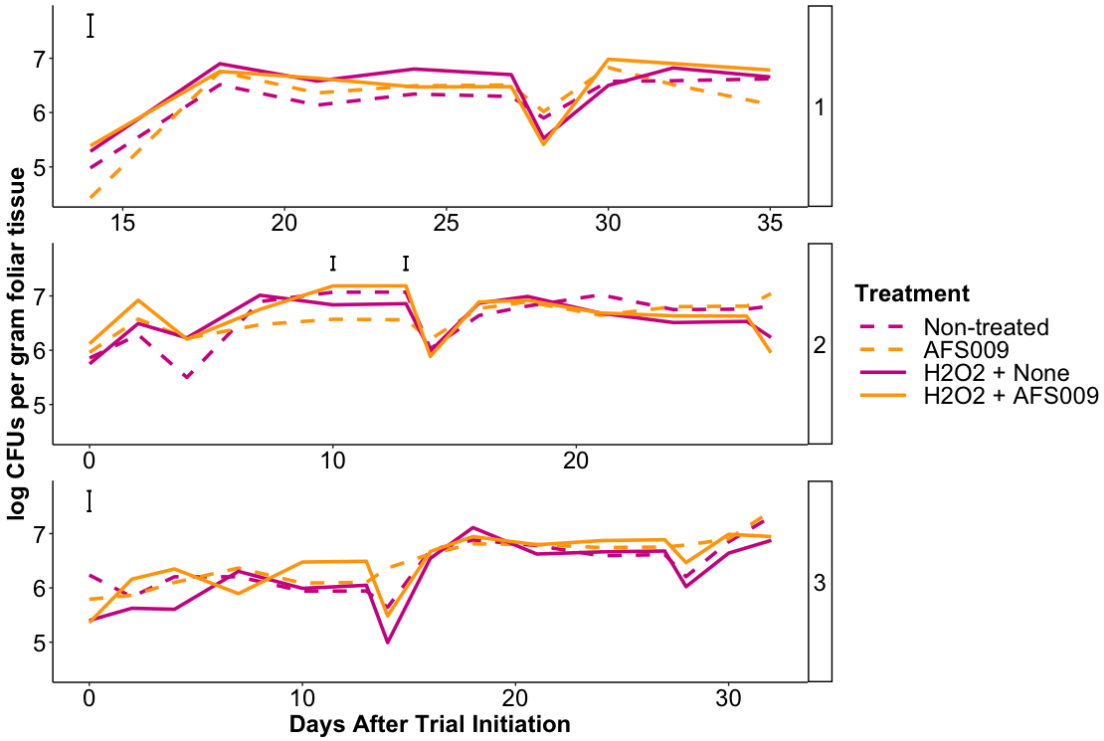

Figure S2.4. Colony forming units (CFUs) of *Pseudomonas* spp., grown on *Pseudomonas* isolation agar, in response to combinations of niche clearing and biological control organism applications. Facets 1, 2, and 3 represent each experimental run. H2O2 and AFS009 refer to the treatments hydrogen peroxide and *Psuedomonas chlororaphis* AFS009, respectively. Error bars represent standard errors of the means for each rating date where statistically significant differences were present ($p \leq 0.05$).

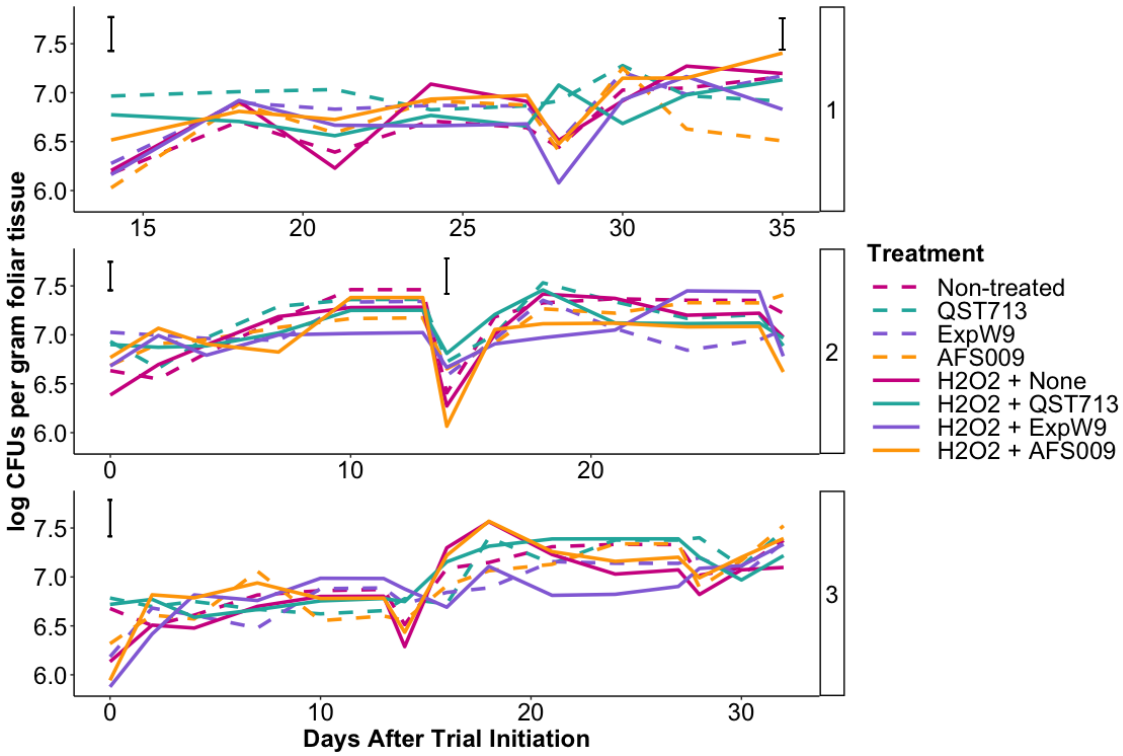

Figure S2.5. Bacterial colony forming units (CFUs), grown on a general nutrient agar, in response to combinations of niche clearing and biological control organism applications. Facets 1, 2, and 3 represent each experimental run. H2O2, QST713, ExpW9, and AFS009 refer to the treatments hydrogen peroxide, *Bacillus subtilis* QST713, *B. subtilis* ExpW9, and *Pseudomonas chlororaphis* AFS009, respectively. Error bars represent standard errors of the means for each rating date where statistically significant differences were present ($p \leq 0.05$).

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