Plant growth-promoting rhizobacteria (PGPR) mediate interactions between abiotic and biotic stresses in cool- and warm-season grasses

by

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Abstract

Non-pathogenic, soil microbes that occupy the rhizosphere can influence plant growth and induce changes in the plant's physiological, chemical, metabolic, molecular activities; influencing plant-microbe interactions with abiotic and biotic stressors. Plants colonized by these microbes express unique plant phenotypes that show increased root and shoot mass, enhanced nutrient uptake, and stress mitigation. Additionally, the microbes may fix nitrogen and phosphate or produce siderophores for plant use. Among the plant-associated microbes, plant growth-promoting rhizobacteria (PGPR) are among the most commonly used as inoculants for biofertilization. Plant growth-promoting rhizobacteria are non-pathogenic, free-living soil and root-inhabiting bacteria that colonize seeds, root tissue (endophytic/epiphytic), or the production of root exudates.

A review of the existing literature related to turfgrasses is provided in Chapter 1. This review provides background information and introduces major concepts that will be referenced throughout the dissertation. The review provides information on turfgrass, turfgrass economics, turfgrass stress related to drought and insect pests.

In Chapter 2, research was designed to track the colonization of rhizobacterial strains as well as determination of beneficial characteristics that may explain the observed growth promotion in bermudagrass from inoculation. Rhizobacterial inoculants have been previously shown to demonstrate growth promotion in bermudagrass, yet mechanisms for growth promotion and colonization of bermudagrass are unknown. Using rifampicin resistant strains of *Bacillus*

spp., colonization and persistence of bacteria under field condition in the rhizoplane, rhizosphere, endorhiza, and endophytic phyllosphere were determined in a loamy sand soil. Strains of *Bacillus pumilus* and *B. sphaericus* were determined to have nitrogenase and phosphate solubilization activity as well as metabolites that resulted in the production of siderophores. These results showed differences between strains of the same species, and phosphate solubilization was greatest under alkaline conditions. The characteristics of the rhizobacterial strains provides greater insights into the growth promotion demonstrated in bermudagrass. All bacterial strains tested were detectable in plant and soil within 24 h after inoculation and persistent through 12 wk post inoculation. Colonization occurred on both external and internal plant structures, but was typically higher in rhizoplane and rhizosphere samples. Populations remained stable for 2 wk after inoculation with drastic declines occurring after 6 wk. *Bacillus sphaericus* was the most prolific colonizer, having the greatest population density per sample and least drastic population decline 12 wk after inoculation. These results provide better understanding of plant-microbe-interactions in amenity grasses and can aid in determining application frequencies and intervals of biostimulants for turfgrass management.

In Chapter 3, I tested the hypothesis that PGPR treatment of bermudagrass would increase the tolerance of bermudagrass to tawny mole crickets. Inoculation of bermudagrass with rhizobacterial biostimulants can increase plant growth and influence relationships with above-ground herbivores. Tunneling and root-feeding behaviors of tawny mole crickets cause severe damage to grass in pastures, golf courses, and lawns. Since bacterial inoculants enhance root growth, the goal of this study was to determine if inoculation of bermudagrass by PGPR can increase the tolerance of hybrid bermudagrass to tawny mole crickets, and if PGPR are compatible with current commonly used insecticides for mole cricket control. In large arenas,

bacteria-treated grass infested with mole crickets produced more shoot and root mass and 128-200% greater root lengths compared to fertilized, infested, and non-infested bermudagrass. Field plots with mole cricket activity were established and treated with PGPR only, a PGPR-bifenthrin insecticide mixture, the insecticide alone, and compared to non-treated control plots. Plots were rated post-treatment for damage. Damage ratings after 3 and 8 weeks were lowest in plots treated with a bacteria-insecticide mixture, with controls having the highest damage. Lab experiments further confirmed that the PGPR used in the field study were compatible with neonicotinoid, phenylpyrazole, and pyrethroid insecticides when mixed in solution for up to 2 wk. Bacterial mediated interactions increase tolerance of bermudagrass applied before, or in response to, damage. Application of PGPR to field plots reduced tunneling relative to control plots and provided comparable reductions to a short residual, synthetic pyrethroid insecticide. Rhizobacterial products or products contained PGPR and certain insecticides may have utility for IPM of root herbivores.

In Chapter 4, I tested the hypothesis that PGPR treatment of grasses would increase tolerance to root-feeding white grubs (Coleoptera: Scarabaeidae). Inoculation of hybrid bermudagrass with PGPR can increase plant growth and influence relationships with above-ground herbivores like Fall armyworms and mole crickets (Chapter 3), however, few experiments have evaluated PGPR applications to tall fescue. Root-feeding white grubs cause severe damage to grasses, especially tall fescue, in pastures, golf courses, and lawns. Since bacterial inoculants enhance root growth, the goal of this study was to determine if inoculation of hybrid bermudagrass by root-colonizing bacteria (PGPR) can increase the tolerance of tall fescue and hybrid bermudagrass to white grubs, and if PGPR are compatible with neonicotinoid insecticides commonly used for white grub control. In trials with tall fescue and hybrid

bermudagrass, grasses were treated with Blend 20 or nitrogen or left non-treated, then infested with Japanese beetle grubs. PGPR and nitrogen fertilized grasses produced significantly more top growth than the non-treated infested controls. Bacteria treated roots tall fescue roots produced greater fresh and dry mass than non-treated and fertilized grasses. Bacterial treated hybrid bermudagrass roots produced greater root mass than non-treated and fertilized roots. No treatment negatively impact grub survival, and weight gains were similar for all treatments. Bacterial mediated interactions increase tolerance of tall fescue and hybrid bermudagrass applied in response to white grub infestation. Application of PGPR to increased root biomass over non-treated and fertilized grasses. Rhizobacterial products have utility for IPM of root herbivores.

Chapter 5 was focused on experimental verification of drought observations made with bermudagrass and PGPR. Drought and water scarcity due to unavailable irrigation are major limiting factors in the productivity of grasses. Rhizobacterial inoculants have been previously shown to mitigate drought stress in crops and grasses. Experiments were designed to determine if a blend of three *Bacillus* strains (Blend 20) could enhance drought stress responses in hybrid bermudagrass varieties with differing drought tolerances compared to fertilized and non-treated controls. Experiments were designed to examine tolerant (Tifway), moderately tolerant (LaPaloma), and susceptible (Yukon) grown pots with sand under greenhouse conditions and treated for 5 wk before being subjected to 3 wk of drought stress, and a recovery period. Drought stress response variables measured RWC, chlorophyll content, EL, and root length and weights. Bacterial inoculated grasses maintained lower RWC during drought periods, but maintained higher content than non-treated grass during recovery. Depending on the variety, bacterial inoculation may enhance chlorophyll content during and post-drought. The most pronounced benefits of bacterial inoculation were on EL and root growth. Bacterial treatment of

bermudagrass could alleviate varietal EL differences between LaPaloma and Yukon varieties. Roots of bacteria-treated grasses often had increased root fresh and dry weight and length over non-treated and fertilized grasses. The results of these experiments confirm the observations that PGPR can mediate or alter abiotic stress responses in hybrid bermudagrass. Furthermore, it provides a better understanding of plant-microbe-interactions in amenity grasses which can aid in incorporation of biostimulants for turfgrass management in areas with reduced water availability.

Chapter 6 provides a summary of the major findings and results. The summary presents future research avenues for PGPR in turfgrass and with insects and drought stress experiments.

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List of Abbreviations

ABA	Abscisic acid
CFU	Colony forming unity
DMSO	Dimethyl sulfoxide
EL	Electrolyte leakage
ET	Evapotranspiration
IAA	Indole-3-acetic-acid
IPM	Integrated pest management
ISR	Induced systemic resistance
PGPR	Plant growth-promoting rhizobacteria
PGPEB	Plant growth-promoting endophytic rhizobacteria
PWP	Permanent wilting point
RTSA	Rifampicin tryptic soy agar
RWC	Relative water content
SEM	Standard error of the mean
SWC	Soil volumetric water content
TSA	Tryptic soy agar
WAT	Weeks after treatment

Chapter 1: Literature Review

Turfgrass: economic impact and biology

The turfgrass industry is economically important, employing nearly a million people and generating revenues over $62 billion annually (Haydu et al. 2005). Turfgrasses, not including pasturelands in the United States cover 16.4 million hectares, an area larger than any other crop which encompass diverse uses from residential, commercial, and recreational purposes (Milesi et al. 2005, Held and Potter 2012). Pasturelands in the United States account for over 45% of all farmland and exceeds 168 million hectares of land coverage (USDA-NASS 2014). Improvements in turfgrass and pasture cultivars for increased adaptability, aesthetic qualities, playability, as well as insect, disease, and stress resistance have been the focus of traditional breeding programs. However, there are a limited number of breeding successes related to grass-feeding insects and particularly root-feeding herbivores (Held and Potter 2012). For example, infection of perennial ryegrass (*Lolium perenne* L.) and fescue (*Festuca* spp.) by fungal endophyte species in the genus *Neotyphodium* enhanced resistance to certain folivores, but the impacts of fungal endophytes appear to have subtler, nonlethal effects on root-feeders (Breen 1994, Grewal et al. 1994). Bacterial mediated interactions with plants that increase pest tolerances by maintaining productivity may yield benefits more easily, faster, and cheaper than traditional or molecular breeding programs (Bashan et al. 2014). The use of rhizobacterial inoculants has allowed for the maintenance of high quality crops, including grasses (Poaceae) under normal and adverse conditions with limited resource input (Omar et al. 2000, Kasim et al. 2013, Bashan et al. 2014, Coy et al. 2014).

The United States is divided into regions based on climatic conditions for recommendations on which turfgrass species, cultivars, and cultural practices are better suited for

a region. The majority of the southeastern United States is categorized as warm, humid or warm, tropical, which is suited for grasses that grow best during the warmer months of late spring and into the fall (Sprague 1982). Grasses that are suited for the southeastern United States are referred to as warm-season or southern turfgrasses. These monocot, C4 photosynthetic, perennial plants typically grow best when air temperatures are between 27-35° C and soil temperature are between 24-27° C. They are dormant during cooler months when soil temperatures are below 10-13° C (Snyder et al. 2008). In this region, *Cynodon* spp. (bermudagrass), *Stenotaphrum secundatum* (Walter) Kuntze (St. Augustinegrass), *Zoysia* spp. (zoyasiagrass), *Eremochloa ophiuroides* (Munro) Hack (centipedgrass), *Axonopus affinis* (carpetgrass), and *Paspalum vaginatum* Swartz (seashore paspalum) are favored grasses because they are heat tolerant, produce thick, lush stands, have deep root systems, and are aggressive growers (Sprague 1982, Duble 1996, Beard 2002).

Bermudagrass (*Cynodon* spp.) is a commonly grown turf in Australia, Africa, India, South America, and the southern United States, and is found in over one hundred countries (Duble 1996). Common bermudagrass (*Cynodon dactylon* (L.) Pers.) is a warm season, perennial turfgrass that reproduces by seed and vegetatively by stolons and rhizomes. *Cynodon dactylon* and its hybrids are the most commonly used bermudagrasses for turf and forage. In the United States, more southern golf course acreage is planted in bermudagrass (*Cynodon* spp.) than any other species, with most of this occurring in the southeast, southwest, and transition zones (Lyman et al. 2007). It is believed that the *Cynodon dactylon* was introduced from Africa or India to the southern states during the colonial period (Duble 1996). Within bermudagrass, Tifway has been the most widely used hybrid bermudagrass on golf courses, sports turf, and

other recreational areas for over 40 years (Beard 2002). The Tifway hybrid (*C. dactylon* x *C. transvaalensis* Burtt-Davey) is a chance hybrid that showed up in *Cynodon transvaalensis* seeds from Johannesburg, South Africa in 1954 (Duble 1996) but was released in 1960 by the Georgia Agricultural Experiment Station and Crops Research Division. Tifway and other hybrid bermudagrasses have been preferred over common bermudagrass because they generally have greater disease resistance, and higher pest tolerance. Further, they produce fewer seed heads, have finer leaf texture, and have better color (darker green) (Foy 1997). Much of the work in this study will focus on Tifway and additional hybrid bermudagrasses.

Plant growth-promoting rhizobacteria (PGPR)

Non-pathogenic, soil microbes that occupy the rhizosphere can influence plant growth and induce changes in the plant's physiological, chemical, metabolic, molecular activities and influence plant-insect interactions. Plants colonized by these microbes express unique plant phenotypes that show increased root and shoot mass, enhanced nutrient uptake, and stress mitigation. Also, the microbes may fix nitrogen and phosphate for plant use (Calvo et al. 2014). Among the plant-associated microbes, plant growth-promoting rhizobacteria (PGPR) are among the most commonly used as inoculants for biofertilization. Plant growth-promoting rhizobacteria are non-pathogenic, free-living soil and root-inhabiting bacteria that colonize seeds, root tissue (endophytic/epiphytic), or root exudates (Kloepper and Schroth 1978, Kloepper 1993). Numerous research papers and review articles summarized the extensive use of PGPR as inoculants in crop and horticultural plants (see review by Bashan et al. 2014). Previous studies of plant-microbe interactions (PMI) in turfgrass have focused on colonization of grass by

endophytic fungi in cool-season grasses for mediation of abiotic stress and effects on insects (Held and Potter 2012). While endophytic fungi have been widely investigated in *Festuca, Lolium,* and *Poa* spp. of grasses, they are not reported in warm-season grasses. Alternatively, roots of bermudagrass, a common warm-season grass, host a diverse group of gram-positive actinomycetes and heat-tolerant bacteria (Elliot et al. 2004). Culturable bacteria associated with plant roots can be re-applied as inoculants for growth benefits. Coy et al. (2014) identified blends of bacteria that, when applied to hybrid bermudagrass (*Cynodon transvaalensis* Burtt Davy × *Cynodon dactylon* (L.) Pers), result in growth promotion relative to non-treated and fertilized (unpublished data) plants.

PGPR and Induced systemic resistance (ISR)

Biostimulation of plants by PGPR can mediate plant defenses against diseases and may influence insect herbivores. Numerous soil fungi and bacteria including PGPR induce systemic resistance (ISR) in plants (van Loon et al. 1998, Bakker et al. 2013) with implications for suppressing plant disease agents (Kloepper et al. 2004) and plant herbivores (van Oosten et al. 2008, Pineda et al. 2012). For example, plant defense signaling pathways that mediate attacks from pathogens or insect herbivores were upregulated when *Arabidopsis thaliana* (L.) was inoculated with *Pseudomonas fluorescens* (van Oosten et al. 2008). While the literature is well-developed and reviewed for plant pathogens (Kloepper et al. 2004, Bakker et al. 2013), only a few studies have considered the influences of PGPR-plant interactions on feeding and oviposition behaviors of above ground insect herbivores (Zehnder et al. 1997, Zehnder et al. 2001, Pineda et al. 2010, Pineda et al. 2012, Biere and Bennett 2013). When PGPR alters plant

growth, the effects on insect herbivores may be subtler, indirect effects, and not inherently lethal. The effects on development, larval weight, and feeding of insect herbivores that are fed PGPR-treated plants may be positive, negative, or non-significant (Pineda et al. 2010). For example, induced systemic resistance from the inoculation of cucumber plants with *Flavomonas oryzihabitans* INR-5 and *Bacillus pumilus* INR-7 altered plant metabolic and defense signaling pathways that mediate attacks from pathogens and insect vectors (Zehnder et al. 2001). The induced systemic resistance in cucumber against bacterial wilt (*Erwinia tracheiphila*) was likely due to changes in plant palatability resulting from decreased cucurbitacin production reducing the number of beetles acquiring and transferring the pathogen. Additionally, the bacteria primed other plant defenses, specifically phytoalexin, and other plant compounds to combat the pathogen after transmission.

Primarily, plant-PGPR interactions have focused on growth promotion and plant pathogen (Kloepper et al. 2004, Bakker et al. 2013,), with interest is growing on the influences of PGPR-plant interactions on insect folivores (Zehnder et al. 1997, Zehnder et al. 2001, Pineda et al. 2012, Pineda et al. 2010, Biere and Bennett 2013, Coy et al. 2017). Thus far, research finding insecticidal strains of PGPR have been in the minority, and determination of insecticidal properties cannot be determined by bacterial systematics alone, and must to be evaluated on a strain by strain basis. The major successes have been in the *Bacillus cereus* group of bacteria, with the discovery of select strains of *Bacillus thuringiensis*. While *B. thuringiensis* strains are insecticidal against caterpillars, white grubs, and mosquitoes, other *Bacillus* species outside of the cereus group also show promise as *B. sphaericus* is larvicidal to mosquitoes (Raun et al. 1966, Singer 1980, Bixby et al. 2007, Lacey 2007). While plant-microbe-insect research is

gaining popularity, virtually no research has been conducted on soil dwelling or root-feeding insects. This lack of research is probably due to the logistical challenges of direct observations of subterranean pests, but the plant-microbe-insect interactions may be more impactful from close associations. With documented success of PGPR inoculants enhancing root growth in bermudagrass (Coy et al. 2014), plant-microbe-insect interactions may be better suited to evaluate changes in plant tolerances to root-feeders in response to bacterial biostimulants.

Turfgrass biotic stress: challenges from root herbivores

Mole crickets

Mole crickets (Orthoptera: Gryllotalpidae) are univoltine, solitary, hemimetabolous, hypogeal insects that cause significant damage to turf, pasture, and other crops in sandy soils throughout the southeastern United States (Walker and Ngo 1982, Bailey et al. 2015). Mole crickets spend most of their life in underground burrows that offer protection for feeding, making direct observations of behavior difficult (Hertl and Brandenburg 2002). The fossorial forelegs of mole crickets aid in subterranean tunneling behavior, which results in direct damage of turfgrass from tunneling activities and root-feeding throughout the soil profile. The tunneling behavior not only damages grass root systems, but displaces soil and disrupts playing surfaces (Frank and Parkman 1999, Bailey et al. 2015). There are three invasive species from the genus *Neoscapteriscus* occurring throughout the southeast (Held and Potter 2012). The southern mole cricket (*Neoscapteriscus borellii* Giglio-Tos), the tawny mole cricket (*Neoscapteriscus vicinus* Scudder), and the short-winged mole cricket (*Neoscapteriscus abbreviatus* Scudder), which has only been reported in Florida and Georgia. Damage severity caused by mole crickets can be

species dependent. Typically, most damage is observed in tawny mole cricket (*N. vicinus* Scudder) infested areas as this species is herbivorous on grass roots and stems while having extensive tunnels; whereas *N. borellii* is carnivorous and produces deeper tunnels in the soil profile (Braman et al. 2000, Thompson and Brandenburg 2005).

Previous research with mole crickets in turfgrasses aimed to find resistant cultivars, alter tunneling behavior in response to inoculation of entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff), or biologically control with parasitoids and predators, including *Euohasiopteryx depleta* (Wiedemann), *Larra bicolor* (Fabricius), *Pheropsophus aequinoctialis* (Linnaeus) and entomopathogenic nematodes (*Steinernema* sp.) (Hudson et al. 1988, Braman et al. 2000, Xia et al. 2000, Barbara and Buss 2005, Thompson and Brandenburg 2005). Resistant cultivar work focused on the susceptibility of bermudagrasses, bahiagrass, St. Augustinegrass, centipedegrass, and zoysiagrass to both the tawny and southern mole crickets, with work yet to identify any highly resistant cultivars to cricket injury or reliable biological controls (Reinert and Busey 1984, Braman et al. 1994, Braman et al. 2000). With the limited number of natural enemies and restrictions on the range of *Larra bicolor* for mole cricket control, manipulation or augmentation of these populations for control is unlikely on a broad scale. While previous work has not found resistant cultivars, it has determined varying tolerances among cultivars within grass species, and avoidance behaviors after applications of entomopathogenic fungi. The changes in tunneling behavior from *B. bassiana* strains suggests that other soil microbes may be able to influence soil microbial ecology and alter grass responses to soil dwelling pests.

Neoscapteriscus mole crickets typically alter turfgrass playability and aesthetic qualities from surface tunneling activities, root-feeding, and soil displacement (Hertl and Brandenburg 2002). Moreover, soil displacement from mole cricket burrows results in biopore formation, which increases water infiltration rates that can reduce fertilizer and pesticide efficacy, and potentially contaminate surface and ground water from runoff (Bailey et al. 2015). Concerns of water contamination from agrochemicals arise from the preferential flow of solutes through the soil profile in response to irrigation or rain events. Agrochemical inputs on high maintenance turfgrasses, especially golf course greens and tees, are where mole cricket activity and damage would be most prevalent, and are areas of concern (Shipitalo et al. 1994, Bailey et al. 2015). The ability of PGPR when used preventively or curatively to increase root biomass and depth, could alleviate concerns of environmental contamination through increased plant growth and bioremediation. The incorporation of PGPR into turfgrass management offers soil benefits through bioremediation that can improve soil health by degrading contaminants (metals, toxins) or by mobilizing solutes in the soil for plant use and uptake (Calvo et al. 2014, Khan and Bano 2016). The successful integration of biostimulants to combat damage from tunneling activity and feeding into turfgrass and pest management, may allow for maintaining grass productivity, aesthetics, and playability under adverse conditions.

Japanese beetles

The Japanese beetle, *Popillia japonica* Newman was introduced into America through southern New Jersey in 1916 (Flemming 1976). Since being introduced, this univoltine, highly polyphagous pest has become problematic in all states east of the Mississippi River, except Florida, as well as spreading to Wisconsin, Minnesota, Iowa, Missouri, Arkansas, and parts of

southern Canada (NAPIS 1998). Additionally, the pest has been partially established in South Dakota, Nebraska, Kansas, Oklahoma, and Texas (USDA-APHIS 2015). Japanese beetles are widespread destructive pests feeding on over 300 plant species as root-feeding grubs and foliage feeding adults (Ladd 1989). Once established in the eastern United States, Japanese beetles exploited the lack of natural enemies and host plant resistance, utilizing large areas of all common species cool-season turf and pasture species, and lawn weeds for larval development (Fleming 1968, Fleming 1976, Crutchfield and Potter 1995a, Crutchfield and potter 1995b, Crutchfield and Potter 1995c). Due to the range of its establishment and host plants, *P. japonica* is one of the most extensive and destructive pests of turf and landscape plants in the eastern United States, with annual control costs exceeding $450 million USD (Potter 1998, Vittum et al. 1999, USDA-APHIS 2015). Japanese beetle larval populations are typically aggregated spatially, occurring in patchy distributions. High larval density areas have been suggested to be correlated with adult feeding sites, and soil with high organic matter low-density areas (Dalthrop et al. 1999, 2000). The commonality and pest status of the Japanese beetle throughout the United States make it a valuable research model for plant-insect interactions. Further, the root-feeding nature of the grubs is highly valuable for gaining insights of soil-dwelling insects for plant-insect-microbe interactions.

Turfgrass abiotic stress: challenges from drought

A current and future challenge for grass grown as turf or pasture will be to maintain quality, growth, and production under sub-optimal climatic conditions that have minimal inputs and environmental impacts. Drought stress is most prevalent during the summer months, often

resulting in a 300% increase in domestic water use for urban landscapes as homeowners struggle to differentiate drought stress and plant survivability during periods of prolonged drought (Steinke et al. 2010). Additionally, drought is challenging to manage due to its unpredictability in nature and the variability of drought scenarios (Kim et al. 2009, Comas et al. 2013). How a plant deals with drought stress has metabolic costs that can exceed 50% of daily photosynthesis and is influenced by the plant's root architecture, as the size of a root system impacts its ability to absorb water and nutrients under varying soil conditions (Comas et al. 2013, Lynch et al. 2014). Soil conditions such as soil type, texture, hydraulic conductivity, and holding capacity play an important role in how a plant responds to drought stress.

A plant's ability to survive and sustain growth during periods of drought stress is loosely defined as drought resistance, which is achieved genetically through the utilization of three strategies: escape, tolerance, and avoidance (Levitt 1980). These drought resistant strategies are not mutually exclusive and plants may use more than one strategy when adjusting to drought conditions. Drought resistance mechanisms that a plant uses depend on drought duration, severity, and grass species. Drought avoidance and tolerance are more desirable characteristics as they are more adaptable traits for breeding and biotechnology.

Drought escape is an adjustment of the plant's life cycle to complete reproduction early under favorable conditions or by going dormant until water is available and is a common drought strategy in the Mediterranean and subtropical climates that experience wet and dry periods (Levitt 1980). Dormancy is a physiological process in which plant leaves may turn brown in response to water stress, but the plant's crowns, stolons, and rhizomes can remain alive for

several weeks to months depending on air temperature and grass species and the grass is able to quickly recover after irrigation or rainfall (Huang et al. 2014). Tall fescue and Kentucky bluegrass are common cool-season grasses that utilize this strategy (Assuero et al. 2002, Fry and Huang 2004).

Drought avoidance occurs when a plant maintains a favorable water status by increasing the capacity for water uptake via the root system and/or reducing water loss from leaves (Levitt 1980). Avoidance may allow for grasses to survive and maintain growth and function during periods of short-term drought until water is replenished or depleted (Huang et al. 2014). Characteristics that are common with drought avoidance are increased root plasticity and root depth into the soil for greater water availability; and/or enhanced leaf pubescence; leaf rolling and folding; and increased stomatal regulation (Duncan and Carrow 1999). Deeper grass roots are a trait that allows for drought avoidance as the plant utilizes water further in the soil profile to delay the dehydration of tissue (Hays et al. 1991, Huang 1998, Duncan and Carrow 1999). This characteristic allows for increased plant survival as the deeper roots are better able to provide continued water and nutrients to the plant even when part of the plant's root system is under dry soil conditions (Bonos and Murphy 1999, Huang 1999). Bermudagrass, buffalograss, and zoysiagrass are common warm-season grasses, and tall fescue, a cool-season grass, commonly use this strategy (Marcum et al. 1995, Volaire and Leliever 2001). Avoidance also changes hormonal balances altering plant responses to environmental stresses. Abscisic acid (ABA) and cytokinins are the primary chemical signals moved from the roots to shoots in response to depleted soil moisture, which results in stomatal closure and a decrease in water loss from transpiration (Assmann and Shimazaki 1999, DaCosta and Huang 2007).

Drought tolerance occurs when a plant maintains active growth and metabolic activity under water deficit conditions. Drought tolerance allows plants to survive prolonged periods of soil moisture deficits through osmotic adjustments, maintenance of root and membrane viability under dehydration, and the accumulation of proteins and metabolites that function in direct and indirect structural stabilization (Nilsen and Orcutt 1996).

Drought stress alters a plant's physiology and metabolic processes which interferes with plant productivity and growth (Kasim et al. 2013). Like food crops, turfgrass and forages require significant amounts of water to maintain high growth and quality (Steinke et al. 2011). Traditional breeding programs geared towards developing drought tolerant cultivars are a strategy to reduce watering and irrigation needs of many turfgrass species and cultivars, but the process is time consuming, expensive, and limited due to a poor understanding of physiological and molecular mechanisms involved in grass stress tolerance (Huang et al. 2014). Biotechnology and the use of microbes to enhance plant and soil health have been studied for mediating stress tolerances to drought in several agronomic crops, and many grasses, (maize, rice, wheat, barley) but similar studies are limited in turfgrass as the physiology and genetics are not well-understood (Rampino et al. 2006, Kasim et al. 2012, Wang and Brummer 2012, Huang et al. 2014).

Turfgrass exposure to biotic and abiotic stress decreases aesthetic quality, functionality playability, or productivity and yield (Hu et al. 2009, Du et al. 2012, Kasim et al. 2013, Huang et al. 2014,). Rarely do abiotic and biotic stresses occur individually, but rather as a combination, making plant management difficult. Environmental stress in warm-season grasses often results from temperature, water, and light stress, or from poor soil quality (Kasim et al. 2013, Huang et

al. 2014). Expectations of increased stress from temperature extremes and water scarcity are linked to climate change and variability, with models predicting global temperature increases of 1-6° C and infrequent rainfall during this century (IPCC 2007). Bermudagrass has evolved and adapted to hot, arid climates and demonstrates drought tolerance (Carrow 1996).

Exposure to abiotic and biotic conditions presents challenges that plants must adapt to and overcome. To deal with this, plants evolved sophisticated physiological, cellular, biochemical, and molecular responses to maintain homeostasis under harsh conditions (Li et al. 2012, Shi et al. 2012, Comas et al. 2013, Kasim et al. 2013,). Drought, salinity, and temperature stresses alter plant physiology and metabolic responses, limiting growth, productivity, survival, and yield (Kim et al. 2009, Shi et al. 2012, Kasim et al. 2013, Manuchehri and Salehi 2014, Yang et al. 2016). Drought is a major consequence of climate change, and models predict that drought will have negative consequences on over 50% of arable lands by 2050 (Vinocur and Altman 2005, Comas et al. 2013,). Insight into plant stress responses from the whole plant to the cellular level is vital for the development of new grass cultivars and for the incorporation of novel technologies into management practices. Bacterial mediated interactions with plants that increase drought tolerance by maintaining productivity may yield results easier, faster, and cheaper than traditional and molecular breeding programs. The use of rhizobacterial inoculants has allowed for the maintenance of high quality crops, including grasses (Poaceae) under adverse conditions with limited resource input (Omar et al. 2000, Kasim et al. 2013, Bashan et al. 2014,).

Soil microbial communities are intimately associated with plants and influence plant health, biomass accumulation, soil quality, nutrient availability and acquisition, as well as pollutant degradation (Johannes et al. 2000, Smalla et al. 2001, Shi et al. 2006, Khan and Bano

2016). Rhizosphere microbial communities evolve over time and are influenced by climate, soil type and characteristics, ground cover, and land use history (Johannes et al. 2000, Smalla et al. 2001, Elliott et al. 2004, Shi et al. 2006). Soil microbes that occupy the rhizosphere can induce changes in plants and influence plant-bacterial interactions. Plant growth-promoting rhizobacteria (PGPR) are non-pathogenic, free living soil and root inhabiting bacteria that colonize seeds and root tissue (Kloepper and Schroth 1978, Kloepper 1993). Plants colonized by PGPR or plant growth-promoting endophytic bacteria (PGPEB) by bacteria in natural plant conditions or added as biostimulants to agronomic and horticultural crops show increased root and shoot mass, enhanced nutrient uptake, and stress mitigation (Suzuki et al. 2003, Calvo et al. 2014). Among plant-associated microbes, PGPR have been linked to drought mitigation through priming plant signaling defenses which alter plant-water regulation, use, and efficiency; the production of larger, more explorative root systems; synthesis of phytohormones (cytokinins, auxins, gibeberellins, ethylene, etc.), or by the production of secondary metabolites (Kasim et al. 2013, Calvo et al. 2014, Halo et al. 2015).

The use of microbes to enhance plant and soil health for mediating drought and salinity stress tolerances have been evaluated in several agronomic crops and grasses, including maize, rice, wheat, barley, ryegrass, bluegrass, and the model grass *Brachypodium distachyon* (Khan et al. 2012, Bashan et al. 2014, Gagné-Bourque et al. 2015, Halo et al. 2015, Kaushal and Wani 2016). However, drought responses in cool-season grasses or model grasses that utilize C_3 photosynthetic systems may offer limited insight to C_4 warm-season grasses as many studies demonstrate physiological and behavioral responses that differ between plant species or cultivars to both microbes and stress (Johannes et al. 2000, Carmo-Silva et al. 2009, Taylor et al. 2011,

Khan et al. 2012, Coy et al. 2014). Bacteria of the genera *Azospirillum*, *Bacillus*, *Methylobacterium*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Sphingobacterium*, and *Sphingomonas* have been documented to mitigate drought stress responses in certain Poaceae crops (Khan et al. 2012, Bashan et al. 2014, Halo et al. 2015). Unfortunately, studies in amenity grasses are limited as the physiology and genetics are not well-understood since cultivars have different parental germplasms and respond and utilize different strategies to tolerate, escape, or avoid drought based on drought severity and duration (Levitt 1980, Rampino et al. 2006, Carmo-Silva et al. 2009, Du et al. 2012, Kasim et al. 2012, Wang and Brummer 2012, Huang et al 2014).

Drought responses in bermudagrass have been associated with larger plant root systems, phytohormones, proline content, antioxidant activity, chlorophyll content, dehydrin accumulation, electrolyte leakage (EL), evapotranspiration (ET) and stomatal regulation, leaf firing, plant biomass, and relative water content (RWC); yet, questions remain as to which root traits and plant responses are most beneficial in understanding drought stress mitigation (DaCosta and Huang 2007, Hu et al. 2009, Du et al. 2012, Shi et al. 2012, Comas et al. 2013). Preliminary drought work with PGPR blends in bermudagrass has shown differences in plant chlorophyll content, EL, and RWC during and after drought stress suggesting these factors may be important for stress mitigation in bermudagrasses (Coy, Held, and Kloepper, unpublished data). Subsequent literature searches linking growth-promotion or drought mitigation to PGPR in bermudagrass have not been successful.

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Chapter 2: Rhizobacterial colonization of bermudagrass by *Bacillus* spp. in a Marvyn loamy sand soil

Abstract

Rhizobacterial inoculants are known to induce growth promotion in grasses, yet mechanisms for growth promotion and colonization are unknown. Using rifampicin resistant strains of *Bacillus* spp., colonization and persistence of bacteria in bermudagrass under field conditions in the rhizoplane, rhizosphere, endorhiza, and endophytic phyllosphere were determined in a loamy sand soil. Lab assays also determined nitrogenase, phosphate solubilization activity, and the production of siderophores among strains of *Bacillus* known to promote growth in bermudagrass. Strains of *Bacillus pumilus* and *B. sphaericus* were determined to have nitrogenase and phosphate solubilization activity as well as metabolites that resulted in the production of siderophores. Differences were noted between strains of the same species, and phosphate solubilization was greatest under alkaline conditions. These characteristics of the rhizobacterial strains suggest mechanisms for observed growth promotion in bermudagrass. All bacterial strains tested were detectable in plant and soil within 24 h after inoculation and persistent through 12 wk post inoculation. Colonization occurred on both external and internal plant structures, but was typically higher in rhizoplane and rhizosphere samples. Populations remained stable for 2 wk after treatment with drastic declines occurring after 6 wk. *Bacillus sphaericus* AP282 was the most prolific colonizer, having the greatest population density per sample and lowest population decline 12 wk after treatment. Although known for associated fungal endophytes, bacterial endophytes of grasses are less commonly reported. These data are the first report of root and phyllosphere colonization of grasses by rhizobacteria and provide new insight into plant-microbe-interactions in grasses and related monocots.

1. Introduction

Plant-microbe interactions have been heavily studied and understanding of these relationships are increasingly important for continued crop production and protection. Microbes, including plant growth-promoting rhizobacteria (PGPR) in the rhizosphere have been among the most heavily studied soil organisms because of their intimate association with plant, root, and soil health (Singh et al. 2011, Calvo et al. 2014). Previous PGPR studies noting beneficial genera for growth promotion or antibiosis have been more prevalent than studies on ecological or population dynamics. Rhizobacteria and endophytes are associated with over 300,000, yet relatively few plant species have been studied in detail in relation to plant colonization (Ryan et al. 2008). Biostimulants, like PGPR increase plant growth and root architecture through improved nutrient cycling, production of plant hormones, reducing or preventing pathogens, as well as changes to plant-water regulations (Compant et al. 2005, Ryan et al. 2008, Calvo et al. 2014, Coy et al. 2014, Gagné-Bourque et al. 2015). While most PGPR work has focused on plant-microbe interactions in the rhizosphere and rhizoplane, PGPR colonization is not restricted to these areas. PGPR or plant growth-promoting endophytic bacteria typically colonize the endorhizosphere within the root cortex or vascular tissue and move into plant foliage (Baldani and Döbereiner 1980, Lalande et al. 1989, van Peer and Schippers 1989, van Peer et al. 1990, Gagné-Bourque et al. 2013, Gagné-Bourque et al. 2015, Santoyo et al. 2016). However, bacterial endophytes may also colonize through the phyllosphere, anthosphere, or spermoshpere (Sturz et al. 2000).

Within the study of plant-microbe interactions of grasses, endophytes are commonly encountered and almost exclusively refer to fungi in cool-season species (Carroll 1988, Funk et

al. 1993, Held and Potter 2012). However, a more appropriate definition of endophytes refers to both fungi and bacteria that complete all or part of their life cycle within the tissues of plants that result in unapparent or asymptomatic infection of plant tissue with no disease symptoms (Sturz et al. 2000, Santoyo et al. 2016). This broader definition of endophyte is important for turfgrass because bacterial endophytes have been previously isolated from three warm-season grasses, kallar grass (*Leptochloa fusca* L.), saltmarsh grass (*Spartina alterniflora* Loisel), and switchgrass (*Panicum virgatum* L.), as well as the cool-season C_3 model grass *Brachypodium distachyon* (McClung et al. 1983, Reinhold-Hurek and Hurek 1998, Gagné-Bourque et al. 2013, Gagné-Bourque et al. 2015). The presence of bacterial endophytes in these grasses is likely an indicator that they are more prevalent and are likely to occur in other species, but have been overlooked.

Endophytic *Bacillus* spp. are commonly reported in corn, cotton, cucumber, grape, peas, soybean, and spruce (Leland et al. 1989, McInroy et al. 1992, Bell et al. 1995, Benhamou et al. 1996, Hallman et al. 1997, Shishido et al. 1999, Reva et al. 2002, Berg et al. 2005, Durham 2013). Species within *Bacillus* that have previously been shown to be endophytic include *B. amyloliquefaciens*, *B. endophyticus*, *B. firmus*, *B. insolitus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilus*. The ability of bacteria to establish internal plant populations within the vascular system may be advantageous, as it allows the bacteria to be in constant contact with plant cells, offers protection from competition with other soil microbes and environmental extremes, which may increase persistence (Shishido et al. 1995, Reinhold-Hurek and Hurek 1998, Santoyo et al. 2016). Additionally, the nutrient rich, low oxygen environments within the plant and rhizosphere offers optimal condition for nitrogenase activity to fix nitrogen for plant use and growth (Reinhold-Hurek and Hurket 1998, Sevilla and Kennedy 2000). Further, PGPR

isolated from internal plant organs are biochemically distinct and more effective plant colonizers (van Peer et al. 1990). The biochemical changes to PGPR strains from endophytic colonization may increase the efficacy of PGPR on plant health. This is likely a result from close associations with plant activities and defenses, demonstrating the adaptability of bacteria to find ecological purposes that form intimate, positive relationships with plants that aid in plant growth or protection from other microbes or abiotic stress (Shishido et al. 1995, Compant et al. 2005).

Plants colonized by endophytic bacteria demonstrate induced systemic resistance, which alters, increases, or prevents stress from disease, insects, and nematodes by altering plant signaling compounds that include jasmonic acid, salicylic acid, or ethylene pathway (van Loon et al. 1998, Kerry 2000, Sturz et al. 2000, Crow 2014, Coy et al. 2017). While the endophytic plant colonization of Bacilli-bacteria is documented (Reva et al. 2002, Gagné-Bourque et al. 2013, 2015, Durham 2013) considerable knowledge gaps remain on the levels of colonization and application frequencies, as well as persistence of biostimulants for plant growth and protection, especially in perennial cropping systems. Grasses grown for forage, hay production, or lawns are limited by nutrients, water, temperature, and pests. Nitrogen, in the forms of ammonium NH_4^+ and nitrate NO_3^- are the most important nutrients for sustaining plant growth in turfgrass, and are abundantly applied to amenity grass (Frank and Guertal 2013a). Losses of nitrogen in fertilized grass can be as high as 50% (Barber 1995, Horgan et al. 2002, Frank and Guertal 2013a). Avenues of loss are leaching, volatilization, and denitrification which releases nitrous oxide, a greenhouse gas leading to environmental concerns (NRC 1993). Use of PGPR and other microbial inoculants could allow for a reduction of nitrogen rates if they can improve nutrient uptake and efficiency while reducing greenhouse gas emissions (Calvo et al. 2013). Phosphorus,

in the form of orthophosphate (PO_4^{3-}), is the third most important nutrient for grass growth and is often applied during turfgrass establishment for increasing seedling vigor and growth (Beard 1973, Frank and Guertal 2013b). In grasses and other crops, there are growing environmental concerns over runoff (Stewart et al. 2006, Bierman et al. 2010) and leaching (Erickson et al. 2005, King et al. 2006). Forms of phosphorus vary by regions and soil conditions. Aluminum and iron phosphate forms are commonly encountered in the northern and southeastern United States, and calcium phosphate common in the western United States (Frank and Guertal 2013b). The production of siderophores from bacterial metabolites has been linked to growth promotion by increasing chlorophyll content, disease suppression, and bioremediation (Sharma and Johri 2003, Sayyed et al. 2013, Calvo et al. 2014).

Using three strains of two *Bacillus* spp., we determined rhizobacterial colonization in the economically important bermudagrass system. Bermudagrass is a common grass in pastures and lawns as well as for hay production in the southeastern and southwestern US. Coy et al. (2014) noted growth promotion in bermudagrass with select rhizobacterial blends, yet the colonization and persistence of rhizobacteria as well as the mechanisms for growth promotion are not clearly defined, particularly for perennial crops like grasses. Understanding fluctuations of bacterial populations over time will benefit efforts to develop application frequencies or intervals in grasses and other perennial crops. Additional experiments were conducted to identify the beneficial characteristics of select rhizobacterial strains to provide a better understanding of the mechanisms which may be used for growth promotion. In lab assays, rhizobacterial strains know to induce growth in bermudagrass were evaluated for qualitative nitrogenase activity and siderophore production, and quantitative phosphate solubilization.

2. Materials and Methods

2.1 Bacterial strains and rifampicin marking of bacteria

Rifampicin resistant mutants of PGPR strains were created and tested for persistence and colonization in soil and *in planta*. Bacterial strains that were stored at -80 °C were transferred from cryovials to plates of tryptic soy agar (TSA) and allowed to grow at 28 °C in an incubator. Rifampicin stock solution (100-ppm) was made by dissolving of 0.5 g of rifampicin into 50.0 ml dimethyl sulfoxide (DMSO) and filter sterilized into a sterile, dark container. Rifampicin stock solution was stored at 4 °C when not being used. Tryptic soy broth (TSB) was made with 30.0 g of TSB per liter of sterile water and autoclaved for 20 min. Once cool, the TSB was augmented with 10 ml of the rifampicin stock solution. To generate rifampicin mutants (McInroy et al. 1996), 100 ml of the TSB-rifampicin (RTSB) solution was transferred into an autoclaved 250 ml flask and a loop full of each bacterium was scrapped off the TSA plate and transferred to the flask. Flasks were then covered with aluminum foil and put on a shaker at 150 rpm and 28 °C for 5 d before being transferred to TSA-rifampicin (RTSA) plates. A RTSA solution was made with 15.0 g of TSA and 18.0 g of agar per liter of sterile water and autoclaved for 20 min, with the addition of 10 ml of rifampicin stock solution after the TSA had cooled. After shaking for 5 d, 50 µl of each strain was plated on RTSA plates and allowed to grow. After 24-48 h, colonies were either scrapped and transferred to RTSA plates or collected in sterile centrifuge tubes (50 ml, VWR, Radnor, PA) containing 40 ml of sterile water, and vigorously shaken to evenly distribute bacterial cells. Rifampicin resistant bacterial strains were prepared for long-term storage in 1.5 ml cryovials. Cryovials contained 1.25 ml of a 100-ppm RTSB with 30% glycerol. A loop full of bacteria is scrapped from a plate of full strength RTSA and transferred to the cryovial. Cryovials

were then shaken at 150 rpm at 27 °C for 12-24 h before being transferred to -80 °C freezer for long-term storage.

2.2 Bacterial strains and inoculant preparation

Three *Bacillus* strains, *Bacillus pumilus* AP 7, *B. pumilus* AP 18, and *B. sphaericus* AP 282, were evaluated individually. The combination of these strains comprises Blend 20, a mixture of equal parts of each bacterium that has previously demonstrated growth promotion in bermudagrass (Coy et al. 2014). Wild-type bacteria (bacterial characteristics experiments) and rifampicin resistant bacterial strains (bermudagrass colonization experiment) that were stored at -80 °C were transferred from cryovials to either plates of TSA or RTSA and allowed to grow at 28 °C in an incubator. After 24-48 h, bacterial lawns were scraped from RTSA plates with inoculating loops and transferred to either new TSA or RTSA plates or to sterile centrifuge tubes (50 ml, VWR, Radnor, PA) containing 40 ml of sterile water, and vigorously shaken to evenly distribute bacterial cells. Serial 10–fold dilutions were then made of each bacterial suspension into sterile water blanks to a final dilution of 10^{-5}.

Bacterial populations (number of colony forming units [CFU]) in the suspensions were determined by plating 50 µl of the serial dilution onto TSA or RTSA plates, incubating plates for 24-48 h and then counting the number of bacterial colonies on each plate. Once the concentrations (CFU per ml) in the prepared suspensions of each strain were determined, these populations were used to make bacterial stock solutions of each bacterium with a final concentration of 6.0×10^9 CFU per ml of each strain.

2.3 Application of rifampicin resistant bacteria to bermudagrass

In June 2017, three field plots for each strain were established using a randomized block design on the campus of Auburn University, Auburn, AL over common bermudagrass (*Cynodon dactylon* (L.) Pers.) in a Marvyn loamy sand (pH 7.3). Plots were 1 m x 1 m with at least 1 m separating plots. Three strains of two rifampicin resistant *Bacillus* species were individually applied using a backpack sprayer (Solo, Newport News, VA) that delivered 500 ml / m^2 of freshly-prepared aqueous bacterial suspension of 6 x 10^9 CFU per ml. The sprayer was thoroughly washed after the application of each strain. After treating, the plots were hand watered with 12.7 L of water (1.27 cm) to move the treatments into the root zone.

2.4 Colonization of rifampicin resistant bacteria in bermudagrass

Experiments were designed to quantify bacterial populations of rifampicin resistant rhizobacterial mutants colonizing internal and external root, shoot, and leaf structures of bermudagrass over time. Field samples were taken at 24, 72 h, 10 d, 2, 4, 6, and 12 weeks after treatment (WAT).

The field experiment quantified bacterial populations per gram of tissue or tissue and soil in the rhizoplane (root surface), rhizosphere (root surface and soil), endorhiza (endophytic root colonization), and phyllosphere (endophytic foliage colonization). All field samples were harvested with a hole cutter (10.8 cm diameter x 20 cm deep). Once roots and shoots were harvested, they were processed with varying extraction methods. Rhizoplane samples had roots extracted and washed with tap water to remove soil particles attached to the root surface. Once the roots were washed, they were cut into smaller sections and placed in test tubes with 9 ml of

sterile water before being agitated for 30 s. Following agitation, the samples were serially diluted and 50 µl of the serial dilution was plated onto RTSA plates and incubated at 28 °C for 48-72 h before colonies were counted. Rhizosphere root samples were harvested as previously described. Roots were then gently shaken to remove excess soil, but leaving behind attached soil particles. Samples were then cut into smaller sections, agitated in sterile water, and serially diluted before plating.

Bacterial colonization of the endorhiza (internal root structure) was assessed under surface sterilized conditions. Endorhiza samples were harvested as described above. Roots were washed with tap water to remove excess soil followed by disinfestation. Roots were surface disinfested in a 96% ethanol solution for 30 s, then transferred to a 20% bleach solution for 60 s, and then rinsed five times in deionized water. Roots were then triturated using a Kleco grinder (Model 4200, Garcia Machine, Visalia, CA) for 90 s. After the samples were pulverized, they were serially diluted before plating. Endophytic phyllosphere (stems and blades) colonization was sampled by cutting top growth to include blades and stems under surface sterilized conditions. Disinfested samples were sterilized as described above before trituration, serial dilution, and plating.

Temporary loss of antibiotic resistance or 'rifampicin masking' has previously been reported from endophytic bacterial colonization of plants (McInroy et al. 1992, McInroy et al. 1996) as well as in the rhizosphere and root surface (Nairn and Chanway 2002). Due to the possibility of 'masking' endophytic bacterial isolates were plated on both TSA and RTSA. Colony transfers from TSA to RTSA were used to confirm bacterial populations when masking occurred. Three replicates were plated for each sampling method. After colony counts, bacterial

populations were enumerated and log transformed. Three control samples of non-treated grasses for each sampling location and interval were plated on RTSA.

2.5 Qualitative determination of nitrogenase activity

The nitrogenase activity of the wild-type of five bacterial strains was determined using a nitrogen-free semisolid media (JNFb) as described in Döbereiner (1995). The nitrogen-free media offers optimal conditions for the bacteria to find a niche within an oxygen gradient to exhibit nitrogen fixation. These strains were grown in JNFb medium, which contained, per liter, 5.0 g of malic acid, 0.6 ml of K_2HPO_4, 1.8 ml of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.2 g of $CaCl_2 \cdot H_2O$, 0.066 g of FeEDTA, 2.0 ml of bromothymol blue, 2.0 ml of micronutrients, 1.0 ml vitamin solution, 0.02 g of yeast extract, and 4.5 g of KOH (pH 5.8). The bromothymol blue solution consisted of 0.5 g bromothymol blue and 1.122 g KOH per 100 ml dH_2O. The micronutrient solution consisted of 0.04 g $CuSO_4 \cdot 5H_2O$, 0.012 g $ZnSO_4 \cdot 7H_2O$, 0.14 g H_2BO_3, 0.1 g $Na_2MoO_4 \cdot 2H_2O$, and 0.15 g $MnSO_4 \cdot H_2O$. The vitamin solution was made with 0.01 g Biotin, and 0.02 g Pyridoxol-HCl in 100 ml dH_2O. After autoclaving, 7.0 ml of the JNFb media was dispensed into 10 ml sterile glass culturable tubes. After hardening, a single colony of bacteria was transferred into each tube or 20 µl of sterile water was injected to a tube for controls. Tubes were then capped, and placed in an incubator at 28 °C for 72-96 h. The formation of a pellicle in the growth media indicated nitrogen fixation of the bacteria. Three replicates were done per strain.

2.6 Qualitative siderophore production

The ability of the wild-type bacterial strains in Blend 20 to produce siderophores was determined with Chrome azurol S (CAS) agar (Schwyn and Neilands 1987, Louden et al. 2011). This media qualitatively determines siderophore production. Bacteria were grown on TSA for 24 hours and then a single colony of bacteria was transferred to the CAS medium that was divided into four quadrants with a sterile inoculating loop. Each quadrant received one bacterial colony. Control plates were inoculated with 10 µl of sterile water. The production of a yellow-orange halo around the growing bacterial colony confirmed siderophore production after 48-72 h incubation. The CAS agar was a mixture of four solutions that were prepared separately and sterilized before mixing. The Fe-CAS indicator solution, buffer solution, and sugar solution were sterilized by an autoclave; the casamino acid solution was filter sterilized with a 0.2 µM filter. Solution 1, the Fe-CAS indicator solution consisted of 10 ml of 1 mM FeCl3·6H2O (in 10 mM HCl), 50 ml of aqueous CAS solution (1.21 mg/ml); and 40 ml of aqueous hexadecyl-trimetylammonium bromide (HDTMA, 1.82 g/ml). Solution 2, the buffer solution, consisted of 750 ml of a salt solution with 0.3 g KH_2PO_4, 0.5 g NaCl 1.0 g NH_4Cl, 30.24 g PIPES (peperazine-N, N'-bis [2-ethanesulfonic acid]), and 15.0 g agar. The final volume was brought to 800 ml with the addition of KOH and the pH was adjusted to 6.8. Solution 3, the sugar solution consisted of 2.0 g of glucose, 2.0 g of mannitol per 70 ml dH_2O. Solution 4, the acid solution consisted of 30 ml filtered-sterilized 10% (W:V) casamino acid. After autoclaving, the sugar and acid solutions (3 and 4) were added to the buffer solution (2). The Fe-CAS solution was added last and then stirred to ensure thorough mixture of the ingredients. The mixture (Fe-CAS dye complex), yielded a blue media.

2.7 Quantitative determination of phosphate solubilization activity

The phosphate solubilization activity of the wild-type bacterial strains in Blend 20 was determined using three versions of a modified Pikovskaya's media (MPIK). Each medium was prepared using a different phosphate source: calcium, iron, or aluminum (0.38% $Ca_3(PO_4)_2$, 0.01% $FePO_4$, and 0.01% $AlPO_4$). Each liter of Pikovskaya's media consisted of 10.0 g glucose, 5.0 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCL, 0.1 g $(NH_4)_2SO_4$. The individual solutions contained 3.8 g $Ca_3(PO_4)_2$ (calcium phosphate); 0.1 g $FePO_4 \cdot 2H_2O$ (iron (III) phosphate); or 0.1 g $AlPO_4$ (aluminum phosphate) as adapted from Lopez *et al.* (2011). The MPIK media was used for quantitative determination of the capacity to solubilize inorganic phosphate under acidic and basic conditions. One loopful of bacteria was inoculated into 25 ml of liquid MPIK media (without yeast extract and agar) in sterile 50 ml centrifuge tubes, which were stirred thoroughly and shaken at 150 rpm at 25-28°C. Bacterial broth samples were taken at 3, 7, and 14 d. Bacteria cells were precipitated by centrifugation at 12,000 rpm for 15 min. The amount of phosphorus in the supernatant was determined using the Molybdenum-blue method (Murphy and Riley 1962). A blue color indicated a positive reaction for phosphate solubilization. Phosphorus concentration was estimated by spectrophotometry, measuring the absorbance at 882 nm and comparison to the standard curve prepared with KH_2PO_4 (sigma Lot# 069K0342). Phosphate solubilization of each strain was expressed in µg / ml for each environmental condition. Three replicates per treatment were per strains per sampling interval.

2.8 Statistical analysis

The bacterial counts of individual rifampicin-resistant bacterial strains were enumerated and log transformed before analysis. The colonization data of an individual strain comparing

colonization of rhizoplane, rhizosphere, endorhiza, and phyllosphere over time were analyzed using linear regression. The data was further analyzed with repeated measures multivariate analysis of variance (MANOVA), orthogonal contrasts ($P < 0.05$, JMP Version 13. SAS Institute Inc., Cary, NC). The colonization data comparing all strains in a plant location at specific time intervals were analyzed using an analysis of variance (ANOVA), Student's *t*-test ($P < 0.05$). The ability of PGPR strains to solubilize phosphate under acidic or basic conditions were analyzed using linear regression and an analysis of variance (ANOVA), Student's *t*-test ($P < 0.05$).

3. Results

3.1 Colonization of rifampicin resistant bacteria in bermudagrass

Soil microbial communities are influenced by soil type, temperature, and moisture (Landa et al. 2004). During the study, the average soil temperature at a 10.2 cm depth was 23.92 °C and the pH was 7.3. Rain events occurred on 25 d during the study resulting in 20.7 cm of precipitation. Significant rain events occurred on June 25 (1.88 cm), 30 (1.75 cm), July 1 (1.57 cm), 15 (1.12 cm), 16 (1.55 cm), 18 (0.89 cm), 26 (1.98), 29 (1.98), 30 (0.58 cm), and September 5 (0.74 cm). Supplemental irrigation was not applied to the grass.

Temporary loss of antibiotic resistance was observed with all endophytic bacterial isolates for each strain at some point during the experiment. No bacterial colonies grew from the non-treated control samples on RTSA plates; however, fungal isolates were often observed. The results of this experiment showed the variation in rhizoplane, rhizosphere, endorhiza, and phyllosphere colonization within and between rhizobacterial strains over a 12 wk period (Tables 2.2-2.6, Figures 2.1-2.3). All rhizobacterial strains were recoverable for the duration of the

experiment (12 wk), but population densities had declined. Populations of bacteria were negatively correlated with time. Linear regression analysis of *B. pumilus* AP 7 population densities determined the colonization of the phyllosphere was the strongest association (F = 132.91, $P < 0.0001$, df =1,20, $R^2 = 0.8749$; Figure 2.1) followed by the rhizosphere, endorhiza, and rhizoplane (F = 61.49, $P < 0.0001$, $R^2 = 0.7640$; F = 53.72, $P < 0.0001$, $R^2 = 0.7387$; F = 23.54, $P = 0.0001$, $R^2 = 0.5534$). Colonization of the endorhiza (F = 63.2, $P < 0.0001$, R2 = 0.7689; Figure 2.2) was the strongest correlation of *B. pumilus* AP 18 populations over phyllosphere, rhizosphere, and rhizoplane populations (F = 53.29, $P < 0.0001$, $R^2 = 0.7372$; F = 32.62, $P < 0.0001$, $R^2 = 0.6319$; F = 23.22, $P = 0.0001$, $R^2 = 0.5499$). Colonization of the phyllosphere (F = 56.9, $P < 0.0001$, $R^2 = 0.7399$; Figure 2.3) was the strongest correlation of *B. pumilus* AP 18 populations over endorhiza, rhizoplane, and rhizosphere populations (F = 49.86, $P < 0.0001$, $R^2 = 0.7241$; F = 42.68, $P < 0.0001$, $R^2 = 0.7034$; F = 18.33, $P = 0.0004$, $R^2 = 0.4910$).

The colonization of bermudagrass over the 12 wk experiment with *B. pumilus* AP 7 determined that the colonization was significantly greater in the rhizosphere than all other locations ($P \leq 0.02$; Table 2.6). Internal plant colonization for AP 7 was significantly greater for endorhiza populations than phyllosphere populations ($P = 0.0185$). External plant colonization was significantly greater than internal colonization ($P = 0.0304$). Rhizosphere colonization for *B. pumilus* AP 18 was significantly greater than the rhizoplane and phyllosphere ($P \leq 0.0005$). Endorhiza colonization of AP 18 was significantly greater than rhizoplane and phyllosphere populations ($P \leq 0.0003$). The rhizoplane colonization by *B. sphaericus* AP 282 was significantly

greater than all other locations ($P \leq 0.0035$). Rhizosphere and rhizoplane colonization were significantly greater than endorhiza and phyllosphere populations ($P \leq 0.0395$).

Colonization of the rhizoplane was not significantly greater than endophytic populations ($P \leq 0.0532$). *B. sphaericus* AP 282 had significantly greater ($P = 0.0122$; Table 2.2) populations than all treatments at 6 and 12 WAT except for *B. pumilus* AP 7 rhizosphere 12 WAT. Typically, populations increased for 2 WAT and were at their highest population densities before populations began to decline at 4 WAT. The exceptions to this were the non-changes or slight increases in *B. pumilus* AP 7 endorhiza populations or rhizoplane and phyllosphere populations of *B. pumilus* AP 18.

Rhizoplane populations significantly varied from one another 24 and 72 h after treatment with *B. sphaericus* AP 282 and *B. pumilus* AP 18 having the highest population densities at the respective sampling intervals. No differences in populations were detected 10 d after treatment. Except for 72 h after treatment, *B. sphaericus* AP 282 had the highest population density during each sampling interval. All bacterial populations were at their lowest at 12 WAT, with *B. pumilus* AP 18 having the lowest population density (log 4.82) and *B. sphaericus* AP 282 having the highest (log 5.87). Populations of *B. pumilus* AP 7 ranged from log 5.51-7.03, *B. pumilus* AP 18 ranged from log 4.82-7.21, and *B. sphaericus* AP 282 ranged from log 5.87-7.23.

Rhizosphere populations significantly varied from one another 24 and 72 h after treatment with *B. pumilus* AP 7 and *B. pumilus* AP 18 having the highest population densities at the respective sampling intervals (Table 2.3). No differences in populations were detected 10 d and 2 WAT. Generally, AP 18 had the highest population densities during the first 4 WAT, but

had significantly lower populations at 6 and 12 WAT. All bacterial populations were at their lowest at 12 WAT, with AP 18 having the lowest population density (log 4.2) and *B. sphaericus* AP 282 having the highest (log 5.87). Populations of AP 7 ranged from log 5.62-7.08, AP 18 ranged from log 5.06-7.25, and AP 282 ranged from log 5.78-7.09.

Endorhiza populations did significantly vary from one another until 72 h after treatment when *B. sphaericus* AP 282 reached its highest population density, having a significantly higher population density than all other treatments (Table 2.4). At 10 d after treatment, *B. pumilus* AP 7 reached its highest population density and was significantly greater than the other bacteria. *B. pumilus* AP 18, at 2 and 4 WAT had significantly greater populations than all other treatments. After 4 WAT, AP 282 populations remained the highest and were significantly greater than the other bacteria. All bacterial populations were at their lowest at 12 WAT, with AP 18 having the lowest population density (log 4.99) and AP 282 having the highest (log 5.72). Populations of AP 7 ranged from log 5.22-7.01, AP 18 ranged from log 4.99-7.42, and AP 282 ranged from log 5.72-7.38.

Endophytic phyllosphere populations of *B. pumilus* AP 7 and *B. sphaericus* AP 282 were significantly greater than *B. pumilus* AP 18 within 24 h after treatment (Table 2.5). However, at 72 h after treatment, populations of AP 7 were significantly lower than AP 18 and AP 282 when both strains reached their highest population densities. At 2 WAT, AP 7 reached its highest population density and both AP 7 and AP 282 were significantly greater than AP 18. At 4 WAT, AP 282 populations remained the highest and were significantly greater than the other bacteria for the remainder of the experiment. All bacterial populations were at their lowest at 12 WAT, with AP 18 having the lowest population density (log 4.66) and AP 282 having the highest (log

5.53). Populations of AP 7 ranged from log 4.98-7.08, AP 18 ranged from log 4.66-7.42, and AP 282 ranged from log 5.53-7.56.

3.2 Qualitative determination of nitrogenase activity

The inoculation of individual bacterial colonies into the semi-solid JNFb media determined that four of the five strains were diazotrophs by the formation a pellicle, indicating nitrogen fixation capabilities. There were differences in nitrogen fixation between strains of *B. sphaericus,* but not *B. pumilus*. All strains of *B. pumilus* (AP 7, 18, 283) and *B. sphaericus* AP 143 were capable of nitrogenase activity. Nitrogenase activity was not observed with *B. sphaericus* AP 282.

3.3 Qualitative siderophore production

The transferring of individual bacterial colonies to quadrants of CAS media that formed a yellow-orange halo on the blue media around the bacterial colony determined that three of the five strains evaluated were capable of siderophore production. Production of siderophores is important for the binding of iron in plants and may enhance plant growth or limit pathogens (Sharma and Johri 2003, Verma et al. 2011). These revealed differences between strains of both species to produce siderophores. Two strains of *B. pumilus* (AP 7, AP 18) and *B. sphaericus* AP 143 produced halos. No siderophore production was observed with *B. pumilus* AP 283 or *B. sphaericus* AP 282.

3.4 Quantitative determination of phosphate solubilization activity

Phosphate solubilization activity of rhizobacterial strains was confirmed by the molybedate-blue method. All rhizobacteria strains solubilized phosphate during the experiments

(Table 2.1, Figures 2.4-2.8). The solubilization of aluminum and calcium phosphate by each rhizobacterial strain was positively correlated with time (Figures 2.4-2.8). Solubilization of iron phosphate for each strain was negatively correlated with time, except for *B. pumilus* AP 18 (Figures 2.4-2.8). Linear regression of *B. pumilus* AP 7 determined that calcium phosphate solubilization ($F = 5.45$, df = 1,8, $P = 0.0528$, $R^2 = 0.4363$; Figure 2.4) activity was most strongly associated with the strain than aluminum and iron phosphate solubilization ($F = 1.19$, $P = 0.3120$, $R^2 = 0.1450$; $F = 0.22$, $P = 0.6564$, $R^2 = 0.0299$). While numerically low, the solubilization of iron phosphate ($F = 8.76$, $P = 0.0211$, $R^2 = 0.5558$; Figure 2.5) by *B. pumilus* AP 18 was more strongly associated with the strain than aluminum and calcium phosphate ($F= 0.01$, $P = 0.9068$, $R^2 = 0.0021$; $F = 4.55$, $P = 0.0704$, $R^2 = 0.4932$). The strongest association of solubilization activity in *B. sphaericus* AP 143 was observed with aluminum phosphate ($F= 25.46$, $P = 0.0015$, $R^2 = 0.7843$; Figure 2.6) then calcium and iron phosphate ($F = 4.09$, $P = 0.0828$, $R^2 = 0.3690$; $F= 0.03$, $P = 0.8714$, $R^2 = 0.0040$). Calcium phosphate solubilization was most strongly associated with *B. sphaericus* AP 282 ($F= 27.84$, $P = 0.0012$, $R^2 = 0.7991$; Figure 2.7) then aluminum and iron phosphate ($F = 3.75$, $P = 0.0941$, R2 = 0.3487; $F = 0.61$, $P = 0.4619$, $R^2 = 0.0796$). The solubilization activity by *B. pumilus* AP 283 was most strongly associated with calcium phosphate ($F = 10.83$, $P = 0.0133$, R2 = 0.6073; Figure 2.8) then iron and aluminum phosphate ($F = 3.29$, $P = 0.1124$, $R^2 = 0.3199$; $F = 0.08$, $P = 0.7877$, $R^2 = 0.0111$). Differences in phosphate solubilization between strains of a species were observed. The greatest phosphate solubilization by rhizobacteria was observed in the calcium phosphate broth (0.026-2.697 µg / ml; Table 2.1), then aluminum phosphate broth (0.104-2.219 µg / ml), and the least activity was observed in iron (III) phosphate broth (0.00-0.283 µg / ml). Solubilization activity

varied by strain, and form of phosphate. Depending on the species, strain, or broth solution, phosphate solubilization activity from 3 to 7 d and 7 to 14 d could either increase or decrease. Typically, 7 d was when the most solubilization activity was observed. Strains of *B. sphaericus* (AP 143 and AP 282) and *B. pumilus* AP 7 at 7 and/or 14 d were the only strains to solubilize > 1.288 µg / ml of aluminum phosphate during the experiment. All strains solubilized > 1.678 µg / ml of calcium phosphate, with was lowest activity at 3 d. While *B. sphaericus* AP 282 had the lowest calcium phosphate solubilization at 7 d (1.167 µg / ml), it had the highest activity at 14 d (2.697 µg / ml). All strains solubilized < 0.3 µg / ml iron phosphate, and all but *B. pumilus* AP 18 solubilized < 0.1 µg / ml at 14 d. *Bacillus pumilus* AP 18 had the longest solubilizing (0.21-0.258 µg / ml) activity at 7 and 14 d. *Bacillus sphaericus* AP 143 had the highest activity at 7 d.

4. Discussion

Rhizobacterial populations of all three strains rapidly colonized soil, as well as external and internal plant tissues of bermudagrass. The presence of bacterial endophytes in model, saltmarsh, and bioenergy grasses have been previously reported, but not in forage or amenity grasses (McClung et al. 1983, Reinhold-Hurek and Hurek 1998, Gagné-Bourgue et al. 2013). Based on our literature review, this is the first study to document inoculation, colonization, and persistence of PGPR in a grass under field conditions. Bacterial populations can be tracked in plants but the rifampicin mutant marking system is the only available technique to enumerate and compare colonization and persistence in plants and over time. The rifampicin marking system is advantageous as it is an inexpensive marking tool that is uncommonly found in soil bacteria. Further, it allows for the isolation and culturing of marked bacterial strains from a non-sterile

environment in the presence of native soil microbes. However, its major disadvantage is that the antibiotic resistance may be temporarily masked when isolating endophytic populations. Masking was noted in at least one endophytic sample after 72 h. Our methodology employed two types of agar for plating to control for masking especially in the endophytic samples.

Generally, bacterial populations increased or remained stable for 2 WAT and then declined over time. The decline of population densities over time is expected, and supports previous research in corn, cotton, lodgepole pine seedlings, potato, and soybean (Shishido et al. 1995, Quadt-Hallmann et al. 1997, Andreote et al. 2009, Durham 2013). The rate of decline was greater in the *B. pumilus* strains (AP 7, AP 18) than *B. sphaericus* AP 282. *Bacillus sphaericus* AP 282 was the 'best' colonizer, as it was the most abundant 12 WAT. Greater colonization occurred in the rhizoplane or rhizosphere versus endophytic colonization in the endorhiza or phyllosphere. Grass plots used in this study were mown at least once per week during this study so leaf tissue and phyllosphere bacteria were partially removed. It is unclear why certain strains would differentially decline. However, the persistence of AP 282 may be indicative of the bacteria's ability to effectively colonize and recolonize post cutting. Under less intense mowing or grazing, persistence may be similar for these bacteria.

Endophytic colonization of plants may be more advantageous for beneficial microbes as it offers protection from the environment, other microbes, and reduces competition for nutrients. The reduction in competition may further benefit the bacteria by prolonging persistence as well as enhanced biocontrol. Endophytic bacteria due to their intimate association with plant functions are better equipped to alter gene expression, plant structures, or elicit induced systemic resistance to abiotic or biotic stress than bacterial populations localized on the root surface or in

soil (Kloepper et al. 2004, Kloepper and Ryu 2006, Gagné and Bourque et al. 2015, Santoyo et al. 2016). The production or secretion of extracellular substances, chitinases, proteases, lytic enzymes, or other secondary metabolites may further inhibit pathogens (Pleban et al. 1995, Beniziri et al. 2001, Graner et al. 2003). Future studies could evaluate microbes that were isolated from endophytic populations to confirm if they are better colonizers and persist longer than isolates of the same strain from external plant colonization as proposed by van Peer et al. (1990).

A limitation of the study is that due to the similarities in colony morphology, the bacterial strains that comprise Blend 20, were applied individually, which could influence how the bacteria interacted, performed, and persisted in the soil and plant. Previous work (Coy et al. 2014, Coy *unpublished*) demonstrated that plant benefits are context dependent and influenced by specific combinations of rhizobacterial strains. Previously, the inoculation of bermudagrass with the individual components of Blend 20 alone, all combinations of two strains, or substitutions of a single strain of the same species did not provide the same level of growth promotion or plant benefits as the blended inoculum (Coy, *unpublished*). For future studies, it could be advantageous to study colonization with and without supplemental fertilization within a strain on different grass species or cultivars in various soil types. For example, Durham (2013) noted different colonization and persistence for *B. firmus* GB126 in corn, cotton, and soybean across soil types. Like our study, it found greater colonization of the rhizoplane and rhizosphere than in the endorhiza. Further, it may be easier to track the populations of bacterial strains that have distinct colony morphologies like *B. mycoides* or coloration like *Serratia* spp. or

Chromobacterium spp. which have been isolated from drought stressed bermudagrasses by our lab group (Coy, *unpublished*) and previously linked to growth promotion (Gray and Smith 2005).

Bacillus sphaericus AP 282 did not demonstrate nitrogen fixation or siderophore production. The inability for nitrogen fixation or siderophore production when paired with its colonization abilities could explain the importance of this strain as a component in Blend 20. The results of the bacterial colonization levels in bermudagrass support previous research that suggested population levels of 10^{5-9} in the rhizoplane and rhizosphere and 10^{6-8} endophytic phyllosphere populations are needed for plant benefits (Benizri et al. 2001, Lindlow and Brandl 2003).

Any bacterium that has the ability to fix nitrogen, solubilize phosphate, or produce siderophores could have the ability to promote plant growth and nutrient acquisition. While nitrogenase activity was determined for specific strains of *B. pumilus* and *B. sphaericus* the amounts nitrogen fixed by each strain were not determined. However, it does provide an explanation for increased bermudagrass growth. Quantitative nitrogenase activity of diazotrophic bacteria could be an area for further development as demonstrated by Xu (2014). The identification of strains with substantial nitrogen fixation or phosphate solubilization abilities could lead to lower fertilizer use rates or needs. Quantitative phosphate solubilization activity of rhizobacteria varied by species and strain, broth condition (alkaline vs acidic), and time. Solubilization was most pronounced in the calcium phosphate broth; however, aluminum and iron phosphate are more common sources of phosphate in acidic soils throughout the northern and southeastern United States (Frank and Guertal 2013b). The strains *B. pumilus* AP 7, *B. sphaericus* AP 143, and *B. sphaericus* AP 282 were isolated from soils in the midwestern United

States, which may better explain why the strains were most efficient at calcium phosphate solubilization. These strains were most competent at solubilizing aluminum phosphate at 7 and 14 d. There was little solubilization activity for any strain in the iron (III) phosphate broth. The phosphate solubilization activity of these rhizobacterial strains were similar to previous work by our lab group (Xu 2014, Liu 2015) and phytase (Hayes et al. 2000), but lower than studies with other rhizobacteria or fungi (Selvakumar et al. 2009, Elias et al. 2016). Siderophore production by bacterial metabolites is an important aspect for biological control as the binding of iron can increase plant growth as well as limit the acquisition of iron by pathogens (Sharma and Johri 2003, Verma et al. 2011). The results of these experiments showed that taxonomic bacterial identification alone cannot explain beneficial characteristics of rhizobacterial strains, as differences occurred within each strain, making it difficult to apply results on a broad scale.

Bacteria that fix nitrogen, solubilize phosphate, or produce siderophores benefit plant and soil heath. This research furthered the understanding of plant-microbe interactions, growth promotion, and nutrient acquisition in grasses. While not all strains were capable of each phosphate solubilization activity, each solubilization activity was demonstrated. The implication of this research for managed grass systems by developing appropriate application intervals or frequencies of rhizobacterial products and provide beneficial characteristics for which to screen. Currently, there are few PGPR products available for use in forage and amenity grasses. Nevertheless, we suspect that plant fertilizers containing PGPR will become available to advance more sustainable management of grasslands and production grasses. At present, the most popular PGPR product in turfgrass is Nortica® (Bayer Environmental Sciences, Research Triangle Park, NC). This granular product is a single strain of *B. firmus* that is used in warm-season grasses for

growth promotion and nematode control has recommended application intervals of 4, 6, and 8 wks. Based on our results, applications frequencies of 2, 4, or 6, may be more beneficial as drastic population declines occur from 4-6 and 6-12 WAT.

This work built on Coy et al. (2014) which previously demonstrated growth promotion in bermudagrass, and adds to the understanding of plant-microbe interactions in warm-season grasses. These experiments were the first to track the bacterial colonization and persistence after inoculation in an economically important warm-season grass under non-sterile, field conditions. Further, it likely represents the first report of endophytic bacterial colonization in warm-season amenity grasses, specifically bermudagrass. During the colonization and persistence experiments, all bacterial strains were detectable for 12 WAT; however, populations were at their lowest densities or near their initial (24 h) colonization levels. The decline of population densities over time is expected, and supports previous research in corn, cotton, lodgepole pine seedlings, potato, and soybean (Shishido et al. 1995, Quadt-Hallmann et al. 1997, Andreote et al. 2009, Durham 2013).

The differences between rhizobacterial strains for colonization across crops and soil types suggests that not all rhizobacteria are effective colonizers in every situation. Failure of rhizobacterial strains to demonstrate growth-promotion across various crops and soil types likely indicates lack of colonization of the host plant or soil (Schippers et al. 1987, Lelande et al. 1989). Further, the reliance on one bacterial strain or an excessive amount (>5) may be risky for products due to competition and antibiotic activities of microbes.

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Table 2.1. Mean (±SEM) quantitative phosphate solubilization by *Bacillus* rhizobacterial strains in a modified Pikovskaya's media liquid broth over 2 weeks.

Strain[§]	Solubilization µg PO_4/ ml (3 d)	Solubilization µg PO_4 / ml (7 d)	Solubilization µg PO_4 / ml (14 d)
	Aluminum Phosphate $AlPO_4$		
Bacillus pumilus AP 7	0.246 ± 0.060a	1.288 ± 0.113b	0.819 ± 0.175b
Bacillus pumilus AP 18	0.582 ± 0.338a	0.775 ± 0.127c	0.581 ± 0.103b
Bacillus sphaericus AP 143	0.186 ± 0.002a	0.528 ± 0.046c	1.460 ± 0.332a
Bacillus sphaericus AP 282	0.214 ± 0.048a	2.219 ± 0.202a	1.741 ± 0.212a
Bacillus pumilus AP 283	0.104 ± 0.016a	0.779 ± 0.062c	0.267 ± 0.088b
Statistics	$P = 0.3034$	$P < 0.0001$	$P = 0.0022$
	Calcium Phosphate $Ca_3(PO_4)_2$		
Bacillus pumilus AP 7	0.325 ± 0.024ab	2.078 ± 0.121a	1.958 ± 0.036b
Bacillus pumilus AP 18	0.263 ± 0.016bc	2.275 ± 0.025a	1.961 ± 0.028b
Bacillus sphaericus AP 143	0.366 ± 0.011a	2.308 ± 0.082a	2.054 ± 0.18b
Bacillus sphaericus AP 282	0.026 ± 0.017d	1.678 ± 0.125b	2.697 ± 0.163a
Bacillus pumilus AP 283	0.225 ± 0.018c	2.165 ± 0.148a	2.140 ± 0.238ab
Statistics	$P < 0.0001$	$P = 0.0378$	$P = 0.0744$
	Iron (III) Phosphate $FePO_4$		
Bacillus pumilus AP 7	0.107 ± 0.064a	0.263 ± 0.189a	0.055 ± 0.022b
Bacillus pumilus AP 18	0.029 ± 0.014a	0.210 ± 0.115a	0.258 ± 0.053a
Bacillus sphaericus AP 143	0.058 ± 0.038a	0.283 ± 0.175a	0.083 ± 0.036b
Bacillus sphaericus AP 282	0.023 ± 0.019a	0.195 ± 0.041a	0.00 ± 0.00b
Bacillus pumilus AP 283	0.126 ± 0.085a	0.159 ± 0.054a	0.064 ± 0.026b
Statistics	$P = 0.4497$	$P = 0.7773$	$P = 0.019$

[§]AP strains contain rhizobacteria unique to the Auburn University PGPR collection.

*Means followed by the same letter are not significantly different from one another (ANOVA, Student's *t*-test, $P < 0.05$, df = 4,14; JMP Version 13. SAS Institute Inc., Cary, NC).

Table 2.2. Mean (±SEM) rhizoplane[†] population density (log CFU / g) of rifampicin mutant rhizobacterial strains after single application[‡] to common bermudagrass in a Marvyn loamy sand soil (pH 7.3) over 12 weeks under field conditions.

Strain[§]	24 h	72 h	10 d	2 wk	4 wk	6 wk	12 wk
Bacillus pumilus AP 7	6.38 ± 0.02b	6.60 ± 0.11b	6.72 ± 0.04a	7.03 ± 0.02b	6.95 ± 0.02b	5.98 ± 0.02b	5.51 ± 0.01b
Bacillus pumilus AP 18	5.86 ± 0.07c	7.21 ± 0.04a	6.69 ± 0.04a	7.02 ± 0.05b	7.01 ± 0.01a	5.97 ± 0.01b	4.82 ± 0.03c
Bacillus sphaericus AP 282	7.48 ± 0.05a	6.91 ± 0.09ab	6.74 ± 0.04a	7.23 ± 0.03a	7.02 ± 0.01a	6.39 ± 0.01a	5.87 ± 0.02a
Statistics	$P = 0.0002$	$P = 0.0294$	$P = 0.775$	$P = 0.056$	$P = 0.0206$	$P = .0001$	$P < 0.0001$

[†]Rhizoplane roots were rinsed to remove attached soil particles to sample root surface populations.
[‡]Rhizobacterial inoculants were applied as liquid treatments at a rate of 500 ml / m^2 and a population density of 6.0 x 10^9 CFU / ml (log 9.78), followed by 1.27 cm of water to move the rhizobacteria into the root zone.
[§]AP strains contain rhizobacteria unique to the Auburn University PGPR collection.
*Means within a column that are followed by the same letter are not significantly different from each other at $P = 0.05$ (df =2, 8).

Table 2.3. Mean (±SEM) rhizosphere[†] population density (log CFU / g) of rifampicin mutant rhizobacterial strains after single application[‡] to common bermudagrass in a Marvyn loamy sand soil (pH 7.3) over 12 weeks under field conditions.

Strain[§]	24 h	72 h	10 d	2 wk	4 wk	6 wk	12 wk
Bacillus pumilus AP 7	6.70 ± 0.04a	6.88 ± 0.01c	6.65 ± 0.06a	7.08 ± 0.03a	6.86 ± 0.002c	6.06 ± 0.01b	5.62 ± 0.03a
Bacillus pumilus AP 18	6.38 ± 0.07b	7.38 ± 0.03a	6.58 ± 0.05a	7.25 ± 0.05a	7.06 ± 0.002a	5.99 ± 0.01c	5.06 ± 0.07b
Bacillus sphaericus AP 282	6.28 ± 0.02b	7.05 ± 0.04b	6.70 ± 0.01a	7.09 ± 0.05a	6.97 ± 0.004b	6.16 ± 0.02a	5.78 ± 0.05a
Statistics	$P = 0.0194$	$P = 0.0011$	$P = 0.3827$	$P = 0.1631$	$P < 0.0001$	$P = 0.0046$	$P = 0.003$

[†]Rhizosphere roots were shaken to removed excess soil, but residual soil particles remained attached to the root surface to sample root surface and soil populations.

[‡]Rhizobacterial inoculants were applied as liquid treatments at a rate of 500 ml / m^2 and a population density of 6.0 x 10^9 CFU / ml (log 9.78), followed by 1.27 cm of water to move the rhizobacteria into the root zone.

[§]AP strains contain rhizobacteria unique to the Auburn University PGPR collection.

*Means within a column that are followed by the same letter are not significantly different from each other at $P = 0.05$ (df =2, 8).

Table 2.4. Mean (±SEM) endorhiza bermudagrass root[†] population density (log CFU / g) of rifampicin mutant rhizobacterial strains after single application[‡] to common bermudagrass in a Marvyn loamy sand soil (pH 7.3) over 12 weeks under field conditions.

Strain[§]	24 h	72 h	10 d	2 wk	4 wk	6 wk	12 wk
Bacillus pumilus AP 7	6.47 ± 0.06a	6.78 ± 0.07b	7.01 ± 0.02a	6.67 ± 0.03c	6.93 ± 0.003b	5.84 ± 0.004c	5.22 ± 0.03b
Bacillus pumilus AP 18	6.65 ± 0.03a	6.97 ± 0.01b	6.75 ± 0.01b	7.42 ± 0.02a	6.95 ± 0.004a	6.08 ± 0.01b	4.99 ± 0.02c
Bacillus sphaericus AP 282	6.65 ± 0.05a	7.38 ± 0.06a	6.73 ± 0.02b	7.27 ± 0.03b	6.88 ± 0.001c	6.26 ± 0.01a	5.72 ± 0.01a
Statistics	P = 0.1543	P = 0.007	P = 0.0007	P = 0.0002	P = 0.0004	P = 0.0133*	P = 0.0001

[†]Roots were rinsed, surface disinfested, and pulverized to sample internal root populations.

[‡]Rhizobacterial inoculants were applied as liquid treatments at a rate of 500 ml / m^2 and a population density of 6.0 x 10^9 CFU / ml (log 9.78), followed by 1.27 cm of water to move the rhizobacteria into the root zone.

[§]AP strains contain rhizobacteria unique to the Auburn University PGPR collection.

*Means within a column that are followed by the same letter are not significantly different from each other at P = 0.05 (df =2, 8).

Table 2.5. Mean (±SEM) endophytic phyllosphere bermudagrass foliage[†] population density (log CFU / g) of rifampicin mutant rhizobacterial strains after single application[‡] to common bermudagrass in a Marvyn loamy sand soil (pH 7.3) over 12 weeks under field conditions.

Strain[§]	24 h	72 h	10 d	2 wk	4 wk	6 wk	12 wk
Bacillus pumilus AP 7	6.92 ± 0.03a	7.01 ± 0.03c	6.8 ± 0.09a	7.08± 0.04a	6.92 ± 0.003b	5.77 ± 0.02c	4.98 ± 0.06b
Bacillus pumilus AP 18	6.31 ± 0.08b	7.42 ± 0.01b	6.72 ± 0.08ab	6.77 ± 0.06b	6.92 ± 0.005b	5.94 ± 0.02b	4.66 ± 0.06c
Bacillus sphaericus AP 282	6.8 ± 0.06a	7.56 ± 0.05a	6.43 ± 0.01b	7.28 ± 0.04a	6.96 ± 0.006a	6.18 ± 0.01a	5.53 ± 0.02a
Statistics	$P = 0.0017$	$P = 0.0005$	$P = 0.08$	$P = 0.009$	$P = 0.0224$	0.0001	$P = 0.0017$

[†]Phyllosphere sampled grass foliage populations, grass blades and stems were rinsed with water, surface disinfested, and pulverized to sample internal foliage populations.
[‡]Rhizobacterial inoculants were applied as liquid treatments at a rate of 500 ml / m^2 and a population density of 6.0 x 10^9 CFU / ml (log 9.78), followed by 1.27 cm of water to move the rhizobacteria into the root zone.
[§]AP strains contain rhizobacteria unique to the Auburn University PGPR collection.
*Means within a column that are followed by the same letter are not significantly different from each other at $P = 0.05$ (df =2, 8).

Table 2.6. Orthogonal contrasts of population densities (log CFU / g) of rifampicin mutant rhizobacterial strains after single application† to common bermudagrass in a Marvyn loamy sand soil (pH 7.3) over 12 weeks under field conditions.

Strain‡	Contrast	Statistics
Bacillus pumilus AP 7	Endophytic§ Root* vs Endophytic Foliage	*P* = 0.0185
Bacillus pumilus AP 7	Endophytic Root vs Rhizoplane#	*P* = 0.4017
Bacillus pumilus AP 7	Endophytic Root vs Rhizosphere*††	*P* = 0.0011
Bacillus pumilus AP 7	Endophytic Foliage vs Rhizoplane	*P* = 0.0532
Bacillus pumilus AP 7	Endophytic Foliage vs Rhizosphere*	*P* = 0.0212
Bacillus pumilus AP 7	All Endophytic‡‡ vs All Epiphytic*‡‡‡	*P* = 0.0304
Bacillus pumilus AP 7	Rhizoplane vs Rhizosphere*	*P* = 0.0021
Bacillus pumilus AP 18	Endophytic Root* vs Endophytic Foliage	*P* = 0.0003
Bacillus pumilus AP 18	Endophytic Root vs Rhizoplane*	*P* = 0.0001
Bacillus pumilus AP 18	Endophytic Root vs Rhizosphere	*P* = 0.3336
Bacillus pumilus AP 18	Endophytic Foliage vs Rhizoplane	*P* = 0.1888
Bacillus pumilus AP 18	Endophytic Foliage vs Rhizosphere*	*P* = 0.0005
Bacillus pumilus AP 18	All Endophytic vs All Epiphytic	*P* = 0.1265
Bacillus pumilus AP 18	Rhizoplane vs Rhizosphere*	*P* = 0.0002
Bacillus sphaericus AP 282	Endophytic Root vs Endophytic Foliage	*P* = 0.3743
Bacillus sphaericus AP 282	Endophytic Root vs Rhizoplane*	*P* = 0.0035
Bacillus sphaericus AP 282	Endophytic Root vs Rhizosphere*	*P* = 0.002
Bacillus sphaericus AP 282	Endophytic Foliage vs Rhizoplane*	*P* = 0.0016
Bacillus sphaericus AP 282	Endophytic Foliage vs Rhizosphere*	*P* = 0.0044
Bacillus sphaericus AP 282	All Endophytic vs All Epiphytic	*P* = 0.8503
Bacillus sphaericus AP 282	Rhizoplane* vs Rhizosphere	*P* = 0.0001

*Indicates which contrast between plant locations which was significantly greater

†Rhizobacterial inoculants were applied as liquid treatments at a rate of 500 ml / m^2 and a population density of 6.0 x 10^9 CFU / ml (log 9.78), followed by 1.27 cm of water to move the rhizobacteria into the root zone.

‡AP strains contain rhizobacteria unique to the Auburn University PGPR collection.

§ Endophytic samples of roots and foliage were washed, surface disinfested, and pulverized to sample internal root populations

#Rhizosphere roots were shaken to removed excess soil, but residual soil particles remained attached to the root surface to sample root and soil populations

†† Rhizoplane roots were washed with tap water to remove attached soil particles to sample populations on the root surface.

‡‡Combination of endophytic root and foliage populations.

‡‡‡Combination of rhizoplane and rhizosphere populations.

Figures

Figure 2.1. *Bacillus pumilus* AP 7 rifampicin mutant mean (± SEM) log populations CFU / g of tissue and linear regression in common bermudagrass (*Cynodon dactylon*) grown under field conditions in Marvyn loamy sand soil (pH 7.3). Populations were sampled from the rhizoplane, rhizosphere, and endophytically from roots and foliage.

Figure 2.2. *Bacillus pumilus* AP 18 rifampicin mutant mean (± SEM) log populations CFU / g of tissue and linear regression in common bermudagrass (*Cynodon dactylon*) grown under field conditions in Marvyn loamy sand soil (pH 7.3). Populations were sampled from the rhizoplane, rhizosphere, and endophytically from roots and foliage.

Figure 2.3. *Bacillus sphaericus* AP 282 rifampicin mutant mean (± SEM) log populations CFU / g of tissue and linear regression in common bermudagrass (*Cynodon dactylon*) grown under field conditions in Marvyn loamy sand soil (pH 7.3). Populations were sampled from the rhizoplane, rhizosphere, and endophytically from roots and foliage.

Figure 2.4 Quantitative phosphate solubilization and linear regression by *Bacillus pumilus* AP 7 in modified Pikovskaya's media liquid broths over 2 weeks.

Figure 2.5 Quantitative phosphate solubilization and linear regression by *Bacillus pumilus* AP 18 in modified Pikovskaya's media liquid broths over 2 weeks.

Figure 2.6 Quantitative phosphate solubilization and linear regression by *Bacillus sphaericus* AP 143 in modified Pikovskaya's media liquid broths over 2 weeks.

Figure 2.7 Quantitative phosphate solubilization and linear regression by *Bacillus sphaericus* AP 282 in modified Pikovskaya's media liquid broths over 2 weeks.

Figure 2.8 Quantitative phosphate solubilization and linear regression by *Bacillus pumilus* AP 283 in modified Pikovskaya's media liquid broths over 2 weeks.

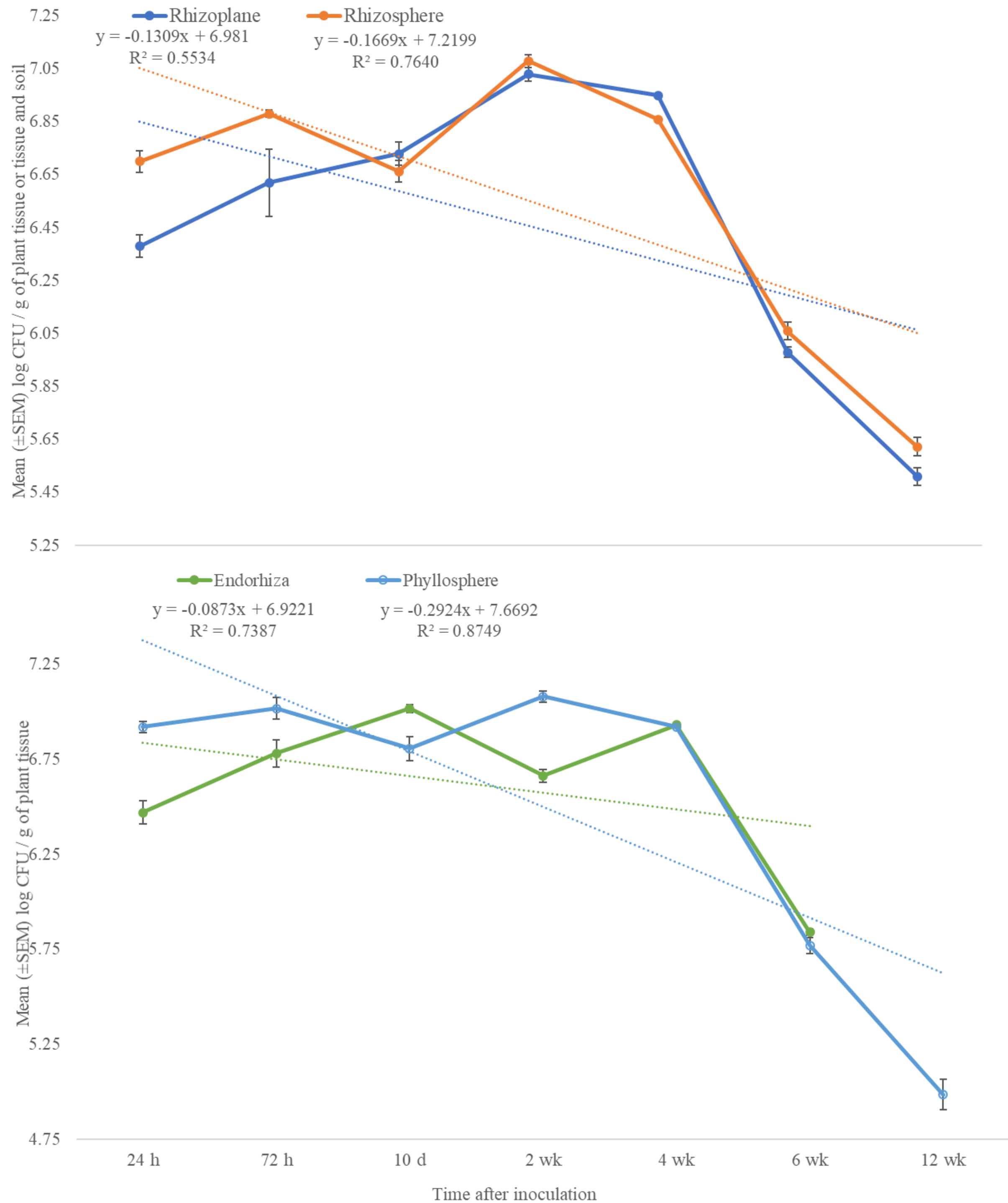


Figure 2.1. *Bacillus pumilus* AP 7 rifampicin mutant mean (± SEM) log populations CFU / g of tissue and linear regression in common bermudagrass (*Cynodon dactylon*) grown under field conditions in Marvyn loamy sand soil (pH 7.3). Populations were sampled from the rhizoplane, rhizosphere, and endophytically from roots and foliage

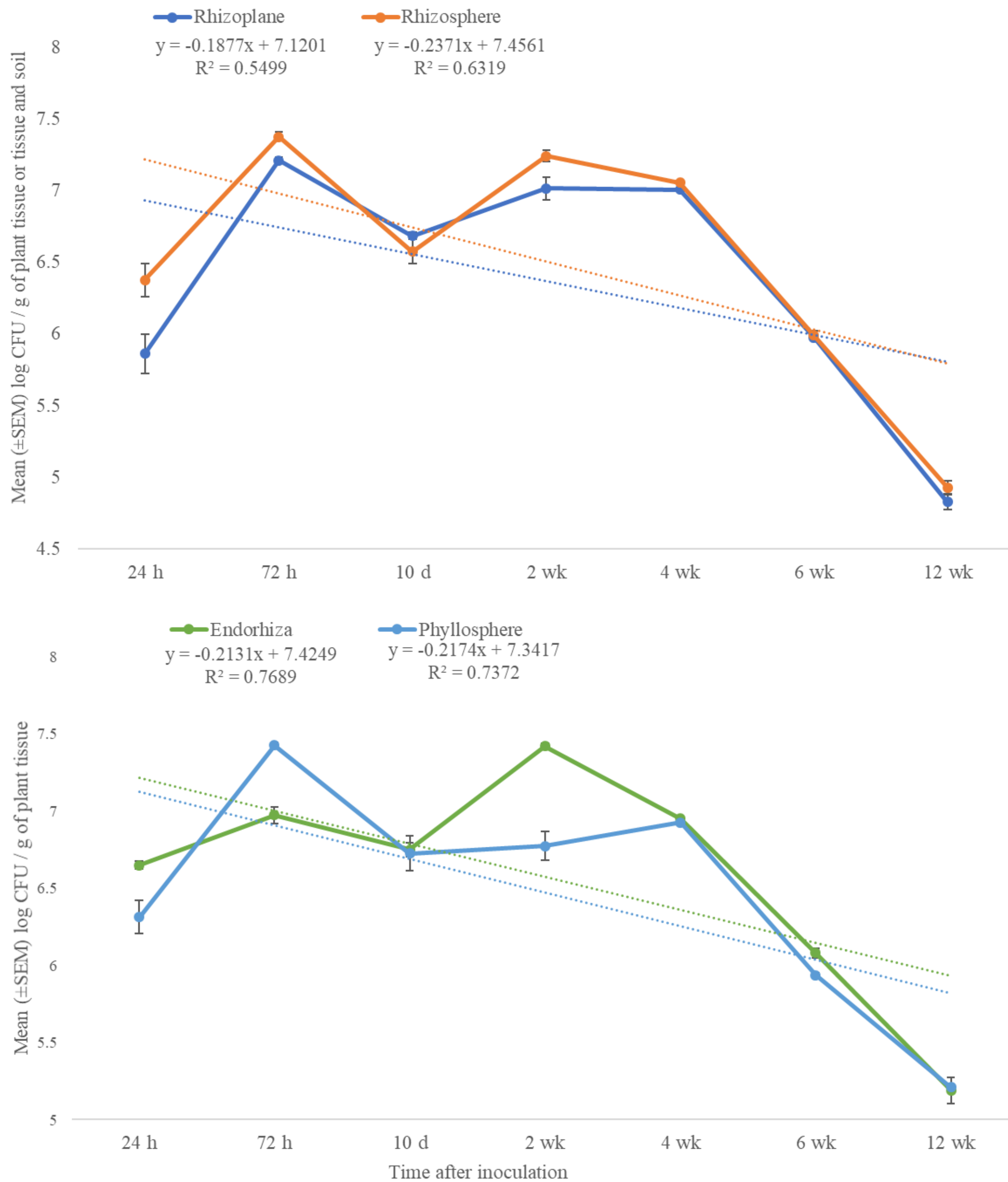
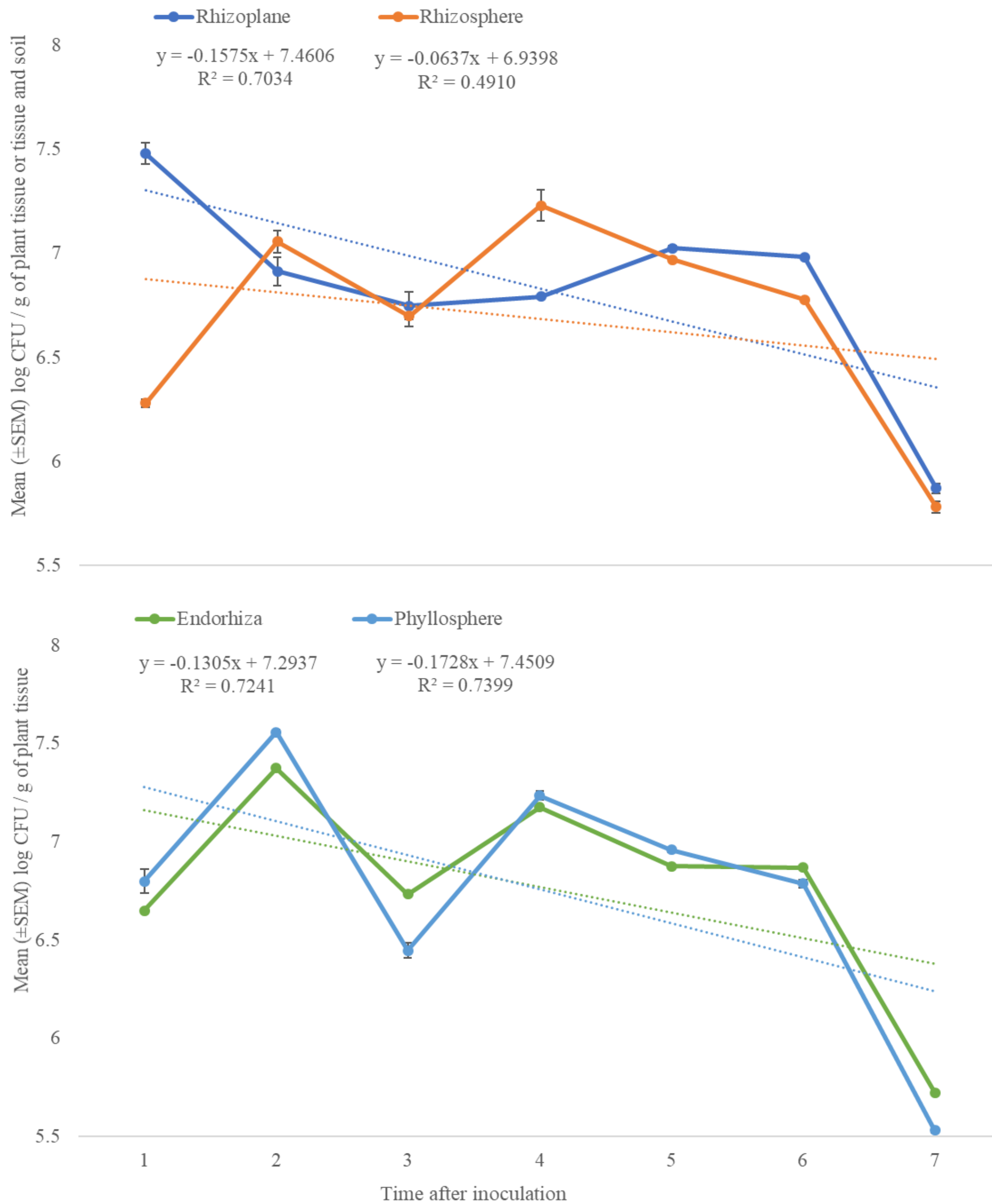


Figure 2.2. *Bacillus pumilus* AP 18 rifampicin mutant mean (± SEM) log populations CFU / g of tissue and linear regression in common bermudagrass (*Cynodon dactylon*) grown under field conditions in Marvyn loamy sand soil (pH 7.3). Populations were sampled from the rhizoplane, rhizosphere (top) and endophytically from roots and foliage (bottom).



CFU / g of tissue in common bermudagrass (*Cynodon dactylon*) grown under field conditions in Marvyn loamy sand soil (pH 7.3). Populations were sampled from the rhizoplane, rhizosphere, and endophytically from roots and foliage.

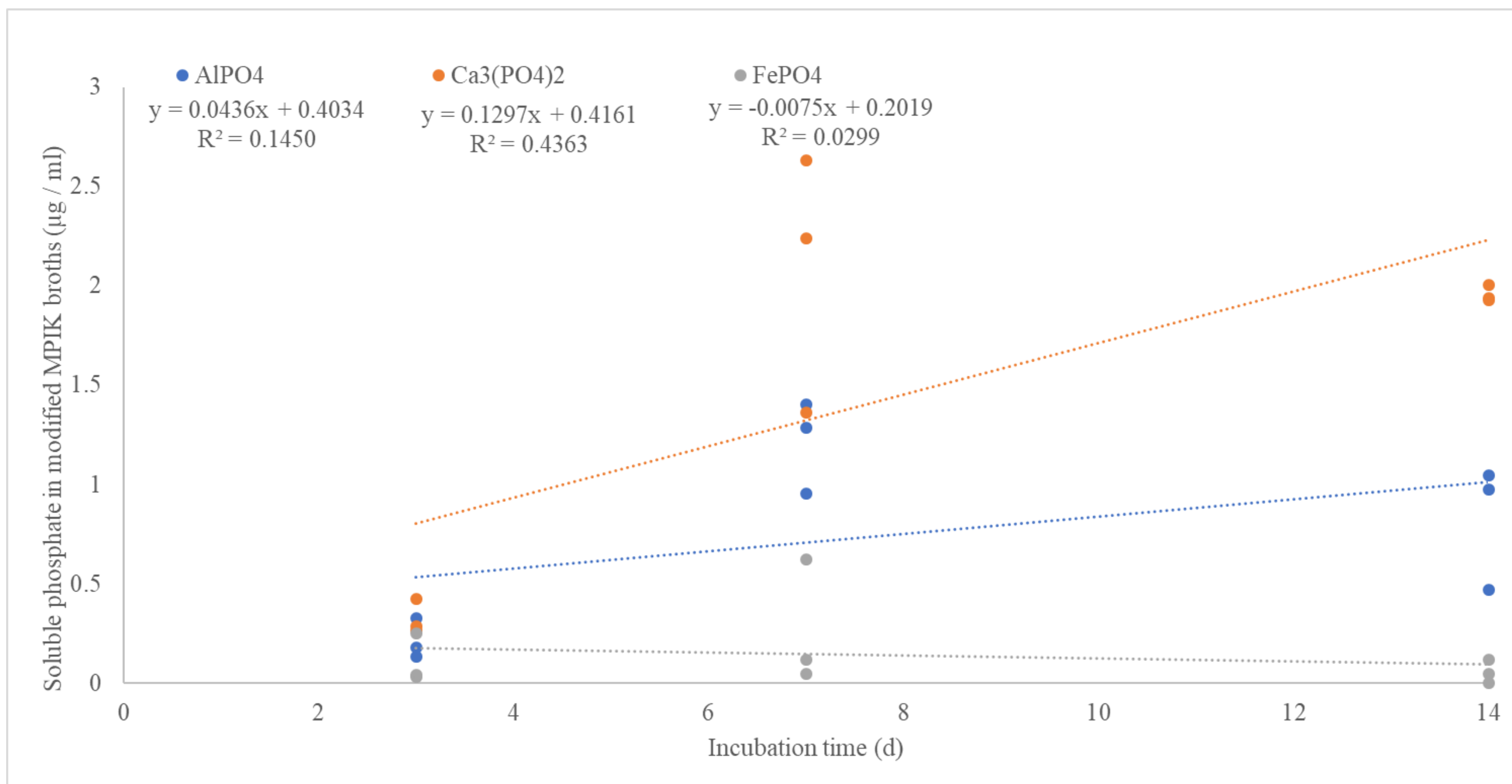


Figure 2.4 Quantitative phosphate solubilization and linear regression by *Bacillus pumilus* AP 7 in modified Pikovskaya's media liquid broths over 2 weeks.

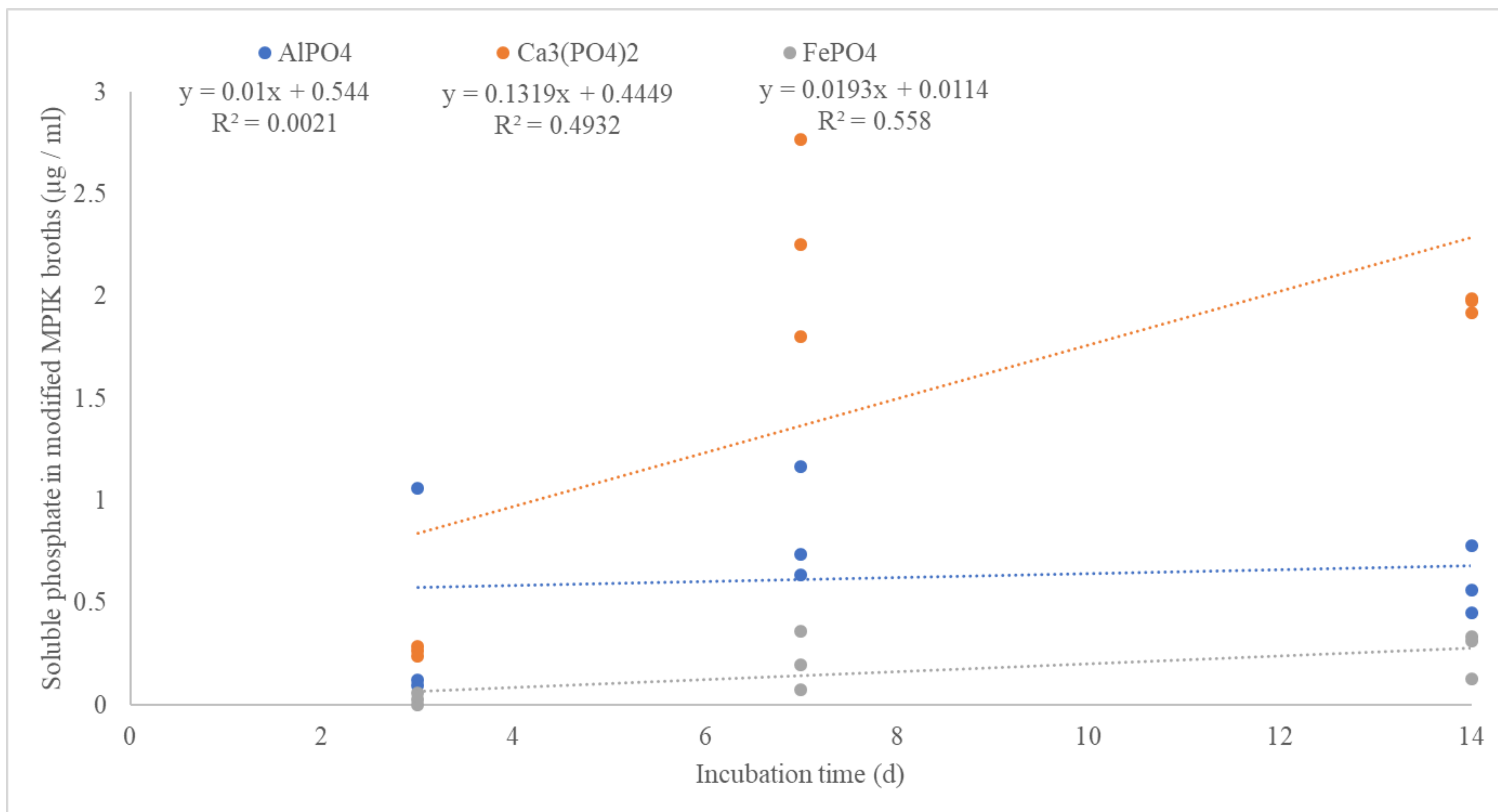


Figure 2.5 Quantitative phosphate solubilization and linear regression by *Bacillus pumilus* AP 18 in modified Pikovskaya's media liquid broths over 2 weeks.

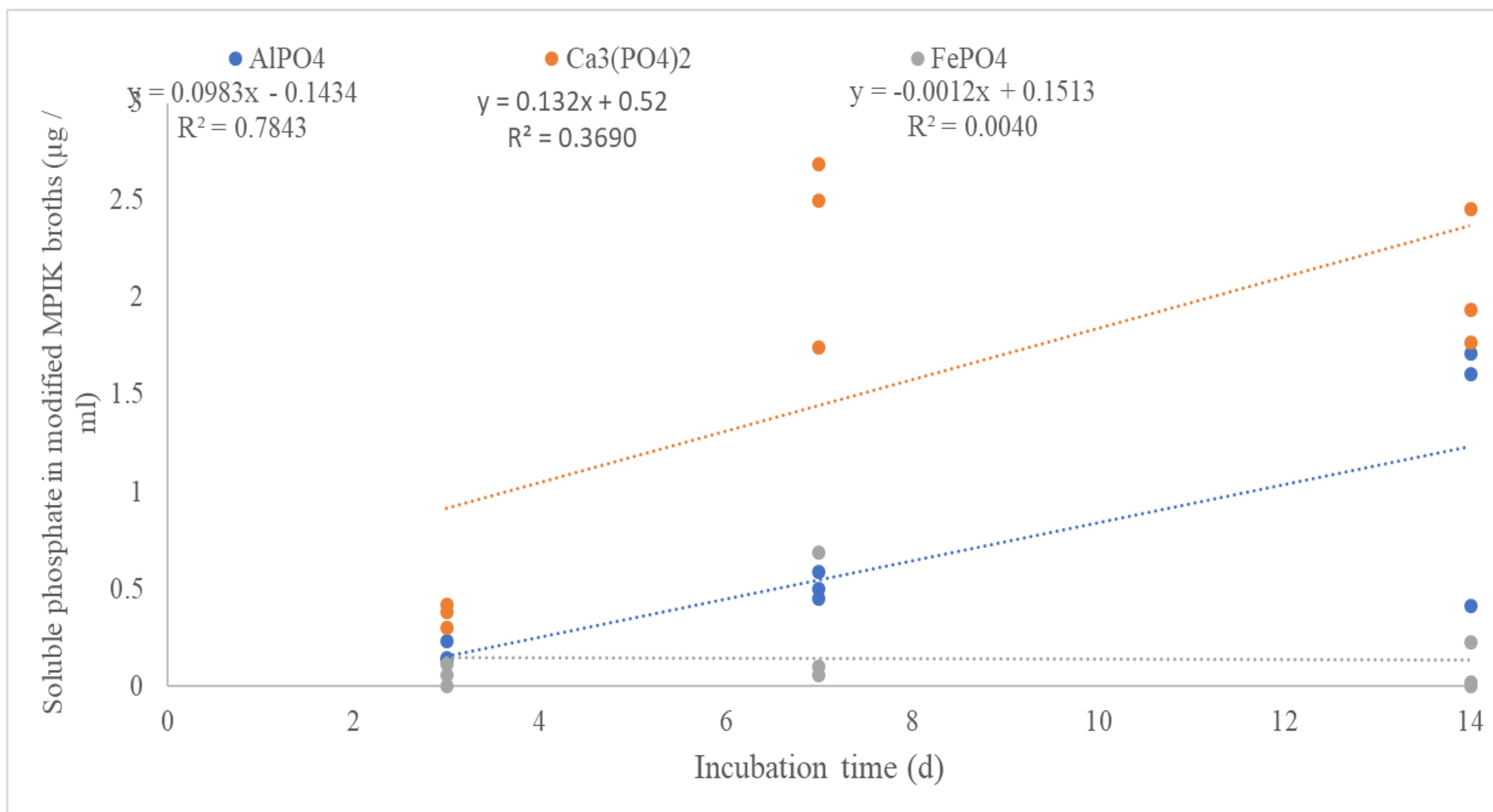


Figure 2.6 Quantitative phosphate solubilization and linear regression by *Bacillus sphaericus* AP 143 in modified Pikovskaya's media liquid broths over 2 weeks.

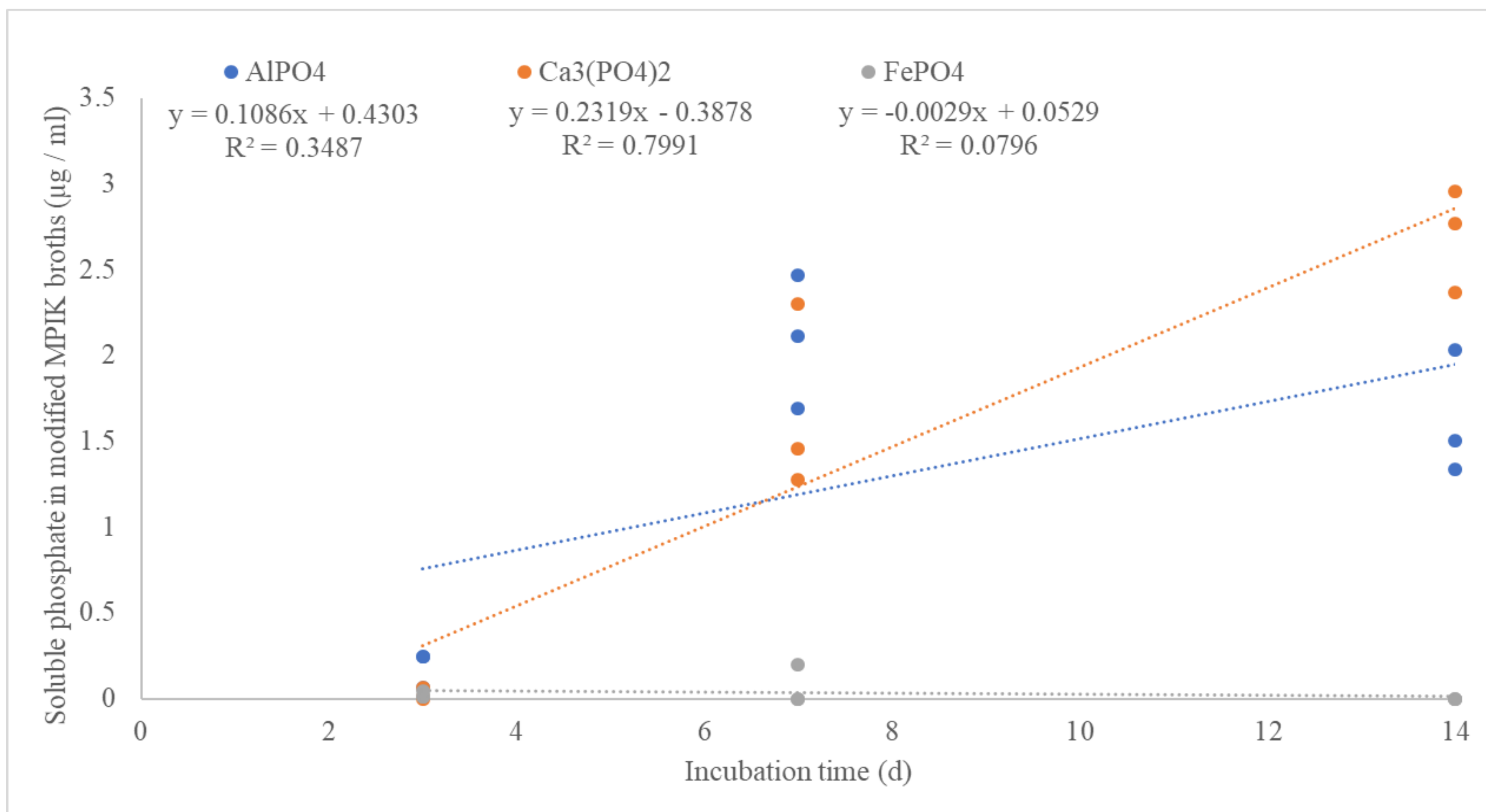


Figure 2.7 Quantitative phosphate solubilization and linear regression by *Bacillus sphaericus* AP 282 in modified Pikovskaya's media liquid broths over 2 weeks.

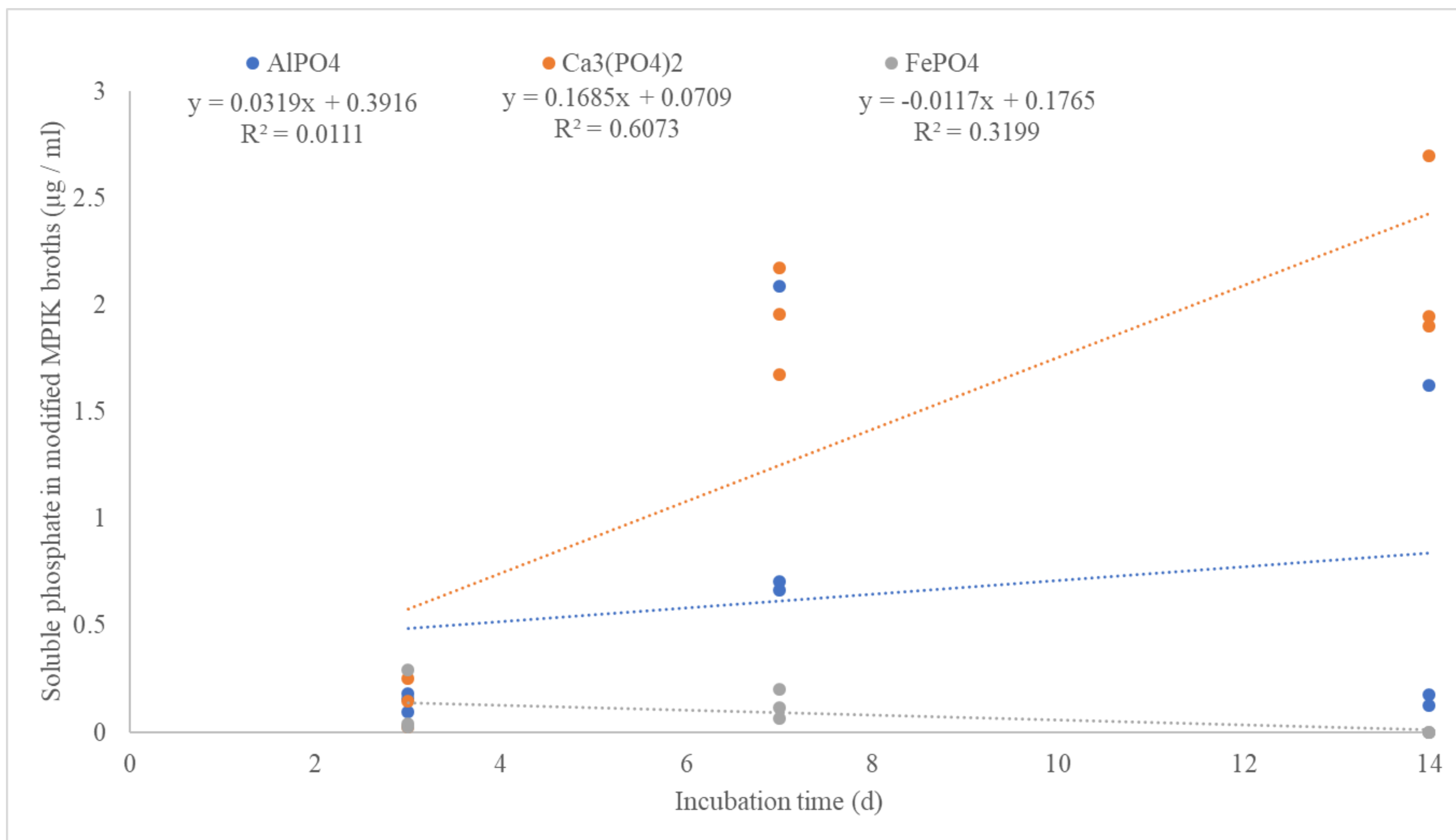
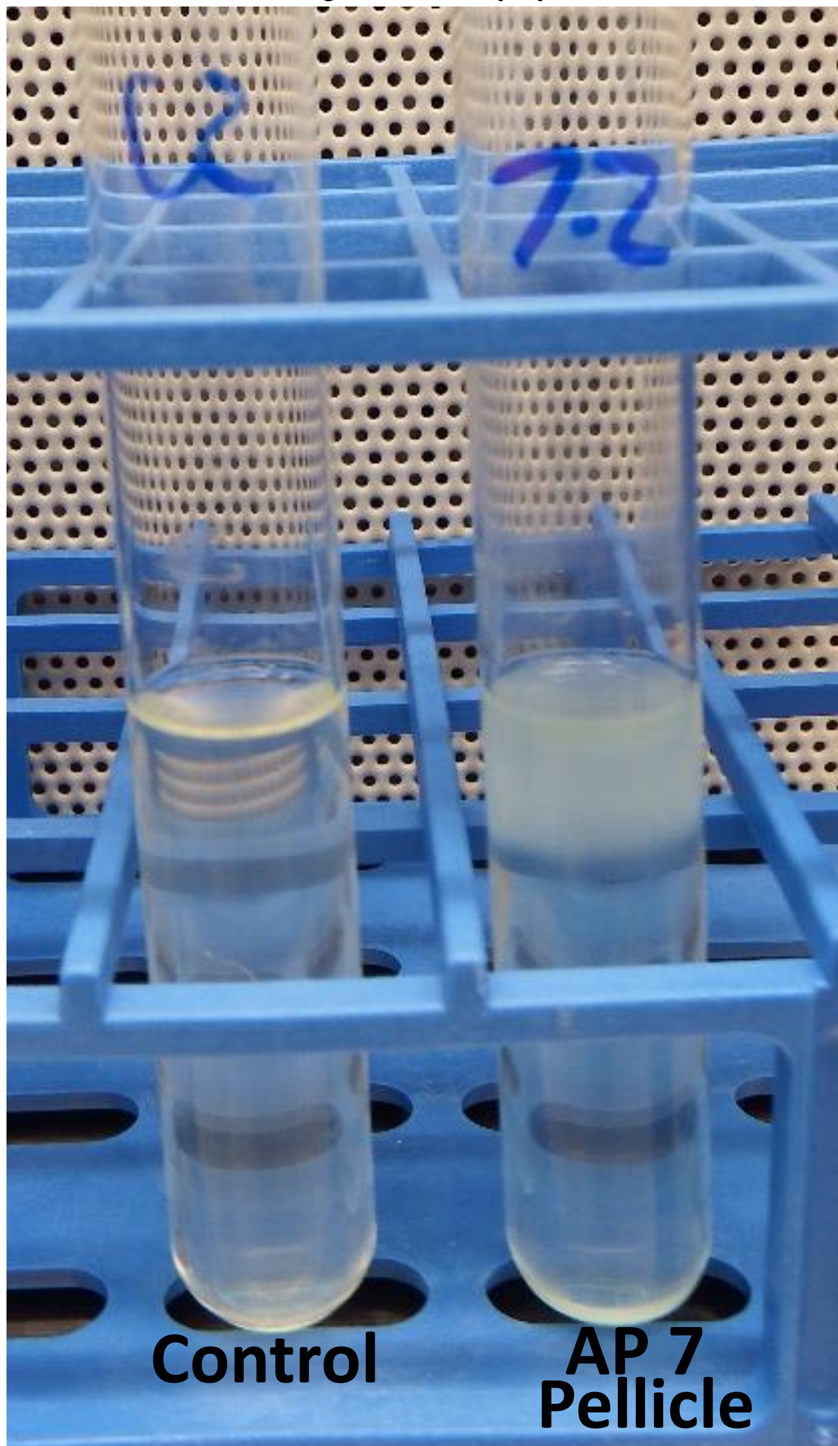


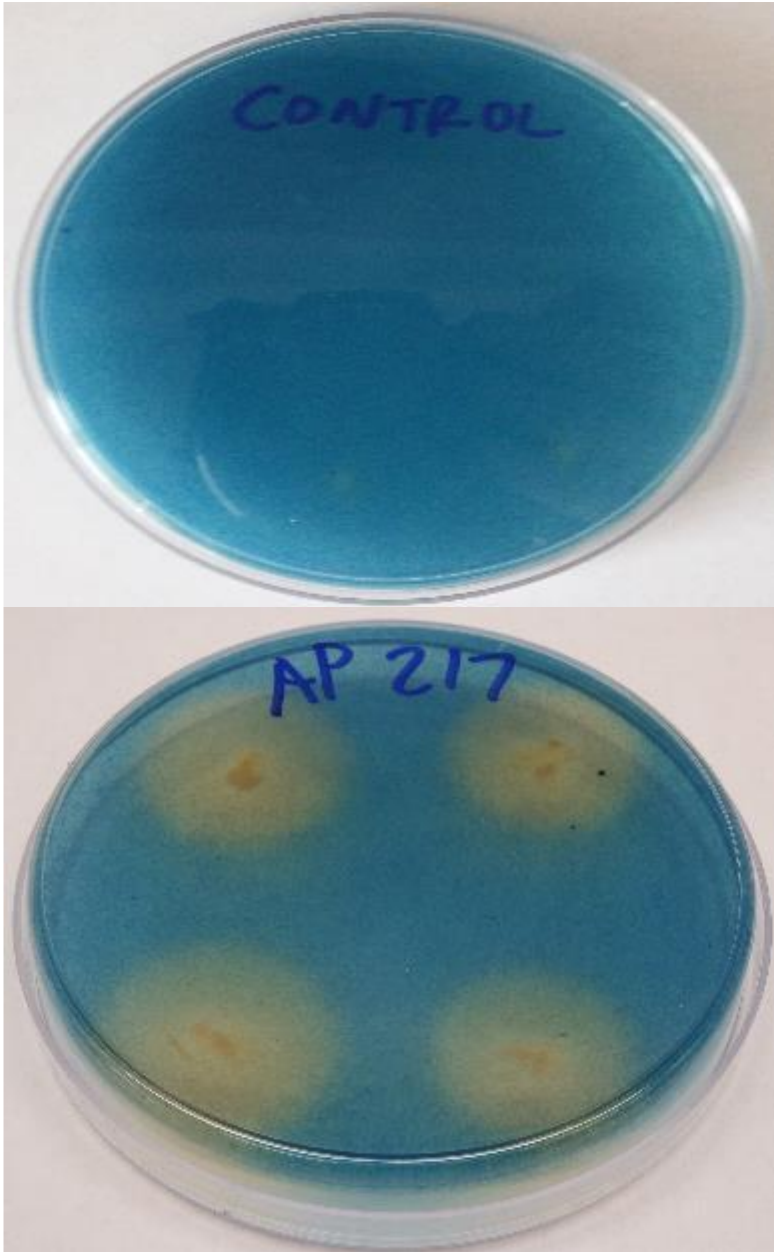
Figure 2.8 Quantitative phosphate solubilization and linear regression by *Bacillus pumilus* AP 283 in modified Pikovskaya's media liquid broths over 2 weeks.

Supplemental Materials

Supplemental Materials Figure 2.1: Determination of qualitative nitrogenase activity using semi-solid JNfB media. Formation of a pellicle after the introduction of a single bacterial colony into the media indicates nitrogenase activity by the strain.

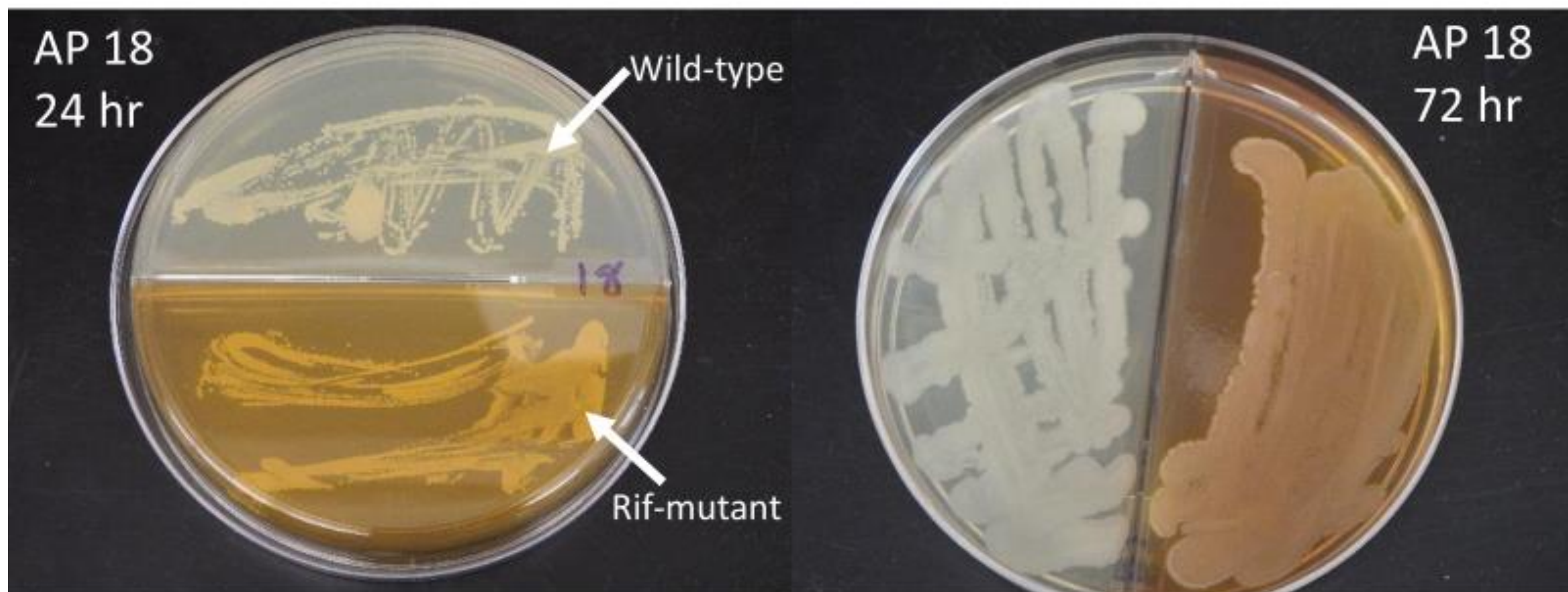


Supplemental Materials Figure 2.2: Qualitative determination of siderophore production using CAS media. Yellow-orange halos surrounding bacterial colonies indicates siderophore production by the strain.



Supplemental Materials Figure 2.3: Growth patterns of wild-type and rifampcin mutants on TSA and RTSA. It is important to select colonies with similar morphogies and growth patterns.

Wild-type and Rifampicin Mutants



Chapter 3: Rhizobacterial treatment of bermudagrass alters tolerance to damage from tawny mole crickets (*Neoscapteriscus vicinus* Scudder)

Abstract

Inoculation of bermudagrass with rhizobacterial biostimulants can increase plant growth and influence relationships with above-ground herbivores. Tunneling and root-feeding behaviors of tawny mole crickets cause severe damage to grass in pastures, golf courses, and lawns. Since bacterial inoculants enhance root growth, the goal of this study was to determine if inoculation of bermudagrass by PGPR can increase the tolerance of hybrid bermudagrass to tawny mole crickets, and if PGPR are compatible with current commonly used insecticides for mole cricket control. In large arenas, bacteria-treated grass infested with mole crickets produced more shoot and root mass and 128-200% greater root lengths compared to fertilized, infested, and non-infested bermudagrass. Field plots with mole cricket activity were established and treated with PGPR only, a PGPR-bifenthrin insecticide mixture, the insecticide alone, and compared to non-treated control plots. Plots were rated post-treatment for damage. Damage ratings after 3 and 8 weeks were lowest in plots treated with a bacteria-insecticide mixture, with controls having the highest damage. Lab experiments further confirmed that the PGPR used in the field study were compatible with neonicotinoid, phenylpyrazole, and pyrethroid insecticides when mixed in solution for up to 2 wk. Bacterial mediated interactions increase tolerance of bermudagrass applied before, or in response to, damage. Application of PGPR to field plots reduced tunneling relative to control plots and provided comparable reductions to a short residual, synthetic pyrethroid insecticide. Rhizobacterial products or products contained PGPR and certain insecticides may have utility for IPM of root herbivores.

1. Introduction

There are environmental concerns about the use of chemicals to sustain growth and productivity of plants facing challenges from abiotic and biotic stresses. These concerns are driving the adoption of management tactics that take a system's based approach, emphasizing conservation and environmental stewardship while incorporating new technologies. Biological technologies that are compatible with current management practices are avenues for development of innovative management strategies. Biologicals that enhance plant resistance or tolerance to abiotic and biotic stresses could minimize environmental consequences while reducing chemical and water input needs.[1] Recently, Myresiotis *et al.* (2) demonstrated the increased root growth and uptake of a neonicotinoid (thiamethoxam) insecticide in corn seedlings in response to treatment with the rhizobacteria *Bacillus subtilis*. Extension of these ideas into grasses could create opportunities for novel solutions to biotic or abiotic stresses especially in high input turfgrasses.

Turfgrasses in the United States cover 16.4 million hectares, an area larger than any other crop, encompassing diverse uses for residential, commercial, and recreational purposes.[3,4] Improvements in turfgrass cultivars for increased adaptability, aesthetic qualities, playability, as well as limited disease, and stress resistance have been the focus of traditional breeding programs. However, there are a limited number of successes related to grass-feeding insects and particularly root-feeding herbivores.[3] Bacterial mediated interactions with plants that maintain productivity despite pest pressure may yield results easier and faster than traditional and molecular breeding programs. Rhizobacterial inoculants are used for the maintenance of high

quality crops, including grasses (Poaceae) under normal and adverse conditions with limited resource input.[5-8]

Mole crickets (Orthoptera: Gryllotalpidae, *Neoscapteriscus* spp.) are solitary, subterranean insects that cause significant damage to turf, pasture, and other crops in sandy soils throughout the southeastern United States.[9,10] The fossorial forelegs of mole crickets aid in subterranean tunneling behavior, which results in direct damage to turfgrass from tunneling activities and root-feeding throughout the soil profile. The tunneling behavior can damage grass roots, displace soil, increase soil infiltration, and disrupt playing surfaces.[9, 11] Three invasive species from the genus *Neoscapteriscus* occur throughout the southeastern United States.[3] The southern mole cricket (*Neoscapteriscus borellii* Giglio-Tos), the tawny mole cricket (*N. vicinus* Scudder), and the short-winged mole cricket (*N. abbreviates* Scudder), which has only been reported in Florida and Georgia. Damage severity caused by mole crickets can be species dependent. Typically, most damage is observed in tawny mole cricket infested areas as this species is herbivorous and produces greater tunnel length than *N. borellii*, which is carnivorous.[9, 12] Cultivar evaluations have focused on the susceptibility of bermudagrasses (*Cynodon* spp.), bahiagrass (*Paspalum notatum* Flugge), St. Augustinegrass (*Stenotaphrum secundatum* (Walt) Kuntz, centipedegrass (*Eremochloa ophiuroides* (Munro) Hack, and zoysiagrass (*Zoysia* spp.) to both the tawny and southern mole crickets, but have not yet identified any cultivar highly resistant to damage.[13-15] The potential for severe damage and lack of alternatives increase the reliance on insecticides to reduce damage from mole crickets.

Primarily, interactions of PGPR with plants have focused on growth promotion and plant pathogens.[16, 17] with more recent work on the influences of PGPR-plant interactions on insect

folivores.[18-23] Thus far, virtually no research with PGPR has been conducted on soil-dwelling or root-feeding insects. This lack of research is probably due to the logistical challenges of direct observations of subterranean pests, however the plant-microbe-insect interactions may be more impactful for subterranean insects in close association and constant exposure to rhizobacteria. Using the tawny mole cricket-bermudagrass system, we determined if inoculation of bermudagrass by root-colonizing bacteria (PGPR) can increase plant tolerance to a below-ground insect herbivore and if PGPR are compatible with current commonly used insecticides for mole cricket control. Considering previous work, we expect that PGPR will be compatible with certain insecticides and alter the grasses response and increase tolerance to mole cricket tunneling and feeding behaviors.

2. Materials and Methods

2.1 Bacterial strains and inoculant preparation

Experiments were conducted with Blend 20, a PGPR blend consisting of three bacterial strains (*Bacillus pumilus* AP 7, *Bacillus pumilus* AP 18, and *Bacillus sphaericus* AP 282) that induces growth promotion in bermudagrass and deters fall armyworm (*Spodoptera frugiperda* J.E. Smith) oviposition.[6,18] Bacterial strains that were stored at -80 °C were transferred from cryovials to plates of tryptic soy agar (TSA) and allowed to grow at 28 °C in an incubator. After 24-48 h, bacterial lawns were scraped from TSA plates with inoculating loops and transferred to either new TSA plates or to sterile centrifuge tubes (50 ml, VWR, Radnor, PA) containing 40 ml of sterile water, and vigorously shaken to evenly distribute bacterial cells. Serial 10–fold

dilutions were then made of each bacterial suspension into sterile water blanks to a final dilution of 10^{-5}.

Bacterial populations (number of colony forming units [CFU]) in the suspensions were determined by plating 50 µl of the serial dilution onto TSA plates, incubating plates for 24-48 h and then counting the number of bacterial colonies on each plate. Once the concentrations (CFU per ml) in the prepared suspensions of each strain were determined, these populations were used to make bacterial stock solutions for each strain. Stock solution of the bacterial blend was prepared by the addition of one liter of equal parts of each bacterium to achieve a blend with a final concentration of 1 x 10^7 CFU per ml of each strain.

2.2 Bacterial strains and insecticide compatibility

The strains of Blend 20, each with a minimum concentration of 1 x 10^8 CFU per ml were individually evaluated for their compatibility with commonly used liquid insecticides. The strains within the blend were evaluated individually to make recovery of each bacterium apparent, as the colony morphologies are similar. Freshly prepared bacteria stock solutions were evaluated for their ability to survive being 'mixed' with three different insecticides mixed in separate 50 ml centrifuge tubes for 1 and 24 h, and 1 wk, and 2 wk at 25 °C under ambient light. The bacteria were evaluated for compatibility with insecticides in three chemical groups: neonicotinoids, phenylpyrazoles, and pyrethroids. The pesticides evaluated were bifenthrin (Talstar Pro, 7.9% active ingredient, FMC Corporation, Philadelphia, PA), fipronil (Termidor SC, 9.1% active ingredient, BASF Corporation, Florham Park, NJ), imidacloprid (Merit 2F, 21.4% active ingredient, Bayer Environmental Sciences, Research Triangle Park, NC). While Termidor SC is not labeled for turfgrass, it was selected for convenience because the active

ingredient (fipronil) is formulated as a granular product and widely used for mole cricket management.[24] Bacteria and pesticide solutions were prepared based on the label recommendations for volume of area covered (bifenthrin, imidacloprid) or amount of active ingredient per volume needed (fipronil). Bifenthrin and fipronil were evaluated at high and low label rates. Bifenthrin was mixed at a rate of 29.6 and 14.8 ml of product per 92.9 m^2 (7.9% active ingredient, 302 g of active ingredient per 3,785 ml). Imidacloprid was mixed a rate of 17 ml of product per 92.9 m^2 (907 g of active ingredient per 3,785 ml). Fipronil was prepared at 47.3 (0.125% dilution) and 23.65 ml of product (0.06% dilution) per 3,785 ml (363 g of active ingredient per 3,785 ml). After the allotted time, the centrifuge tubes were vigorously shaken to evenly distribute bacterial cells before serial 10–fold dilutions. Serial dilutions were made of each bacterial suspension into sterile water blanks to a final dilution of 10^{-5}. Bacterial populations (number of CFU) in the suspensions were determined by plating 50 µl of the 10^{-5} serial dilutions onto three TSA plates, incubating plates for 24 h and then counting the number of CFU on each plate.

2.3 Sources of insects and preparation

Tawny mole crickets were locally obtained using soapy water flushes over infested areas on golf course greens and tee boxes.[25] After emerging from the soap flush, mole crickets were rinsed free of soap in the field and placed in deep Petri dishes (100 mm x 25 mm, VWR, Radnor, PA) filled with moist sand. In the lab, mole crickets were provided freeze-dried mealworms (Coleoptera: Tenebrionidae; Fluker Farms, Port Allen, Louisiana) and organic carrots as food sources. Soil moisture and food sources were replaced as needed. Until they were needed, mole crickets were maintained in a growth chamber at 26.7° C.

2.4 Arena experiment

Two trials were conducted outdoors in the summer (Trial 1) and fall (Trial 2) of 2016. Treatments included one PGPR blend (Blend 20) with mole crickets, a fertilized control with mole crickets, a non-treated control with mole crickets, and a non-treated control without mole crickets. Each treatment was replicated four times per trial in a randomized complete block design using large PVC arenas similar to Bailey *et al.* (9). For these trials, plugs of Tifway hybrid bermudagrass (3.8 cm diameter) were harvested from the Auburn University Turfgrass Research Unit, Auburn, AL. After harvesting, plugs were washed free of field soil and transplanted into clean, fine sand. In the first trial, ten hybrid bermudagrass plugs were planted and established in arenas (PVC cylinders, 25.4 cm diameter x 45.2 cm high). Arenas were held above ground outdoors on a landscape fabric mat under overhead irrigation for the duration of the experiment. After transplanting in Trial 1, grasses received 1.45 g of product / m^2 granular ammonium sulfate fertilizer (PRO fertilizer, 21-0-0; Harrell's Inc., Lakewood, FL) weekly followed by 675 ml (1.27 cm) of water after fertilizer was applied. All arenas were fertilized and plants cut weekly to a height of 3.7 cm for 4 wk until treatments were applied. For Trial 2, individual grass plugs were grown in a plastic pot (7.6 cm diameter x 20.3 cm high; MT38 Mini-Treepot, Stuewe and Sons, Tangent, OR) for 4 wk before 10 plugs were transplanted into each PVC cylinder. While growing in the plastic pots, grasses received 1.45 g of product / m^2 granular ammonium sulfate fertilizer weekly followed by 75 ml (1.27 cm) of water after fertilizer was applied. During this time, grasses were cut weekly to a height of 3.7 cm. Treatments were applied after transplanting in Trial 2. Except when applications were made, grasses were watered as needed.

Arenas were randomly assigned to each treatment and the following treatment methods were used in both trials. Those assigned to the bacteria treatment received weekly inoculations of 26.5 ml (500 ml / m^2) of a freshly-prepared aqueous bacterial suspension of 1 x 10^7 CFU per ml applied to the growing media of each pot followed by 675 ml of water for 6 wk. The same volume of distilled water was applied to the control plants each time bacteria were applied. Pots assigned to the fertilizer treatments received 1.45 g of product / m^2 granular ammonium sulfate fertilizer weekly and 675 ml water after fertilizer was applied. After two applications of each treatment, tawny mole crickets were placed into each of the infested treatments (PGPR, fertilizer, and control). Mole crickets were placed on the surface and allowed to burrow into the soil. Each of these arenas was infested with 6 mole crickets in Trial 1 and five mole crickets in Trial 2. The non-infested controls were free of insects to determine grass productivity in the absence of herbivory.

Weekly top growth beyond 3.7 cm was cut, collected, and weighed. Fresh weights of grass clippings were recorded before samples were oven dried at 70º C for 72 h and weighed again for dry weight.[6] A week after the sixth application of each treatment, the arenas were destructively sampled and the mole crickets were collected, counted, and weighed. The root system of each arena was collected and washed in the lab. After washing, root fresh weights were recorded before digital image analysis of the linear root structure was conducted using a root scanning system (Regent Instruments, Inc. Sainte-Foy, Quebec) which consisted of a scanner (LA 1600+) and WinRhizo software (2004a). Based on image analysis, the software calculated total root length. After scanning the root systems, the roots were dried in an oven at 70 ºC for 72 h. The data collected were used to compare root growth and top growth in arenas

treated with Blend 20 and infested, fertilized and infested, non-treated and infested, and non-treated and non-infested plants.

2.5 Field Experiment

On 26 March 2017, field plots were established at the Auburn University Turfgrass Research Unit, Auburn, AL over a mixed stand of hybrid and common bermudagrass infested with tawny mole crickets. The site is on a Marvyn loamy sand, and plots were 3 m x 2 m with at least 2 m separating plots. Field plots were assessed for mole cricket damage based on the rating system of Cobb and Mack.[26] A 1 m x 1 m frame divided into a grid with 9 subsections was used to score the plots on a scale of 0-9, where 0 indicates no damage and 9 indicates activity of surface mounding or tunneling in each section. After the initial assessment, plots were assigned to a treatment group based on damage ratings. There were 6 replicates per treatment. Damage ratings were completed nine times on Day 0, 14, 21, 28, 33, 42, 46, 52, and 56. Damage assessments were taken from seven locations (center, top left, top right, bottom left, bottom right, middle left, and middle right), with one location sampled during each damage assessment period for all treatments. The experiment evaluated four treatments, PGPR-treated (Blend 20), bifenthrin-treated, PGPR + bifenthrin-treated, and non-treated control. On 27 March 2017 (Day 0) and 23 April (Day 27) plots were treated with a backpack sprayer. Bacteria-treated plots received 3 L (500 ml / m^2) freshly-prepared aqueous bacterial suspension of 1 x 10^7 CFU per ml. Bifenthrin-treated plots were treated at a rate of 29.6 ml of product per 92.9 m^2. The sprayer applied 244 ml of this mixture per plot. PGPR+ bifenthrin plots were treated with the 3 L of bacteria and 244 ml of bifenthrin mixed together in the same tank. The non-treated plots were treated with 3 L of distilled water coincident with treatment of the other plots. After treating, the

plots were hand watered with 76.2 L of water (1.27 cm) to move the treatments into the root zone.

2.6 Statistical Analysis

Top growth (fresh and dry mass) in the arena experiments trials were analyzed separately using repeated measures of multivariate analysis of covariance (MANCOVA) due to trial being a significant factor ($P < 0.05$, JMP Version 13. SAS Institute Inc., Cary, NC). Root fresh and dry mass, and length were analyzed using orthogonal contrasts ($P < 0.05$). The number of mole crickets recovered and mole cricket weights from each treatment were used as covariants. Mole cricket weights were analyzed using analysis of variance (ANOVA), Student's *t*-test. The number of mole crickets recovered and mole cricket weights within treatments were not significant in either arena experiment. For the field trial, mole cricket damage ratings were compared using LSMeans, Student's *t*-Test (LSD, $P < 0.05$) to determine recovery from existing activity by treatment.

3. Results

3.1 Bacteria and Insecticide Compatibility

In this trial, each strain that comprises Blend 20 (*B. pumilus* AP 7, *B. pumilus* AP 18, and *B. sphaericus* AP 282) was mixed with insecticides commonly used to control mole crickets. Strains were not negatively impacted, and remained stable when mixed with a neonicotinoid (Imidacloprid), phenylpyrazole (Fipronil), and pyrethroid (Bifenthrin) insecticide for 2 wk (Figures 3.1-3.3). Slight variations in populations were noted, but this likely resulted from bacterial distribution in the centrifuge tubes, serial dilutions, and growth times of bacterial

colonies on growth media. All populations remained within the standard errors of the initial populations.

3.2 Arena Experiment

These experiments evaluated if PGPR application before mole cricket infestation and repeated applications after infestation would increase the tolerance of bermudagrass to tawny mole crickets. Recovery of live mole crickets in the first trial was 55.5% and 60% in the second trial, with no replicates having less than 40% recovery. Final weights of mole crickets increased relative to initial weight in both trials. In Trial 1, final weights ranged from 550-596 mg and from 852-873 in Trial 2. There was no difference between treatments in mole cricket weights in either Trial (Trial 1, F = 0.35, df = 2, 39, $P = 0.709$; Trial 2, F= 0.05 df = 2, 35, $P = 0.947$).

Plant growth response parameters in Trial 2 were greater than in Trial 1, resulting in a significant trial effect (F= 10.48, df = 1, 26, $P = 0.003$). In Trial 1, fresh and dry mass of top growth were not significant across treatments (fresh mass, F= 0.52, df = 3, 11, $P = 0.675$; dry mass, F = 0.48, df = 3, 11, $P = 0.703$). In Trial 2, the infested control arenas produced the lowest amount of top growth (Figure 3.4). The arenas treated with Blend 20 or fertilizer, and the non-infested control arenas had significantly greater top growth than the infested controls (F= 13.90, df = 3, 11, $P < 0.001$). Bermudagrass treated with Blend 20 or nitrogen-fertilized produced similar amounts of top growth (fresh mass, F = 0.18, df = 3, 11, $P = 0.68$; dry mass, F= 0.07, df = 3, 11, $P = 0.8$). Both Blend 20 and nitrogen treatments produced significantly more fresh weight of top growth than the non-infested controls (PGPR fresh mass, F = 15.62, $P = 0.002$; Nitrogen fresh mass, F = 16.32, $P = 0.002$), but only PGPR produced significantly more dry mass than the infested control (dry mass, F = 15.3, $P = 0.002$).

Root length, fresh and dry mass were analyzed separately by trial (Table 3.2). Infesting bermudagrass with mole crickets reduced total root length on average by 203 cm ($P = 0.053$, df = 1, orthogonal contrast) and 101 cm ($P = 0.643$, df = 1) for Trials 1 and 2, respectively. PGPR-treated bermudagrass produced the greatest root fresh mass and length in both trials and the greatest dry mass in Trial 2 (Tables 3.1-3.2). In Trial 1, PGPR-treated bermudagrass infested with tawny mole crickets produced nearly 300 cm more in total root length than bermudagrass held under similar conditions without mole crickets (orthogonal contrast, $P \leq 0.078$, df = 1). Relative to treatments infested with mole crickets, PGPR-treated bermudagrass produced >500 cm of total root length more than bermudagrass treated with either nitrogen or non-treated controls (Trial 1, orthogonal contrast, $P = 0.001$, df = 1). Because growth was greater in Trial 2, the magnitude of the treatment differences was greater. Bermudagrass treated with PGPR and infested with tawny mole crickets produced >180% more in total root length relative to bermudagrass held under similar conditions with or without mole crickets (orthogonal contrast, $P < 0.001$, df = 1). PGPR-treated bermudagrass infested with mole crickets also produced more total root length than bermudagrass treated with nitrogen (Trial 2, orthogonal contrast, $P = 0.002$, df = 1).

Root dry mass was not significantly different for any treatment comparison in Trial 1 (Table 3.1, means; Table 3.2, orthogonal contrast, $P > 0.05$). In Trial 2, root dry masses were significantly greater for bermudagrass infested with mole crickets treated with either nitrogen or PGPR (Table 3.2, $P \leq 0.015$) compared to bermudagrass infested with no treatment. PGPR and nitrogen produced roots with similar dry masses in Trials 1 and 2. For root fresh mass, PGPR-treatment of bermudagrass was not different from any other treatment at $P < 0.05$ in Trial 1.

However, at $P < 0.1$, PGPR-treated fresh masses were greater than all treatments. Differences in root fresh mass were observed in Trial 2, with PGPR-treated grass having the greatest root mass of all treatments. Fresh root mass of bermudagrass treated with either PGPR or nitrogen and infested with mole crickets, and non-infested bermudagrasses were similar, and all were significantly greater than the infested control ($P \leq 0.043$).

3.3 Field Experiment

Tunneling activity of mole crickets is dependent on soil moisture.[27] During the study, rain events occurred on 17 days during the study resulting in 23.69 cm of total precipitation. Significant rain events occurred on April 28 (4.52 cm, Day 32), May 1 (3.43 cm, Day 35), May 5 (1.32 cm, Day 39), May 13 (1.19 cm, Day 47), and May 21 (1.68 cm, Day 55). The average soil temperature at a 10.2 cm depth was 17.99 °C (ranged from 14.44-21.67 °C). Soil moisture appeared adequate to maintain damage ratings ≥ 7 (out of 9) in non-treated control plots through 21 d. Following the second application on day 27, damage ratings in control plot were generally lower.

All field plots had similar damage ratings at the beginning of the experiment (Days 0 and 14; $P > 0.05$; Table 3.3, Figure 3.5) and non-treated plots had the highest damage ratings throughout the study. The damage ratings of PGPR-only and bifenthrin-only treated plots were never significantly different from one another ($P \geq 0.13$, df = 3). Beginning at 21 DAT, one or more treatments had significantly lower damage ratings than control plots ($P < 0.05$; Table 3.3, Figure 3.5). Among treated plots, the combined PGPR and bifenthrin treatment had the lowest damage ratings except for the first sample following re-application (28 DAT). Damage to plots treated with PGPR mixed with bifenthrin were never significantly different from plots where

only PGPR was applied. Plots treated with PGPR mixed with bifenthrin had significantly less damage then bifenthrin-only treated plots at 21 and 56 DAT ($P \leq 0.03$, df = 3). These two treatments had similar damage ratings on all other sample dates.

4. Discussion

The effects of PGPR inoculation of grasses have focused mainly on growth promotion,[6,28] nematode suppression or mitigation,[29] and other work from our lab with insect folivores[18]. In bermudagrass, Coy *et al.* (18) noted certain PGPR strains and blends may selectively promote root growth and those may be better suited to evaluate changes in plant tolerances to root-feeders. The purpose of this study was to determine if inoculation of bermudagrass by root-colonizing bacteria (PGPR) can increase plant tolerance to a below-ground insect herbivore and if PGPR are compatible with current commonly used insecticides for mole cricket control. We used the tawny mole cricket as the model insect herbivore because it is a significant pest of turf and pasture grasses. We hypothesized that PGPR inoculation of bermudagrass would increase the plants tolerance to mole cricket activity and would remain stable when tank mixed with certain insecticides. Based on our literature review, this study represents the first to investigate PGPR inoculation and show positive effects on plants infested with a below-ground insect herbivore.

Foliage growth of bermudagrass treated with either PGPR or nitrogen was sustained or enhanced even when infested with mole crickets. Production of foliage or top growth in grasses is one metric used to determine relative performance of grasses subject to insect feeding injury.[13] Grass productivity (root and top growth) was lower in general in Trial 1 of the arena experiment,

so differences in mass of top growth produced were only evident in Trial 2 (Figure 3.4). These trial differences could be attributed to time of year or grass establishment methods, but the trends of increased root and top growth with PGPR were consistent with previous work using Blend 20.[6] In Trial 2 of the arena experiment, grasses treated with nitrogen or PGPR and infested with mole crickets continued to produce more top growth than either infested or non-infested grass. When applied before mole crickets are present, growth promotion with fertilizer or PGPR appear to mitigate the negative effects on top growth over 4 wk when bermudagrass is infested with mole crickets. Nitrogen and PGPR application produced similar top growth in the presence of mole crickets in Trial 2, supporting our hypothesis that growth promotion produces tolerance to mole crickets in bermudagrass. Blend 20 can increase root and shoot mass in grass but performance is not always comparable to synthetic fertilizers.[30] Fike (30) compared seasonal production of foliar biomass of Coastal bermudagrass, a pasture type grass, treated with either Blend 20 or a full (56 kg/ha) or half rate (28 kg/ha) of ammonium sulfate. In that study, both rates of synthetic fertilizer outperformed Blend 20. While foliar biomass is desired in certain situations (e.g., pastures), the top growth produced by Blend 20 was still comparable to nitrogen despite the stress imposed by feeding and tunneling of mole crickets. Furthermore, excessive foliar growth may not desirable in low-cut grass situations, like golf courses or lawns.

Root data from the arena experiment also supported our hypothesis that PGPR can create tolerance to root feeding insects. Nitrogen-fertilized and PGPR-treated grasses infested with mole crickets produced similar root mass yet significantly greater root mass than infested, non-treated grass (Tables 3.1-3.2). However, only grasses treated with Blend 20 and subjected to mole crickets produced greater root fresh mass than non-infested grass. Treatment differences

were more obvious with total root length. PGPR-treatment of bermudagrass produced total root lengths 501-870 cm greater than nitrogen fertilized grass. Bermudagrass inoculated with PGPR produced 200-1475 cm more of total root length than non-infested, and 543-1576 cm more than non-treated bermudagrass infested with mole crickets. The PGPR in Blend 20 is reported to increase and change root architecture in hybrid bermudagrass[6], but this work was in the absence of insect herbivory. In their review of microbe-plant-insect interactions, Pineda *et al.* (31) suggested that the effects of microbes on plants may be strengthened under biotic or abiotic stress. The mechanisms are not well understood but microbes are likely acting in ways to stimulate biosynthetic pathways related to stress.[32] This may explain the greater total root length produced in bermudagrass inoculated with PGPR and subjected to tawny mole crickets compared to grass that was infested and treated with nitrogen.

The use of bacterial biostimulants may result in increases root biomass and length when applied before pest problems exist, but the field experiment evaluated PGPR applied to an active infestation. Mole crickets (*Neoscapteriscus* spp.) typically alter turfgrass playability and aesthetic qualities from surface tunneling activities, root-feeding, and soil displacement.[3,9,27] Mitigation of surface tunneling and root disruption are the primary goals of mole cricket management. The data from the field experiment showed that application of PGPR alone can lead to reductions in tunneling relative to control plots and comparable reductions to an application of a short residual, synthetic pyrethroid insecticide (Table 3.3, Figure 3.5). Furthermore, we hypothesized that a mixture of PGPR and the same insecticide would reduce tunneling and hasten the recovery of the infested grass. In two samples (21 and 56 DAT), plots treated with PGPR plus bifenthrin had significantly lower damage ratings than bifenthrin alone.

These two sample dates were about 3-4 wk after the treatment application to the plots, but no differences between treatments were observed within the first 2 wk following application. There are a few possible explanations for this. First, all plots were under heavy pressure for the first 3 wk of the experiment which likely reduced root mass (not measured). Where roots were limited, PGPR may not have been able to rapidly colonize under these conditions.[17] Secondly, PGPR may require an inoculation strategy for application.

In this study and past work, we have used an inoculation strategy to introduce PGPR to bermudagrass. This strategy relies on multiple applications of population densities (1x 10^7 CFU per ml) to build up soil populations of the desired bacterial strains. Bacterial colonization of plants is speculated to occur between 10^{6-8} bacterial cells per cm^2 of plant tissue.[33] PGPR inoculation into field conditions may create the potential for competition for sites along roots with existing soil bacteria.[17] Under field conditions, the results of this study suggest that turfgrass infested with mole crickets may require more frequent or 'booster' applications of PGPR to establish new or augment populations.

Ongoing research in our lab is investigating colonization and application frequency of PGPR applications to turf and pasture grasses. In the field experiment, two applications of bacteria, insecticide, or a bacteria-insecticide combination were separated by 4 wk intervals, but a shorter time frame (2 wk) may be better suited for more rapid growth responses and recovery from mole cricket damage. It is unlikely that PGPR-alone would provide comparable control and responses to a long residual soil insecticide like fipronil (phenylpyrazole), but PGPR may be used in conjunction with insecticides to positively affect plant health or uptake of systemic insecticides.[2]

The populations of *Bacillus* spp. in Blend 20 are compatible and stable when mixed with liquid formulations of neonicotinoid, phenylpyrazole, or pyrethroid insecticides commonly used for control of mole crickets (Figures 3.1-3.3). This is not surprising, given previous bacterial work with *Bacillus subtilus* has confirmed endophytic colonization by the bacterium in corn and cotton, and shown to increase plant growth and uptake of thiamethoxam in corn.[2,34] Additionally, bacteria have positive relationship with fertilizers and may increase efficiency of fertilizer use, which may allow for lower use rates of many agrochemicals.[35] Although PGPR appear to not be impacted by the insecticide formulations that were evaluated in this study, PGPR are not likely compatible with all pesticides, extracts, or formulations. Populations of *Pseudomonas fluorescens*, for example, remain stable when mixed with the insecticides avermectin, carbofuran, chlorpyrifos, and endosulfan, but not with indoxacarb.[36-38] Similarly, *P. fluorescens* mixed with the fungicides carbendazim and thiram, or certain plant extracts like, neem seed kernel extract (NSKE)[36] were not negatively impacted, but were negatively impacted by cotton seed treatments with imidacloprid[37] or when mixed with the fungicides mancozeb, captan, propiconazole.[36,39] The population stability of PGPR, measured by survival or shelf life, when mixed with pesticides will likely also be influenced by PGPR strain, application method, and formulation.[40,41] More research will be needed with different bacterial genera, strains, formulations and pesticides to determine compatibility.

The demands for near perfect aesthetics and playability on golf courses and the lack of reliable cultural and biological controls or host plant resistance for mole crickets drive reliance on insecticides for management.[3,13,42] Highly maintained areas may receive up to three chemical applications per year for mole crickets. Insecticide applications are made in response to early

spring (February-April) or fall (September-October) activity of adults or during egg hatch and early nymphal stages (May-July).[24] Frequent pesticide applications on a univoltine insect can place substantial selection pressures on populations that may lead to reduced chemical efficacy and resistance. Application of PGPR as a biostimulant to increase the tolerance of bermudagrass to mole crickets could reduce selection pressure on these univoltine pests from repeated exposure to the same or similar mode of action. PGPR applied frequently (monthly or weekly) as a biostimulant may enhance the vigor of bermudagrass such that insecticide applications for mole crickets may be reduced or unnecessary. Previous work in our lab suggests that these applications may also positively influence IPM by inducing changes in oviposition behaviors or fall armyworms,[18] or tolerance to other root-feeding insects like white grubs (Coy RM, *unpublished data*). Our data further suggest that PGPR, if used as a biostimulant in managed turfgrass, would not likely be adversely affected by insecticides present in the soil or applied as needed. Now, there are fewer PGPR products available for use in pasture or amenity grasses than for food or fiber crops. The global market for biostimulants, including PGPR is estimated to be $2 billion (USD) in 2018.[35] As this and previous work demonstrates the utility of PGPR for plant growth promotion and IPM, we anticipate products containing these beneficial microbes to become more widely available.

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Tables

Table 3.1. Mean ± SEM root mass and total root length of Tifway bermudagrass in PVC arenas after 5 wk with tawny mole cricket infested treatments or a non-infested control

Trial	Treatment	FW[a] (g)	DW[a] (g)	Length (cm)[b]
1	Non-infested	37.12 ± 1.20	5.91 ± 0.22	1,217.16 ± 168.45
1	Infested	37.49 ± 9.16	5.76 ± 1.27	1,019.96 ± 199.78
1	Nitrogen[c]	41.12 ± 6.83	6.67 ± 1.04	1,036.02 ± 182.82
1	PGPR[d]	53.06 ± 4.32	6.57 ± 0.11	1,557.89 ± 77.20
2	Non-infested	100.98 ± 4.35	18.34 ± 0.93	1,679.20 ± 122.33
2	Infested	73.30 ± 6.96	12.24 ± 0.74	1,578.45 ± 150.93
2	Nitrogen	103.44 ± 17.64	19.58 ± 4.23	2,284.23 ± 319.97
2	PGPR	111.06 ± 4.24	21.56 ± 1.28	3,154.85 ± 143.04

[a] Root mass as fresh weight (FW) or dry weight (DW)
[b] Total root length (cm) as determined by digital image analysis using WinRhizo software
[c] Ammonium sulfate applied weekly at a rate of 5.81 g/ m^2 and infested
[d] Blend 20 (*Bacillus pumilus* AP 7, *Bacillus pumilus* AP 18, *Bacillus sphaericus* AP 282) applied at a rate of 500 ml / m^2 and infested

Table 3.2. Orthogonal contrasts comparing root fresh (FW) and dry weights (DW) of infested, bacteria-treated, and fertilized (Tifway bermudagrass) after 5 wk with tawny mole crickets or non-infested control bermudagrass

Trial	Contrast[a]	FW (g)	DW (g)	Length (cm)
1	PGPR[b] vs Non-Infested	*P* = 0.062	*P* = 0.459	*P* = 0.077
1	PGPR* vs Infested	*P* = 0.071	*P* = 0.450	*P* = 0.001*
1	PGPR* vs Nitrogen[c]	*P* = 0.087	*P* = 0.675	*P* = 0.001*
1	Non-Infested vs Infested	*P* = 0.950	*P* = 0.987	*P* = 0.053
1	Nitrogen vs Non-Infested	*P* = 0.745	*P* = 0.733	*P* = 0.069
1	Nitrogen vs Infested	*P* = 0.915	*P* = 0.745	*P* = 0.886
2	PGPR* vs Non-infested	*P* = 0.442	*P* = 0.278	*P* < 0.001*
2	PGPR* vs Infested	*P* = 0.009*	*P* = 0.006*	*P* < 0.001*
2	PGPR* vs Nitrogen	*P* = 0.582	*P* = 0.652	*P* = 0.002*
2	Non-Infested vs Infested	*P* = 0.043*	*P* = 0.055	*P* = 0.643
2	Nitrogen* vs Non-Infested	*P* = 0.824	*P* = 0.517	*P* = 0.022*
2	Nitrogen* vs Infested	*P* = 0.028*	*P* = 0.015*	*P* = 0.009*

* denotes which treatment was significantly different between treatments from orthogonal contrasts ($P < 0.05$, df = 1; JMP Version 13. SAS Institute Inc., Cary, NC).

[a] PGPR, Non-infested, Infested, Nitrogen refer to all cylinders within a treatment for contrasts

[b] Blend 20 (*Bacillus pumilus* AP 7, *Bacillus pumilus* AP 18, *Bacillus sphaericus* AP 282) applied weekly at a rate of 500 ml / m^2 and infested

[c] Ammonium sulfate applied weekly at a rate of 5.81 g of product / m^2 and infested with 6 mole crickets (Trial 1) or 5 mole crickets (Trial 2)

Table 3.3. Mean field plot damage ratings from active tawny mole cricket activity during curative field study (2017) evaluating non-treated, insecticide-treated, bacteria-treated, and insecticide and bacteria-treated bermudagrass

	Mean Mole Cricket Damage Ratings, Days After Treatment (Days After Re-Treatment)								
Treatment	0	14	21	28 (1)	33 (5)	42 (15)	46 (19)	50 (23)	56 (29)
Non-treated	7.71a	8.47a	7.29a	4.10a	7.24a	4.62a	4.86a	3.67a	6.48a
Bifenthrin[a]	7.52a	7.95a	7.14a	1.71b	5.10ab	3.90ab	1.48b	1.86b	4.43b
PGPR[b]	7.52a	7.57a	6.71ab	3.38ab	5.67ab	3.71ab	2.24b	2.29ab	3.00bc
Bifenthrin + PGPR[c]	7.81a	7.33a	4.05b	2.24ab	4.14b	2.52b	3.62ab	1.43b	1.71c
Statistics	$P = 0.966$	$P = 0.33$	$P = 0.026$	$P = 0.037$	$P = 0.035$	$P = 0.043$	$P = 0.03$	$P = 0.041$	$P = 0.032$

Mole cricket damage ratings ranged from 0-9, 0 = no damage, 9 = severe damage.
Means followed by the same letter are not significantly different (LSMeans Student's *t*-test, $P < 0.05$, df = 3; JMP Version 13. SAS Institute Inc., Cary, NC).
[a] Talstar® Pro applied at a rate of 29.6 ml formulated product per 92.9 m^2. The sprayer applied 244 ml of this mixture per plot
[b] Blend 20 (*Bacillus pumilus* AP 7, *Bacillus pumilus* AP 18, *Bacillus sphaericus* AP 282) applied weekly at a rate of 500 ml / m^2. The sprayer applied 3 L of PGPR per plot.
[c] Combination of Talstar® Pro and Blend 20. The sprayer applied 3.244 L of this mixture per plot.

Figures

Figure 3.1. Mean (±SEM) log populations of *Bacillus pumilus* AP 7 mixed with bifenthrin (FMC, Talstar® P Professional, high and low label rate), fipronil (BASF Corp., Termidor® SC, high and low label rate), and imidacloprid (Bayer Environmental Sciences, Merit® 2F) over two weeks.

Figure 3.2. Mean (±SEM) log populations of *Bacillus pumilus* AP 18 mixed with bifenthrin (FMC, Talstar® P Professional, high and low label rate), fipronil (BASF Corp., Termidor® SC, high and low label rate), and imidacloprid (Bayer Environmental Sciences, Merit® 2F) over two weeks.

Figure 3.3. Mean (±SEM) log populations of *Bacillus sphaericus* AP 282 mixed with bifenthrin (FMC, Talstar® P Professional, high and low label rate), fipronil (BASF Corp., Termidor® SC, high and low label rate), and imidacloprid (Bayer Environmental Sciences, Merit® 2F) over two weeks.

Figure 3.4. Mean (±SEM) of top growth (g) of Trial 2 Tifway bermudagrass foliage from PVC arenas infested with tawny mole crickets for 4 wk. Treatments evaluated non-treated, non-infested; non-treated, infested; PGPR treated, infested, or fertilized, infested grasses. Top: foliage top growth above 3.7 cm fresh mass; Bottom: foliage top growth above 3.7 cm dry mass.

Figure 3.5. Mean (±SEM) of curative tawny mole cricket damage ratings based on Cobb and Mack (1989) evaluating non-treated, PGPR-treated, bifenthrin treated, and PGPR + bifenthirn over 56 days.

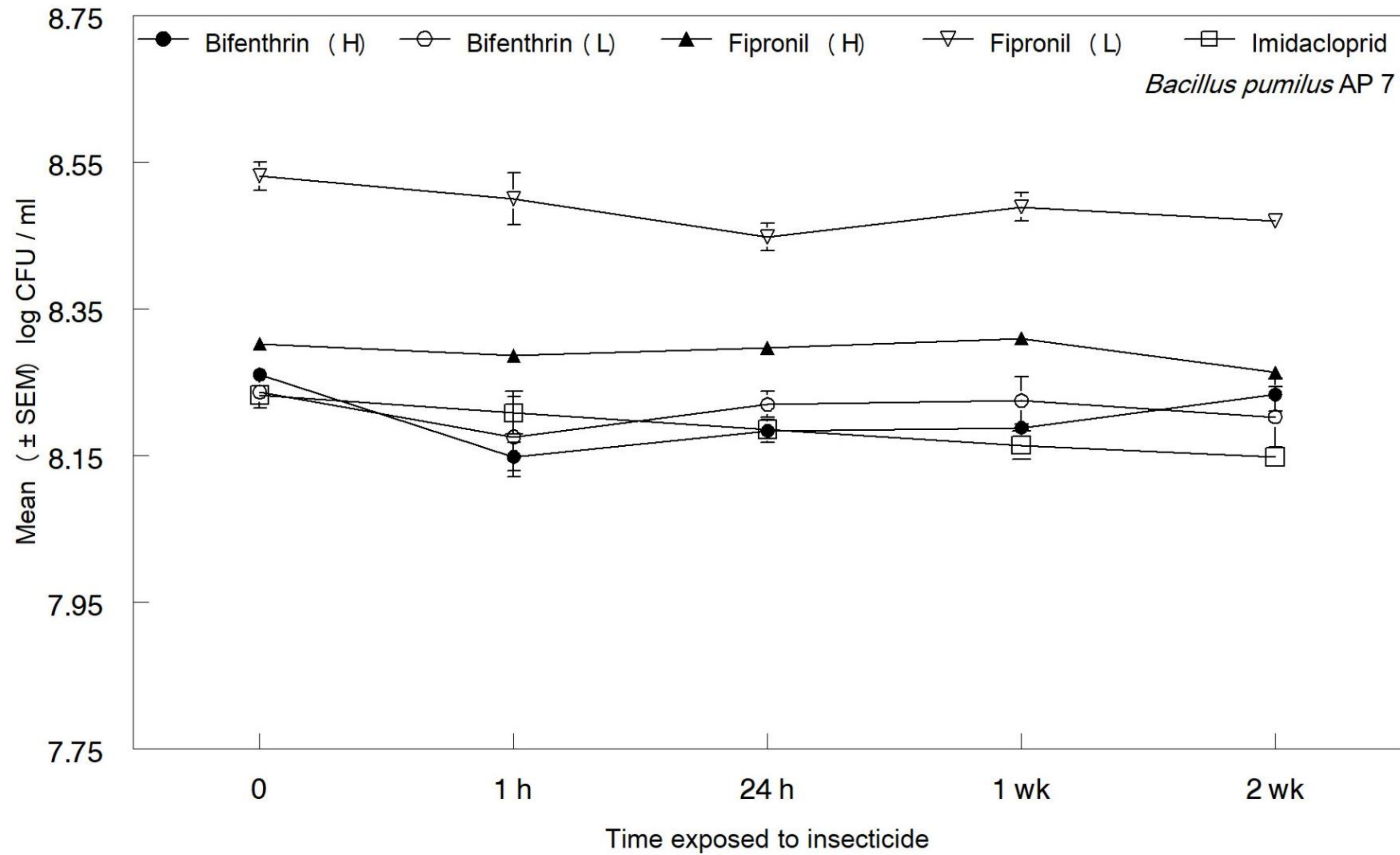

Figure 3.1. Mean (±SEM) log populations of *Bacillus pumilus* AP 7 mixed with bifenthrin (FMC, Talstar® P Professional, high and low label rate), fipronil (BASF Corp., Termidor® SC, high and low label rate), and imidacloprid (Bayer Environmental Sciences, Merit® 2F) over two weeks.

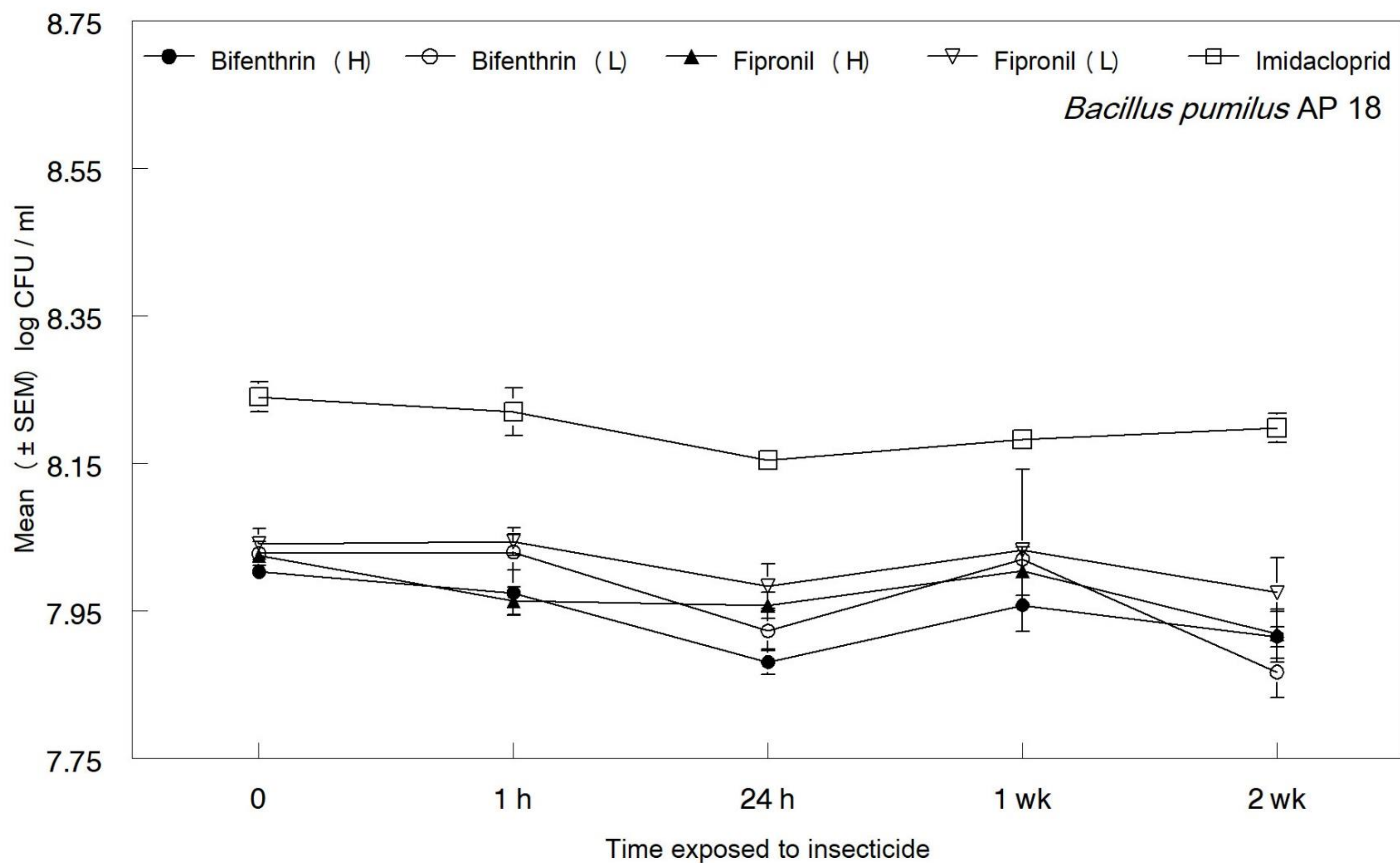

Figure 3.2. Mean (±SEM) log populations of *Bacillus pumilus* AP 18 mixed with bifenthrin (FMC, Talstar® P Professional, high and low label rate), fipronil (BASF Corp., Termidor® SC, high and low label rate), and imidacloprid (Bayer Environmental Sciences, Merit® 2F) over two weeks.

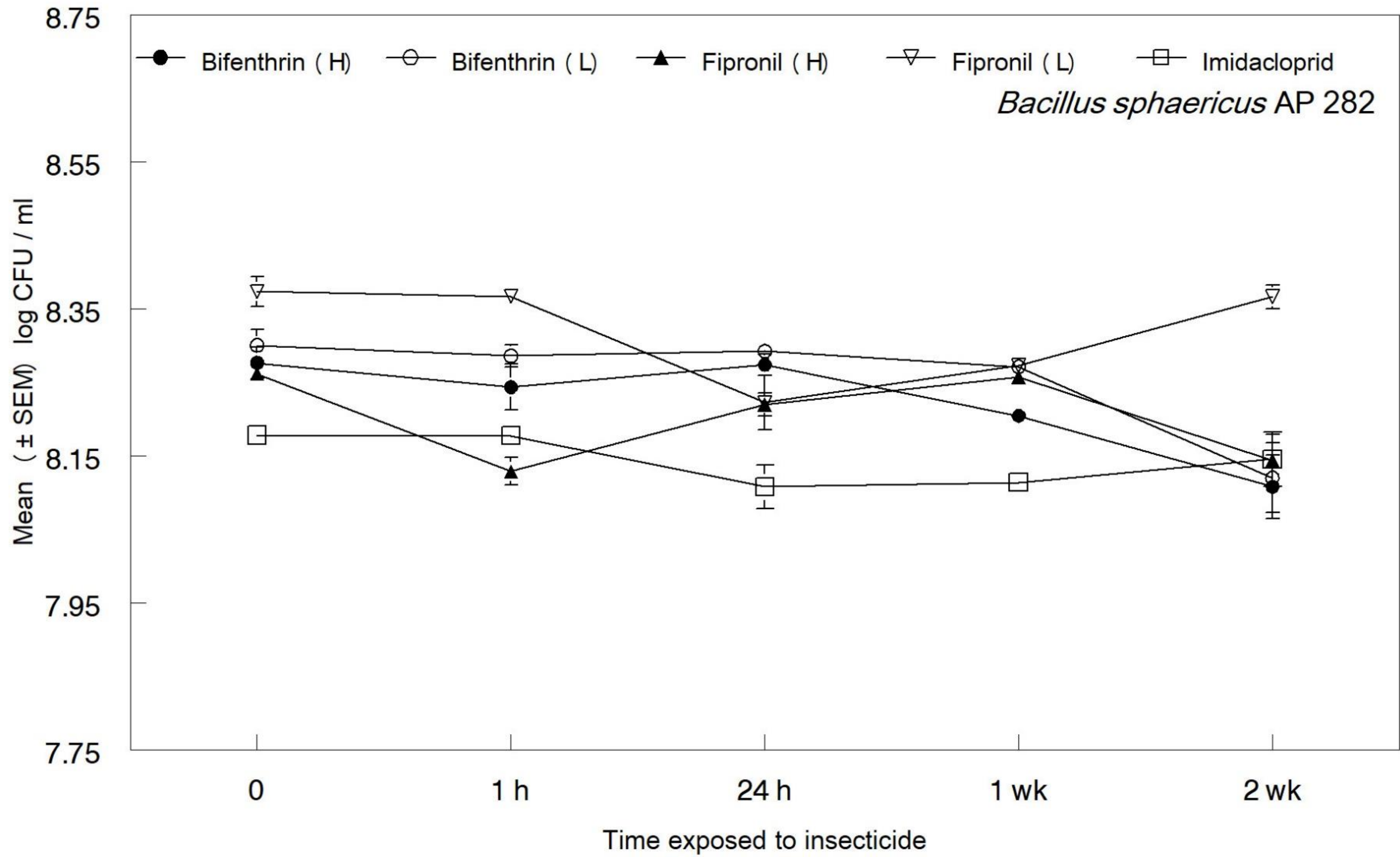

Figure 3.3. Mean (±SEM) log populations of *Bacillus sphaericus* AP 282 mixed with bifenthrin (FMC, Talstar® P Professional, high and low label rate), fipronil (BASF Corp., Termidor® SC, high and low label rate), and imidacloprid (Bayer Environmental Sciences, Merit® 2F) over two weeks.

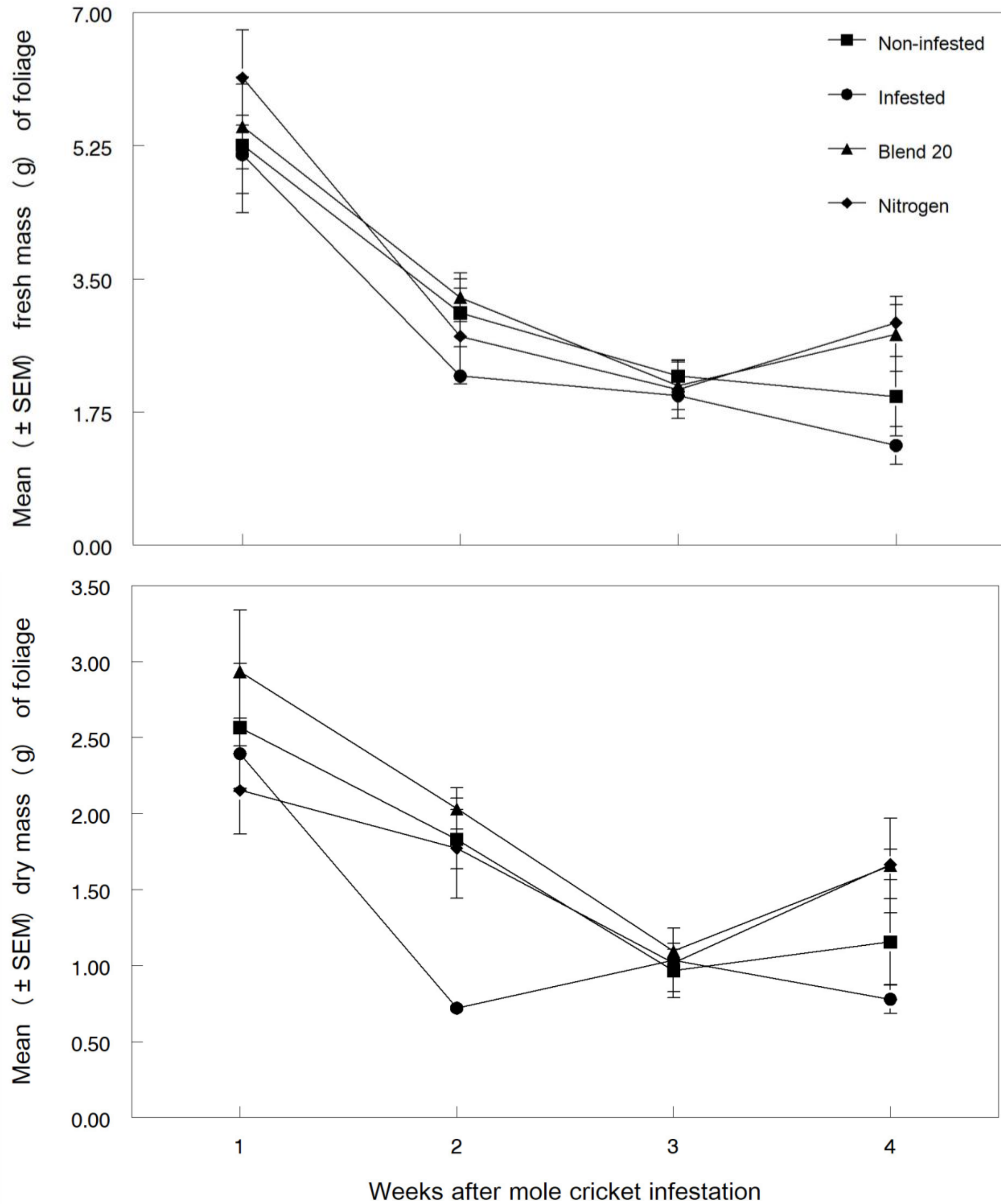

Figure 3.4. Mean (±SEM) of top growth (g) of Trial 2 Tifway bermudagrass foliage from PVC arenas infested with tawny mole crickets for 4 wk. Treatments evaluated non-treated, non-infested; non-treated, infested; PGPR treated, infested, or fertilized, infested grasses. Top: foliage top growth above 3.7 cm fresh mass; Bottom: foliage top growth above 3.7 cm dry mass.

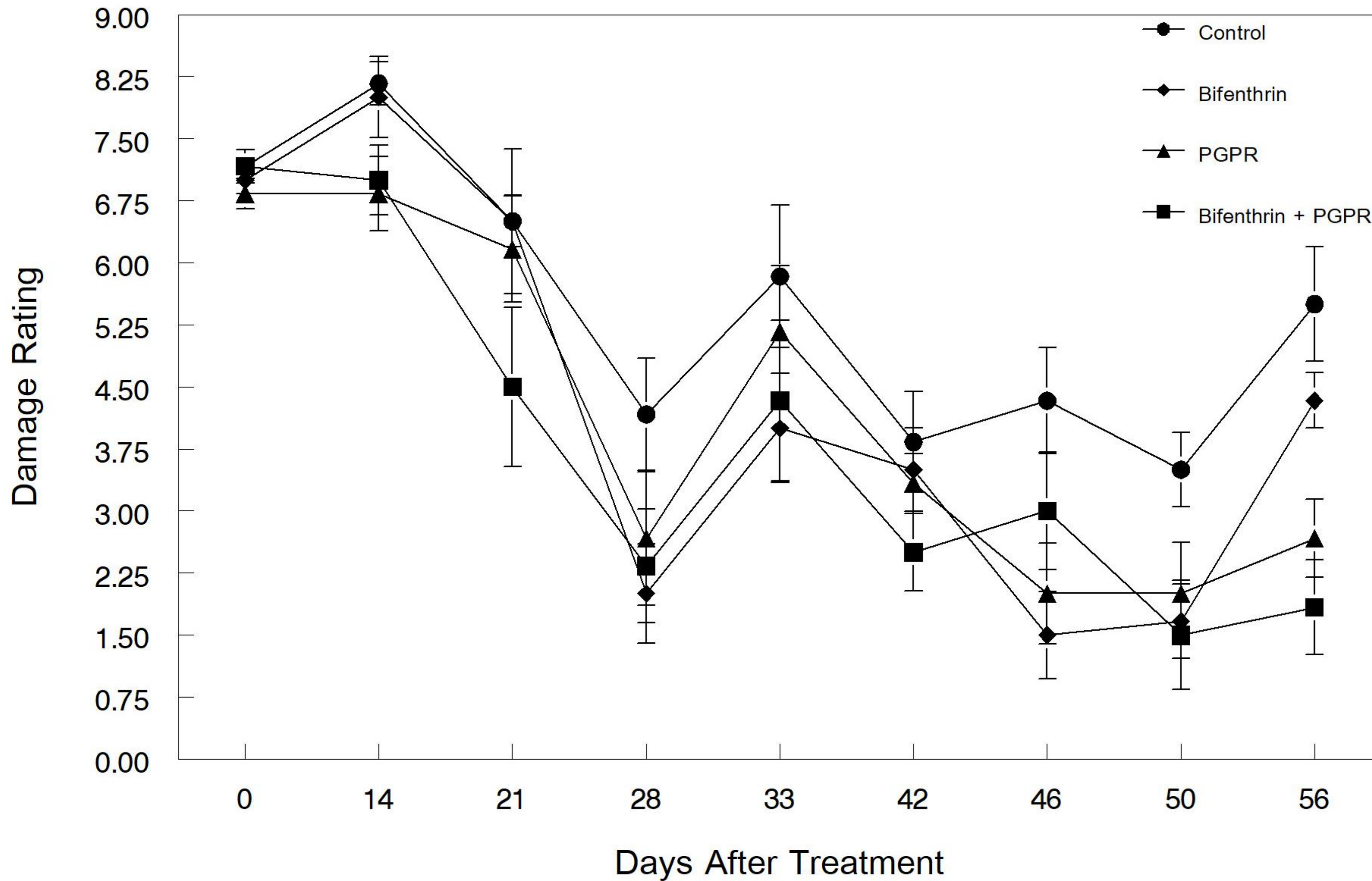

Figure 3.5. Mean (±SEM) of curative tawny mole cricket damage ratings based on Cobb and Mack (1989) evaluating non-treated, PGPR-treated, bifenthrin treated, and PGPR + bifenthrin over 56 days.

Chapter 4: Rhizobacterial treatment of tall fescue and bermudagrass alters tolerance to damage from white grubs

Abstract

Inoculation of hybrid bermudagrass with PGPR can increase plant growth and influence relationships with above-ground herbivores like Fall armyworms and mole crickets (Chapter 3), however, few experiments have evaluated PGPR applications to tall fescue. Root-feeding white grubs cause severe damage to grasses, especially tall fescue, in pastures, golf courses, and lawns. Since bacterial inoculants enhance root growth, the goal of this study was to determine if inoculation of hybrid bermudagrass by root-colonizing bacteria (PGPR) can increase the tolerance of tall fescue and hybrid bermudagrass to white grubs, and if PGPR are compatible with neonicotinoid insecticides commonly used for white grub control. In trials with tall fescue and hybrid bermudagrass, grasses were treated with Blend 20 or nitrogen or left non-treated, then infested with Japanese beetle grubs. PGPR and nitrogen fertilized grasses produced significantly more top growth than the non-treated infested controls. Bacteria treated roots tall fescue roots produced greater fresh and dry mass than non-treated and fertilized grasses. Bacterial treated hybrid bermudagrass roots produced greater root mass than non-treated and fertilized roots. No treatment negatively impact grub survival, and weight gains were similar for all treatments. Bacterial mediated interactions increase tolerance of tall fescue and hybrid bermudagrass applied in response to white grub infestation. Application of PGPR to increased root biomass over non-treated and fertilized grasses. Rhizobacterial products have utility for IPM of root herbivores.

1. Introduction

White grubs (Coleoptera: Scarabaeidae) are serious pests of grasses grown for pasture, golf courses, sod production, and lawns.[1-3] Most species are univoltine developing underground as root-feeding larvae for 9-10 months. As larvae, particularly larger 2nd and 3rd instars, feeding increases on roots, and grasses are unable to maintain normal water relations resulting in wilting or even stand loss.[1] Due to the subterranean nature and feeding of white grubs, damage can be unnoticed until substantial root loss has occurred, resulting in abrupt and severe damage.[1] Further, control of soil insects is challenging as the insecticide must move through the turf canopy and thatch to enter the root or contact the pest directly, often requiring post-treatment irrigation for efficient control. Potential losses from white grubs in turfgrass drives control practices that focus on damage prevention. Preventative control measures rely on the application of insecticides, often neonicotinoids, around egg hatch but before white grubs are detected.[1,3] Neonicotinoid insecticides applied for preventive control of white grubs have consequences for insect pollinators and beneficials, and soil-dwelling invertebrates.[4-7] These non-target impacts of grub control create opportunities for alternatives such as increasing plant tolerance to damage.

Biologicals that enhance plant resistance or tolerance to abiotic and biotic stresses could minimize environmental consequences while reducing chemical and water input needs.[8] Traditionally, improvements in turfgrass cultivars for increased adaptability, aesthetics, and playability, as well as limited disease and stress resistance have been the focus of grass breeding programs. However, there are a limited number of successes related to grass-feeding insects and particularly root-feeding herbivores.[9] For example, infection of perennial ryegrass and fescue by fungal endophyte species in the genus *Neotyphodium* enhanced resistance to certain folivores,

but the impacts of fungal endophytes appear to have subtler, nonlethal effects on root-feeders.[10,11]

Inoculation of turfgrasses with elicitors or biostimulants may result in increased pest tolerances or desirable plant physiologies by maintaining color, productivity, and playability despite pest pressure. Potentially, products applied to existing grass when needed would yield results easier and faster than traditional breeding programs. Rhizobacterial inoculants are used for the maintenance of high quality crops, including grasses (Poaceae) under normal and adverse conditions with limited resource input.[12-14] In previous work, Coy et al. (12) noted greater root mass, volume and length of bermudagrass treated with rhizobacteria compared to non-treated bermudagrass. However, greater root mass alone may not always convey tolerance to white grubs.[15-17] Cultivars of seashore paspalum (*Paspalum vaginatum* Swartz), a warm-season grass were more tolerant of feeding by Japanese beetle grubs, but did not have a greater root mass[16]. Cool-season grass varieties with larger root biomass had greater tolerance to grubs of European chafer (*Rhizotrogus majalis* Razoumosky), but also yielded larger grub mass.[15,17] Larger root masses may have proportionately the same loss as smaller root systems but yield large grubs.

Studies of plants and plant-growth promoting rhizobacteria (PGPR) in the literature are primarily focused on growth promotion and suppression of plant pathogens.[18,19] More recently, studies have extended these interactions to determine the effects on insect folivores.[20-26] Thus far, virtually no research has been conducted on soil-dwelling or root-feeding insects. This lack of research is probably due to the logistical challenges of direct observations of subterranean pests, but the plant-microbe-insect interactions may be more impactful for subterranean insects in close association and constant exposure to rhizobacteria. Because of their association with grass roots,

white grubs are an interesting model system to explore these effects with an economically-important pest. Due to the range of its establishment and host range, *P. japonica* is one of the most extensive and destructive pests of turfgrass and landscape plants in the United States, with annual control costs exceeding $450 million USD.[3,27] Japanese beetles utilize all common species cool- and warm-season grasses, and lawn weeds for larval development.[28-32] Using Japanese beetle grubs in tall fescue and bermudagrass systems, we determined if inoculation of these grasses by rhizobacteria (PGPR) can negatively impact Japanese beetle survival and increase plant tolerance to a below-ground insect herbivore. We also determined if PGPR would be compatible with current commonly used insecticides for Japanese beetle control.

2. Materials and Methods

2.1 Bacterial strains and inoculant preparation

Experiments were conducted with Blend 20, a PGPR liquid mixture, containing equal parts from three bacterial strains (*Bacillus pumilus* AP 7, *B. pumilus* AP 18, and *B. sphaericus* AP 282) that induce growth promotion in bermudagrass.[12, 20] Bacterial strains that were stored at -80 °C were transferred from cryovials to plates of tryptic soy agar (TSA) and allowed to grow at 28 °C in an incubator. After 24-48 h, bacterial lawns were scraped from TSA plates with inoculating loops and transferred to either new TSA plates or to sterile centrifuge tubes (50 ml, VWR, Radnor, PA) containing 40 ml of sterile water, and vigorously shaken to evenly distribute bacterial cells. Serial 10–fold dilutions were then made of each bacterial suspension into sterile water blanks to a final dilution of 10^{-5}.

Bacterial populations (number of colony forming units [CFU]) in the suspensions were determined by plating 50 µl of the serial dilution onto TSA plates, incubating plates for 24-48 h and then counting the number of bacterial colonies on each plate. Once the concentrations (CFU per ml) in the prepared suspensions of each strain were determined, these populations were used to make bacterial stock solutions for each strain. Stock solution of the bacterial blend was prepared by the addition of one liter of equal parts of each bacterium to achieve a blend with a final concentration of 1 x 10^7 CFU per ml of each strain.

2.2 Bacterial strains and insecticide compatibility

The strains that comprise Blend 20, each with a minimum concentration of 1 x 10^8 CFU per ml were individually evaluated for their compatibility with commonly used liquid neonicotinoid insecticides. The strains within the blend were evaluated individually to make recovery of each bacterium apparent, as the colony morphologies are similar. Freshly prepared bacteria stock solutions were evaluated for their ability to survive being mixed with three different insecticides mixed in separate 50 ml centrifuge tubes for 1 and 24 h, and 1 and 2 wk at 25 °C under ambient light. Bacteria and pesticide solutions were prepared based on the label recommendations for volume of area covered. The pesticides evaluated were imidacloprid (Ferti-lome® Tree and Shrub Systemic Insect Drench, 1.47% active ingredient, Voluntary Purchasing Groups, Inc., Bonham, Texas), imidacloprid (Merit® 2F, 21.4% active ingredient, Bayer Environmental Sciences, Research Triangle Park, NC), and imidacloprid and clothianidin (Bayer Advanced 12 Month Tree and Shrub Protect and Feed II®, 0.74% imidacloprid and 0.37% clothianidin, Bayer Environmental Sciences). The Ferti-lome® product was mixed at a rate of 89

ml of product per 3,785 ml. Merit® 2F was mixed at a rate of 17 ml of product per 92.9 m^2. Bayer Advanced Tree and Shrub Protect and Feed II® was mixed at a rate of 89 ml product per 3,785 ml.

After the allotted time, the centrifuge tubes were vigorously shaken to evenly distribute bacterial cells before serial 10–fold dilutions. Serial dilutions were made of each bacterial suspension into sterile water blanks to a final dilution of 10^{-5}. Bacterial populations (number of colony forming units [CFU]) in the suspensions were determined by plating 50 µl of the 10^{-5} serial dilutions onto three TSA plates, incubating plates for 24 h and then counting the number of CFU on each plate.

2.3 Sources of insects and preparation

First and second instar Japanese beetle grubs were field collected from infested turf. Grubs were collected in groups of 25 and placed in containers containing a 1:1 ratio of Sunshine #2 Natural and Organic (Sun Gro Horticulture, Agawam, MA) and Fafard Canadian Sphagnum Peat Moss (Sun Gro Horticulture, Agawam, MA) and placed in a cooler for transport back to the lab. The organic matter from the mixture of Sunshine #2 and peat moss was the food source for the developing grubs. Once in the lab, grubs were transferred to individual containers with the same soil mixture until the experiment started and held in a growth chamber at 23.5 °C 14:10 (L:D) photoperiod. At the start of the experiments, grubs were weighed individually and grouped based on initial mass for each replicate. Each container of grass (tall fescue or bermudagrass) was infested with one grub (~17 grubs per 0.1 m^2). At the end of the experiments, grasses were destructively sampled and Japanese beetle grubs re-collected and weighed to determine differences in weight by treatment.

2.4 Assessment of PGPR on white grub survival

Two experiments were conducted to determine possible insecticidal activity of the strains present in Blend 20 against first and second instar grubs of Japanese beetles. Since white grubs consume roots and soil during feeding that may contain rhizobacteria, these experiments determined if contact with PGPR in soil or if incidentally consumed would impact survival of Japanese beetle grubs. In the direct application experiment, 25 grubs with an average mass of 38.7 g were selected and had 1 ml of 1 x10^7 CFU / ml of Blend 20 pipetted directly over its body before being returned to its individual container. Applications were made to each grub twice during the first week, and grubs were monitored for 3 wk for survival. The experiment evaluating the effects of PGPR in the soil placed 25 grubs in individual containers with the 1:1 ratio of Sunshine mix #2 and peat moss. A solution of PGPR was the only source of moisture in these containers forcing grubs to contact and consume PGPR. Soil moisture was replenished as needed with a hand sprayer of PGPR stock solution and survival was monitored for 3 wk. Initial weights in this study ranged from 35.4-88.7 mg. After 3 wk, grubs were removed from each cup and survival assessed.

2.5 Evaluation of tolerance to white grubs in tall fescue and bermudagrass

Two trials during the summer of 2016, evaluated one PGPR blend (Blend 20), nitrogen-fertilized, and non-treated cool and warm-season grasses. The first trial evaluated 'KY 32,' a cool-season, endophyte free tall fescue (*Festuca arundinacea* Schreb) variety and the second trial conducted evaluated 'Tifway' hybrid bermudagrass (*Cynodon dactylon* (L.) x *C. transvaalensi*s Burtt-Davy), a warm-season variety. The selection of a non-endophytic tall fescue

(KY 32) was deliberate for this work to avoid possible interactions between PGPR and fungal endophytes in tall fescue. The tall fescue trial had 30 replicates per treatment and the bermudagrass trial had 20 replicates per treatment in a randomized complete block design.

The tall fescue trial was conducted in a growth chamber set at 23.5 °C and a 14:10 (L:D) photoperiod. Styrofoam cups (9.0 cm diameter x 15.5 cm depth) were filled with a local loamy sand field soil and seeded at a rate of 33.6 kg / ha. Seeded cups were placed in the growth chamber maintained at 16-22% soil moisture by volume. The grasses were grown for 3 wk before being infested. During that time, plants were fertilized twice with granular ammonium sulfate fertilizer (5.81 g of product / m^2, PRO fertilizer, 21-0-0; Harrell's Inc., Lakewood, FL). Plants were not cut until new grass growth exceeded 5 cm. After 3 wk, grasses were infested with one Japanese beetle grub with an average weight of 66.67 mg. Within each replicate, grub weights were within 1-2 mg of one another. Coincident with infesting cups with grubs, treatments were randomly assigned and the first treatments started. Grasses assigned to PGPR treatments received weekly treatments of 3 ml (500 ml / m^2) of bacterial suspension for 4 wk. The same volume of distilled water was applied to the control plants each week. Grasses assigned to the fertilizer treatment received 5.81 g of product / m^2 granular ammonium sulfate fertilizer weekly. After each treatment application, each cup received 80 ml (1.27 cm) of water to move the treatment to the root zone. Except when applications were made, cups were weighed and then watered as needed.

For the bermudagrass trial, Tifway hybrid bermudagrass plugs (3.8 cm diameter) were harvested from the Auburn University Turfgrass Research Unit, Auburn, AL. After harvesting, plugs were washed free of field soil and transplanted into square plastic pots (7.6 cm diameter x

20.3 cm depth; MT38 Mini-Treepots, Stuewe and Sons, Tangent, OR). Plants were grown in a greenhouse with an average temperature of 28.6 ± 5 °C, 14:10 (L:D), 50% average relative humidity. The grass was grown for 3 wk during which time plants were cut weekly to a height of 3.7 cm and fertilized weekly with ammonium sulfate fertilizer at rate of 5.81 g of product / m^2. After 3 wk, grasses were infested with one Japanese beetle grub with an average weight of 46.35 mg. Within each replicate, grub weights were within 1-2 mg of one another. Coincident with infesting cups with grubs, treatments were randomly assigned and the first treatments started. Grasses assigned to PGPR treatments received weekly treatments of 3 ml (500 ml / m^2) of bacterial suspension for 8 wk. The same volume of distilled water was applied to the control plants each week. Grasses assigned to the fertilizer treatment received 5.81 g of product / m^2 granular ammonium sulfate fertilizer weekly. After weekly treatment applications, pots received 75 ml (1.27 cm) of water to move the treatment to the root zone. Except when applications were made, pots were watered as needed.

2.6 Impact of Japanese beetle, PGPR, and fertilizer on grass growth

Once treatments began, tall fescue plants were cut weekly to a height of 5 cm and Tifway bermudagrass was cut every other week to a height of 3.7 cm. Top growth was collected and weighed for leaf fresh mass and then oven dried at 70 °C for 72 h before being reweighed for dry mass.[12] To avoid any temperature or lighting bias, grass plants in cups (growth chamber) and pots (greenhouse) were rotated on a weekly basis. At the end of each trial, plants were destructively sampled to recover Japanese beetle grubs and root systems. Grubs were collected and reweighed for final mass. The root system of each plant was washed in the lab and weighed for fresh mass and then oven dried at 70 °C for 72 h before being reweighed for dry mass.

2.7 Statistical analysis

Top growth (fresh and dry mass) in each trial were analyzed separately using repeated measures of multivariate analysis of variance (MANOVA), orthogonal contrasts ($P < 0.05$, JMP Version 13. SAS Institute Inc., Cary, NC). Japanese beetle grub mass, root fresh and dry mass were analyzed using analysis of variance (ANOVA), Student's *t*-test ($P < 0.05$).

3. Results

3.1 Assessment of PGPR on white grub survival

The direct application of PGPR to Japanese beetle grubs and continued feeding and exposure of grubs for 3 wk to PGPR was not found to be toxic. All grubs exposed to either treatments were alive at the end of the experiment. Additionally, direct observation of grubs did not reveal any symptomology (e.g., discoloration). After 3 wk and two topical applications, white grubs increased in mass from an average of 38.7 mg to a final average weight of 103.7 mg.

3.2 Bacteria and insecticide compatibility

In this trial, all strains within Blend 20 (*B. pumilus* AP 7, *B. pumilus* AP 18, and *B. sphaericus* AP 282) were mixed neonicotinoid with insecticides commonly used to control Japanese beetles. Strains were not negatively impacted, and remained stable when mixed with either formulation of imidacloprid or the mixture of imidacloprid and clothianidin for 2 wk (Figures 4.1-4.3). Slight variations in populations occurred but were likely a result from bacterial distribution in the centrifuge tubes, serial dilutions, and growth time of bacterial colonies on growth media.

3.3 Impacts of PGPR on Japanese beetle weight gain and survival

These experiments evaluated if PGPR application after Japanese beetle grub infestation and repeated PGPR applications after infestation would increase the tolerance of tall fescue and bermudagrass to white grubs. Recovery of live Japanese beetle grubs in the tall fescue trial was 100% and 78.3% in the bermudagrass trial, with no treatment having less than 75% survival and recovery. Final weights of Japanese beetle grubs increased relative to initial weights in both trials. In the tall fescue trial, final larval weights ranged from 176.77-183.17 mg, and 151.5-164.94 mg in the bermudagrass trial. There were no significant differences between treatments in the final weights of grubs in either trial (tall fescue, $F = 0.158$, $df = 2, 89$, $P = 0.906$; bermudagrass, $F = 1.76$, $df = 2, 46$, $P = 0.184$).

3.4 Impact of infested arenas and grass growth

Top growth in containers infested with grubs declined over time in response to Japanese beetle infestation with the non-treated grasses producing the lowest shoot and root masses in both trials (Table 4.1, Figures 4.4-4.5). In both trials, significant treatment differences were observed. In the Fescue trial, differences in fresh mass of foliage was significant between treatments, but dry mass was not (fresh mass, $F = 12.04$ $df = 2, 87$, $P < 0.0001$; dry mass $F = 0.24$, $df = 2, 87$, $P = 0.79$). PGPR-treated tall fescue produced significantly more fresh mass than all treatments ($F = 23.75$, $df = 1,87$, $P < 0.0001$). Nitrogen fertilized grasses produced more top growth than the non-treated tall fescue ($F = 3.74$, $df = 1, 87$, $P = 0.057$).

In the Bermudagrass trial, fertilized grasses produced significantly more fresh and dry masses of top growth than all treatments (fresh mass, $F = 19.13$, $df = 2,57$, $P < 0.0001$; dry mass, $F = 10.5$, $df = 2,57$, $P = 0.002$). Both PGPR-treated and fertilized grasses had significantly more

fresh and dry mass top growth than the non-treated bermudagrass over the 8 wk period (fresh mass, F = 18.05, df = 1, 57, $P < 0.0001$; dry mass, F = 16.03, df = 1, 57, $P = 0.0002$). Fertilized grass produced significantly more fresh and dry top growth than the non-treated control (fresh mass, F = 24.79, df = 1, 57, $P < 0.0001$; dry mass, F = 13.65, df = 1, 57, $P = 0.0005$). Treatment of bermudagrass with a nitrogen fertilizer resulted in significantly greater fresh top growth than treatment with PGPR (F = 6.75, df = 1, 57, $P = 0.012$), but not greater dry mass (F = 2.58, df = 1,57, $P = 0.11$). Treatment of grass with PGPR resulted in significantly more fresh and dry top growth than the non-treated control (fresh mass, F = 5.67, df = 1, 57, $P < 0.02$; dry mass, F = 5.74, df = 1, 57, $P = 0.019$).

Root fresh and dry mass were analyzed separately by trial (Table 1). PGPR-treated tall fescue and bermudagrass produced the greatest root fresh and dry mass in both trials. PGPR-treatment of grass infested with Japanese beetle grubs produced fresh masses that were 146-180% greater and dry masses that were 145-267% greater than non-treated, infested grasses. Tall fescue treated either with PGPR or fertilizer produced significantly greater fresh and dry root mass than non-treated controls (fresh mass, F = 21.11, df = 2, 89, $P < 0.0001$; dry mass, F = 25.5, df = 2, 89, $P < 0.0001$). PGPR treatment of tall fescue produced significantly more fresh and dry root mass than fertilized tall fescue (fresh mass, F = 21.11, df = 2, 89, $P < 0.029$; dry mass, F = 25.5, df = 2, 89, $P < 0.019$). Bermudagrass treated with PGPR produced significantly greater root fresh and dry mass than non-treated controls (fresh mass, F = 6.96, df = 2, 59, $P = 0.0006$; dry mass, F = 2.35, df = 2, 59, $P = 0.002$). Nitrogen fertilized grass produced numerically greater root fresh mass, but only significantly greater dry mass than non-treated bermudagrass (fresh mass, F = 6.96, df = 2, 59, $P = 0.055$; dry mass, F = 2.35, df = 2, 59, $P =$

0.043). PGPR-treated and fertilized bermudagrass produced fresh and dry masses that were similar and not significantly different (fresh mass, F = 6.96, df = 2, 59, *P* = 0.088; dry mass, F = 2.35, df = 2, 59, *P* = 0.226).

4. Discussion

White grubs are one of the key pests of grasses in pastures, lawns, golf courses, and sod production[1-3] As they consume the roots of their host, the grass becomes weakened, losses turgor, and can die. Because a larger root mass may enable grasses to tolerate damage from white grubs, this study sought to compare the growth responses of a cool- and warm-season grass to white grubs when treated with rhizobacteria or a synthetic fertilizer to sustain promotion during root herbivory. Root and foliar growth of tall fescue or bermudagrass treated with either PGPR or fertilizer were greater relative to non-treated controls. Growth promotion in hybrid bermudagrass was expected based on previous work with Blend 20[12,20] but this likely represents the first demonstration of growth promotion in tall fescue with a PGPR.

Foliar growth in both infested grasses declined steadily over time with treatment differences most apparent in the first week of each trial. This decline in top growth over time is likely due to the high survival and increasing size of the white grubs. Grub survival was ≥75% and not affected directly by the treatment, as noted in the grass trials and the separate topical and constant exposure experiments. It should be noted that PGPR are soil bacteria related to common microbials with insecticidal properties. Although we did not observe insecticidal effects or reduced survival, this should not be interpreted that PGPR lack possible direct effects on white grubs. For example, when evaluated for effects on the fall armyworm, *Spodoptera frugiperda* JE

Smith, larvae fed bermudagrass treated with certain blends of PGPR had lower larval and pupal weights and adult eclosion. Bermudagrass treated with the same PGPR, Blend 20, used in this study with white grubs, actually yielded greater fall armyworm pupal weights and shorter development time than the non-treated controls.[20] Previous research with Japanese beetles in turfgrass have found the susceptibility of grubs to soil bacterium *Paenibacillus popilliae* the causal agent of milky disease and *Bacillus thuringiensis* serovar *japonensis* strain Buibui (*Btj*).[33-36] Further investigations of PGPR for plant-microbe or plant-insect-microbe interactions could yield new discoveries, because it is not possible to know the ecology of these bacteria based on systematics. For example, species of *Bacillus* and *Paenibacillus* are noted insect pathogens but these genera also contain species that cause growth promotion or indirect effects on insects.[12, 20, 39, 43-46]

On average, grubs tripled their mass during each experiment, but the final weights and weight gain (data and analysis not shown) were not different between treatments. Grass species (tall fescue or bermudagrass) did not appear to impact the weight gain or survival of grubs as noted by Braman and Pendley (37), which noted higher survival for Japanese beetle and southern masked chafer (*Cyclocephala lurida* Oliver) grubs on Tifway bermudagrass, but increased larval weights on 'KY 31,' an endophyte-infected tall fescue. Additionally, the treatment of grasses with PGPR likely did not change the palatability of the grass roots as weight gains between treatments were similar, supporting similar work with *P. japonica* grubs and fungal endophytes.[38] The greater root mass of the PGPR and fertilized grasses did not appear to produce greater mass of white grubs in those treatments. In other studies considering host plant resistance to white grubs among grasses,[15-17] it is suggested that larger root systems in grasses produce

larger white grubs. We did not find evidence of this in our experiment. The previous work may have been confounded by variation across cultivars and species of grasses being examined.

Interestingly, tall fescue and bermudagrass infested with white grubs produced 12-15% greater root masses when treated with PGPR compared to fertilized. A synthetic fertilizer was included to provide a control for greater root mass in both grass trials. The tall fescue trial suggests an interaction with PGPR that stimulates roots in the presence of herbivory. In their review of microbe-plant-insect interactions, Pineda *et al.* (22) suggested that the effects of microbes on plants may be strengthened under biotic or abiotic stress. The mechanisms of plant-microbe-insect interactions are not well understood, but microbes are likely acting in ways to stimulate biosynthetic pathways related to stress.[39]

In both grasses, our data suggest that bacteria or fertilizer can mitigate damage from root feeding white grubs. While the use of bacterial biostimulants is a novel approach for mitigating damage from root-infesting pests in turfgrass, the use of fertilizer or plant hormones to alleviate stress from root-feeding is not novel.[40-41] Blanco-Montero and Ward (41) evaluated weekly applications of commercial cytokinin products that increased root biomass of Kentucky bluegrass (*Poa pratensis* L.). The grass infested with masked chafer (*Cyclocephala pasadenae* Casey) grubs that received these treatments compensated for the white grub feeding. Commercially-available PGPR products (e.g., Nortica, Bayer Environmental Sciences) are available for use in turfgrass but the labeling of those products does not currently include stress mitigation due to root feeding insects. In related work in our lab, we have also shown similar results when Blend 20 was used to mitigate damage from mole crickets under lab and field conditions (Coy RM, *unpublished*).

The *Bacillus* spp. used to produce Blend 20 were compatible and stable when mixed with liquid formulations of neonicotinoid insecticides commonly used for control of Japanese beetles (Figures 4.2-4.4). The combination of PGPR and insecticides for prolonged periods (2 wk) represents a scenario where the two tactics would either be mixed before application or interact in the soil. The stability of the PGPR in Blend 20 mixed with insecticides is not surprising, given that *Bacillus subtilus* used with thiamethoxam in corn seeds increases pesticide uptake and plant growth.[42] There do not appear to be products formulated containing both bacteria and pesticide(s), however our data suggest this approach may be a novel and integrated approach for pest management. With the environmental concerns over neonicotinoids used in agricultural and commodity crops, including turfgrasses, increased pesticide efficiency and post-treatment irrigation could alleviate some concerns and may enable lower use rates. As noted previously, the lack of negative consequence for the rhizobacteria in Blend 20 when mixed with these insecticide formulations should be interpreted cautiously. Other PGPR may not be compatible with all pesticides or inert ingredients in formulations. Populations of *Pseudomonas fluorescens* remained stable when mixed with the insecticides avermectin, carbofuran, chlorpyrifos, and endosulfan, but not with indoxacarb.[43-46] PGPR populations may be more sensitive to fungicide exposure. *Pseudomonas fluorescens* populations were negatively impacted when mixed with the fungicides mancozeb, captan, propiconazole.[43,47] Individual trials would be needed with different bacterial genera, strains, formulations and pesticides to determine compatibility.

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Table 4.1. Mean ± SEM of grass roots fresh and dry mass after 4 wk (KY 32) and 8 wk (Tifway) of Japanese beetle grub infestation

Species	Cultivar	Treatment	FW (g)	DW (g)
Tall Fescue	KY 32	Control	9.24 ± 0.5c	2.09 ± 0.27c
Tall Fescue	KY 32	Nitrogen[a]	14.38 ± 0.7b	4.66 ± 0.24b
Tall Fescue	KY 32	Blend 20[b]	16.67 ± 0.55a	5.58 ± 0.23a
Bermudagrass	Tifway	Control	4.99 ± 0.32b	1.04 ± 0.07b
Bermudagrass	Tifway	Nitrogen	6.22 ± 0.33ab	1.33 ± 0.08a
Bermudagrass	Tifway	Blend 20	7.30 ± 0.42a	1.51 ± 0.09a

Numbers presented are treatment means. For each Trial, means in the same column followed by the same letter are not significantly different from each other ($P < 0.05$; JMP; ANOVA, Student's *t*-test).

[a]Ammonium sulfate applied weekly at a rate of 5.81 g/ m^2 and infested with a single white grub

[b]Blend 20 (*Bacillus pumilus* AP 7, *Bacillus pumilus* AP 18, *Bacillus sphaericus* AP 282) applied weekly at a rate of 500 ml / m^2 and infested with a single white grub

Figures

Figure 4.1. Mean (±SEM) log populations of *Bacillus pumilus* AP 7 mixed with 1.47% imidacloprid (Voluntary Purchasing Groups, Inc, Ferti-lome® Tree and Shrub Systemic Insect Drench), 21.4% imidacloprid (Bayer Environmental Sciences, Merit® 2F), and 0.74% imidacloprid and 0.37% clothianidin (Bayer Environmental Sciences, Bayer Advanced 12 Month Tree and Shrub Protect II®) over two weeks.

Figure 4.2. Mean (±SEM) log populations of *Bacillus pumilus* AP 18 mixed with 1.47% imidacloprid (Voluntary Purchasing Groups, Inc, Ferti-lome® Tree and Shrub Systemic Insect Drench), 21.4% imidacloprid (Bayer Environmental Sciences, Merit® 2F), and 0.74% imidacloprid and 0.37% clothianidin (Bayer Environmental Sciences, Bayer Advanced 12 Month Tree and Shrub Protect II®) over two weeks.

Figure 4.3. Mean (±SEM) log populations of *Bacillus sphaericus* AP 282 mixed with 1.47% imidacloprid (Voluntary Purchasing Groups, Inc, Ferti-lome® Tree and Shrub Systemic Insect Drench), 21.4% imidacloprid (Bayer Environmental Sciences, Merit® 2F), and 0.74% imidacloprid and 0.37% clothianidin (Bayer Environmental Sciences, Bayer Advanced 12 Month Tree and Shrub Protect II®) over two weeks.

Figure 4.4. Mean (±SEM) of top growth (g) of KY 32 tall fescue foliage from Styrofoam cup arenas infested with a Japanese beetle grub for 4 wk. Treatments evaluated non-treated, PGPR-treated, or fertilized grasses. Top: foliage top growth above 5.0 cm fresh mass; Bottom: foliage top growth above 5.0 cm dry mass.

Figure 4.5. Mean (±SEM) of top growth (g) of Tifway bermudagrass foliage from plastic pot arenas infested with a Japanese beetle grub for 8 wk. Treatments evaluated non-treated, PGPR-treated, or fertilized grasses. Top: foliage top growth above 3.7 cm fresh mass; Bottom: foliage top growth above 3.7 cm dry mass.

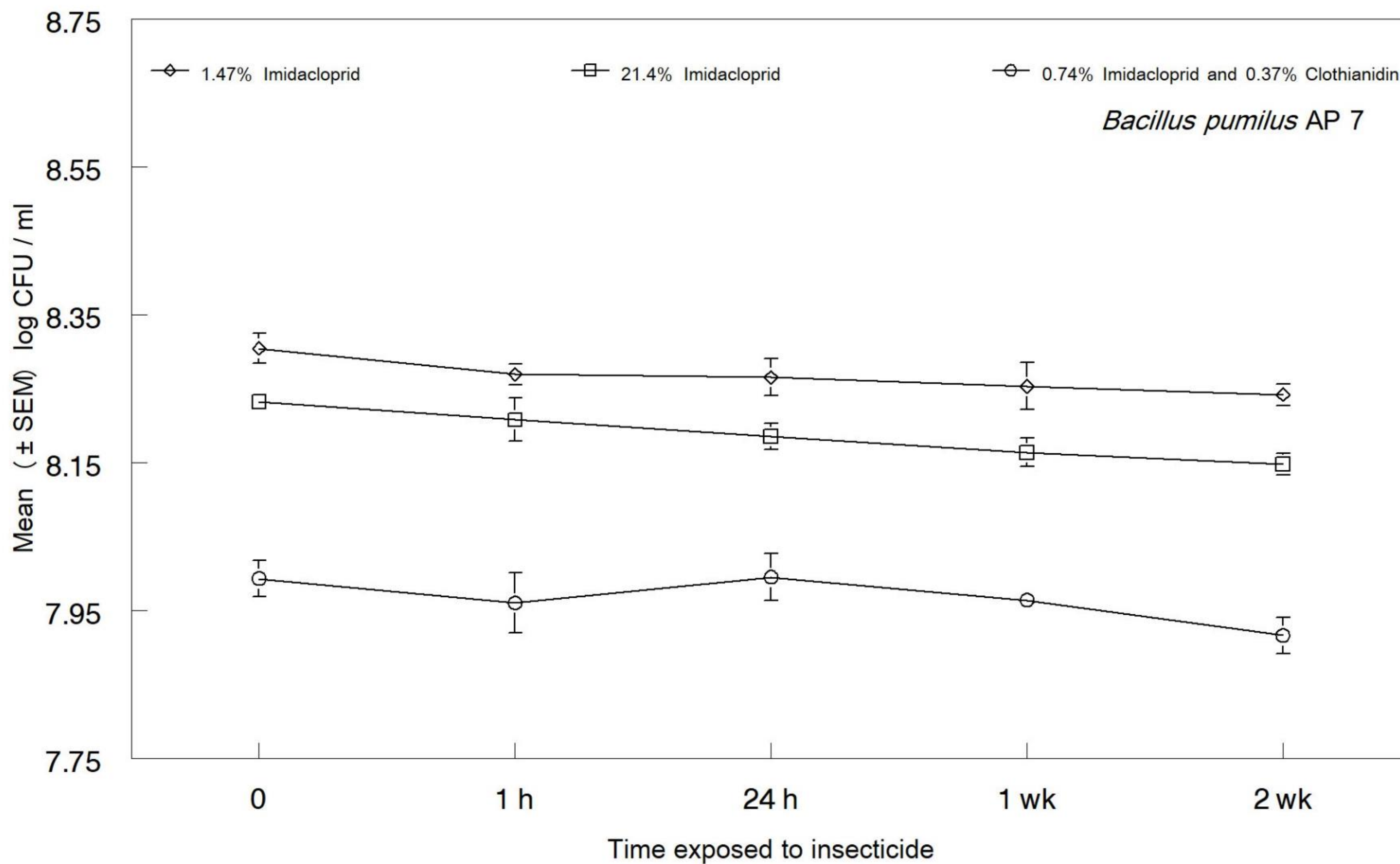

Figure 4.1. Mean (±SEM) log populations of *Bacillus pumilus* AP 7 mixed with 1.47% imidacloprid (Voluntary Purchasing Groups, Inc, Ferti-lome® Tree and Shrub Systemic Insect Drench), 21.4% imidacloprid (Bayer Environmental Sciences, Merit® 2F), and 0.74% imidacloprid and 0.37% clothianidin (Bayer Environmental Sciences, Bayer Advanced 12 Month Tree and Shrub Protect II®) over two weeks.

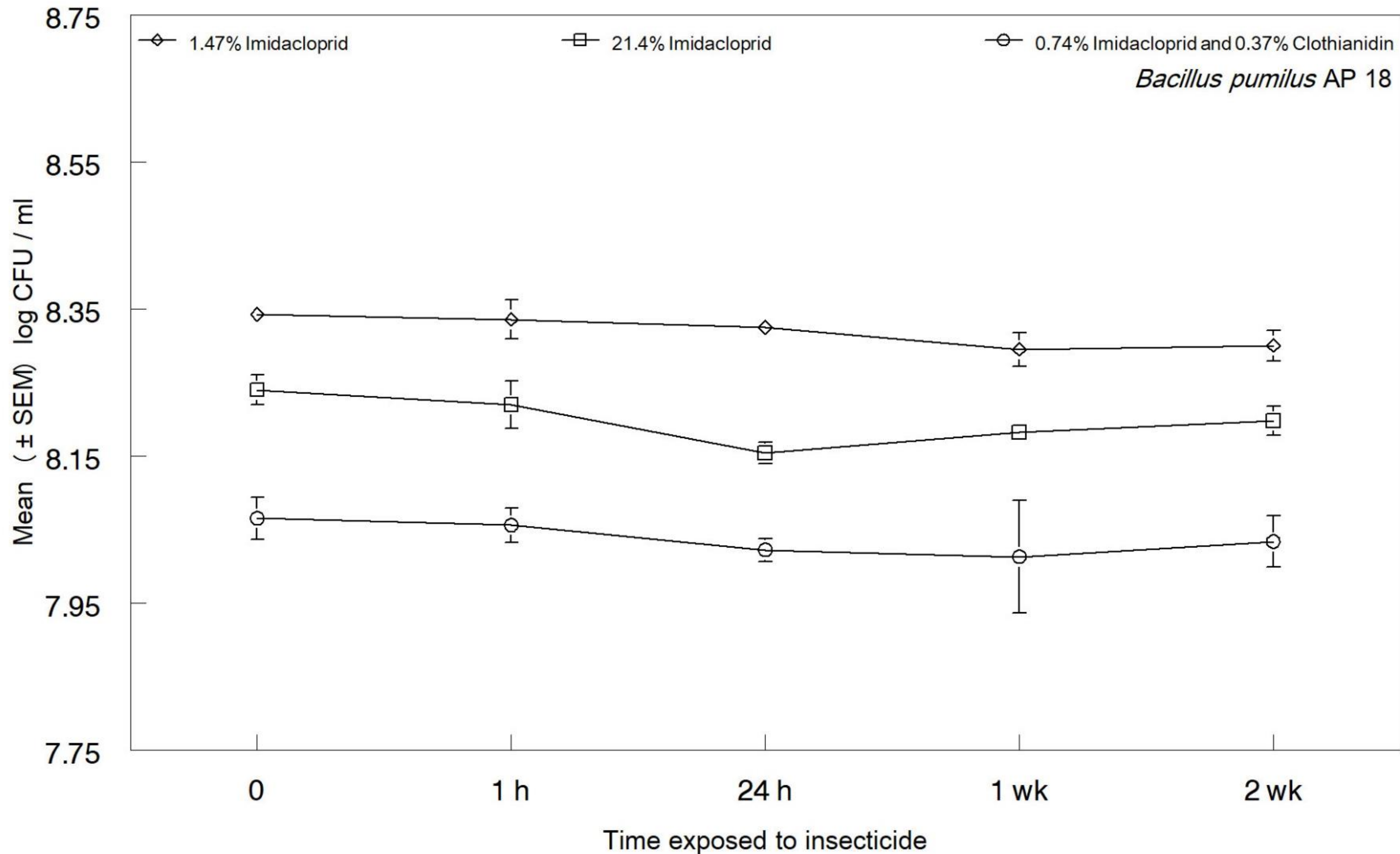

Figure 4.2. Mean (±SEM) log populations of *Bacillus pumilus* AP 18 mixed with 1.47% imidacloprid (Voluntary Purchasing Groups, Inc, Ferti-lome® Tree and Shrub Systemic Insect Drench), 21.4% imidacloprid (Bayer Environmental Sciences, Merit® 2F), and 0.74% imidacloprid and 0.37% clothianidin (Bayer Environmental Sciences, Bayer Advanced 12 Month Tree and Shrub Protect II®) over two weeks.

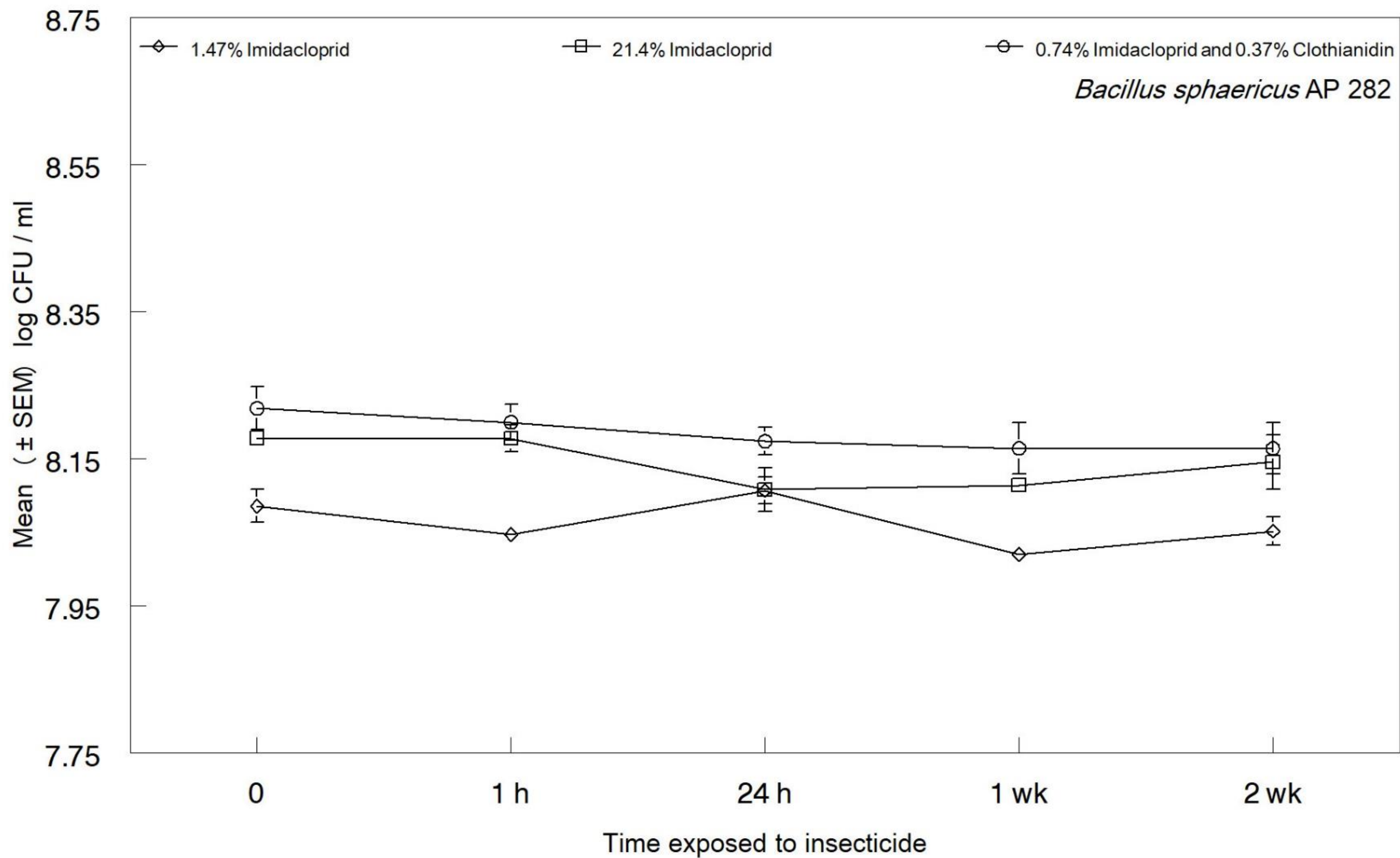

Figure 4.3. Mean (±SEM) log populations of *Bacillus sphaericus* AP 282 mixed with 1.47% imidacloprid (Voluntary Purchasing Groups, Inc, Ferti-lome® Tree and Shrub Systemic Insect Drench), 21.4% imidacloprid (Bayer Environmental Sciences, Merit® 2F), and 0.74% imidacloprid and 0.37% clothianidin (Bayer Environmental Sciences, Bayer Advanced 12 Month Tree and Shrub Protect II®) over two weeks.

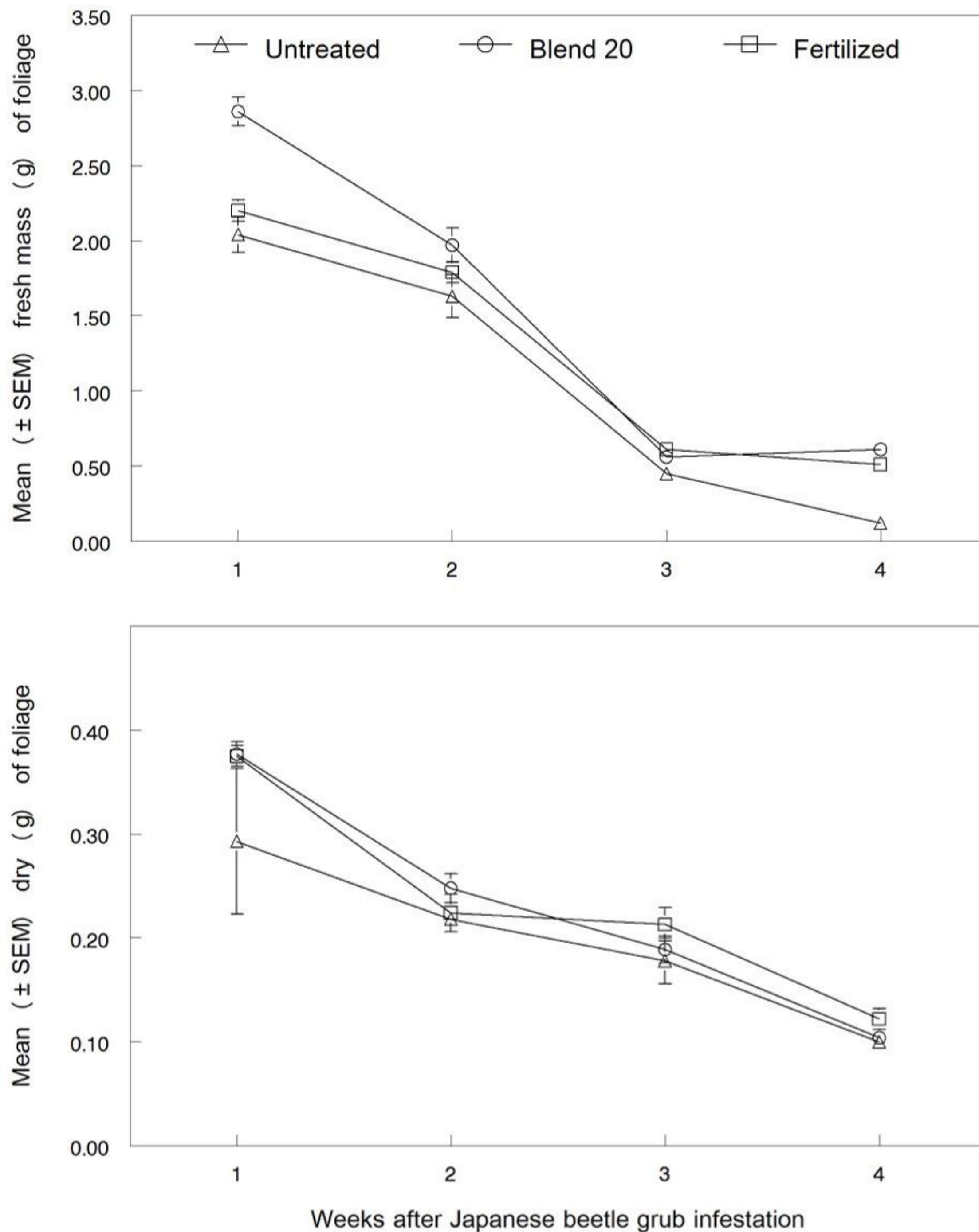

Figure 4.4. Mean (±SEM) of top growth (g) of KY 32 tall fescue foliage from Styrofoam cup arenas infested with a Japanese beetle grub for 4 wk. Treatments evaluated non-treated, PGPR-treated, or fertilized grasses. Top: foliage top growth above 5.0 cm fresh mass; Bottom: foliage top growth above 5.0 cm dry mass.

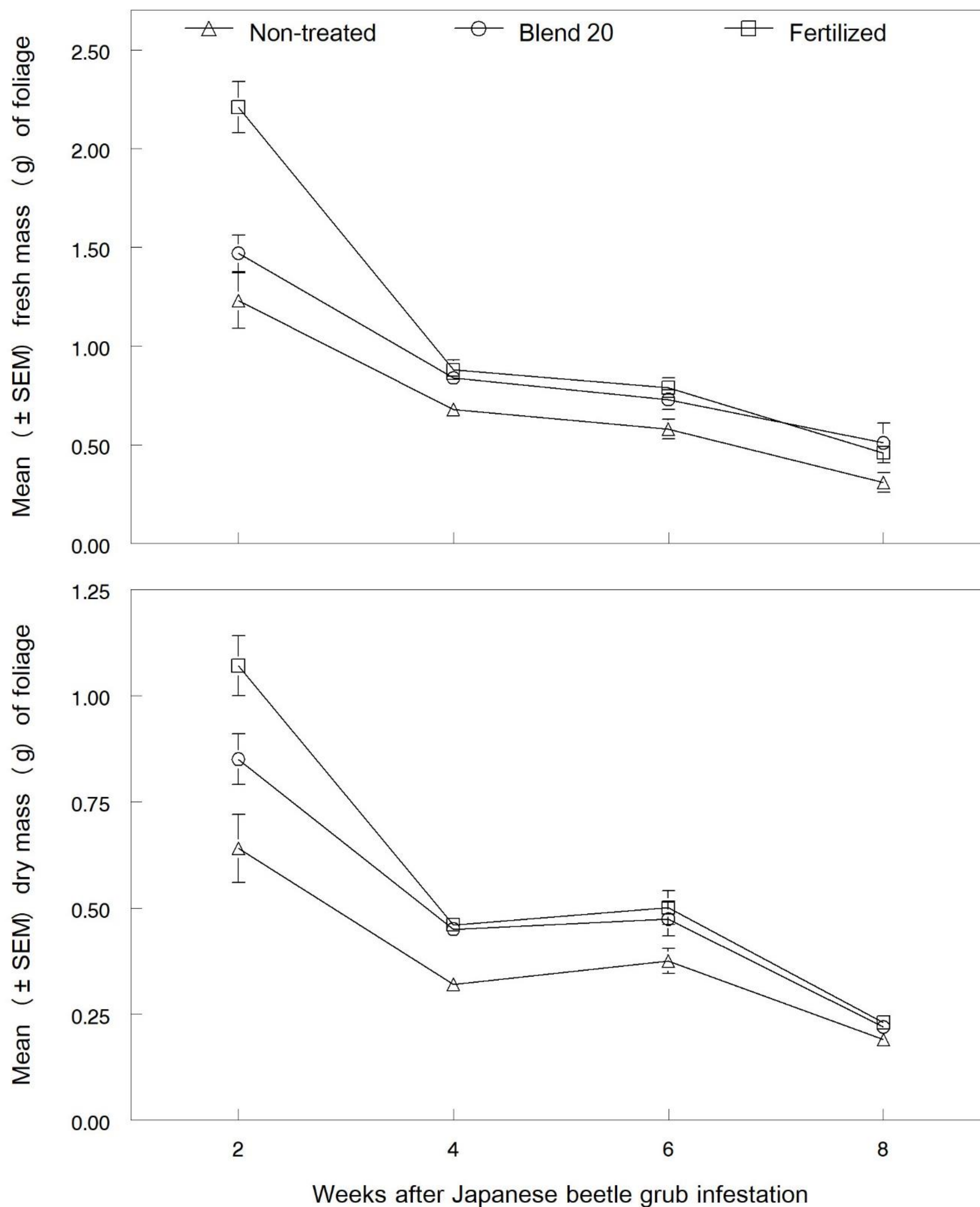

Figure 4.5. Mean (±SEM) of top growth (g) of Tifway bermudagrass foliage from plastic pot arenas infested with a Japanese beetle grub for 8 wk. Treatments evaluated non-treated, PGPR-treated, or fertilized grasses. Top: foliage top growth above 3.7 cm fresh mass; Bottom: foliage top growth above 3.7 cm dry mass.

Chapter 5: Rhizobacterial inoculants alter bermudagrass drought stress response

Abstract

Rhizobacterial inoculants have been previously shown to mitigate drought stress in crops and grasses. Experiments were designed to determine if a blend of three *Bacillus* strains (Blend 20) could enhance drought stress responses in hybrid bermudagrass varieties with differing drought tolerances compared to fertilized and non-treated controls. Experiments were designed to examine tolerant (Tifway), moderately tolerant (LaPaloma), and susceptible (Yukon) grown pots with sand under greenhouse conditions and treated for 5 wk before being subjected to 3 wk of drought stress, and a recovery period. Drought stress response variables measured relative water content, chlorophyll content, electrolyte leakage, and root length and weights. Bacterial inoculated grasses maintained lower relative water contents during drought periods, but maintained higher content than non-treated grass during recovery. Depending on the variety, bacterial inoculation may enhance chlorophyll content during and post-drought. The most pronounced benefits of bacterial inoculation were on electrolyte leakage and root growth. Bacterial treatment of bermudagrass could alleviate varietal electrolyte leakage differences between LaPaloma and Yukon varieties. Roots of bacteria-treated grasses often had increased root fresh and dry weight and length over non-treated and fertilized grasses. The results of these experiments confirm the observations that PGPR can mediate or alter abiotic stress responses in hybrid bermudagrass. Furthermore, it provides a better understanding of plant-microbe-interactions in amenity grasses which can aid in incorporation of biostimulants for turfgrass management in areas with reduced water availability.

1. Introduction

Turfgrasses are the most important groundcover plants cultivated in the United States, covering an area three times greater than any other irrigated crop (Milesi et al. 2005, Manuchehri and Salehi 2014). Environmental and ecological benefits of turfgrass results from nutrient cycling, soil and water conservation, pollutant removal, and erosion control (Bronick and Lal 2005). Turfgrass exposure to abiotic and biotic stress decreases productivity, aesthetic quality, and functionality (Hu et al. 2009, Du et al. 2012, Huang et al. 2014). Rarely do abiotic and biotic stress occur individually, but rather as a combination of stressors, making management difficult. Environmental stress in warm-season grasses often results from high temperatures, excessive light, low water availability, or poor soil quality (Kasim et al. 2013, Huang et al. 2014). The use of water resources in amenity grasses is a growing concern as water resources are facing greater demands from agriculture, industry, domestic uses, as well as climate variability (Seager et al. 2009). Expectations of increased stress from temperature extremes and water scarcity are linked to climate variability, with models predicting increases in temperature and drought severities, frequencies, and durations (Vinocur and Altman 2005, IPCC 2007, Ault et al. 2013).

Exposure to abiotic and biotic stress presents challenges that plants must adapt to and overcome. To deal with stress, plants evolved sophisticated physiological, cellular, biochemical, and molecular responses to maintain homeostasis under harsh conditions (Li et al. 2012, Shi et al. 2012, Comas et al. 2013, Kasim et al. 2013). Drought, salinity, and temperature stresses alter plant physiological and metabolic responses; limiting growth, productivity, and survival (Kim et al. 2009, Shi et al. 2012, Comas et al. 2013, Kasim et al. 2013, Manuchehri and Salehi 2014, Yang et al. 2016). Insight into plant stress responses from the whole plant to the cellular level is

vital for the development of new grass cultivars and for the incorporation of novel technologies into management practices. Bacterial mediated interactions with plants that can be selectively applied that alter drought responses by maintaining productivity may yield results easier and faster than traditional breeding programs.

Use of rhizobacterial inoculants has allowed for the maintenance of high quality crops, including grasses (Poaceae) under adverse conditions with limited resource input (Omar et al. 2000, Kasim et al. 2013, Bashan et al. 2014, Gagné-Bourque et al. 2015,). A plant's ability to survive and sustain growth during periods of drought stress is loosely defined as drought resistance. Drought resistance is achieved genetically through the utilization of three strategies: avoidance, escape, or tolerance. (Levitt 1980). Drought resistance mechanisms utilized are not mutually exclusive and depend on drought duration, severity, as well as grass type, species, and cultivar (Carmo-Silva et al. 2009, Taylor et al. 2011, Khan et al. 2012, Shi et al. 2012). In turfgrass, drought tolerance or avoidance strategies are preferable as they are more adaptable traits for breeding and biotechnology. Drought escape is not as preferable for amenity turfgrass, as it results in grass dormancy, which interferes with aesthetics and playability until water resources are replenished (Assuero et al. 2002, Fry and Huang 2004, Huang et al. 2014).

Drought avoidance occurs when a plant maintains a favorable water status by increasing the capacity for water uptake via the root system or by reducing water loss from leaves (Levitt 1980). Common drought avoidance characteristics include increased root plasticity and depth; enhanced leaf pubescence; and increased stomatal regulation (Duncan and Carrow 1999). Deeper grass roots are better able to provide continued water and nutrients to the plant and may delay the

dehydration of tissue even when part of the plant's root system is under dry soil conditions (Hays et al. 1991, Bonos and Murphy 1999, Duncan and Carrow 1999, Huang 1999).

Drought tolerance occurs when a plant maintains active growth and metabolic activity under water deficit conditions (Levitt 1980). Drought tolerance allows plants to survive prolonged periods of soil moisture deficits through osmotic adjustments, maintenance of root and membrane viability under dehydration, and the accumulation of proteins and metabolites that function in direct and indirect structural stabilization (Nilsen and Orcutt 1996).

Soil microbial communities evolve over time and influence plant health; biomass accumulation; nutrient availability and acquisition; and soil structure and quality (Johannes et al. 2000, Smalla et al. 2001, Shi et al. 2006, Khan and Bano 2016). Soil microbes in the rhizosphere can induce changes in plants and influence stress responses. Colonization of plants by plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting endophytic bacteria (PGPEB) in natural plant conditions or added as biostimulants to agronomic and horticultural crops show increased root and shoot mass, enhanced nutrient uptake, chlorophyll content, and stress mitigation (see reviews by Calvo et al. 2014, Ngumbi and Kloepper 2016, Santoyo et al. 2016). Among plant-associated microbes, PGPR have been linked to drought mitigation through priming plant defenses; altering plant-water regulation, use, and efficiency; or the production of larger, more explorative root systems (Kasim et al. 2013, Halo et al. 2015).

The use of microbes to enhance plant and soil health for mediating drought and salinity stress tolerances have been evaluated in several agronomic crops and grasses, including maize, rice, wheat, barley, ryegrass, bluegrass, and the model C_3 grass *Brachypodium distachyon* (Khan

et al. 2012, Comas et al. 2013, Bashan et al. 2014, Gagné-Bourque et al. 2015, Halo et al. 2015, Kaushal and Wani 2016). Bacteria of the genera *Azospirillum*, *Bacillus*, *Methylobacterium*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Sphingobacterium*, and *Sphingomonas* have been documented to mitigate drought stress responses in certain Poaceae crops (Khan et al. 2012, Comas et al. 2013, Halo et al. 2015). Studies in amenity grasses are limited as the physiologies and genetics are not well understood since cultivars have different parental germplasms, which respond and utilize different strategies to avoid, escape, or tolerate drought based on drought severity and duration (Levitt 1980, Rampino et al. 2006, Carmo-Silva et al. 2009, Du et al. 2012, Kasim et al. 2012, Wang and Brummer 2012, Huang et al. 2014). Drought responses in bermudagrass have been associated with larger plant root systems, phytohormones, proline content, antioxidant activity, chlorophyll content, dehydrin accumulation, electrolyte leakage (EL), evapotranspiration (ET), and stomatal regulation, leaf firing, plant biomass, and relative water content (RWC); yet, questions remain as to which root traits and plant responses are most beneficial in understanding drought stress mitigation (DaCosta and Huang 2007, Carmo-Silva et al. 2009, Hu et al. 2009, Du et al. 2012, Shi et al. 2012, Comas et al. 2013).

Coy et al. (2014) noted growth promotion in the economically important bermudagrass system with select rhizobacterial blends. Subsequent experiments suggested increased drought stress mitigation in bermudagrass with PGPR. Using Blend 20, a mixture of three *Bacillus* strains we determined if inoculation of bermudagrasses with a biofertilizer can alter drought responses of bermudagrass cultivars similar to a synthetic fertilizer regarding chlorophyll content, RWC, and EL.

2. Materials and Methods

2.1 Bacterial Strains and Inoculant Preparation

Blend 20, a PGPR consisting of equal parts of *Bacillus pumilus* AP 7, *B. pumilus* AP 18, and *B. sphaericus* AP 282 reported to induce growth promotion in bermudagrass was evaluated for drought interactions. Bacterial strains that were stored at -80 °C were transferred from cryovials to plates of tryptic soy agar (TSA) and allowed to grow at 28 °C in an incubator. After 24-48 h, bacterial lawns were scraped from TSA plates with inoculating loops and transferred to either new TSA plates or to sterile centrifuge tubes (50 ml, VWR, Radnor, PA) containing 40 ml of sterile water, and vigorously shaken to evenly distribute bacterial cells. Serial 10–fold dilutions were then made of each bacterial suspension into sterile water blanks to a final dilution of 10^{-5}.

Bacterial populations (number of colony forming units [CFU]) in the suspensions were determined by plating 50 µl of the serial dilution onto TSA plates, incubating plates for 24-48 h and then counting the number of bacterial colonies on each plate. Once the concentrations (CFU per ml) in the prepared suspensions of each strain were determined, these populations were used to make bacterial stock solutions for each strain. Stock solutions of bacterial blends (e.g. Blend 20) were prepared by the addition of one liter of equal parts of each bacterium to achieve a blend with a final concentration of 1×10^7 CFU per ml of each strain.

2.2 Greenhouse grass establishment and evaluations of drought tolerance

Two trials conducted in the fall of 2015 and summer of 2017 evaluated non-treated control, PGPR-treated, and synthetic fertilizer-treated bermudagrass varieties under simulated

drought conditions. The 2015 trial evaluated Tifway hybrid bermudagrass, a drought tolerant cultivar treated with either Blend 20, ammonium sulfate fertilizer, or non-treated control (Hu et al. 2010, Shi et al. 2012). The trial had 6 replicates per treatment. Plugs of Tifway bermudagrass (3.8 cm diameter) were harvested from the Auburn University Turfgrass Research Unit, Auburn, AL. After harvesting, plugs were washed free of field soil and transplanted into clean, fine sand and grown in square plastic pots (10.2 cm diameter x 34.3 cm high; CP413CH Treepots, Stuewe and Sons, Tangent, OR). Plants were grown in a greenhouse with an average temperature of 29.0 ± 6 °C, 14:10 (L:D), 70% average relative humidity. The grass acclimated for 3 wk during which liquid fertilizer (284 ppm Nitrogen, Peterson's 20N-20P-20K; Alix, Alberta, Canada) was mixed and applied weekly at a rate of 5.4 g (1 tsp) per 3.78 L. During acclimation, plants were cut weekly to a height of 3.7 cm. Post-acclimation, grasses assigned to PGPR treatments received weekly treatments of 4 ml (500 ml / m^2) of bacterial suspension of 1 x 10^7 CFU per ml for 5 wk. Grasses assigned to the fertilizer treatment received 5.81 g of product / m^2 granular ammonium sulfate fertilizer (PRO fertilizer, 21-0-0; Harrell's Inc., Lakewood, FL) weekly after acclimation. Post-acclimation, grasses received weekly treatments of bacteria or fertilizer for 5 wk. The same volume of distilled water was applied to the control plants each time bacteria was applied. After weekly treatment applications, pots received 130 ml (1.27 cm) of water to move the treatment to the root zone. Except when applications were made, pots were watered as needed. Plants were cut to a height of 3.7 cm at the beginning of the experiment, then cut as needed during the drought trial. Top growth was collected to determine baseline RWC and EL among treatments before drought.

After 5 wk of treatment, all supplemental watering was stopped and grasses were drought stressed for 21 days. During the drought stress period, plants were cut weekly to a height of 3.7 cm. The top growth was collected to determine leaf water content. Plant chlorophyll measurements of the turfgrass canopy were taken weekly with a FieldScout CM 1000 NDVI chlorophyll meter (Spectrum Technologies, Inc., Aurrora, IL). After 21 days of drought stress, plants were watered and maintained under normal water conditions and allowed to recover. Plants in each treatment group were re-treated 7 days after drought and allowed to recover for 5 wk. After 5 wk of recovery, the pots were destructively sampled and the root system was collected and washed in the lab. After washing, root fresh weights were recorded before digital image analysis of the linear root structure was conducted using a root scanning system (Regent Instruments, Inc. Sainte-Foy, Quebec) which consisted of a scanner (LA 1600+) and WinRhizo software (2004a). Based on image analysis, the software calculated total root length. After scanning the root systems, the roots were dried in an oven at 70 ºC for 72 h. The data collected were used to compare root growth of non-treated, PGPR-treated, and fertilizer-treated Tifway bermudagrass.

The 2017 trial evaluated two seeded hybrid bermudagrass varieties with differing drought tolerances. The trial had 16 replicates per treatment. Varieties evaluated were LaPaloma, a moderately tolerant bermudagrass and Yukon, a susceptible bermudagrass (Shi et al. 2012) under similar environmental conditions previously described. Grasses were seeded in square plastic pots (7.6 cm diameter x 20.3 cm high; MT38 Mini-Treepot, Stuewe and Sons, Tangent, OR) at a rate of 14.65 g / m^2 in clean, fine sand. Grasses in this trial were grown in smaller pots to allow for more replicates than the 2015 trial. After germination, grasses were established for 4 wk.

During establishment, all grasses were fertilized based on rates previously described. Grasses were not cut until top growth exceeded 5 cm. Grasses were cut to a height of 3.7 cm and then cut weekly. Top growth was collected to determine baseline RWC and EL among treatments before drought. The trial was replicated 16 times per treatment. Five bermudagrass treatments evaluated 2 PGPR treatments, 2 fertilizer treatments, and a non-treated control. PGPR treatments were Blend 20 by itself, Blend 20 with 50% of recommended nitrogen fertilization. Fertilized plants were treated monthly at the recommended rate of nitrogen and 50% of recommend rate. Post-acclimation, grasses assigned to PGPR treatments received weekly treatments of 3.8 ml (500 ml / m^2) of bacterial suspension of 1 x 10^7 CFU per ml for 5 wk. The same volume of distilled water was applied to the control plants each time bacteria was applied. Grasses assigned to the 50% of recommended fertilizer treatments received 11.6 g of product / m^2 of granular ammonium sulfate fertilizer monthly after acclimation. Treatment applications were followed by 75 ml (1.27) cm of water.

After 5 wk of treatment, all supplemental watering was stopped and grasses were drought stressed for 21 days. During the drought stress period, plants were cut weekly to a height of 3.7 cm. The top growth was collected to determine RWC and EL. Four replicates of RWC and EL were done for each treatment. Plant chlorophyll measurements were taken weekly. After 21 days of drought stress, plants were watered and maintained under normal water conditions and allowed to recover for 3 wk. Plants in each treatment were subdivided into groups of 8, which were either re-treated with their treatment or left untreated during recovery. After 3 wk of recovery, the pots were destructively sampled and the root system was collected and washed in the lab. After washing, root fresh weights were recorded before digital image analysis of the

linear root structure was conducted using a root scanning system previously described. Based on image analysis, the software calculated total root length. After scanning the root systems, the roots were dried in an oven at 70 ºC for 72 h. The data collected were used to compare root growth of non-treated, PGPR-treated, PGPR + 50% nitrogen-treated, and fertilizer-treated LaPaloma and Yukon bermudagrass.

2.3 Measurement of relative water content (RWC) in bermudagrass shoots

Relative water content was measured by weighing out ~200 mg of fresh leaf tissue and placing it in distilled water for 24 h. After rehydrating, the sample was reweighed for turgid weight, and then placed in an oven at 70º for 72 h before dry weight was measured. RWC was then calculated using the equation RWC (%) = (FW - DW) / (TW - DW) x 100%.

2.4 Measurement of electrolyte leakage (EL) in bermudagrass shoots

Electrolyte leakage was measured by obtaining 300 mg of FW shoots cut into 1 cm segments. Clippings were triple rinsed in deionized water before being placed in 50 ml centrifuge tubes with 30 ml of deionized water and shaken for 24 h. After shaking, the initial conductivity (C_i) of the sample was measured using a YSI 3200 Conductivity Instrument (YSI Inc., Yellow Springs, OH). Leaves were then autoclaved for 20 min at 121 ºC, and then shaken for another 24 h. After shaking, the final conductivity (C_f) was measured as previously described. Electrolyte leakage was calculated from the formula (C_i / C_f) x 100%. This experiment was conducted before, during, and after drought conditions.

2.5 Statistical Analysis

Measurements used to determine chlorophyll content, RWC, and EL were separated by drought and recovery periods and analyzed using repeated measures multivariate analysis of

variance (MANOVA), orthogonal contrasts ($P < 0.05$, JMP Version 13. SAS Institute Inc., Cary, NC). Relative water content and EL data were arcsine square root transformed before analysis. Analyses were used to determine treatment by variety and treatment by recovery interactions in the LaPaloma and Yukon trial. Root fresh and dry masses and length were analyzed by treatment, variety, and variety by treatment using analysis of variance (ANOVA), Student's *t*-test ($P < 0.05$,).

3. Results

3.1 2015 Tifway bermudagrass Trial

Prior to the initiation of drought, all treatments maintained RWC > 85%. Relative water contents of grasses decreased with drought duration resulting in significant treatment differences (F = 14.19, $P = 0.0003$, df =2, 15; Table 5.1). During drought, nitrogen fertilized Tifway bermudagrass maintained significantly higher RWC than control or Blend 20 treated bermudagrass (MANOVA, repeated measures; $P = 0.0003$, df = 1,15). The impacts of drought on RWC were evident as early as week 1 of drought. The lowest observed RWC occurred after 3 wk of drought. Nitrogen fertilized grasses had a lowest observed RWC of 22.6 % which was nearly double the water content of control and Blend 20 grasses (12.7 and 12.4 % respectively). During the recovery period, RWC ranged from 17.1-80.0 % for all treatments and were not significantly different (F = 3.09, $P = 0.0752$, df = 2,15). All grasses recovered to nearly pre-drought RWC (80%) after 4-5 weeks.

Chlorophyll contents averaged over 210 for all treatments before drought initiation. Chlorophyll contents decreased within 1 wk of drought duration. There were significant

differences between treatments during the drought and recovery periods (F = 11.93, $P = 0.0008$, df = 2, 15; F = 13.65, $P = 0.0004$). Tifway bermudagrass treated with Blend 20 maintained significantly higher chlorophyll contents (184.9-87.7) than either control (132.6-84.3) or nitrogen fertilized (132.8-92.6) grasses during the drought and recovery periods ($P = 0.0002$; $P = 0.0001$; Table 5.1). Control and nitrogen fertilized grasses were not different in chlorophyll contents ($P > 0.05$).

Although Tifway bermudagrass treated with Blend 20 produced numerically greater root fresh weight and length, it was not different from either control or nitrogen fertilized grass ($P > 0.05$; Table 5.5). However, Blend 20 produced significantly greater root dry weight than control and nitrogen fertilized grasses ($P < 0.0001$).

3.2 2017 LaPaloma and Yukon Trial

Prior to the initiation of drought, all treatments maintained RWC > 90% (Figures 5.1-5.2). Leaf RWC of grasses in all treatments decreased over time, with significant reductions in water content occurring from 2-3 wk of drought (Figures 5.1-5.2). At the end of the drought period, RWC ranged from 19.85-42.66% for LaPaloma treatments and 27.9-52.59% for Yukon (MANOVA, repeated measures; F = 9.75, $P = 0.0026$, df = 1,70; Table 5.2). During the drought period, significant treatment effects were observed (MANOVA, repeated measures; F = 4.23, $P = 0.004$, df = 4, 70). Only the half rate of nitrogen fertilized treatment maintained a significantly greater leaf water content than the control during drought ($P = 0.0267$; Table 5.2). Blend 20 + 50% nitrogen maintained the lowest water content; significantly lower than Blend 20 alone, 50% nitrogen, and fully fertilized ($P \leq 0.0065$) during drought.

A treatment by variety interaction was not significant during drought (MANOVA, repeated measures; F = 1.55, $P = 0.1961$; Table 5.3). Although this treatment by variety interaction was not significant overall, it was significant within contrasts comparing treatments (Table 5.3). During drought, LaPaloma treated with Blend 20 + 50% nitrogen or 50% nitrogen was lower in water content than the same treatments applied to Yukon ($P \leq 0.0099$; Table 5.3). This suggests that these treatments during drought had a greater impact on Yukon than LaPaloma. Post-drought, RWC increased over time, but were not significantly affected by grass variety or treatment during recovery (reapplication of pre-drought treatments or non-treated) (F = 1.26, $P = 0.2654$; F = 0.65, $P = 0.6287$). Once watering continued, grasses receiving any treatment (nitrogen, bacteria or a combination of both) recovered better than the non-treated control grasses ($P \leq 0.0005$; Tables 5.2, 5.4). During recovery, RWC were not significantly different if grasses recovered with or without their respective treatment (F = 1.51, $P = 0.2233$, df = 1, 70; Table 5.4).

Prior to the initiation of drought, all grasses had chlorophyll contents over 230 (Figures 5.3-5.4). Chlorophyll content of grasses decreased with duration of drought and was significantly affected by treatment (F = 35.93, $P < 0.0001$, df = 4, 144; Table 5.2) but not variety (F = 1.34, $P = 0.2493$, df = 1, 144). During the drought period, Blend 20 + 50% nitrogen, 50% nitrogen, and nitrogen-treated grasses maintained significantly higher chlorophyll contents than Blend 20 and the non-treated control ($P < 0.0001$; Table 5.2), which had the lowest chlorophyll contents. Both Blend 20 + 50% nitrogen and the full rate of nitrogen treatments maintained higher chlorophyll contents than the half rate of nitrogen treatment ($P \leq 0.0076$). During the drought period, there was a significant interaction of treatment and grass variety (F = 14.02, $P < 0.0001$, df = 4, 144;

Table 5.3). During the drought period, non-treated controls, Blend 20, and 50% nitrogen treatments of LaPaloma maintained significantly greater chlorophyll contents than the same treatments in Yukon ($P \leq 0.0016$; Table 5.3).

Post drought, varietal responses were not different (MANOVA, repeated measures; F= 0.01, $P = 0.9092$), but treatments were affected by drought recovery (F = 2.96, $P = 0.0218$). Chlorophyll contents of grasses post-drought were significantly greater than non-treated control grasses for all treatments applied before drought occurred (MANOVA, repeated measures; $P = 0.0268$; Table 5.2). The application of any nitrogen fertilizer treatment before drought was imposed resulted in significantly greater chlorophyll content during drought relative to all other treatments (Table 5.2). During the post drought period, grasses that received their assigned treatment had greater chlorophyll contents than grasses recovering with only the application of water (F = 5.02, $P = 0.0266$, df = 1,144).

There were significant differences between treatment (those applied before drought was imposed and those applied during recovery) and grass variety, as well as the interaction of treatments applied pre- and post-drought (MANOVA, repeated measures; F = 5.58, $P = 0.0003$, df = 4, 144). For treatments applied before drought was imposed, the treatment by variety interaction was significant (F = 4.91, $P = 0.001$) for chlorophyll contents measured post-drought. LaPaloma treated before drought with Blend 20 + 50% nitrogen and the full rate of nitrogen treatments had higher chlorophyll contents than Yukon grasses with the same treatments ($P \leq 0.0254$; Table 5.3). Chlorophyll contents of LaPaloma treated before and after drought with either 50% nitrogen or Blend 20 + 50% nitrogen had significantly greater chlorophyll contents

than Yukon grasses that received the same pre- and post-drought treatments ($P \leq 0.0012$; Table 5.4).

As expected, EL was low (< 10%) for all treatment before drought was imposed (Figures 5.5-5.6). Electrolyte leakage increased for all treatments as the duration of drought increased. At the end of the drought period, EL was > 60% for non-treated control grasses of either variety, and 29.4% for the full rate of nitrogen in both grass varieties. During the drought period, Yukon experienced greater EL than LaPaloma grasses (MANOVA, repeated measures; F = 20.58, $P < 0.0001$, df = 1, 70). During the drought period, there were significant treatment differences (F = 3.06, $P = 0.0222$ df = 4, 70) as well as a treatment by variety interaction (F = 21.64, $P < 0.0001$, df = 4,70). Among the treatments, grasses fertilized with the full rate of nitrogen had less EL during drought than the non-treated controls, Blend 20 + 50% nitrogen, and 50% nitrogen treatments ($P \leq 0.0247$; Table 5.3). However, the low EL in grasses treated with the full rate of nitrogen was not different from Blend 20 applied in either grass variety (Table 5.3). Yukon non-treated controls, and Yukon treated with 50% nitrogen or full rate of nitrogen have greater EL than LaPaloma treated with the same treatments.

Post drought, as grasses recovered, significant differences in EL were observed relative to treatment (F = 18.17, $P < 0.0001$ df = 4,70), variety (F = 9.04, $P = 0.0037$, df = 1, 70). LaPaloma experienced greater EL than Yukon but this difference was mainly observed in the first week of the post-drought period (Figures 5.5-5.6). Non-treated control grasses and grasses treated with Blend 20 +50% nitrogen experienced more EL than Blend 20, 50% nitrogen, and the full rate of nitrogen treatments ($P \leq 0.02$; Table 5.3). The rates of nitrogen did not differ in EL but grasses treated with Blend 20 had significantly more EL than one receiving 50% nitrogen ($P = 0.0307$).

There was a significant treatment by variety interaction (F = 23.09, $P < 0.0001$, df = 4, 70; Table 5.3) but there was no interaction between treatments applied pre- and post-drought (F = 1.96, $P = 0.1657$, df = 1,70; Table 5.4). Yukon non-treated control grasses had lower EL than LaPaloma non-treated controls during recovery (Table 5.3; Figures 5.5-5.6). LaPaloma treated with the full rate of nitrogen had less EL than Yukon receiving the same treatment. The effect of Blend 20 was consistent across both grass varieties.

Roots were sampled after grasses were subjected to drought and recovery. Root fresh weights were significant for treatment (F = 16.42, $P < 0.0001$, df = 4) and treatment by variety interaction (F = 10.81, $P < 0.0001$), but not for variety (F = 3.51, $P = 0.065$, df = 1). Root fresh weights were not affected by the reapplication or withholding of treatments post drought (F = 1.18, $P = 0.3220$). The non-treated controls had the lowest fresh weights for each variety. Treatment of Yukon with Blend 20 produced significantly more root fresh weight than all other treatments ($P \leq 0.0309$) and the full rate of nitrogen produced the greatest root fresh weight in LaPaloma (Table 5.5). Blend 20 + 50% nitrogen and Blend 20 applied to LaPaloma produced root fresh weights similar to the full rate of nitrogen applied to LaPaloma.

Roots dry weights were significant for treatment and treatment by variety interaction (F = 9.57, $P < 0.0001$; F = 10.86, $P < 0.0001$), but were not for variety (F = 1.64, $P = 0.2043$). Root dry weights of grasses re-treated post-drought were significantly greater than grasses not receiving post-drought treatments (3.23 g vs 3.03 g; F = 2.51; $P = 0.0332$). Yukon bermudagrass treated with Blend 20 and LaPaloma treated with either Blend 20 + 50% Nitrogen or the full rate of nitrogen produced the greatest root dry weights and were not different from each other (Table 5.5). Interestingly, LaPaloma treated with Blend 20 did not differ from the non-treated controls.

Root lengths were significant for treatment (F = 9.0, $P < 0.0001$), variety (F = 10.9, $P = 0.0015$), and treatment by variety interaction (F = 10.07, $P < 0.0001$). Root lengths were not affected by the reapplication or withholding of treatments post drought (F = 0.61, $P = 0.6945$). LaPaloma produced greater total average root lengths (2,782.1 cm) that were 117% longer than Yukon roots (2,385.7 cm). Root lengths were the lowest in the non-treated control grasses of each variety. LaPaloma bermudagrass treated with Blend 20 + 50% nitrogen produced greater root lengths than all treatments ($P \leq 0.0013$, Table 5.5). Yukon treated with Blend 20 produced greater root lengths than the Yukon control ($P \leq 0.0342$) but similar root lengths to LaPaloma treated with Blend 20 ($P = 0.2642$).

4. Discussion

Drought and water scarcity are major challenges facing crop production and protection. Types of drought encountered in the United States vary by region, ranging from persistent to cyclic threats. Historically, the southeastern United States experiences shorter drought periods, lasting weeks to a few years (Seager et al. 2009). However, due to commonality of sandy soils in the region, short summer drought periods are often severe due to the limited water holding capacities of soils and high temperatures. Recent droughts in the region occurred from 1998-2002, 2006-2009, and 2016-2017, resulting in crop losses in the billions (Manuel et al. 2008, Seager et al. 2009, Williams et al. 2017). Microbial biofertilizers benefit crop and soil qualities, and their influence may be more evident in sandy soils, as the microbial communities readily produce available C-sources and stabilize soil aggregates (Kiem and Kandeler 1997). Soil structure and rhizosphere microbial communities evolve over time and can be managed to

enhance nutrient cycling and water availability (Bronick and Lal 2004, Shi et al. 2006). Research was designed for this region to determine if PGPR biofertilization of bermudagrasses could alter plant responses to drought by measuring RWC, chlorophyll content, EL, and root growth.

Water requirements of turfgrasses vary by species and variety, function, soil texture, and climate (Kopp and Jiang 2013). When soil water content is depleted or insufficient, turfgrasses deal with water stress from whole plant to molecular responses by altering shoot and root growth, water use, photosynthetic rates, or nutrient and structural changes (Fry and Huang 2004, Hu et al. 2009). The environmental conditions potted hybrid bermudagrasses were exposed to in the greenhouse represented a short, severe drought. Bermudagrasses were impacted by a restricted rooting depth and the limited water holding capacity of sand. Turfgrasses subjected to 3 wk of drought stress in either the 2015 Tifway or 2017 LaPaloma and Yukon trials resulted in significant declines in quality, as grass stands thinned, decreased leaf water and chlorophyll contents, and increased EL. In both trials, no treatment prevented the bermudagrass treatments from utilizing the escape drought strategy, as all grasses were dormant by day 21 (see supplemental materials at the end of the chapter documenting drought responses). Future studies could shorten the drought duration, replenish, or maintain grasses at or below the permanent wilting point. However, the time it took for grasses to go dormant varied by treatment and cultivar. Grasses under water deficit stress may be in different physiological, metabolic, or biochemical states (Kopp and Jiang 2013), as was indicated by treatment and variety differences in RWC, chlorophyll content, and EL observed during and post-drought. Grass survival for all treatments in both Trials was 100%, but speed of recovery varied by treatments and variety.

Nitrogen, water, and pests are the main factors limiting turfgrass growth and productivity. Turfgrasses with insufficient nitrogen experience a more rapid decline than properly fertilized grasses (Kopp and Jiang 2013). While nitrogen-fertilized and control grasses responded similarly in the 2015 Tifway Trial, control grasses of LaPaloma and Yukon were typically outperformed by PGPR and nitrogen treatments in 2017. Nitrogen was determined to be impactful on plant quality, helping bermudagrass during and post-drought. Nitrogen was critical for increasing root depth, chlorophyll content, and reducing cell membrane disruption, as indicated by lower EL.

Relative water content of leaves is an indicator of how a plant's tissue is handling water status under dehydration (Abraham et al. 2004). Typically, plants that maintain higher RWC are assumed to be maintaining normal plant functions over plants that decrease RWC (DaCosta and Hunag 2007, Hu et al. 2010, Merewitz et al. 2010, Du et al. 2012, Shi et al. 2012). However, do to the unpredictability of drought, it may be beneficial in grasses to adjust leaf water content to lower levels to ensure grass survival, but that strategy may result in drought escape responses. Previous work with PGPR has shown increased RWC during drought with sunflower (*Helianthus annuus* L.) and henbane (*Hyoscyamus niger* L.) (Castillo et al. 2013, Ghorbanpour et al. 2013). PGPR treatment of grass typically resulted in lower RWC than control and fertilized grasses during drought. Varietal differences in water contents between LaPaloma and Yukon during and post-drought could help further explain the differences in the drought responses between the grasses. Post-drought, the addition of water, not PGPR or nitrogen, was most impactful on restoring normal water balances. There were no differences between grasses retreated and non-treated, suggesting that prior application of treatments or combinations of them were responsible for why they performed better than non-treated grasses during recovery.

Maintaining green color is a desirable phenotype for drought stressed grasses, and previous experiments of PGPR have demonstrated increased chlorophyll content after colonization and during drought (Zhang et al. 2008, Grover et al. 2014). Measurements of chlorophyll content suggested that PGPR alone may (Tifway) or may not (LaPaloma and Yukon) increase chlorophyll content during drought, and that results may be dependent on variety. It is important to note that the chlorophyll measurements of grasses grown under greenhouse conditions were consistently 2-3 times lower than grasses grown under field conditions. The addition of nitrogen is the most important nutrient for increasing grass color. However, it is important to note that the PGPR + 50% nitrogen treatment may help increase chlorophyll content during drought. This treatment was comparable to the full rate of nitrogen and significantly higher than the 50% nitrogen treatment, suggesting the result was not just a fertilizer affect. The post-drought period suggested that grasses previously treated all recovered better than the non-treated control grasses. It also revealed varietal and recovery treatment differences as certain LaPaloma treatments outperformed Yukon treatments, and the re-treatment of LaPaloma and Yukon grasses maintained higher chlorophyll content than the same Yukon treatments.

Plants under water stress experience structural and metabolic changes causing cell membrane disruptions causing the leakage of solutes, that may continue post-drought even with rehydration (Hopkins 1995). Electrolyte leakage is a useful parameter to measure the level of disruption grasses experience under drought (Hu et al. 2010, Du et al. 2012, Shi et al 2012). The impact of PGPR on aboveground drought responses was most pronounced in EL. Grasses treated with fertilizer and Blend 20 experienced less disruption of cell membranes during and post-drought. Interestingly, varietal differences of EL were significant for control and fully fertilized

grasses, but these varietal differences were negated with Blend 20, 50% nitrogen, or Blend 20 + 50% nitrogen. While treatments experienced less EL in the post-drought interval, the retreatment or non-treatment of grass with Blend 20 or fertilizer did not produce differences in responses. These results suggest that the use of PGPR products could alleviate varietal differences between moderately tolerant and susceptible lines, and enhance drought tolerance. Future experiments should further evaluate EL, proline content, and soluble sugars of grasses treated with PGPR during and post drought.

Fundamental to turfgrass breeding for drought tolerance is increasing root length, depth, and surface area (Comas et al. 2013). PGPR, including Blend 20, have been shown to consistently increase root growth (Coy et al. 2014). The increases in root length and weights are useful indicators of whole plant stress responses (Kopp and Jiang 2013). While root lengths and weights of Blend 20 grasses were not always significantly greater in all parameters, they were consistently numerically greater. The main benefit of rhizobacterial products is enhanced root growth, which increases the plants water uptake and scavenging abilities. In the LaPaloma and Yukon trials, the post-drought re-treatment was found to have a significant impact on grass recovery, as grasses re-treated had dry masses that were about 0.25 g greater than non-treated plants of the same treatment. PGPR and nitrogen are critical to increase plant growth during drought and recovery periods.

It is possible that the removal of tissue and new growth during the drought and post drought periods negatively impacted grass responses and recovery. However, all treatments were exposed to the same cultural practice of ‘mowing,’ which affects grass growth, physiology, and ability to tolerate stress (Fry and Huang 2004). The removal of new, green growth is necessary

for accurate measurements of RWC and EL, but the removal of this tissue, deprives the plant of newly formed cells with chloroplasts, chlorophyll, and stomata (Kopp and Jiang 2013). The loss of this tissue is likely to negatively impact cell membranes, photosynthesis, and plant nutrients and carbohydrates (Xu and Huang 2004). Future studies could benefit from increasing the recovery interval, time between cuttings, or increasing the height of cut of grasses during and post drought.

The treatment of bermudagrass with PGPR likely involved all three drought resistance strategies during the experiments. While the escape strategy was the most obvious, avoidance and tolerance were implanted through increases in root growth and weights, reductions in RWC and EL, and changes in chlorophyll contents during (Tifway) and post drought (Tifway, LaPaloma, Yukon) than non-treated control plants. Further, PGPR by itself or with nitrogen may alleviate varietal drought differences in bermudagrasses as indicated by negating varietal interactions of RWC, chlorophyll content, or EL during or post drought. In the 2015 Tifway Trial, Blend 20-treated grasses maintained lower RWC and higher chlorophyll content than the non-treated control and fertilized grass during and post drought. It is likely that the bacteria in Blend 20 do not represent the best candidate strains for alleviating drought symptoms in bermudagrass, but they do demonstrate that bacteria can mediate and alter grass responses to abiotic stress. The general alterations that PGPR have on grass stress responses are likely to be similar, but the impact is likely to be dependent on bacterial strain or blend identity, grass genotype, and environmental conditions. The drought of 2016 in the southeastern United States offered the opportunity to sample native and amenity grasses as well as weeds that maintained desirable physiologies during and post drought. Microbial communities of localized areas of

grasses that remained productive may have stronger associations or correlations with stress tolerance and explain why some grasses remained green and actively growing. In all, over 600 bacterial isolates were recovered from these samples.

Turfgrass management benefits from applied research that aims to incorporate novel technologies that increase or enhance stress responses. As the demand for high quality turfgrass remains unchanged, but water resources decline, new management practices will need to be developed. The use of biological products, like PGPR that can be selectively applied may benefit management by manipulating turfgrass physiology or phenotype by maintaining desired characteristics under abiotic or biotic stress. Microbial manipulated plant responses may be achieved easier and faster with the use of PGPR than from the development of new germplasms that must be planted prior to drought. Further, future germplasms could incorporate the benefits demonstrated by microbes to enhance drought tolerance or resistance.

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Table 5.1. All orthogonal contrasts of non-treated, rhizobacteria-treated, and nitrogen-fertilized Tifway bermudagrass drought stress responses during 3 wk of drought and 5 wk of drought recovery

Comparisons[a]	RWC[b] Drought	RWC Recovery	Chlorophyll[c] Drought	Chlorophyll Recovery
Blend 20* vs Control	*P* = 0.111	*P* = 0.2628	*P* = 0.0005	*P* = 0.0004
Blend 20* vs Nitrogen	*P* = 0.0012	*P* =0.0658	*P* = 0.001	*P* = 0.0004
Blend 20* vs Control and Nitrogen	*P* = 0.0051	*P* = 0.0895	*P* = 0.0002	*P* = 0.0001
Control vs Nitrogen	*P* = 0.0474	*P* = 0.558	*P* = 0.64	*P* = 0. 7794

* denotes which treatment was significantly different between treatments from orthogonal contrasts ($P < 0.05$, df = 2, 15; JMP Version 13. SAS Institute Inc.)

[a]Comparisons between treatments were orthogonally contrasted using multivariate analysis of variance (MANOVA), repeated measures

[b]Relative water content (RWC) as determined by leaf fresh, turgid, and dry weights

[c]Plant chlorophyll content was determined using a FieldScout CM 1000 NDVI Chlorophyll meter

Table 5.2. All orthogonal contrasts of treatments of LaPaloma and Yukon bermudagrasses responses during 3 wk of drought and 3 wk of drought recovery

Comparisons[a]	RWC[b] Drought	RWC Recovery	Chlorophyll[c] Drought	Chlorophyll Recovery	EL[d] Drought	EL Recovery
Control vs Blend 20*#$	$P = 0.1756$	$P = 0.0005$	$P = 0.4898$	$P = 0.0017$	$P = 0.5635$	$P < 0.0001$
Control vs Blend 20 + 50 % Nitrogen*#$	$P = 0.1546$	$P < 0.0001$	$P < 0.0001$	$P = 0.0047$	$P = 0.7346$	$P = 0.1475$
Control vs Nitrogen*#$	$P = 0.1602$	$P < 0.0001$	$P < 0.0001$	$P = 0.0268$	$P = 0.0247$	$P < 0.0001$
Control vs 50% Nitrogen*#$	$P = 0.0267$	$P < 0.0001$	$P < 0.0001$	$P = 0.0166$	$P = 0.3471$	$P < 0.0001$
Blend 20 vs Blend 20 + 50% Nitrogen*#$	$P = 0.0065$	$P = 0.0004$	$P < 0.0001$	$P = 0.7239$	$P = 0.3604$	$P = 0.0021$
Blend 20 vs Nitrogen*#$	$P = 0.9592$	$P = 0.0149$	$P < 0.0001$	$P = 0.2968$	$P = 0.0908$	$P = 0.0984$
Blend 20$ vs 50% Nitrogen*#	$P = 0.374$	$P = 0.0009$	$P < 0.0001$	$P = 0.4001$	$P = 0.1313$	$P = 0.0307$
Blend 20 + 50% Nitrogen* vs Nitrogen$	$P = 0.0056$	$P = 0.2164$	$P = 0.1451$	$P = 0.4894$	$P = 0.0103$	$P < 0.0001$
Blend 20 + 50 % Nitrogen*# vs 50% Nitrogen$	$P = 0.0004$	$P = 0.7846$	$P = 0.0076$	$P = 0.6249$	$P = 0.5463$	$P < 0.0001$
Nitrogen#$ vs 50% Nitrogen	$P = 0.4019$	$P = 0.334$	$P < 0.0001$	$P = 0.8394$	$P = 0.0018$	$P = 0.5979$

*, #, $ denotes which treatment was significantly different between treatments of relative water content, chlorophyll content, and electrolyte leakage from orthogonal contrasts ($P < 0.05$; JMP Version 13. SAS Institute Inc.

[a]Comparisons between treatments were orthogonally contrasted using multivariate analysis of variance (MANOVA), repeated measures

[b]Relative water content (RWC) as determined by leaf fresh, turgid, and dry weights (df = 4, 70)

[c]Plant chlorophyll content was determined using a FieldScout CM 1000 NDVI Chlorophyll meter (df = 4, 144)

[d]Electrolyte leakage was determined using a YSI 3200 conductivity instrument (df = 4, 70)

Table 5.3. All orthogonal contrasts of treatments by variety of LaPaloma and Yukon bermudagrasses responses during 3 wk of drought and 3 wk of drought recovery

Comparisons	RWC[b] Drought	RWC Recovery	Chlorophyll[c] Drought	Chlorophyll Recovery	EL[d] Drought	EL Recovery
LaPaloma Control*# vs Yukon Control$	$P = 0.5286$	$P = 0.0022$	$P = 0.0001$	$P = 0.1619$	$P < 0.0001$	$P < 0.0001$
LaPaloma Blend 20# vs Yukon Blend 20*	$P = 0.9949$	$P < 0.0001$	$P = 0.0016$	$P = 0.1649$	$P = 0.7137$	$P = 0.1115$
LaPaloma Blend 20 + 50% Nitrogen*# vs Yukon Blend 20 + 50% Nitrogen	$P = 0.0099$	$P = 0.4293$	$P = 0.5348$	$P = 0.0062$	$P = 0.1712$	$P = 0.1563$
LaPaloma Nitrogen# vs Yukon Nitrogen$	$P = 0.3618$	$P = 0.5267$	$P = 0.747$	$P = 0.0254$	$P < 0.0001$	$P = 0.0133$
LaPaloma 50% Nitrogen*# vs Yukon 50% Nitrogen$	$P = 0.0071$	$P = 0.9352$	$P < 0.0001$	$P = 0.0867$	$P < 0.0001$	$P = 0.6833$

*, #, $ denotes which treatment was significantly different between treatments of relative water content, chlorophyll content, and electrolyte leakage from orthogonal contrasts ($P < 0.05$; JMP Version 13. SAS Institute Inc.)

[a]Comparisons between treatments were orthogonally contrasted using multivariate analysis of variance (MANOVA), repeated measures

[b]Relative water content (RWC) as determined by leaf fresh, turgid, and dry weights (df = 4, 70)

[c]Plant chlorophyll content was determined using a FieldScout CM 1000 NDVI Chlorophyll meter (df = 4, 144)

[d]Electrolyte leakage was determined using a YSI 3200 conductivity instrument (df = 4, 70)

Table 5.4. All orthogonal contrasts of treatments by recovery of LaPaloma and Yukon bermudagrasses responses during 3 wk of drought recovery

Comparisons	RWC Recovery	Chlorophyll Recovery	EL Recovery
Retreated Control vs Non-treated Control	$P = 0.5716$	$P = 0.9000$	$P = 0.5986$
Retreated Blend 20 vs Non-treated Blend 20	$P = 0.5507$	$P = 0.1306$	$P = 0.5254$
Retreated Blend 20 + 50% Nitrogen* vs Non-treated Blend 20 + 50% Nitrogen	$P = 0.9402$	$P = 0.0012$	$P = 0.0045$
Retreated Nitrogen vs Non-treated Nitrogen	$P = 0.0727$	$P = 0.5964$	$P = 0.6458$
Retreated 50% Nitrogen* vs Non-treated 50% Nitrogen	$P = 0.7513$	$P = 0.0002$	$P = 0.6132$
All treatments* vs Controls	$P < 0.0001$	$P = 0.0004$	$P = 0.0003$
Control vs Blend 20*	$P = 0.0128$	$P = 0.0006$	$P = 0.0053$
Control vs Blend 20 + 50% Nitrogen*	$P < 0.0001$	$P = 0.0014$	$P = 0.1032$
Control vs Nitrogen*	$P = 0.0002$	$P = 0.0119$	$P = 0.0007$
Control vs 50% Nitrogen*	$P < 0.0001$	$P = 0.0091$	$P = 0.0002$

* denotes which treatment was significantly different between treatments from orthogonal contrasts ($P < 0.05$; JMP Version 13. SAS Institute Inc.)

[a]Comparisons between treatments were orthogonally contrasted using multivariate analysis of variance (MANOVA), repeated measures

[b]Relative water content (RWC) as determined by leaf fresh, turgid, and dry weights (df = 4, 70)

[c]Plant chlorophyll content was determined using a FieldScout CM 1000 NDVI Chlorophyll meter (df = 4, 144)

[d]Electrolyte leakage was determined using a YSI 3200 conductivity instrument (df = 4, 70)

Table 5.5. Mean (±SEM) of bermudagrass cultivars root fresh weight, dry weight, and length after 3 wk drought and recovery under greenhouse conditions

Year	Variety	Treatment	Fresh Weight (g)	Dry Weight (g)	Length[a] (cm)
2015[b]	Tifway[d]	Control	6.07 ± 0.86a	0.38 ± 0.12b	642.62 ± 86.61a
2015	Tifway	Blend 20[f]	8.91 ± 0.86a	1.36 ± 0.15a	690.85 ± 133.33a
2015	Tifway	Nitrogen[g]	7.76 ± 1.09a	0.61 ± 0.06b	881.21 ± 177.28a
		Statistics	F = 2.29, *P* = 0.1359	F = 19.22, *P* < 0.0001	F = 0.84, *P* = 0.4503
2017[c]	LaPaloma[e]	Control	14.52 ± 1.32e	2.07 ± 0.22f	2,495.06 ± 202.90bc
2017	LaPaloma	Blend 20[f]	20.84 ± 0.86bcd	2.34 ± 0.14ef	2,615.68 ± 174.49bc
2017	LaPaloma	Blend 20 + 50% Nitrogen[h]	23.00 ± 1.07bc	4.14 ± 0.21a	3,791.53 ± 198.02a
2017	LaPaloma	Nitrogen[i]	23.97 ± 1.75b	3.74 ± 0.29ab	2,664.00 ± 181.77bc
2017	LaPaloma	50% Nitrogen[j]	18.55 ± 1.42d	2.85 ± 0.19cde	2,344.04 ± 165.21c
2017	Yukon[e]	Control	13.90 ± 1.24e	2.42 ± 0.17ef	1,371.78 ± 1130.64d
2017	Yukon	Blend 20	28.34 ± 2.01a	4.38 ± 0.36a	2,914.60 ± 142.90b
2017	Yukon	Blend 20 + 50% Nitrogen	19.13 ± 1.04cd	3.42 ± 0.17bc	2,430.56 ± 223.88bc
2017	Yukon	Nitrogen	17.32 ± 1.09de	3.25 ± 0.24bcd	2,657.99 ± 176.80bc
2017	Yukon	50% Nitrogen	14.27 ± 1.30e	2.71 ± 0.27def	2,553.71 ± 183.62bc
		Statistics	*P* < 0.0001	*P* < 0.0001	*P* < 0.0001

Letters connected by the same letter are not significantly different from each other. Analysis of variance (ANOVA), Student's *t*-test ($P < 0.05$; 2015, df = 2, 17; 2017 df = 9, 159; JMP Version 13. SAS Institute Inc.)

[a]Total root length (cm) as determined by digital image analysis using WinRhizo software

[b]2015 drought recovery was 5 wk

[c]2017 drought recovery was 3 wk

[d]Tifway was established from plugs (3.8 cm) and established for 3 wk and treated for 5 wk before drought initiation

[e]LaPaloma and Yukon were seeded at a rate of 14.65 g/ m^2 and established for 4 wk and treated for 5 wk before drought initiation

[f]Blend 20 (*Bacillus pumilus* AP 7, *Bacillus pumilus* AP 18, *Bacillus sphaericus* AP 282) applied weekly at a rate of 500 ml / m^2

[g]Ammonium sulfate applied weekly at a rate of 5.81 g/ m^2

[h]Blend 20 applied weekly with ammonium sulfate applied monthly at rate of 11.6 g / m^2

[i]Ammonium sulfate applied monthly at rate of 23.2 g / m^2

[j]Ammonium sulfate applied monthly at rate of 11.6 g / m^2

Figures

Figure 5.1. Mean (± SEM) relative water contents of non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses during 3 wk of drought (left) and drought recovery (right). Yukon bermudagrass (top) and LaPaloma bermudagrass (bottom).

Figure 5.2. Mean (± SEM) relative water contents of Yukon and LaPaloma bermudagrasses during drought and recovery. The experiment evaluated non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses.

Figure 5.3 Mean (± SEM) chlorophyll contents of non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses during 3 wk of drought (left) and drought recovery (right). Yukon bermudagrass (top) and LaPaloma bermudagrass (bottom).

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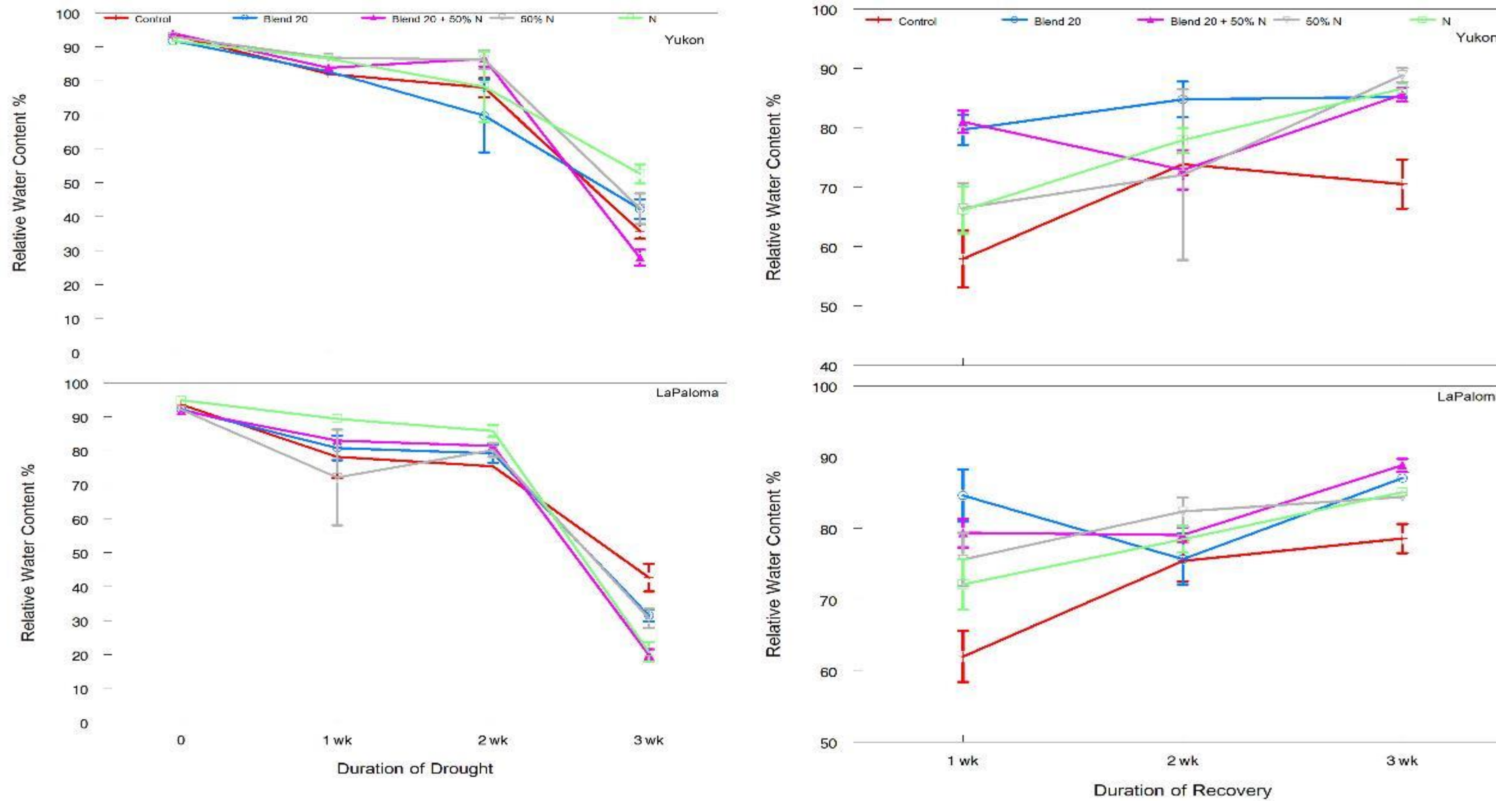
Figure 5.5. Mean (± SEM) electrolyte leakage of non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses during 3 wk of drought (left) and drought recovery (right). Yukon bermudagrass (top) and LaPaloma bermudagrass (bottom).

Figure 5.6. Mean (± SEM) electrolyte leakage of Yukon and LaPaloma bermudagrasses during drought and recovery. The experiment evaluated non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses.



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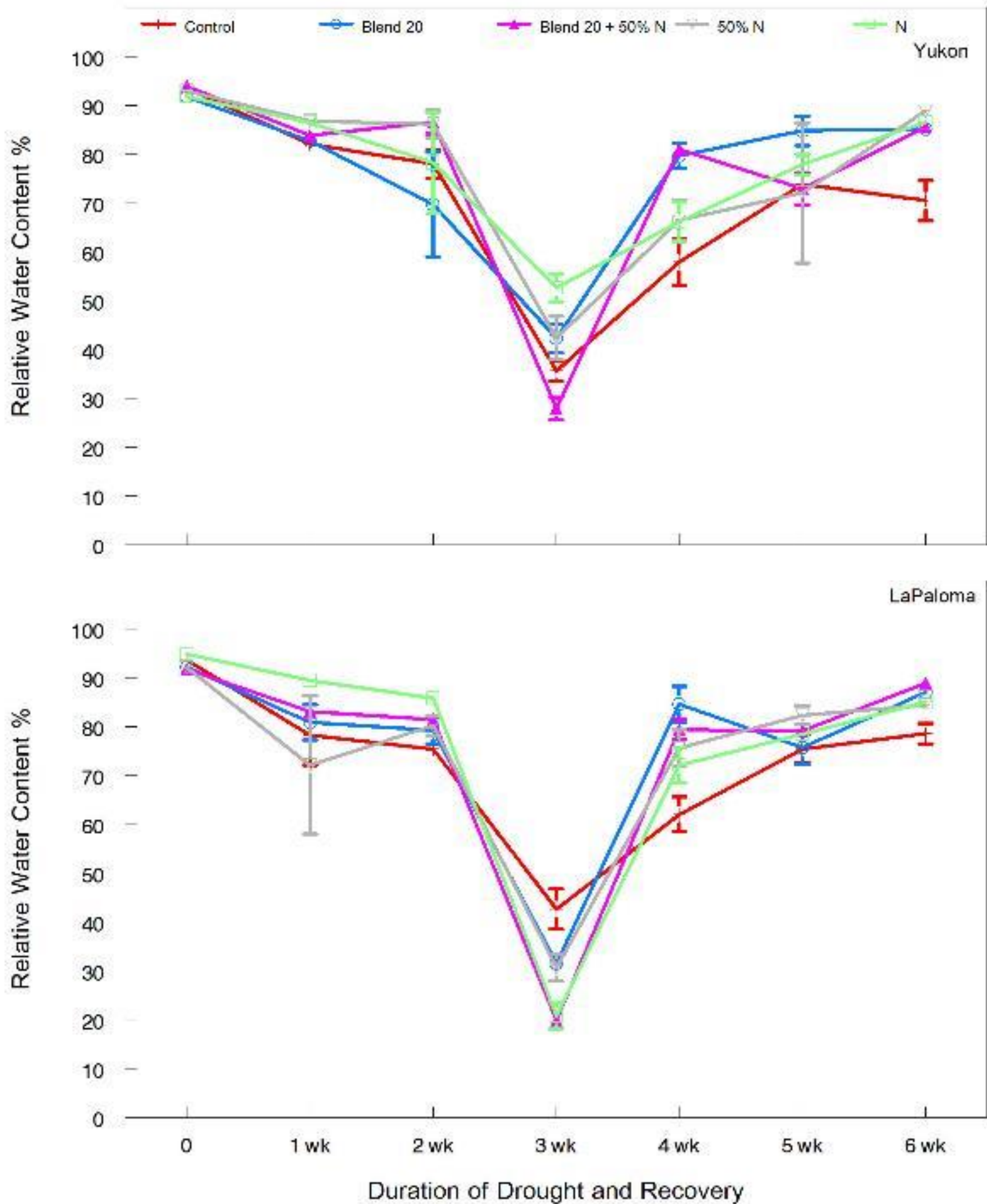

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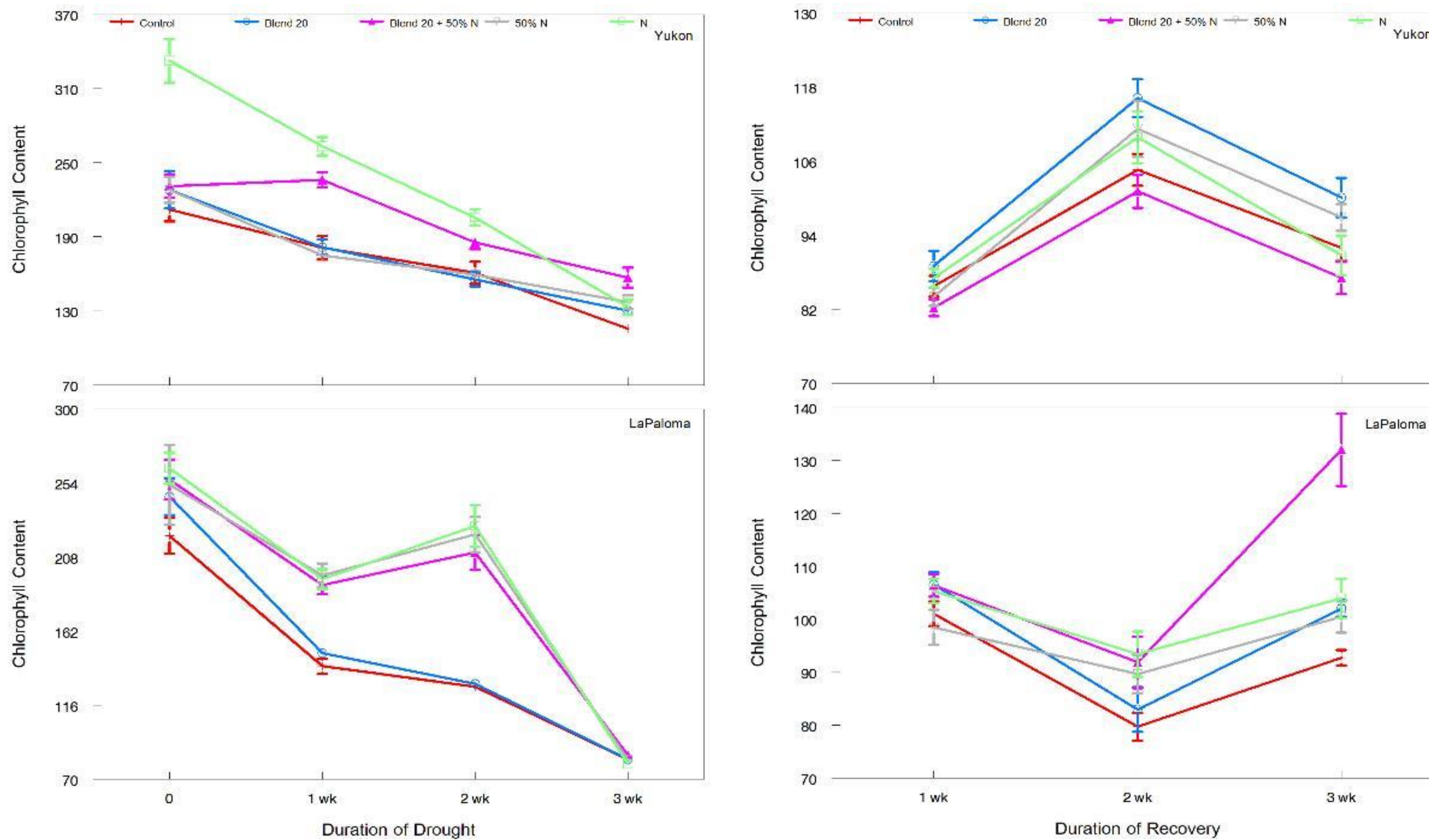
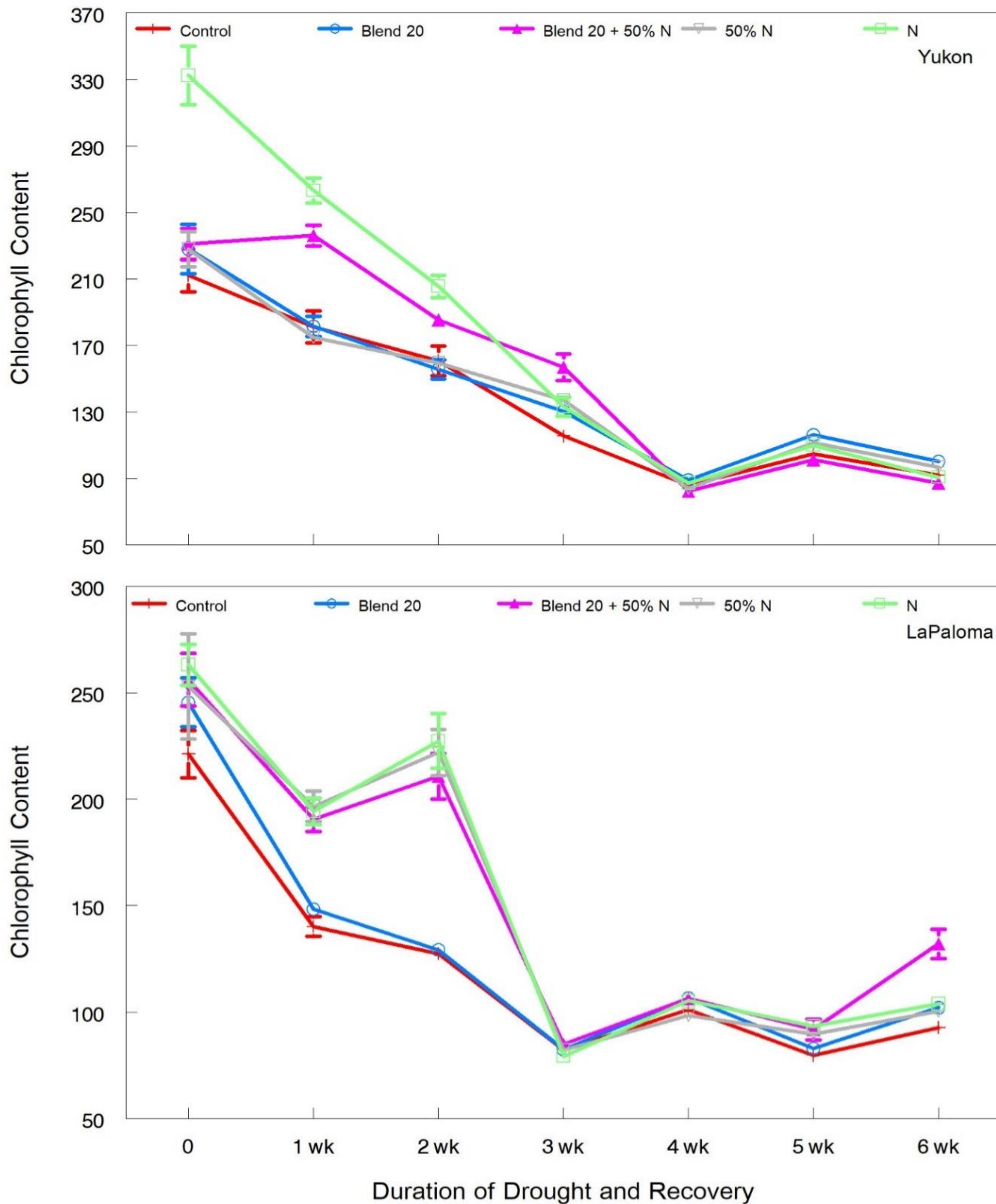


Figure 5.3 Mean (± SEM) chlorophyll contents of non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses during 3 wk of drought (left) and drought recovery (right). Yukon bermudagrass (top) and LaPaloma bermudagrass (bottom).



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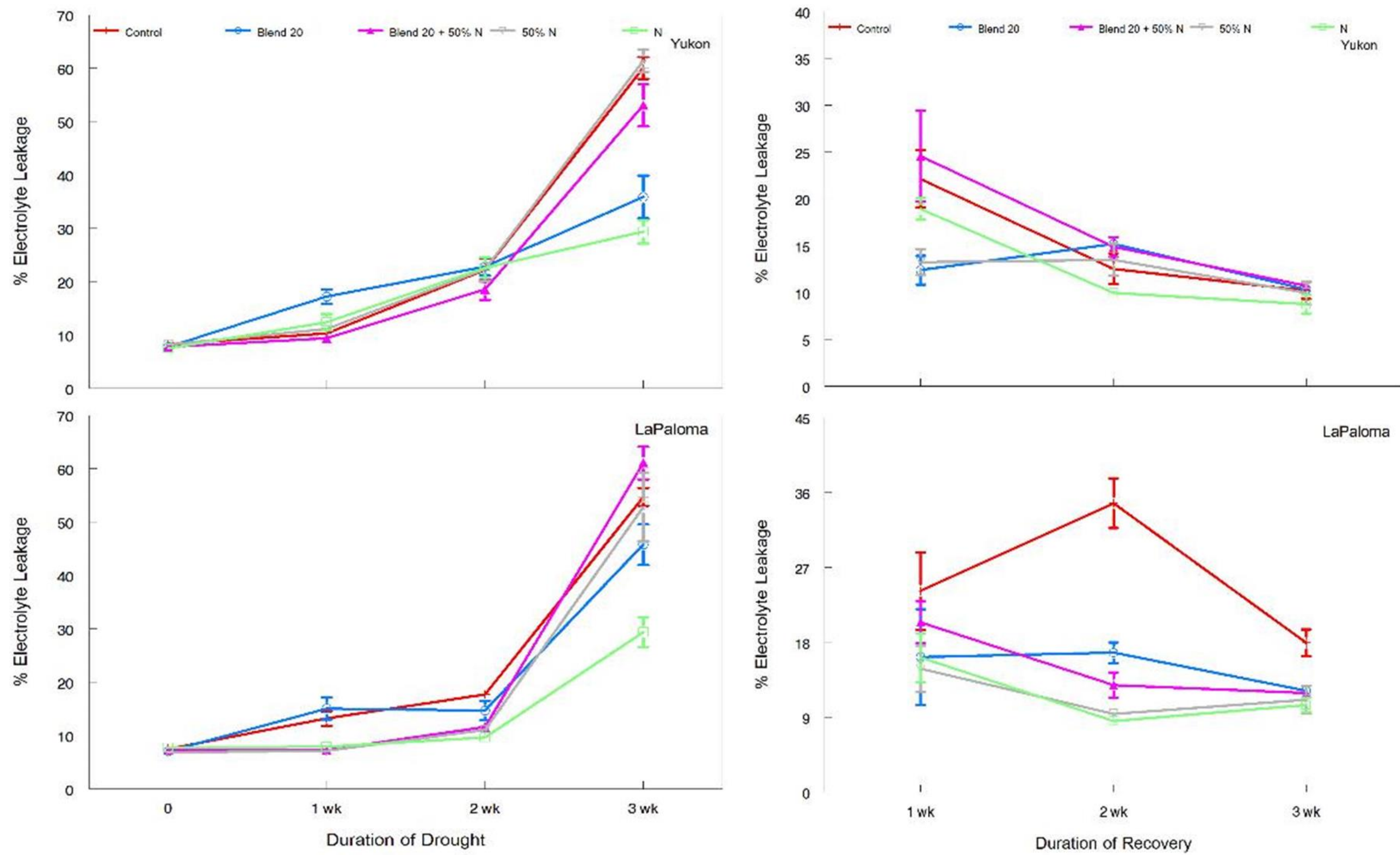

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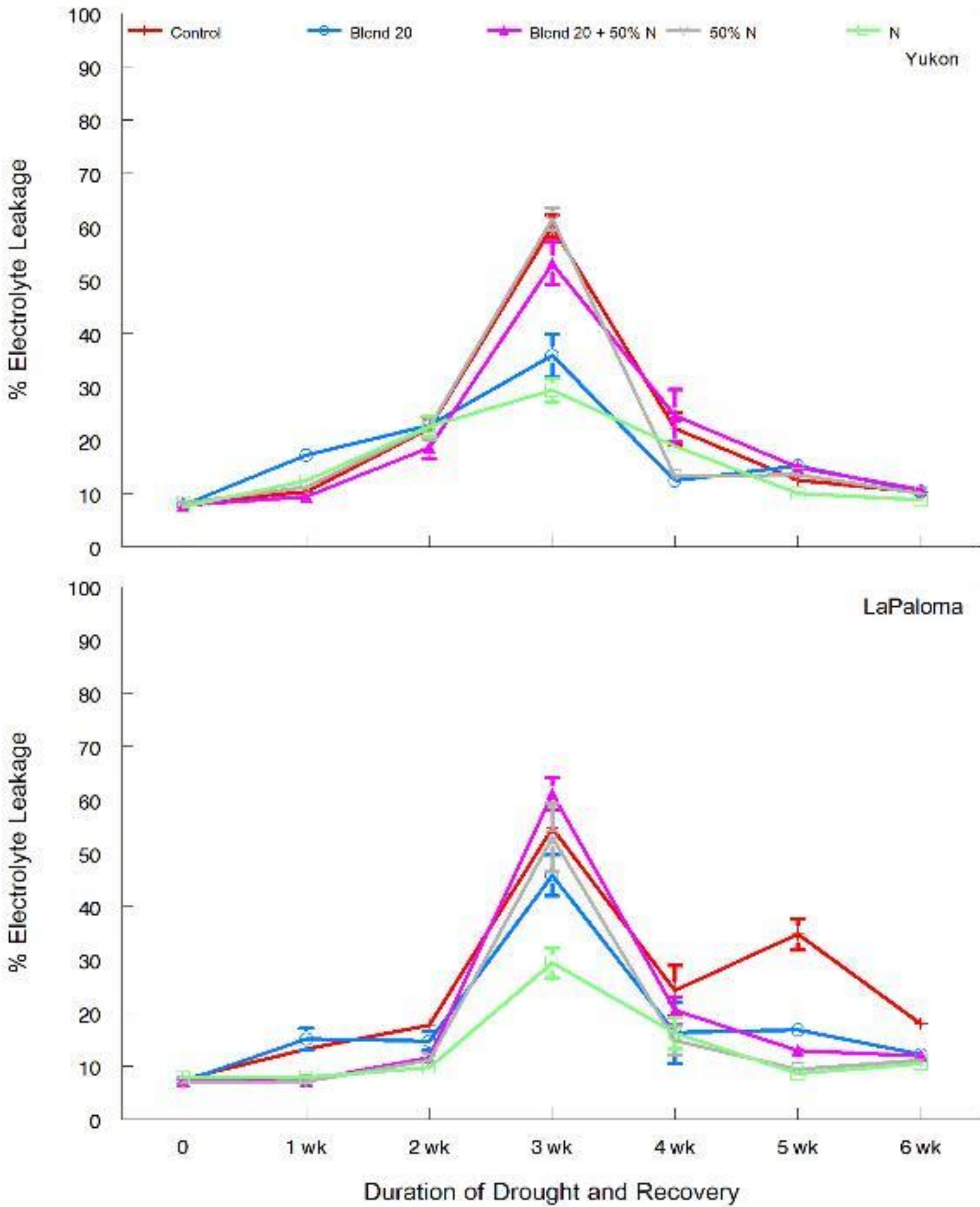
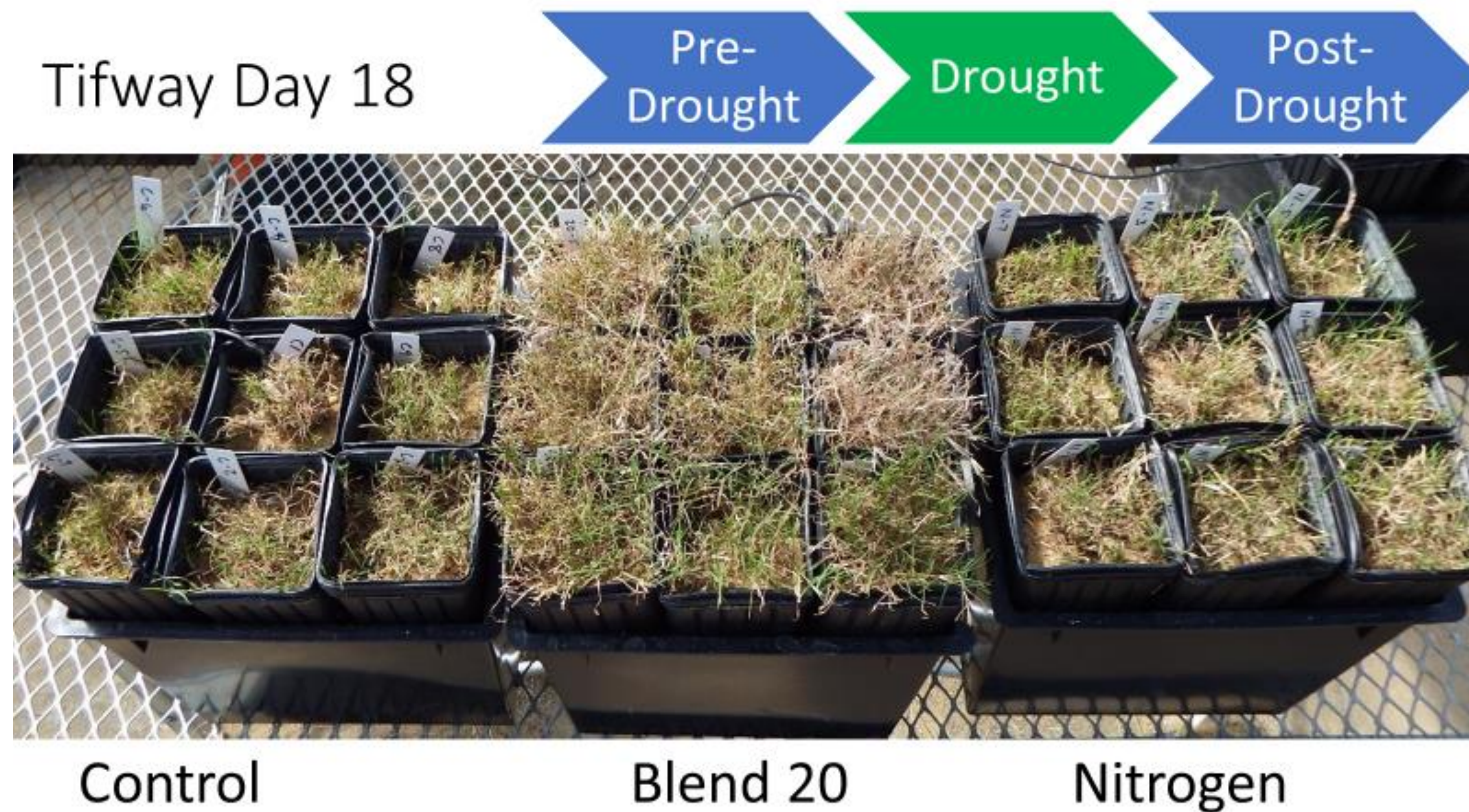


Figure 5.6. Mean (± SEM) electrolyte leakage of Yukon and LaPaloma bermudagrasses during drought and recovery. The experiment evaluated non-treated, Blend 20, Blend 20 + 50% Nitrogen, Nitrogen, and 50% Nitrogen bermudagrasses.

Supplemental materials: Pictures of bermudagrass during drought

Supplemental materials Figure 5.1: Shows the 2015 Tifway Drought Experiment and treatment differences between non-treated, bacteria-treated, and nitrogen fertilized grasses. Grasses treated with Blend 20 retained color and stands do not think as drastically as the control and fertilized grasses.



Supplemental materials Figure 5.2. Shows that by day 21 of the experiment all grasses had gone dormant.

Tifway Day 21

Control Blend 20 Nitrogen

Supplemental materials Figure 5.3: Shows the recovery of Tifway bermudagrass 17 d after water and treatments were reapplied.

Tifway Day 38

Control Blend 20 Nitrogen

Supplemental materials Figure 5.4: Shows the recovery Tifway bermudagrass at the conclusion of the experiment.

Day 56

Nitrogen Blend 20 Control

Supplemental materials Figure 5.5: Shows the 2017 LaPaloma and Yukon drought experiment after 1 wk of drought.

Supplemental materials Figure 5.6: Shows the 2017 LaPaloma and Yukon drought experiment after 2 wk of drought.

Supplemental materials Figure 5.7: Shows the 2017 LaPaloma and Yukon drought experiment after 3 wk of drought.

Supplemental materials Figure 5.8: Shows the 2017 LaPaloma and Yukon drought experiment after 3 wk of drought and 1 wk of recovery.

During the recovery period, the front 8 pots of each treatment did not receive a reapplication of the pre-drought treatment, but the back 8 pots were re-treated with their respective pre-drought treatment.

Supplemental materials Figure 5.9: Shows the 2017 LaPaloma and Yukon drought experiment after 3 wk of drought and 2 wk of recovery.

During the recovery period, the front 8 pots of each treatment did not receive a reapplication of the pre-drought treatment, but the back 8 pots were re-treated with their respective pre-drought treatment.

Supplemental materials Figure 5.10: Shows the 2017 LaPaloma and Yukon drought experiment after 3 wk of drought and 3 wk of recovery.

During the recovery period, the front 8 pots of each treatment did not receive a reapplication of the pre-drought treatment, but the back 8 pots were re-treated with their respective pre-drought treatment.

Supplemental materials Figure 5.11: Shows the 2017 LaPaloma and Yukon drought experiment after 3 wk of drought and 3 wk of recovery.

LaPaloma re-treated with PGPR + 50% Nitrogen visually demonstrated the best drought recovery and re-greening during the experiment.

Control Blend 20 + 50% Nitrogen Nitrogen

Chapter 6: Summary and future research directions

1. Summary

Since 2011, our knowledge of interactions of PGPR with turfgrass, insects, and drought stress has vastly grown. During my Master's Thesis, we identified blends of rhizobacteria that did and did not result in growth promotion of Tifway bermudagrass. This lead to the conclusion that results of PGPR with crops, including grasses is largely context dependent and that strain and blend composition is highly important for consistent results. Further, it is highly likely that some PGPR blends may result in more root or shoot growth, or vice versa, suggesting that the PGPR used should be tailored to desired outcome. The main benefit of Blend 20 was increased root growth, with occasional increases in top growth. Depending on the turfgrass and purpose, it may be more desirable for increased root growth over shoot growth (sports turf) or shoot growth over roots (pasture). This may require more research efforts to identify more candidate strains and blends. While growth promotion was previously observed, mechanisms for growth promotion were not fully known or understood. The identification of beneficial bacterial characteristics such as qualitative nitrogenase, quantitative phosphate solubilization, and siderophore production provided reasonable explanations for growth benefits. Future research should aim to identify quantitative nitrogenases and indole-3-acetic-acid (IAA) activity that may provide further explanations of growth-promotion and stress mitigation.

Results from the rifampicin rhizobacterial colonization work demonstrated the rapid colonization and persistence of the rhizosphere, rhizoplane, and endophytic populations of *Bacillus* spp. in bermudagrass in a loamy sand. This research was likely the first to demonstrate endophytic bacterial colonization in bermudagrass and may benefit turfgrass management by forming more precise application intervals for PGPR products. While colonization data are now

available for *Bacillus* spp. in bermudagrass in a loamy sand soil, these data may not be directly applicable to other soil types, as Durham (2013) noted differences in colonization based on crop and soil type. More research will be needed in different soil conditions and even nutrient management practices to fully understand colonization and persistence in grasses. While it may be easier from a production standpoint to rely on one strain, this may be a risky approach to rely on for all soil conditions, and could explain product failures in the field.

Previous work from my Master's Thesis (Coy 2014) and Coy et al. (2017) noted minimal impacts of PGPR on insect folivores; however, more meaningful relationships were observed with root-feeding herbivores, from increased root growth. The PGPR tested were not found to be insecticidal or detrimental, even with direct topical applications to white grubs. While the use of certain PGPR blends may deter fall armyworm oviposition or negatively impact larval and pupal weights, which in turn mat impact adult eclosion, these interactions are likely not significant for fall armyworm management purposes and nearly impossible to implement on a large scale for generalist moths. However, the use of PGPR to disrupt or deter oviposition by selective, specialist moths, like the black cutworm (*Agrotis ipsilon* Hufnagel) may be more impactful in preventing oviposition and later infestation of grasses (e.g., bentgrass) and crops. The only meaningful investigation with generalist folivores may be feeding assays to determine if a strain negatively impacts development or is a feeding deterrent. There are several candidate strains in the DH collection that could serve this purpose; however, I do not feel like that is a high-level research priority. Even with deterred oviposition, which is a very interesting ecological observance, females will still lay eggs and larvae will likely hatch near a host plant, negating its management significance. While some theorize that PGPR treatment of plants may alter the

attractiveness of plants to beneficial, parasitoid insects, based on my experience in turfgrass, I do not believe that to be true in managed, amenity grass. Over the course of several years of field evaluations, treatment of bermudagrass with PGPR did not increase the number of parasitoids in yellow pan traps, nor did it increase parasitism. In over 11,000 attempts of parasitism of fall armyworm larvae and eggs, only two instances of parasitism were observed. The lack of parasitoid attraction and parasitism could be influenced by management practices (mowing) as the grasses were typically cut three times per week. However, preliminary volatile work has not shown major differences between cut and uncut grasses. The amount of rhizobacterial strains that have insecticidal properties are likely restricted to certain species, with few and far between, as demonstrated by the relatively few strains previously identified. It has been proposed that insect interactions with PGPR could influence relationships with phloem and sap feeders, but I do not believe that to be a successful avenue with the presently used PGPR strains. Observations have been made on spittle bugs, rhodesgrass mealybugs, aphids, and spider mites feeding on bermudagrass under greenhouse and field conditions with no apparent impact on these arthropods. In my opinion, the most meaningful relationship of PGPR in turfgrass with herbivores is restricted to root-feeders like white grubs, mole crickets, and nematodes. While PGPR may or may not alter the acceptance or palatability of the plant to insect pests, the increased root growth from PGPR may help the plant to outgrow or better tolerate root-herbivory. Until there is further identification of bacterial strains that have direct, negative impact insects, relationships should focus on increasing tolerance to root-feeders, insecticide compatibility and uptake. It would be very interesting to know if the PGPR in Blend 20 or the other PGPR libraries increase pesticide uptake and efficiency of systemic pesticides like

insecticides or fungicides. Experiments could easily be modeled after Myresiostis *et al.* (2015) and would be very useful in understanding PGPR-pesticide interactions for future product development.

I believe the most significant interaction of PGPR identified in this work was with abiotic stress, specifically drought stress. Blend 20 is likely not the best PGPR for drought stress, but it served as a great model for developing preliminary methods and identifying meaningful interactions to investigate. Drought experiments designed for this research and other observational studies suggest Blend 20 plays a role in drought responses, but the drought response observed is not always consistent. This may be due to time of year, environmental conditions, or colonization. In our drought evaluations, no treatment prevented the grasses from using the escape drought strategy by 3 wk. Since these experiments were conducted under a greenhouse setting, it may be more useful to decrease the drought length or to alter the drought severity. Withholding water in a restricted depth pot for 3 wk likely compound or complicate the drought observances. It may be more insightful to withhold water for 1-2 wk and then maintain grasses at the permanent wilting point (PWP) or to selectively add specific amounts of water based on ET rates to observe plant water regulations under limited water settings (field conditions). Further, if the goal of PGPR inoculation is to increase drought tolerance, maintaining some type of soil moisture treatment differences may be more pronounced and yield results faster. Once candidate strains and blends are identified, the experiments need to be replicated under field conditions.

The drought of the summer of 2016 provided a great opportunity to sample and isolate bacteria from drought stressed amenity and native grasses and weeds. During the drought and

drought recovery period, over 600 bacterial strains were isolated from bermudagrass, zoysia, St. Augustine, centipede, bahia, johnsongrass, muhly grass, sea oats, and crabgrass that maintained color and productivity. The use of PGPR offers the advantage of being able to selectively apply before or in response to stress. While many PGPR can offer plant benefits, it is highly probable that blends tailored to a specific response will have greater utility, than a blanket, generic product. Considering the duration of the drought, and novelty of the approach of selecting grasses with desirable phenotypes, this work may identify beneficial strains easier than traditional or molecular breeding programs. I truly believe that DH collection will identify bacterial strains linked to drought stress that increase drought tolerance and that are superior to Blend 20. Using methods previously developed and modified since Coy et al. (2014), the DH collection could be screened under growth chamber conditions to speed up initial screening efforts to identify beneficial drought strains before greenhouse and field evaluations. Due to the cost, it is likely more cost effective to first screen and then identify successful strains after proof of concept.

2. Future research directions

Coy et al. (2014) demonstrated the successful colonization of PGPR of Tifway bermudagrass that resulted in increased shoot and root growth, and supplemental studies have shown increased drought tolerance in bermudagrass with the use of bacterial inoculants. Subsequent literature searches linking growth-promotion or drought mitigation to PGPR in bermudagrass have not been successful. Building on previous work with Blend 20 and new strains DH 25, 27, 32, 35, 37, and 44 the objectives of these studies are to 1) identify and

characterize bacterial strains related to drought mitigation, 2) evaluate turf and forage bermudagrasses for growth-promotion and drought tolerance, 3) determine transcriptome level changes in PGPR inoculated bermudagrass under drought conditions.

Overall Hypothesis

We hypothesize that certain PGPR blends and strains can offer plants enhanced drought tolerances by altering plant responses and inducing changes in key plant pathways, noted previously in *Cynodon* to abiotic stress. Drought tolerance in turfgrass has been the subject of several studies considering changes in grass physiology as well as transcriptomic changes, with relatively few studies examining the role of PGPR in grass stress mitigation. Gagné-Bourque e*t al.* (2015) noted increased drought stress and mitigation with *Bacillus subtilis* in the model C_3 grass, *Brachypodium dactylon*. Grasses with rhizomous root systems, like bermudagrass are more likely to survive longer under periods of drought. Although more examples exist for cool-season grasses (fescue, ryegrass, and bluegrass), bermudagrass exhibits drought avoidance characteristics; increasing capacity for water uptake or reducing water loss. In general, drought stress in grasses results in a loss of soluble proteins except for the accumulation of stress-related proteins under drought conditions (Huang et al. 2014). Most gene expression studies have compared creeping bentgrass, a cool season turfgrass, to transformed lines overexpressing the *ipt* (isopentenyl transferase) gene (Merewitz et al. 2010a, 2010b, 2011). These studies implicate ABA and cytokinins as key hormones related to drought stress in grasses. Levels of these hormones do not differ for well-watered grasses but decrease significantly under drought

conditions in creeping bentgrass (Merewitz et al. 2010a). Transcriptomic data relative to other grasses, particularly bermudagrass, under drought conditions are severely lacking.

Objectives

1. Isolation, identification, and characterization of bacterial strains
2. Evaluation of PGPR and drought tolerance in turf and forage bermudagrass
3. Determine transcriptome level changes in PGPR inoculated bermudagrass

Objective Hypotheses

1. A system's based approach to isolate and characterize microbial communities within bermudagrass will provide greater chances of selecting bacterial strains that demonstrate successful colonization, growth-promotion, and superior drought tolerances than bacterial strains isolated from other agricultural systems then applied to turfgrass.
2. The use of PGPR blends and strains will alter plant defenses and increase drought tolerances of bermudagrass cultivars.
3. Transcriptomic changes in gene expression will be altered in grasses treated with PGPR. The identification of up and down regulated genes will provide further insights into how bermudagrass cultivars respond to drought conditions based on drought severity and longevity.

Methods Overview

Isolation, identification, and characterization of new bacterial strains
Rationale

It is logical to hypothesize that a microbial community evolves and adapts with climate, soil type, and crop history or crop cover, and that a microbial community becomes more established and stable over time. Numerous studies have demonstrated that soil temperature, moisture status, and nutrient availability control, in part, the timing and duration of root growth (Pregitzer et al. 2000, Xu and Huang 2000, Fry and Huang 2004). In addition to the studies documenting soil conditions to plant growth, I believe that soil types also relate to the successful colonization and persistence of PGPR. I think that different bacterial species and strains have evolved with soil types based on the soils physical and chemical properties. It is documented that

rhizobacteria in the rhizoplane and rhizosphere have a greater influence on plant and root activity in sandier soils than clay soils (Kiem and Kandeler 1997). Preliminary experiments in sand, sandy loam, loamy sand soils have yielded different growth responses in turf and pasture bermudagrasses. I suspect that bacteria will respond differently based on soil type and sand content. Therefore, by applying this logic, we could take a system's based approach to develop new bacterial collections that are crop, soil, and response specific.

To test this hypothesis, a new bacterial collection was created during the summer of 2016. Root microbial samples were isolated from turf type grasses (centipede, bermuda, zoysia, St. Augustine) from a Marvyn loamy sand soil as well as from forage and native grasses (bahia, crabgrass, johnsongrass, muhly grass, sea oats) under drought and drought recovery conditions. Plants that had maintained plant vigor and color during 21+ days of drought were sampled in Auburn, AL. By selecting grasses that were demonstrating desired physiological and phenotype responses we hope to speed up the process of identifying beneficial bacterial strains in a bermudagrass system for drought tolerance. A total of 604 bacterial strains were isolated from 25 samples (15 drought, 10 drought recovery).

Preliminary growth chamber Petri dish screenings are under way, but 6 potential strains (DH 25, 27, 32, 35, 37, and 44) have been isolated from bermudagrass that increase root and shoot growth as well as provide drought tolerance possibly superior to Blend 20 (Ngumbi, Coy, Held, and Kloepper, *unpublished data*). I would like to suggest continuing screening bacterial strains, but the priority should be on samples isolated from bermudagrass. Additionally, while I do not think it is necessary to know the strain identity for the purposes of screening (getting our desired outcome is more important), I think it could be beneficial to get a 16S rDNA bacterial

gene sequence for species ID which would further aide in literature searches and publication purposes. For example, DH 27 has a preliminary ID of *Sphingomonas*, and *Sphingomonas* spp. isolated from poplar and willow trees were associated with gibberellins and able to promote growth of roots and shoots while increasing drought tolerance in vegetables and perennial rye grass (Khan et al. 2012, Halo et al. 2015). By knowing the strain ID of promising drought tolerant strains, we may be able to put together a more focused screening effort and have literature precedence for drought enhancement by these strains. I would purpose for that we thoroughly evaluated these 6 strains and future others for characteristics that provide further insights to their behavior with plants. While many unknowns remain about which plant responses and root traits are most important, especially in bermudagrass for drought responses, a large initial battery of tests may help focus future research efforts and identify if bermudagrass cultivars vary in responses. I will suggest some tests for bacterial characteristics I think we should screen strains for before drought evaluations, but due to the length of the methods and protocols I will not include all of them here. I would like to screen these bacteria for qualitative and quantitative nitrogen fixation, phosphate solubilization, siderophore production, IAA activity, ABA activity, cytokinins, changes in sugars and amino acids (specifically proline content), and EL (Bajii et al. 2001, Du et al. 2012, Manuchehri and Salehi 2014).

Molecular identification of bacterial isolates using 16s rDNA

Taxonomic classification of each strain was based on the partial sequence of 16S rDNA. Bacterial DNA was extracted and amplified using three universal bacterial primers: 8F (5’-AGAGTTTGATCCTGGCTCAG -3’), 907R (5’- CCG TCA ATT CCT TTG AGT TT -3’), and 1492R (5’-ACGGCTACCTTGTTACGACTT - 3’). A Lucigen EconoTaq Plus Green 2X master

mix was used for PCR (Lucigen Corp., Middleton, WI) with the following cycling parameters: initial denaturation at 95°C for 5 m; 31 cycles of 94°C for 1 m, 57°C for 45 s, 70°C for 2 m; and a final extension at 70°C for 10 m. After all sequences were blasted against the type strains in the ribosomal database project to identify bacterial taxa of each strain.

Measurement of electrolyte leakage (EL) in bermudagrass shoots

Electrolyte leakage can be measured by obtaining 300 mg of FW shoots cut into 1 cm segments. Clippings should be triple rinsed in distilled water before being placed in 50 ml centrifuge tubes with 20 ml of distilled water and shaken for 24 hrs. After shaking, the initial conductivity (C_i) of the sample will be measured in a YSI 3200 Conductivity Instrument (YSI Inc., Yellow Springs, OH). Leaves will then be autoclaved for 30 min at 121° C, and then shaken for another 24 hrs. After shaking, the final conductivity (C_f) will be measured as previously described. Electrolyte leakage will be calculated from the formula (C_i /C_f) x 100%. This experiment should be conducted before, during, and after drought conditions.

Evaluation of proline content and reducing sugars

Proline content and reducing sugars can be determined following the methods of Kim *et al.* (2009) and Manuchehri and Sakehi (2014) which have been adapted from Dubois *et al.* (1956) and Bates *et al.* (1973). Proline content and reducing sugars can be measured using a spectrophotometer at 520 nm and 490 nm wavelengths respectively and comparing the proline and glucose standard curves. Reducing sugars can be measured by oven drying 200 mg of root and shoot tissue (separate samples) for 72 hrs and then grinding the samples into a fine powder and centrifuging the sample in an 80% ethanol solution. After centrifugation, the sample volume

should be brought to 25 ml with an 80% ethanol solution. One ml of the sample should be added to separate test tube containing 1 ml of a 5% phenol and 5 ml of sulfuric acid, stirred, and then read in the spectrophotometer.

Evaluation PGPR application to turf and forage types of bermudagrass for drought tolerance.

Experiments will evaluate the drought response of bermudagrass to conditions previously observed. This greenhouse experiment, will be a split-split plot design with whole plot treatments for water status: well-watered (daily or every 2-3 d) or drought, grass cultivar the first subplot, and PGPR treatment the second subplot level. This allows for all cultivars to be evaluated with and without PGPR and under well-watered and drought conditions. PGPR blends (as determined by the initial experiment) and a non-treated control will be used. Initially there will be no difference between the whole plot treatment as all plants will receive adequate watering. Each cultivar subplot treatment will be replicated 16 times and the experiment will run 5 weeks. Bermudagrass cultivars with varying drought tolerances will be evaluated Tifway (tolerant), TifEage (tolerant) LaPaloma (moderately tolerant), Riviera (moderately tolerant), Yukon (susceptible), and Wrangler (susceptible) are varieties that could be considered. As previously described, plugs will be harvested from the field, transplanted in cone-tainers and allowed to acclimate in the greenhouse for 3 wk, and seeded varieties will be planted based on seeding rates. During acclimation, fertilizer (Harrell's 21N-0P-0K) will be applied weekly at the recommended rate (5.81 g of product / m^2). After acclimation, fertilization will continue monthly (23.2 g of product / m^2), and PGPR applications will be made weekly. Following acclimation, 4 ml of a freshly prepared aqueous bacterial suspension of 10^7 colony forming units (cfu/ml) from PGPR stock solutions will be applied to the growing bermudagrass plants for 5 weeks. Weekly

top growth above 5 cm will be collected, dried and weighed. At the end of the eighth week (5 applications), all water will be withheld from the drought whole plot treatments for 18 d (or 28-35 d if maintaining PWP, ET, or other water supplementation). To avoid accidental watering, two separate greenhouse benches (one for each whole plot treatment) will be used.

The soil volumetric water content (SWC) will be determined for each whole plot-cultivar combination using Onset ECHO soil moisture data logging probes. Data will be logged every 15 min throughout the entire experiment. At 3, 7, 14, 18 d samples will be collected to determine leaf RWC; a measure of foliar hydration status (Merewitz et al. 2010a, 2010b). Leaf RWC uses leaf fresh weight, a turgid weight, and dry weight. To determine turgid weight, a harvested sample of leaf tissue (~0.1 g) is weighed fresh (FW), then soaked in de-ionized water for 12 h at 4 °C. The foliage is then blotted dry and re-weighed (turgid weight; TW) then dried at 70 °C in an oven for at least 72 h then finally weighed (dry weight; DW). Leaf RWC is (FW-DW)/(TW-DW) x 100. Leaf chlorophyll content will be determined using a chlorophyll meter for 2 subsamples on each plant initially then repeated every 2 d after drought conditions begin (Merewitz et al. 2010a, 201b). Number of dead or dormant plants per treatment will be noted every 2 d concurrent with leaf chlorophyll samples. The experimental design should allow determination of main effects and interactions between cultivar and PGPR treatment. Each variable can be used to determine the main effects and interactions over time.

After this experiment, PGPR that demonstrated superior drought tolerance should be evaluated in microplates or under field conditions. The methods for these field and microplot experiments could follow those set forth by Steinke *et al.* (2010, 2011) as they are thorough and could be easily replicated. In these experiments cultivars of Bermudagrass, St. Augustine, and

buffalograss were evaluated for drought tolerance at two soil depths (10.2 cm (municipal guideline for new developments) and an unrestricted rooting depth that had irrigation withheld for 60 d under field conditions during the summer in San Antonio, Texas. Additional response variables for this experiment could be turf visual ratings, proline content, RWC, chlorophyll content, and EL.

Determination of transcriptome level changes in bermudagrass treated with PGPR and subjected to drought conditions.

A preliminary experiment could be designed to determine if the rifampicin resistant strains alter grass drought responses similarly to the wild-type bacteria. If the rifampicin resistant strains perform similarly, then colonization and persistence data could be generated during and post drought. Grasses and soil could be sampled as previously described in Chapter 2. It is likely that the bacterial populations would survive by producing dormant, vegetative spores, but populations in the soil and *in planta* would likely decline during prolonged drought. Grasses will be grown in pots in the greenhouse, like what was previously described for assessing drought tolerance. Due to sequencing costs and budget limits, it is not feasible to analyze transcriptome changes in PGPR-treated and control plants for all cultivars and PGPR combinations. For that reason, this experiment will use Tifway bermudagrass and a PGPR blend known for growth and drought responses. There are four key time points for sampling; 1) before adding bacteria, 2) before drought is imposed, 3) after 18 d of drought conditions, 4) after 2-3 wk of drought recovery. For each plant, root and leaf tissue will be assessed separately for well-watered and drought plants yielding 12 samples. Plants in this experiment will be monitored for soil volumetric water content, but leaf tissue will not be cut to avoid possible confounding effects and molecular noise. Leaf and root tissue will be collected for RNA extraction and immediately

frozen in liquid nitrogen and stored at −80°C. Four, 100 mg tissue samples for PGPR-treated and control plants at each time point will be used. Leaf and root samples will be prepared for sequencing on the Illumina hi-Seq platform by the Auburn University Sequencing Center. Total RNA was isolated from the leaf and root samples using RNeasy Plant Mini-kit RNA isolation kit (GE Healthacre, NJ, USA) following the manufacturer's protocol. The cDNA library will be created by AU Genomics and Sequencing lab with TruSeq Stranded mRNA Sample Prep Kits from Illumima Inc.

The following methods (Held, *grant proposal*) should be followed for assessing transcriptome changes. Separate transcriptomes will be assembled for roots and shoots for PGPR-treated and control plants at each time. Transcripts from each will be aligned to a database containing all proteins from *Cynodon* spp. using BLASTx, e-value threshold of 10e-10, to compare assembled transcripts. For *Cynodon dactylon,* GenBank listed >21,000 nucleotide sequences, >20,000 EST sequences (GenBank, accessed Apr 27, 2015). Most of these sequences have been provided by the researchers associated with the University of Georgia breeding programs for turfgrass and forage-types of bermudagrass. A search for *Cynodon* and drought returned 444 EST associated with 5 or 10 d drought conditions in either Tifway or common bermudagrass. Transcripts will be assigned to unigene clusters based on their best gene hit.

Any transcripts without a BLASTx hit will be compared to a database of *Agrostis* spp. proteins. Creeping bentgrass (*Agrostis palustris* Huds.), a popular cool season turfgrass with published studies on drought tolerance, has >43,000 available nucleotide sequences (GenBank, accessed Apr 27, 2015). These transcripts will also be assigned to unigene clusters based on their best BLASTx hit to this secondary reference protein database. The gene accession names will be

used to label the unigene clusters. Any remaining transcripts not having a significant BLASTx hit to will be removed from the transcriptome. Additionally, Chen et al. (2015) used RT-PCR to identify and quantify target gene expression in bermudagrass roots and shoots under drought, salinity, cold, and heat stress and identified 8 target genes. Comparing transcriptomic changes of PGPR treated grasses to results observed in this study may provide additional clarity to how our PGPR alter plant responses.

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