Potential of plant growth-promoting rhizobacteria (PGPR) as a biological control agent against warm-season turfgrass pests

by

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are non-pathogenic, beneficial bacteria that colonize seeds and roots of plants to enhance plant growth. Despite work in agronomic crops, there has been little emphasis on development of PGPR for grasses. Accordingly, experiments were designed to evaluate novel bacterial inoculants for growth promotion in hybrid bermudagrass, *Cynodon dactylon (L). Pers. x Cynodon transvaalensis Burtt-Davy* (Tifway). Replicated laboratory and greenhouse experiments compared various blends of bacteria genera and species applied as weekly root inoculations. Growth promotion was assessed by measuring foliar growth from 3–8 wk and root growth at 8 wk after the first treatment. In all experiments, at least one bacterial treatment resulted in significantly increased top growth and greater root growth (length, surface area, volume, or dry weight). Blends 20 and MC3 caused the greatest growth promotion of roots and shoots. These results suggest that the bacterial strains could be used in strategies to help reduce nitrogen or water inputs to turf.

Non-pathogenic, soil microbes can induce changes associated with phytohormones that may influence plant-insect interactions. Much of this work has focused on mycorrhizal fungi or certain rhizobacteria. Soil microbes may deter herbivore oviposition, influence performance of above ground herbivores, while attracting natural enemies. Only a few studies have explored these interactions. Blends previously studied were screened for their ability to deter oviposition in no-choice greenhouse assays, negatively impact larval development in growth chamber conditions, and recruit natural enemies and parasitoids of the fall armyworm (FAW) in field. FAWs deposited most of their eggs on the grass in the control plants and ≤ 29% on the

inoculated treated grass, suggesting that microbes can mediate interactions between females and oviposition hosts. Three blends negatively impacted larval weights and two of these blends negatively impacted pupal weight and eclosion. Inoculants have shown to increase the attraction of natural enemies in laboratory settings, however the results failed to demonstrate this under field conditions. These experiments were some of the first to examine parasitoid recruitment to plants treated with bacterial inoculants under field conditions and the first attempt to use microbial inoculants to manipulate natural enemies in turfgrass. Induced resistance in plants from microbe inoculation to plant pathogens is well documented in the literature, but whether these interactions extend to herbivores, like insects, remains inconclusive.

Nitrogen (N) is the most important macronutrient for sustaining plant growth in turfgrass, and is abundantly applied to amenity grass. Plant N use efficiency is estimated to be only 50%. Avenues of loss are leaching, immobilization, and denitrification which releases nitrous oxide, a greenhouse gas. Use of PGPR and other microbial inoculants could allow for a reduction of N rates if they can alter the soil microbe community by improving nutrient uptake and efficiency while reducing greenhouse gas emissions. Replicated field study experiments evaluated varying N rates with and without a bacterial inoculant were conducted on a golf course. Parameters evaluated were foliar N content, visual turfgrass ratings, chlorophyll content, and tensile strength. We failed to demonstrate that the addition of PGPR to an N fertility management practice increased bermudagrass quality based on the parameters evaluated. Factors like site selection, weather, and N rates evaluated may have negatively impacted this study, and may explain why differences were not observed. Differences may have been observed if the study had been conducted in a less managed turfgrass setting, like a home lawn or pasture.

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List of Abbreviations

PGPR	Plant growth-promoting rhizobacteria
FAW	Fall Armyworm
cfu	Colony Forming Unit
ISR	Induced Systemic Resistance
SA	Salicylic Acid
JA	Jasmonic Acid
ET	Ethylene
PR	Pathogenesis-Related
TGRU	Auburn University Turfgrass Research Unit
RTJ	Robert Trent Jones
RBD	Randomized Block Design
NTEP	National Turfgrass Evaluation Program
NDVI	Normalized Difference Vegetation Index
MC	Murphey Coy

Chapter 1
Introduction and Literature Review

Plant growth-promoting rhizobacteria (PGPR)

Rhizobacteria can have deleterious, neutral, or beneficial effects on the host plant (Tuzun and Kloepper 1995, Elliot et al. 2004). The rhizosphere is the layer of soil that is influenced by the plant root and it has a greater density of organic carbon and bacteria than the rest of the bulk soil (Dimkpa et al 2009). This allows for the plant's roots to secrete root exudates and metabolites that can be used as plant nutrients (Lutenberg and Kamilova 2009). Initially, the term PGPR was used to describe strains of soil bacteria of the genus *Pseudomonas* but has now been broadened to include bacteria from the genera of *Azotobacter, Arthrobacter, Bacillus, Clostridium, Hydrogenophaga, Enterobacter, Serratia, Azospirillum, Paenibacillus,* and *Rhizobium* (Benizri et al. 2001). This grouping of genera is broad, and these genera contain species that cause pathogens too.

PGPR have been further developed from strains, individual bacteria species, to blends containing two or more strains. This was done to accomplish more consistent field results by having multiple beneficial strains available for the plant. Commercially-available PGPR products include Ag Blend®, Bioyield®, Equity®, PGA®, and Soil Builder®, which targeted agricultural and horticultural crops (Calvo et al. 2013, Burkett-Cadena et al. 2008). After purchasing the rights to *Bacillus firmus* in 2009, Bayer Environmental Sciences introduced Nortica® in 2011, the first PGPR product targeted for use on warm season turfgrasses for enhanced growth and nematode control.

Since the bacterial community is richer around the rhizosphere, competition exists among soil microbes for limited soil nutrients and space that can be colonized on the root.

Therefore, to have a positive impact on the plant, bacterial inoculants must be competitive in the soil. PGPR inoculation can result in antibioisis a mechanism of biocontrol that secretes antibiotics along the root to suppress pathogens (Lutenberg and Kamilova 2009).

In order for PGPR to have a positive effect on the plant, the following characteristics of the strains are crucial: ability to survive inoculation onto the plant, multiply in the rhizosphere in response to plant exudates, attach to the root surface, and colonization of the developing root system (Kloepper 1993). When colonizing the root, PGPR demonstrate both endophytic (capable of living within the plant tissue) and epiphytic characteristics (capable of living on plant surfaces), but isolation via surface washing or disinfestation is required to determine which mechanism is utilized by each bacterium (Hallmann et al 1997, Hirano and Upper 1997, Bressan and Borges 2004). The impact that PGPR has on plants and their yield is accomplished through both direct and indirect mechanisms. However, the specific means by which this is done has not been fully understood (Whipps 2001, Nelson 2004). The reason for this lack of understanding is that studies focus on PGPR's ability to mediate tolerance to abiotic stresses have focused on the evaluation of the plants' growth rather than the modes of action by which it is accomplished (Dimkpa et al. 2009). The direct impact that PGPR has on a plant results in growth promotion (biofertilization) in the absence of plant pathogens and pests. Under these conditions, they benefits they offer to plants can be easily demonstrated. The indirect benefits PGPR offer is demonstrated when it is able to reduce deleterious effects of plant pathogens and pests (Dimkpa et al. 2009, Lutenberg and Kamilova 2009, Nelson 2004, Okon et al. 1998, Whipps 2001).

The benefits of PGPR applications to crops are not limited to enhancing plant growth. There are verified biocontrol abilities in certain PGPR strains and blends on certain crops (Tuzan and Kloepper 1995). While PGPR offer growth promotion and biocontrol aspects, these two

mechanisms are not always elicited by the same strain. PGPR have been demonstrated to reduce damage from insect feeding and plant parasitic pests through induced systemic resistance (van Loon et al. 1998, Ramamoorthy et al. 2001, Kloepper et al. 2004, Burkett-Cadena et al. 2008), suppression of soil and foliar pathogens (Backman et al. 1997, van Loon et al. 1998, Siddiqui and Shaukat 2002, Kloepper et al. 2004, Cortes-Barco et al. 2010), synthesis of anti-fungal metabolites (Benizri et al. 2001), and increased ability of plants' metabolisms towards optimization of nutrient acquisition (Benizri et al. 2001, Dimkpa et al. 2009).

Induced systemic resistance (ISR) that is elicited by PGPR is a result of physiological and biochemical reactions and structural changes of the plant cells that produce defensive compounds of the host plant against pathogens. Plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are vital in plant defense signaling pathways, and the production of these hormones varies according to the invading pathogen or insect pest. However herbivorous insects are usually resisted by defenses dependent on JA, ET, or both (Bostock 2005, Glazebrook 2005, van Oosten et al. 2008). Accumulation of pathogenesis-related proteins (PR proteins), synthesis of phytoalexin, and other secondary metabolites suggest that these compounds have important contributions of the defensive capacities of induced plant tissues (van Loon 1998, Ramamoorthy et al. 2001). The defensive compounds' mechanisms of action have been unknown or identified as hydrolyzing fungal cell walls, or producing antimicrobial activities. The treatment of a plant with PGPR may also result in rapid structural changes within the cell walls of the plant. The result may be an increased line of defense against a pest or pathogen. Structural changes of the cell walls can induce cell thickening, lignification, appositions, or the accumulation of phenolic compounds that will act as barriers to protect the plant (Ramamoorthy et al. 2001). Additionally, PGPR may inhibit soil-borne pathogens by competing with the pathogens for limited resources,

like iron in the soil. An iron deficiency of *Fusarium* spp. will result in reduction of fungal spore germination and hyphal growth, which will lower the chances of plants being infected with a disease and disease severity (van Loon 1998).

Research involving PGPR is relatively new. Thus far, PGPR research studies have been focused primarily on agronomic and horticultural crops even though one of the first observations of soil microorganisms interacting with plants that promoted growth, resistance, and recovery from disease was demonstrated in turfgrass as early as 1938 (van Loon 2007). Although turfgrass is a crop, its value differs from agronomic and horticultural crops because it is based on its aesthetic qualities, rather than its yield. Because of this, maintenance of amenity turfgrass is intensive and expensive. This study will address the knowledge gap about PGPR and turfgrass interaction, focusing on bermudagrass (*Cynodon* spp. L.C. Richard).

PGPR strains have been studied in published and unpublished work and identified for their favorable attributes with cool-season turfgrasses (Bigelow et al. 2003, Suzuki et al. 2004, Elliot et al. 2004). Studies that evaluate PGPR strains' impact on warm-season turfgrasses (bermudagrass) have not been conducted. Yet studies have monitored rhizosphere bacteria population fluxions over several years in bermudagrass and grouped the strains based on bacterial characteristics (Gram-negative, Gram-positive, Fluorescent, etc.) (Elliot et al. 2004). A large initial screening process to identify potentially beneficial strains of PGPR on bermudagrass.

Growing environmental concerns by the public and increasingly stringent government regulations and bans on certain chemical inputs used for managing crops, including turfgrass, have given rise to the need for more integrated and sustainable management tactics and strategies

that are less harmful to the environment. The environmental concerns have focused on the overuse of water, fertilizer, greenhouse gas emissions, pesticides, surface and groundwater quality, and nontarget terrestrial and aquatic organisms (Haydu and Hodges 2002, Held and Potter 2012). PGPR offer an alternative as a biocontrol agent or biological elicitor, but their use, efficiency, or benefits have yet to be established in turfgrass. Biocontrol has been loosely defined as the use of a selected living organism to control a pest through herbivory, parasitism, predation, or other natural mechanisms. This project will focus on determining which strains are the most beneficial for growth promotion and biocontrol aspects in turfgrass management.

Plant growth promotion mediated by these soil microbes may directly or indirectly influence performance of above ground herbivores. A few studies have explored these interactions using *Arabidopsis* (van Oosten et al. 2008, Pineda et al. 2012). In *Arabidopsis*, *Pseudomonas fluorescens* positively affects weight gain of *Myzus periscae* (Pineda et al. 2012), but the same microbe-plant combination negatively affects development of *Spodoptera exigua* (van Oosten et al. 2008). The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are important in plant defense signaling pathways that mediate plant defenses to pathogens and heribivores (van Oosten et al. 2008). Typically, JA and ET are involved in plant defenses from herbivorous insect injuries (Bostock 2005). These direct defenses to insect feeding result in the production of chemicals that act as toxins or feeding deterents to the herbivores (Howe 2004). Indirectly, the plant is primed for defense from herbiovry by emiting a blend of volatiles that may attract natural enemies (Turlings et al. 1995). Impacts of soil microbes on herbivores may also be indirect via ovipositional deterrence of female *Cameraria* sp. leafminer on *Quercus emoryi* (Wilson and Faeth 2001) and of *Spodoptera exigua* on cotton (Nangle 2012)

or by recruitment of natural enemies through changes in plant volatiles in *Arabidopsis* and cotton (van Oosten et al. 2008, Ngumbi et al. 2009)

Warm Season Turfgrass

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 particular area. 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). Within these climatic regions, additional microclimatic factors influence these recommendations. Microclimatic conditions, such as light, temperature, precipitation, and wind in a particular area, have additional influences in species adaptation and selection for turf managers as well as soil conditions like varying soil types, pH's, and microbial activities (Sprague 1982, Beard 2002).

The grasses that are suited for these regions within the southeastern United States are referred to as warm-season or southern turfgrasses. These monocot, C_4 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 and their cultivars are heat tolerant, produce thick, lush stands, have deep root systems, and are aggressive growers (Sprague 1982, Duble 1996, Beard 2002).

Bermudagrass

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 grasses for turf and forage. In the United States, more golf course acreage is planted in bermudagrass (*Cynodon* spp.) than any other species, with the majority 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).

Common bermudagrass is the most widely cultivated turfgrass in warm climates around the world (Pessarakli 2008). Within the genus *Cynodon*, there are eight additional species, ten varieties, and numerous cultivars (Taliaferro 1995). This research will be conducted using *Cynodon dactylon (L). Pers. x Cynodon transvaalensis Burtt-Davy*, a hybrid that is commonly known as Tifway or Tifway. Because Tifway is a hybrid bermudagrass, it does not produce any viable seeds and must be established through sprigs (rhizomes, stolons, and stems), plugs, or as sod.

Tifway has been the most widely used bermudagrass on golf courses, sports turf, and other recreational areas for over 40 years (Beard 2002). The Tifway hybrid 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 pest

tolerances. Further, they produce fewer seed heads, have finer leaf texture, and have better color (darker green) (Foy 1997).

Nutrient Management in Turfgrass

Amenity turfgrass is a high value, high input system. Temperature, soil moisture, and plant available nitrogen (N) are the most common limiting factors of growth. Of all the macronutrients that are required for turfgrass establishment and maintenance, nitrogen is the most crucial resource for sustaining plant growth. Therefore, it is the most abundantly applied chemical to turfgrass (Frank and Guertal 2013). It is estimated that only 50% of applied N is used by plants in temperate, humid climates where half of the applied N can be lost from leaching, denitrification, and immobilization (NRC 1993). In bermudagrass, nitrogen is commonly applied monthly at a rate of 0.45 kg / 93 m^2 (1 lb / 1,000 ft^2) to maintain the aesthetics of a dense, green stand that is tolerant of environmental and pest stressors during the growing season (Carrow et al 2001). Urea, ammonium sulfate, potassium nitrate, and ammonium nitrate are commonly used soluble nitrogen sources in turfgrass.

Dinitrogen (N_2) is the most abundant natural gas and makes up about 78% of the earth's troposphere. However, this form of nitrogen (N) cannot be utilized by plants. A series of complex reactions, referred to as the nitrogen cycle, transform N_2 into a plant available nutrient. Mineralization, atmospheric deposition of N by lightning, and N fixation by nonsymbiotic organisms create plant available N. Immobilization, denitrification, leaching, and volatilization are reactions and processes in which N is lost. These chemical reactions and processes are impacted by environmental conditions pertaining to temperature, soil moisture, and soil microbe activity (Frank and Guertal 2013). Therefore, plant available nitrogen is often a limiting factor

for plant uptake and growth (Bernhard 2012). Plant available N sources that are created during the N cycle are ammonium (NH_4^+) and nitrate (NO_3^-). Bermudagrass can absorb and utilize both NH_4^+ and NO_3^-, which are the result of nitrification by nitrogen fixing bacteria in the soil. N can be applied by foliar application, but the majority of N uptake is through the roots. During periods of rapid growth, N is delivered to the shoots but enters the roots and rhizomes otherwise (Wherley et al. 2009).

Greenhouse gas emissions have increased during the past several decades. Nitrous oxide (N_2O) is a common greenhouse gas produced from denitrification in turfgrass with the use of fertilizers. According to the Environmental Protection Agency (EPA), N_2O accounts for 5% of the world's greenhouse gas emissions, and of that 5%, about 3% comes from non-agricultural fertilizer use (Terry and Kirby 1997). Loss of N by denitrification can range from 20-50% of applied N if the application is followed by heavy precipitation (Horgan et al. 2002, Barber 1995). Denitrification is favored by high temperatures with soil conditions being wet and oxygen poor (Frank and Guertal 2013).

The use of PGPR and other soil microbial inoculants could alter the soil microbe community and improve the uptake of nutrients by the plant, allowing for a reduction or alternative to fertilizer use (Calvo-Velez 2013, Adesemoye et al 2009). PGPR can act as a biofertilizer and supply the plant with nutrients. The inoculation of *Azospirillum*, a N_2- fixing bacteria, in wheat, sorghum, and maize resulted in a yield increase that was attributed to increased root development and rates of water and mineral uptake (Okon et al. 1998). In bermudagrass, the use of N_2-fixing bacteria inoculants (*Azospirillum* and *Azotobacte*r) resulted in a 21% increase of N in top growth and 13% increase in total harvested plant material when N rates were low, but had no effect when N rates were high (Baltensperger et al 1978).

Pest Management in Turfgrass

The rapid growth of warm-season turfgrasses during the spring, summer, and into the fall, coupled with the improved characteristics that hybrid bermudagrass offers, provide these grasses with the tolerance of higher pest densities. While these grasses may be able to withstand greater pest pressures, managed turf differs from other crops in that its value determination is derived from its aesthetic qualities. Due to the demand for perfectly maintained turfgrass by consumers, there is zero or low pest tolerance, which makes it difficult for turf managers to use action and economic thresholds. Because of this, turf managers must use a myriad of tactics to manage their turf, often relying heavily on the use of chemical inputs. The heavy reliance on chemical inputs for turf management can disrupt the ecosystem leading to soil compaction, excessive thatch accumulation, pest resurgence, or secondary pest outbreaks (Held and Potter 2012).

Chemical control options can be placed into two basic categories: preventative or curative. Preventative applications are aimed to prevent a pest problem. These applications are specific in their timing and application as to be most effective in the control of the specific pest(s). A curative application is applied in response to an outbreak of a pest within the season. Typically, these applications are applied with higher rates of active ingredients and do not provide long term control activity. Recently, the demand for a more integrated pest management approach (IPM) has gained momentum with about 30% of 18-hole golf courses having a voluntary environmental stewardship initiative, and turf managers are looking to adopt new and improved management tactics to add to their management strategy (Held and Potter 2012). For IPM tactics to be adopted, they must be cost effective, easy to implement, fit the management requirements, and be reliable. A few of the tactics that turf managers have been relying on are

mapping of pest occurrences on the course, use of resistant turfgrass strains, and conservation biological control to manage turf pests (Held and Potter 2012).

Fall Armyworm

The fall armyworm (FAW) (Lepidoptera: Noctuidae), *Spodoptera frugiperda* (Smith), is a multivoltine noctuid moth that has three to four generations per year in the southern United States (Cranshaw 2004). The FAW is in the same family as the cutworms and other armyworms which are pests of many plants and damage crops by feeding directly on plant tissue. While the FAW is a polyphagous insect, it has become a serious pest with it being particularly injurious to grasses, corn, and sorghum. Occasionally it is also a pest of legumes, certain vegetables, fruit trees, and flowers. Of the 60 plus host species, the caterpillars of fall armyworms prefer lush, dense, green fertilized bermudagrass the most. This fertilized bermudagrass is a preferred ovipositional site for adult females (Lynch 1984).

Biology of Research Taxa

Figure 1: Adult female FAW

Spodoptera frugiperda is a tropical, migratory species that only over winters in tropical areas of the United States (southern Texas and Florida), as it does not have a diapausing mechanism (Sparks 1979, Lewis and Nordlund 1984). FAW migrates each spring to the United States from Mexico, Central and South America, and the West Indies where it is a continuous inhabitant (Cobb 1995, Cranshaw 2008). The adult FAW is a small, dark ash-gray moth with a wingspan near 1½ inches with a noticeable light or whitish spot near the wing tip. The adults live for 2-3 weeks and become active around twilight

and feed on nectar. The female moth lays egg masses at night and can lay up to four hundred eggs at a time and about one thousand eggs in its lifetime (Cobb 1995, Cranshaw 2008).

Egg masses can be laid directly on the host plant. However, eggs are also laid on inanimate objects adjacent to turf when population densities are high, and oviposition is indiscriminate on surfaces like goal posts, flagging, fences, sheds, tree trunks, and light colored undersurfaces of other foliage (Sparks 1979, Cobb 1995, Cranshaw 2008). When searching for an oviposition site, the adult female FAW preferentially oviposits on undamaged plants over damaged plants (Carroll et al. 2006). The egg masses will be covered with hairs from the female moth. The infestations caused by FAW occur from mass egg hatching 2-10 days after egg laying.

The FAW has two distinct strains, the corn and rice strains. The distinction of the two strains is derived from their host plant feeding preferences as larvae and ovipositon behavior as adults. The corn strain has shown a preference to feed and oviposit on corn and sorghum, and the rice strain has been shown to prefer bermudagrass and rice in choice tests. While the strains have shown differences in oviposition behavior, the corn strain is able to develop equally well on corn, sorghum, and bermudagrass, but the rice strain develops best on bermudagrass. The larvae and pupae of the two strains also differ in weight, with the corn strain producing heavier larvae and pupae (Whitford et al. 1988).

Young larvae eat the remains of their shells and then disperse either by spinning down to the ground on silk threads or by crawling from where they hatch to turf. Dispersal of the larvae is a behavioral characteristic that has been attributed to competition for limited food and overcrowding due to the mass egg hatch in a small area. However, recent studies have shown that FAW larvae may be attracted to olfactory cues from linalool and 4,8-dimethyl-1,3,7-

nonatriene (Herbivore Induced Plant Volatiles, HIPVs) emitted by damaged corn seedlings for host plant location (Carroll et al. 2006). The young larvae are small and light green colored with a black head. As the larvae molt, they become darker (brown to black) in color and have three main stripes running longitudinally down the body. The head of the larvae has a distinct yellow or white inverted "Y" that continues into the midstripe followed by the two other stripes on the outside. Each abdominal segment of the FAW caterpillar has four dark colored dorsal spots, with the arrangement of the spots on the second to last abdominal segment having the spots in a square format (Potter 1988). When fully grown (two to three weeks after egg hatch), the larvae can be up to 1½ inches long before they burrow into the soil to pupate. The pupae of the FAW form a loose cocoon that is reddish brown in the soil, and the adults emerge 10-14 days later.

Figure 2: FAW larva with inverted "Y" leading into midstripe.

Damage to Turfgrass

The larvae of the FAW are the injurious stage of the pest. They can be active feeders at any point during the day (Potter 1998). The damage caused by FAW does not usually kill the grass, but it does weaken the plant, making it more susceptible to other stresses. In addition to weakening the plant, the damage lowers the aesthetic qualities and value of the grass. The damage can resemble drought stress when the grass appears to thin and shows brown spots that can rapidly expand (Potter 1998). Usually, FAW feeding is most active in the early morning and late afternoon. When they are not feeding, they hide in silk tunnels or burrows in the soil (Cobb 1995).

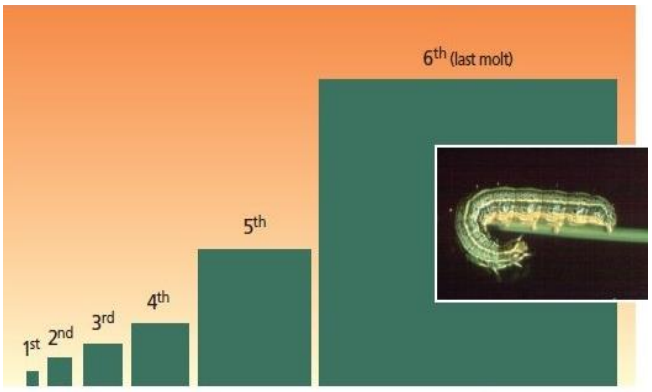


Figure 3: Relative amounts of food eaten by fall armyworm. Retrieved from Flanders et al. 2011

The larvae cause damage to turfgrass by feeding on all above ground plant parts, giving the grass a ragged look until re-growth occurs. They can also damage root systems from their underground activity. The younger larvae of FAW feed only on a single side of a leaf blade making it transparent in appearance (leaf skeletonization); older larvae are general feeders and will consume entire areas of the leaf (Cobb 1995). The majority of the feeding damage by FAW is caused by older larvae in the 5th and 6th instar stages, as these stages consume the most grass (Figure 3; Flanders et al. 2011).

Plants emit volatile organic compounds (VOCs) that may vary quantitatively and qualitatively in response to herbivore feeding and plant treatment. The volatile compounds released by plants can be divided into constitutive compounds and herbivore induced plant volatiles (HIPVs). Constitutive compounds are constantly present and are released due to mechanical damage or immediately after herbivore feeding begins. HIPVs are delayed in their release after from herbivore feeding damage, (Ngumbi et al. 2009) and are assumed to be detrimental to the herbivore through indirect defense mechanisms because they attract natural enemies of herbivores (Carroll et al. 2006). The manipulation of VOCs by turfgrass managers could enhance the biological management of FAW with natural enemies by attraction of parasitoids. Although tested in other systems, this hypothesis has not been investigated in hypothesis in turf, except for a few studies.

Damage caused by FAW will often appear in outbreak cycles that occur about once a month during the spring and fall, which is due to the peaking of adult activity and egg laying. In

a typical year, it is the second and third generations that cause the most damage to turfgrass (Potter 1998). The overlapping of generations and quick reproduction and life cycle rates of the pest result in the worst damage being evident from August through November as populations of the pest grow. However, when a season has drought conditions, it is common to have damage as early as July. The damage the FAW causes is usually scattered within a field as circular or irregular shaped patches. When high populations occur, grasses may be eaten to the soil surface (Beard 2002) and FAW may move in mass across a field (Carroll et al. 2006).

The FAW can cause indirect damage to turfgrass by attracting flocks of birds, especially the cattle egret (*Bubulcus ibis* Linnaeus) (Flanders et al. 2011), which feeds on the caterpillars and pecks holes into the turf, further lowering the aesthetic value of the grass.

Management of Fall Armyworm in Turfgrass

Cultural: Cultural control of FAW has relied on specific, but not the most effective, management tactics. Modifying irrigation patterns and frequent mowing are the best options to culturally combat FAW. Frequent mowing mechanically destroys larvae and reduces the depth of the turfgrass to ensure that better coverage of the grass by an insecticide chemical spray is obtained. With this practice it is useful to withhold irrigation for 24 hours to ensure greater chemical coverage of the turfgrass (Cobb 1995, Potter 1998).

Chemical: Monitoring and scouting are the best practices for turf managers to use to decide if chemical applications of insecticides are needed to control FAW. Scouting turfgrass for FAW populations and presence can help in detecting the pest before economic damage is caused. In areas where birds are congregating or turf appears dead, the turf should be checked for caterpillars. A soap or pyrethrin flush should be used in areas that show FAW damage to bring larvae up from the thatch to determine if a chemical treatment is necessary (Potter 1998). If no

caterpillars are present, the thatch should be further examined for small larvae, gray-cottony egg masses, and green pellets of frass (Cobb 1995, Potter 1998, Flanders et al. 2011).

Monitoring FAW flight with synthetic sex lures and sticky traps is a tactic that is often implemented to determine the peak flight of an adult generation. With this knowledge, turf managers will monitor for larvae presence and activity about a week after the peak flight period (Potter 1998). Action thresholds used as chemical treatment guidelines for FAW have been established as 3 or more FAW per square foot in turfgrass (Flanders et al. 2011). Insecticide applications should be spot-treatments on an "as needed" basis, as a blanket spray or preventative treatment may be ineffective as well as harmful to natural enemy populations (Potter 1998). Numerous insecticides are effective against FAW, but certain chemicals (e.g., pyrethroids and Acelypryn) are more commonly used by turf managers. Before applying an insecticide, the area should be mowed to lower the turfgrass height to allow for better coverage. Effective insecticides should be applied in the early morning or late afternoon when the FAW is most likely to be active (Flanders et al. 2011). In addition, liquid insecticide applications that leave residues on the foliage are the preferred choice. After the application, irrigation and mowing of the treated area should be withheld for up to 24 hours to prevent leaching and degradation of the chemical treatment (Potter 1998).

Biological: Over 50 species have been reported to be parasitoids (Diptera and Hymenoptera) of the FAW, with many more species of invertebrates and vertebrates being predators (Lewis and Nordlund 1984). As a result, FAW populations are often effectively reduced by natural enemies (Potter 1998). Among parasitoids, flies are mostly in the family Tachinidae (Delfín-González et al. 2007). *Cotesia marginiventris* (Cresson) and *Chelonus insularis* (Cresson) in the family Braconidae are the most commonly encountered parasitic wasps of FAW (Lewis and Nordlund

1984). Parasitoids rely on semiochemicals to locate their prey, and studies have suggested that herbivore feeding and PGPR applications to crops can alter the production of these plant chemicals (volatile organic compounds) to higher levels, which attract more parasitoids to the pests' locations (Lewis and Nordlund 1984, Ngumbi et al. 2009).

Long-term goals

The long-term goals of this project are to increase knowledge of PGPR's relationship with warm-season turfgrass and to offer turf managers additional management tactics that emphasize biocontrol. The aim is to add knowledge that identifies beneficial PGPR strains that have a positive impact on turf quality, durability, and pest tolerances and increasing the understanding of the chemical interactions between PGPR, turfgrass, and pests. PGPR may offer the ability to reduce chemical inputs, some of which are slow, ineffective, costly, and detrimental to the environment, while producing similar or better yielding turf.

Chapter 2

Effects of PGPR treatments on Tifway bermudagrass growth

Abstract

Plant growth-promoting rhizobacteria (PGPR) are non-pathogenic, beneficial bacteria that are able to colonize seeds and roots of plants to enhance plant growth. Despite work in agronomic crops, there has been little emphasis on development of PGPR for grasses in pastures or turf. These experiments were designed to evaluate novel bacterial inoculants for growth promotion in hybrid bermudagrass (Tifway). Replicated laboratory and greenhouse experiments compared blends of bacteria applied as weekly root inoculations or distilled water to control plants. Foliar and root growth were then compared to determine relative growth promotion. In all experiments, at least one bacterial treatment of bermudagrass resulted in increased top growth and greater root growth (length, surface area, or volume). Blend 20 as well as MC3 provided greater positive impacts on roots and shoots. These two strains could positively impact grass growth while also potentially reducing nitrogen or water inputs.

Introduction

Plant growth-promoting rhizobacteria (PGPR) are non-pathogenic, beneficial, free-living soil and root inhabiting bacteria that are able to colonize seeds and roots (rhizosphere) (Kloepper and Schroth 1978, Kloepper 1993). These rhizobacteria can have deleterious, neutral, or beneficial effects on the host plant (Tuzun and Kloepper 1995, Elliot et al. 2004). Rhizobacteria, fungi, and associated microbes in the soil community influence the nitrogen cycle through nitrification, denitrication, fixation, and mineralization (Calvo 2013, Adesemoye et al. 2009). Enhancement of the soil microbe community is common with the use of microbial inoculants.

Inoculation of *Azospirillum*, a N_2- fixing bacteria, increases root development in wheat, sorghum, and maize and increases yield (Okon et al. 1998). Rhizobacterial inoculants interact with plant root exudates and metabolites to promote plant growth (Lutenberg and Kamilova 2009). Commercially-available inoculants are developed from individual strains into blends containing two or more strains. Blends are used to increase more consistent field results relative to single strains (Burkett-Cadena et al. 2008).

In order for inoculants to be successful, the bacteria must be able to survive inoculation, multiply in the rhizosphere in response to plant exudates, attach to the root surface, and colonize the developing root system (Kloepper 1993). The impact that PGPR have on plants and their yield is accomplished through both direct and indirect mechanisms. However, the specific interactions are not fully understood (Whipps 2001, Nelson 2004). The common direct impact of inoculants is growth promotion (biofertilization) in the absence of plant pathogens or pests where growth promotion is easily demonstrated. Indirectly, PGPR are able to reduce severity of pathogens and herbivore feeding through plant acquired induced systemic resistance (ISR) or systemically acquired resistance (SAR) by utilizing plant metabolic pathways such as jasmonic acid or ethylene (Dimkpa et al 2009, Lutenberg and Kamilova 2009, Howe 2004, Nelson 2004, Okon et al. 1998, Whipps 2001).

Bermudagrass (*Cynodon dactylon* (L.) Pers.) is a warm season, perennial grass found in over one hundred countries that reproduces by seed and vegetatively by stolons and rhizomes (Duble 1996). *Cynodon dactylon* and its hybrids are the most commonly used grasses for turf and forage. On golf courses in the United States, bermudagrass (*Cynodon* spp.) is the most widely cultivated grass species, with most land coverage in the Southeast, Southwest, and transition zones (Lyman et al. 2007). The Tifway hybrid (*Cynodon dactylon x Cynodon transvaalensis*

Burtt-Davey) is a chance hybrid discovered in seeds from Johannesburg, South Africa in 1954 (Duble 1996) and released in 1960 by the Georgia Agricultural Experiment Station and Crops Research Division. Tifway has been the most widely used bermudagrass hybrid on golf courses, sports turf, and other recreational areas for over 40 years (Beard 2002). Tifway and other hybrid bermudagrasses have been preferred over common bermudagrass because they generally have greater disease resistance, pest tolerances, produce fewer seed heads, have finer leaf texture, and have better color (darker green) (Foy 1997).

Although there has been extensive research in agronomic crops with microbial inoculants, relatively few studies have considered the influence of endemic microbial community or inoculants in grasses. Turfgrasses support rich microbial communities in spite of manipulation of soil profiles and intense use of pesticides on creeping bentgrass (*Agrostis stolonifera* Huds.) and bermudagrass putting greens (Bigelow et al. 2002, Elliot et al. 2004). Only one previous published study evaluating microbial inoculants for growth promotion in grass was found to establish literature precedence. Baltensperger et al. (1978) evaluated nitrogen-fixing bacterial inoculants (*Azospirillum* and *Azotobacter*) with eight genotypes of bermudagrass in a greenhouse. Although there was no difference in response among the different genotypes, bacterial inoculantions increased foliar nitrogen and plant biomass under zero N fertility conditions (Baltensperger et al. 1978). Bacterial inoculants and low fertilizer rates seem to be an option for lower input, more sustainable turfgrass management; yet studies evaluating this potential have not been conducted. Bacterial inoculants from Auburn University's Plant Pathology department have demonstrated growth promotion and pathogen resistance in different crop systems, and some are being commercially developed. The objectives of these experiments were to screen blends with commercial potential for growth promotion in bermudagrass in a

growth chamber and then evaluate successful blends under greenhouse conditions similar to Baltensperger et al. (1978).

Materials and Methods

Bacterial Strains and Inoculant Preparation. Bacterial strains listed in Table 1 were transferred from cryovials maintained at -80C (-112F) for long-term storage to plates of tryptic soy agar (TSA). After incubation at 28C (82.4F) for 48 hrs, bacteria were scraped from TSA plates with inoculating loops, transferred to tubes (20 ml Glass Culturable, VWR, Radnor, PA) of sterile water that contained 10 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 in the suspensions were determined by plating 50 µl of the serially diluted bacterial suspensions onto TSA plates, incubating plates for 24–48 hrs, and then counting the number of bacterial colonies that grew on each plate. Once the concentrations in the prepared suspensions of each strain were determined, the populations of all strains were used to make a bacterial stock solution. Stock solutions 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 colony forming units $(cfu) \cdot ml^{-1}$ of each strain.

Experiment 1. Growth Chamber Trials. Twelve PGPR blends (Table 1) as well as a control were used in the initial screening. Plugs of bermudagrass hybrid Tifway were collected from the Auburn University Turfgrass Research Unit (TGRU). After harvesting, the plants were washed to remove field soil and then surface sterilized (1 minute bleach, 3 minutes EtOH, rinsed 3 times distilled water) before a single stolon was transplanted into plastic Petri dishes (160 x 15 mm; VWR, Radnor, PA) that contained a 1/10 Murashigie & Skoog media, 1.5% agar media (Figure

4; Mandyam et al 2010). Transplanted plugs were grown in a growth chamber at 28.6 (83.5F) ± 5C, 14:10 (L:D) photoperiod, and 50 ± 10% relative humidity for 1 wk to adjust to the new conditions. After this period, plugs were standardized using an adaptation of Braman et al. (2002). At the start of the experiment, plants were cut to a height of 5 cm. Each week 2 ml of an aqueous bacterial suspension of 10^7 cfu·ml^{-1} was applied to the growing bermudagrass plants for 5 wks. After the 6th wk (5 applications), the bermudagrass plugs were destructively sampled to determine root growth impacts. This experiment was replicated six times for each treatment in two separate trials.

Experiment 2. Greenhouse trials: After identifying potential candidate blends of PGPR in experiment 1, we tested those strains using plugs planted in a sandy textured soil in a greenhouse. Four PGPR blends as well as a control were evaluated in Greenhouse Trial 1, and 8 blends (Table 2) were evaluated in the Greenhouse Trial 2. The Murphey Coy (MC) blends were designed using strains that were associated with top and root growth from the original blends and had shown growth promotion in maize and rice.

In this experiment, Tifway plugs (3.8 cm) from the TGRU were harvested on April 1 and July 24, 2013, for each trial. After harvesting, plugs were washed free of field soil and transplanted. Bermudagrass plugs were grown in SC7 Stubby cone-tainers (3.8 x 14 cm, Stuewe & Sons, Tangent, OR) in a greenhouse facility (Auburn University) set at 28.6 (83.5F) ± 5C, 14:10 (L:D) photoperiod, and 50 ± 10% relative humidity. Bermudagrass plugs were planted in 100% clean sand. After this period, plugs were standardized using an adaptation of Braman et al. (2002). At the start of the experiment, plants were cut to a height of 5 cm. The plants were given 3 wks to acclimate to the new conditions. During the 3 wks of acclimation, fertilizer (305 ppm Nitrogen, Peterson's 20N-20P-20K; Alix, Alberta, Canada) was mixed weekly at a rate of 5

mg·3.78 L^{-1}, and 50 ml were applied to each plant. After acclimation (4^{th} week), the plants were cut to a height of 5 cm, and supplemental fertilization was stopped. Each week 2 ml of a freshly prepared aqueous bacterial suspension of 10^7 colony forming units (cfu·ml^{-1}) from PGPR stock solutions were applied to the growing media of each pot followed by 30 ml of tap water (Figure 6). Plants were watered daily to field capacity, except when PGPR applications were made. At the end of the 8th week (5 applications), the bermudagrass plugs were destructively sampled. Each trial had 12 replicates for each treatment.

Evaluations of Growth Promotion. Each week the grass foliage was cut to a height of 5 cm, and top growth was collected (Figure 6). Foliage was dried in an oven at 70C (158F) for 40 min, and dry mass was recorded. For the experiment conducted in Petri dishes, it was not possible to completely extract roots, so a rating of root coverage was used to make comparisons between bacterial inoculants and with the control. Coverage was rated visually by dividing each dish into eight equal sections. If the root structure was present in 50% or more of a section, then it was rated. Each replicate was rated (0–8) based on the sections containing roots.

For experiments with plants in cone-tainers, the root system of each plant was washed in the lab. After washing, analysis of 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 (version 2004a). Based on image analysis, the software computed the following parameters: root length, root surface area, root volume, root tips. Plant roots were dried and weighed to determine root dry weight (Figure 4). The data collected were used to compare root growth and shoot growth to determine if strains and blends caused growth promotion in bermudagrass relative to the controls.

Statistical Analysis. Top growth for all trials was analyzed using repeated measures of multivariate analysis of variance (MANOVA) ($P < 0.05$, JMP Version 10. SAS Institute Inc., Cary, NC, 1989-2007). Root ratings for the growth chamber trials were transformed (log + 0.5) before the analysis of variance (ANOVA). Means were subject to Student's t-test ($P < 0.05$, JMP) for comparisons. Root growth parameters for the first greenhouse trial were analyzed using analysis of variance (ANOVA). Treatment means for this trial were compared only to the control using Dunnett's test ($P < 0.05$, JMP). Root growth for the second greenhouse trial was analyzed using analysis of variance (ANOVA) Student's t-test ($P < 0.05$, JMP).

Results

Experiment 1. Growth Chamber Trials. Treatment with eight blends (Blends 8, 12, 14, 15, 16, 18, 19, and 20; Table 1) resulted in more top growth relative to the control (Figure 5). These blends produced 158-345% more dry weight in top growth. Treatment with six of these blends (Blends 8, 14, 16, 18, 19, 20; 236-345% increase in top growth) resulted in significantly greater top growth relative to the control ($P <0 .01$; Figure 5). Treatment with blends 9, 11, and 17 resulted in less top growth relative to the control (37–98% of control). Roots from Blends 8, 18, 19, and 20 covered more than half the Petri dish on average (ratings >5) compared to control plants, which had a median rating of 3.67 (Table 3). Root rating of plants treated with blends 8 and 20 were significantly greater ratings of the controls ($P <0.01$).

Experiment 2. Greenhouse Trials. In Trial 1, treatment with blends 8, 18, 19, and 20 resulted in significantly more total dry weight top growth (158-197%) relative to control plants (Figure 8). Blend 8 resulted in significantly greater dry weight relative to the control (11% increase). Blend 20 had a slightly (4%) greater dry weight relative to the control. Inoculation of bermudagrass resulted in roots that were 141-157% longer than the control (Table 3, Figure 7).

For all measured root parameters, Blend 18 was never significantly different from the control (Table 4). Only Blend 20 had a significantly greater root length ($P< 0.03$). All blends increased mean root surface area by 146-172% relative to the control, but only Blends 19 and 20 resulted in significantly greater root surface areas. Similarly, Blends 19 and 20 had 150-186% greater root volume relative to the controls ($P< 0.05$). Root volume and root length for Blends 8 and 18 were numerically greater than controls ($P\leq 0.06$), but these pairwise comparisons were marginally beyond our set level ($\alpha =0.05$).

In Greenhouse Trial 2, all blends increased top dry growth by 122–134% relative to the control. Certain blends unique to Trial 2 (MC 1, MC 2, and MC 3) had significantly greater top growth than the control (Figure 8), but MC 4 and Blend 19 did not differ significantly from control. In general, root length and root surface area were generally lower in Trial 2. All blends except 8 resulted in root systems that were 124-157% longer relative to the control ($P\leq 0.05$; Table 4). Blend 8 resulted in the lowest root length and surface area ($P<0.05$). Blends MC 3 and 19 resulted in the greatest root surface area and length, and both blends were significantly greater than the controls. None of the blends in Trial 2 resulted in a root volume that was significantly greater than the control ($P\leq 0.05$). Treatment with MC2 resulted in root dry weight that was significantly greater than the control and all other treatments ($P\leq 0.05$).

Discussion

In this study we demonstrate significant root and shoot promotion in bermudagrass using bacterial inoculants from the Auburn University collection. Of the 12 blends tested, six resulted in significantly greater top growth (Blends 8, 14, 16, 18, 19, and 20). Initial screenings of bacterial inoculants were done in a growth chamber with an M-S media bioassay. Positive results from this assay were similar to those obtained in the greenhouse trials, suggesting that this

method is beneficial for a high-throughput, rapid screening of initial bacterial candidates in the future. However, M-S media assays limit the amount of data that can be collected from root systems. For example, it was impossible to extract the roots from the media for analysis using the scanning system, which resulted in the use of a more rudimentary visual rating system (Table 3, Figure 4).

The treatment of grass with all PGPR blends (8, 18, 19, and 20) resulted in significantly greater top growth than the control, and all blends except for Blend 18 had a significant impact on one or more root parameters. All PGPR treated plants averaged about 150% greater root length than the control (Figure 7). An additional greenhouse screening was performed with the same blends (8, 18, 19, and 20) as well as four new blends (MC 1, MC 2, MC 3, and MC 4). These results may have important implications for turf and pasture grasses with low fertility inputs. In pastures, an increase in top growth makes the pasture more productive as forage. In our experiments, inoculants were applied weekly. However, Durham (2013) found that a single application to cotton persisted and was detectable up to 12 weeks post inoculation. These bacteria could be applied 1–2 times per season, perhaps in a granular form similar to Poncho Votivo (Bayer Environmental Sciences, 2013), to provide season long growth promotion. In turf settings, applications are easier to make and could be tank mixed with fertilizer applications, possibly allowing for nitrogen rate reductions and enhanced nutrient uptake (Baltensperger 1978). This hypothesis is currently under further investigation. The increase in root growth when inoculants are present could increase nutrient and water acquisition from soils, which could enhance drought tolerance (RMC, *unpublished data*) and decrease water inputs for turfgrass managers.

In general, greenhouse Trial 2 had less growth than Trial 1. One limitation to these experiments is that we are not fully aware of the root mass of the plants before beginning the inoculation. The plants in each trial were harvested and grown in the greenhouse at different times of the year. We tried to compensate for this variation through larger numbers of replicates. Each trial, for example, typically had 12 replicates, and many treatments were repeated across multiple trials. Interestingly, Blend 8 was marginally significant for certain root parameters in Trial 1, yet plants with that inoculant in Trial 2 were typically less vigorous than controls. In Trial 2, only Blend 19 and MC 3 increased root length and surface area, and MC 2 increased root dry weight. However, a shade cloth was placed over the greenhouse between weeks 1 and 2 of the study, which resulted in a reduction in growth between the two greenhouse trials (Figure 8).

Blends (MC1–4) were designed by combining PGPR strains that had previously demonstrated the capacity to promote growth in bermudagrass (growth chamber trial) or in similar monocot crops (maize and rice), data not shown. Different blends from the Auburn University PGPR collection may offer growth promotion in one crop, but the same benefits may not be seen in another crop (Kloepper, personal communication). Performance of bacterial inoculants may be crop dependent and influenced by not only the blend's strains but their interactions with plant physiology (C_3 vs. C_4) or root morphology. For example, Blend 9 has been credited with increased root growth in cotton (Nangle 2012) but was one of the weakest performers in turfgrass in regards to top and root growth parameters. Also, a more fibrous monocot root system may be easier to colonize for some strains than the tap roots of dicots.

Bacterial inoculants in the Auburn University collection were selected based on success in root colonization (endophytically or ectophytically), and some are being commercially developed for use in different plant production systems. Certain companies already have

bacterial inoculants available for use in turfgrass. Nortica (*Bacillus firmus* (strain I-1582), Bayer Environmental Sciences) is labeled for control of plant parasitic nematodes in bermudagrass (Bayer 2013). Bacterial inoculants represent a biological alternative to pesticides for plant health management. In this study we present data indicating that Blends 20, MC3, and MC4 should be further evaluated for use in pasture and amenity grass systems. Further research will consider the performance of individual strains compared to blends as well as field performance of these bacterial inoculants in relation to fertility and pest management.

While many questions about PGPR's relationship with bermudagrass were answered in this study, more questions still remain unaddressed and will be evaluated in future work. Further considerations that should be addressed relate to activity of individual bacterial strains in blend identity, application rate, number of applications as they correspond to root colonization and persistence, and whether bacterial inoculants colonize the root system endophytically or ectophytically.

Table 1: Identity of rhizobacterial blends and components evaluated in bermudagrass.

Name	Identity of Formulation	Bacterial species present
Blend 8	AP188, AP209, AP217, AP218	*Paenibacillus macerans, Bacillus atrophaeus, Brevibacillus brevis, Bacillus subtilis*
Blend 9	AP136, AP188, AP219, AP295	*Bacillus subtilis, Paenibacillus macerans, Bacillus subtilis, Bacillus vallismortis*
Blend 11	AP3, AP279, AP280, AP282	*Bacillus pumilus, Bacillus subtilis, Bacillus pumilus, Bacillus sphaericus*
Blend 12	AP272, AP282, AP283	*Bacillus mycoides, Bacillus sphaericus, Bacillus pumilus*
Blend 13	AP3, AP278, AP279, AP282	*Bacillus pumilus, Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus sphaericus*
Blend 14	AP7, AP271, AP282	*Bacillus pumilus, Bacillus megaterium, Bacillus sphaericus*
Blend 15	AP32, AP33, AP34, AP40, AP50	*Bacillus circulans, Bacillus pumilus, Bacillus megaterium*

Blend 16	AP188, AP204, AP209, AP217, AP218	*Paenibacillus macerans, Bacillus amyloliquefaciens, Bacillus atrophaeus, Brevibacillus brevis, Bacillus subtilis*
Blend 17	AP136, AP153, AP188, AP204, AP219	*Bacillus subtilis, Brevibacillus laterosporus, Paenibacillus macerans, Bacillus amyloliquefaciens, Bacillus subtilis*
Blend 18	AP143, AP153, AP204, AP217, AP218	*Paenibacillus macerans, Brevibacillus laterosporus, Bacillus amyloliquefaciens, Brevibacillus brevis, Bacillus subtilis*
Blend 19	AP223, AP279, AP282, AP283	*Bacillus circulans, Bacillus subtilis, Bacillus sphaericus, Bacillus pumilus*
Blend 20	AP7, AP18, AP282	*Bacillus pumilus, Bacillus pumilus, Bacillus sphaericus*

Table 2: List of PGPR blends to be tested in Greenhouse Trials

Trial	Name	Identity of Formulation	Bacterial species present
1,2	Blend 8	AP 188, AP 209, AP 217, AP 218	*Paenibacillus macerans, Bacillus atrophaeus, Brevibacillus brevis, Bacillus subtilis*
1,2	Blend 18	AP 143, AP 153, AP 204, AP 217, AP218	*Paenibacillus macerans, Brevibacillus laterosporus, Bacillus amyloliquefaciens, Brevibacillus brevis, Bacillus subtilis*
1,2	Blend 19	AP 223, AP 279, AP 282, AP 283	*Bacillus circulans, Bacillus subtilis, Bacillus sphaericus, Bacillus pumilus*
1,2	Blend 20	AP 7, AP 18, AP 282	*Bacillus pumilus, Bacillus pumilus, Bacillus sphaericus*
2	MC 1	AP 188, AP 209, AP 282	*Paenibacillus macerans, Bacillus atrophaeus, Bacillus sphaericus*
2	MC 2	AP 7, AP 188, AP 209, AP 282	*Bacillus pumilus, Paenibacillus macerans, Bacillus atrophaeus, Bacillus sphaericus*
2	MC 3	AP 18, AP 188, AP 209, AP 282	*Bacillus pumilus, Paenibacillus macerans, Bacillus atrophaeus, Bacillus sphaericus*
2	MC 4	AP 188, AP 204, AP 209, AP 282	*Paenibacillus macerans, Bacillus amyloliquefaciens, Bacillus atrophaeus, Bacillus sphaericus*

Table 3: Median values of root ratings (0–8) from growth chamber screenings

Name	Rating
C	3.67cd
8	5.92a
9	1.75e
11	2.25de
12	2.33de
13	1.67e
14	4.33bc
15	2.75de
16	4.58abc
17	4.58abc
18	5.58ab
19	5.33ab
20	5.75ab

Means presented are actual means. Within a column, means followed by the same letter are not significantly different from each other ($P < 0.05$; JMP; ANOVA Student's t-Test; [SAS Institute Inc., Cary, NC. 1989-2007]).

Table 4. Bermudagrass root measurements after 5 wk exposure to bacterial inoculants

Treatment	Trial	Mean (± SEM) values of root measurements (N=12)[a]			
		Length (cm)	Surface area (cm^2)	Volume (cm^3)	Dry weight (g)
Control	1	849.1 ± 127.3b	138.9 ±10.0b	1.83 ± 0.27b	2.34 ± 0.03b
Blend 8	1	1,213.1 ± 212.9ab	204.1± 35.3ab	2.75 ± 0.47ab	2.61 ± 0.08a
Blend 18	1	1,207.4 ± 117.2ab	203.9 ± 19.0ab	2.76 ± 0.27ab	2.38 ± 0.02b
Blend 19	1	1,199.5 ± 187.6ab	214.0 ± 35.1a	3.05 ± 0.53a	2.40 ± 0.02b
Blend 20	1	1341.2 ± 175.4a	239.4 ± 26.0a	3.41 ± 0.41a	2.45 ± 0.03b
Statistics		F=4.55, *P* =0.033	F=3.83, *P* = 0.012	F=3.11, *P* = 0.048	F=1.94, *P* =0.001
Control	2	365.4 ± 35.1d	126.3 ± 7.7c	3.71 ± 0.40a	2.33 ± 0.02bc
Blend 8	2	192.0 ± 13.1e	88.8 ± 4.6d	3.30 ± 0.23ab	2.29 ± 0.01c
Blend 19	2	575.3 ± 26.1ab	169.3 ± 8.1a	3.91 ± 0.34a	2.30 ± 0.01c
Blend 20	2	469.4 ± 26.8c	138.5 ± 8.1c	3.22 ± 0.31abc	2.33 ± 0.01bc
MC 1	2	455.8 ± 53.2bc	129.0 ± 8.8c	2.53 ± 0.17c	2.32 ± 0.01bc

MC 2	2	543.5 ± 32.3bc	137.4 ± 6.7c	2.90 ± 0.16bc	2.46 ± 0.04a
MC 3	2	655.1 ± 41.8a	162.0 ± 5.2ab	3.28 ± 0.18abc	2.36 ± 0.01b
MC 4	2	573.8 ± 52.8bc	145.4 ± 11.2bc	3.17 ± 0.28abc	2.32 ± 0.02bc
Statistics		F=19.17, *P* = 0.012	F=9.99, *P* = 0.012	F=2.52, *P* = 0.012	F=6.77, *P* = 0.0001

[a]Means presented are actual means (±SEM). For each Trial, means in the same column followed by the same letter are not significantly different from each other (Trial 1: $P < 0.05$; JMP; Dunnett's; Trial 2: $P < 0.05$; JMP; Student's t-Test).

Figure 4: Bermudagrass growing in M-S media bioassay to initially screen rhizobacteria for growth promotion.

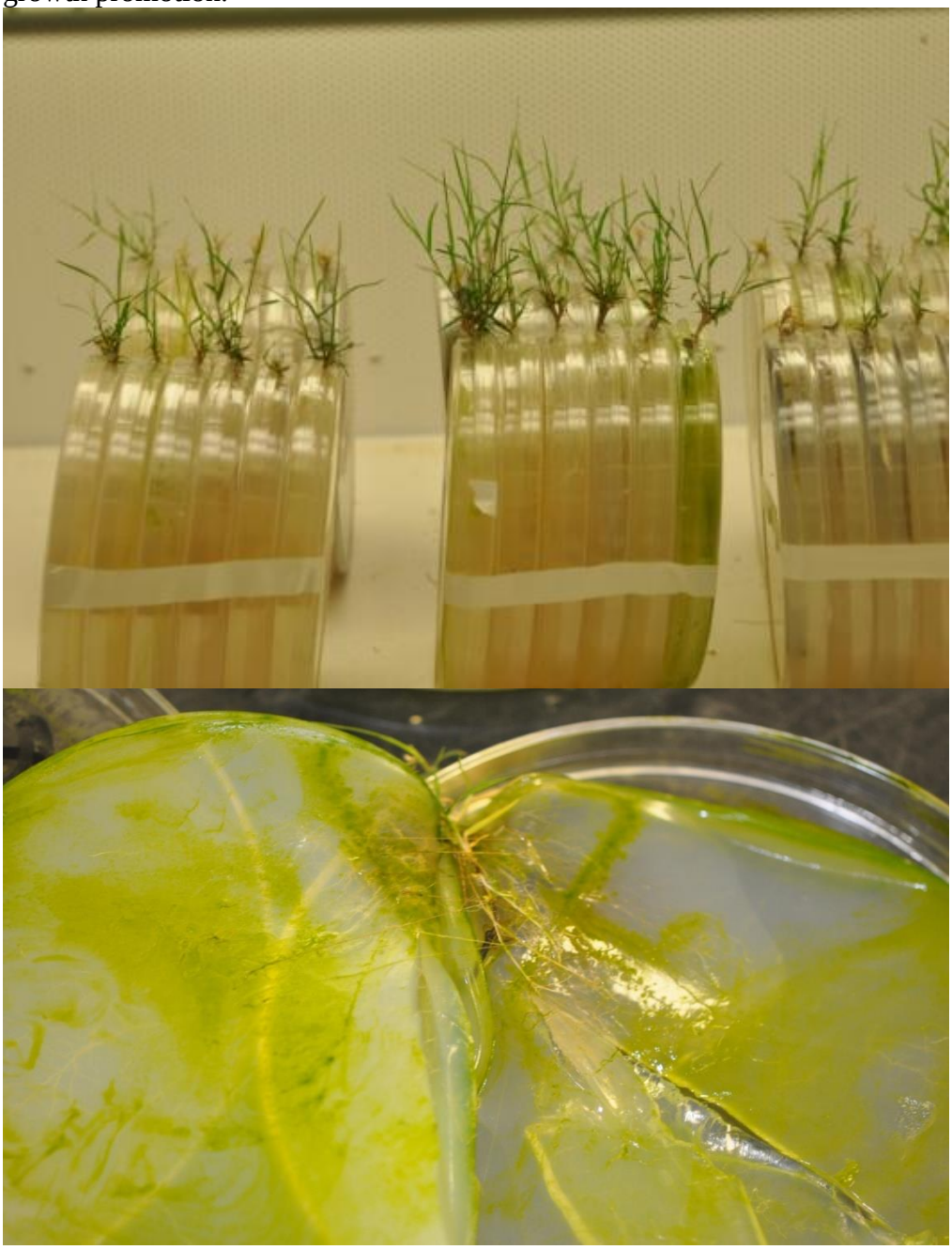


Figure 5: Weekly dry top growth (mg/stolon) during growth chamber bioassay

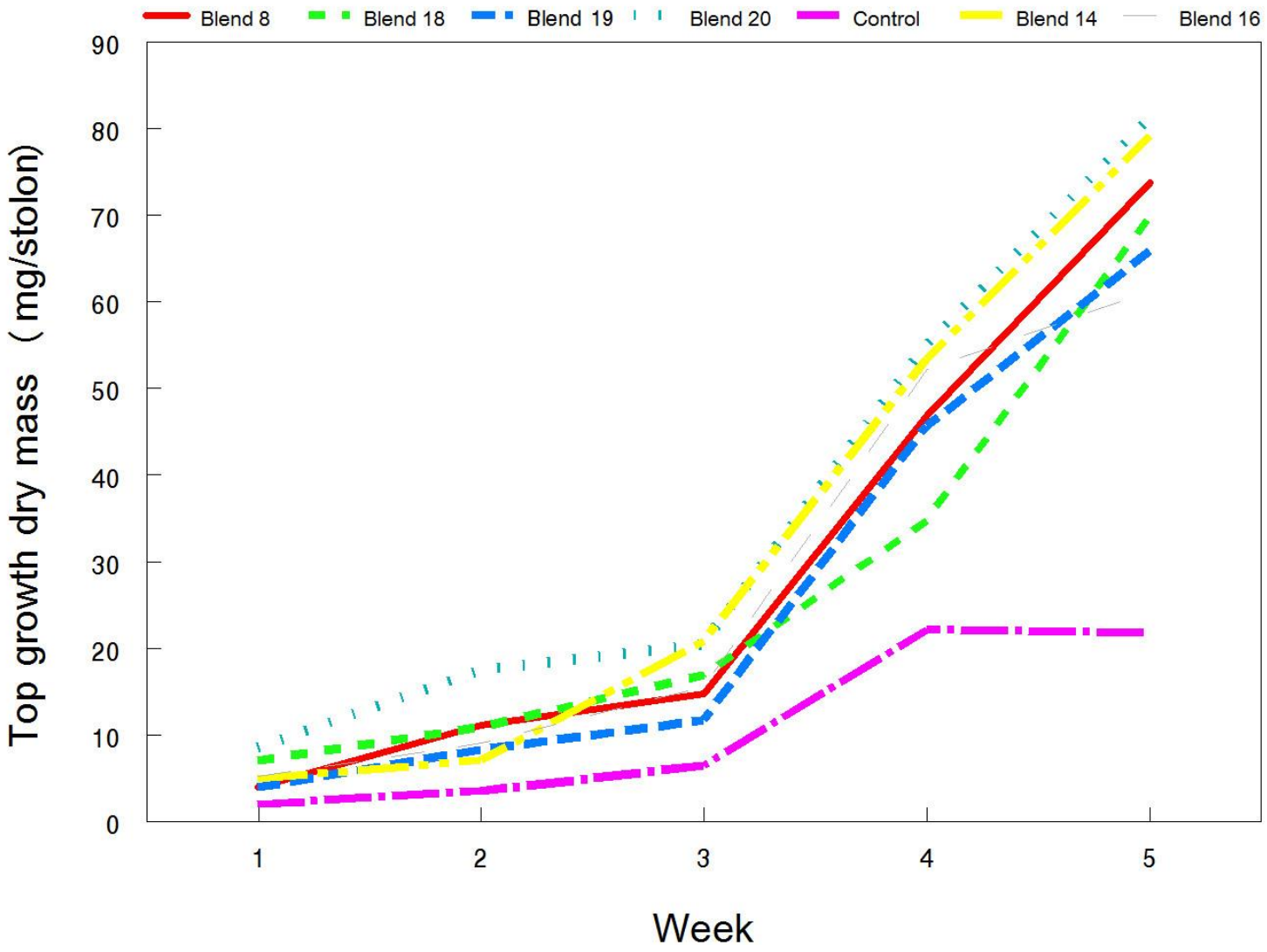


Figure 6: PGPR application and top growth clippings

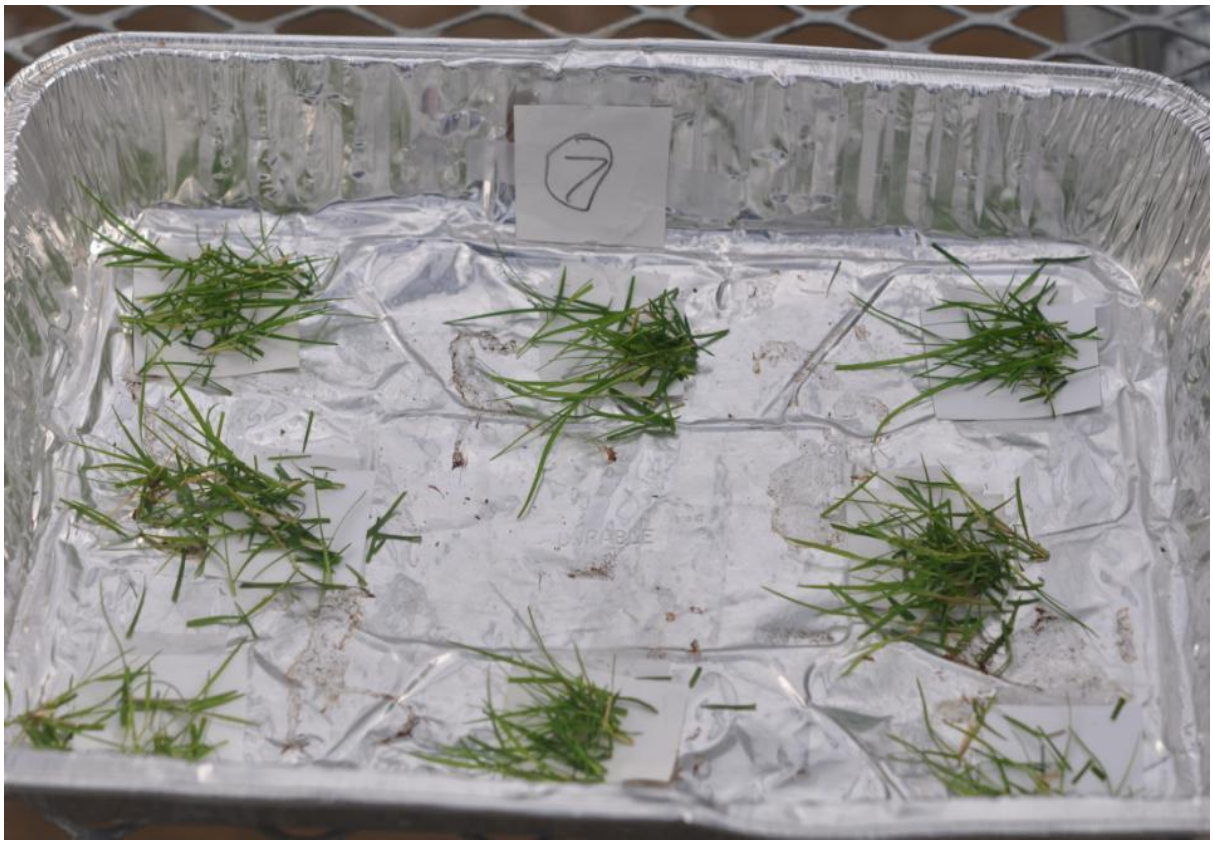


Figure 7: Average WinRhizo root scans from Greenhouse trial 1

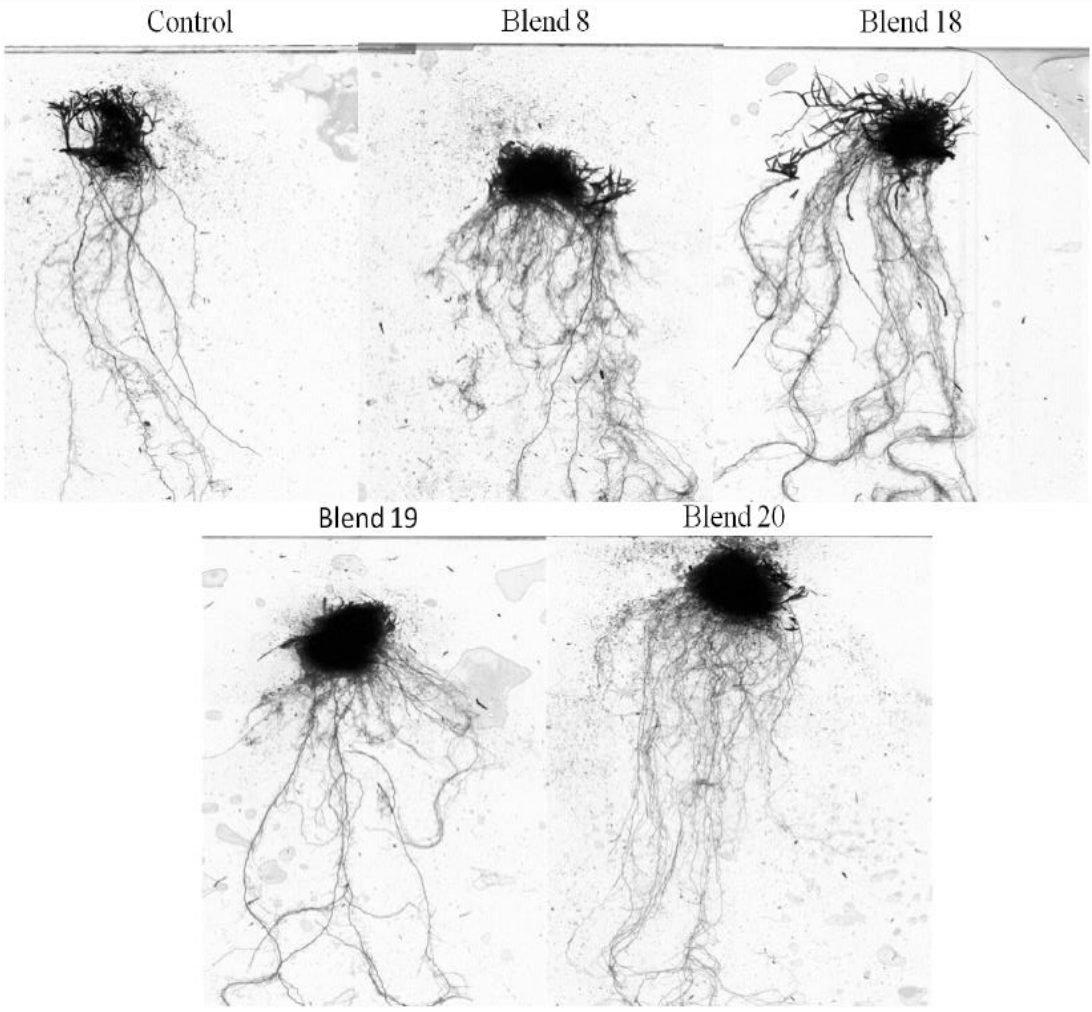
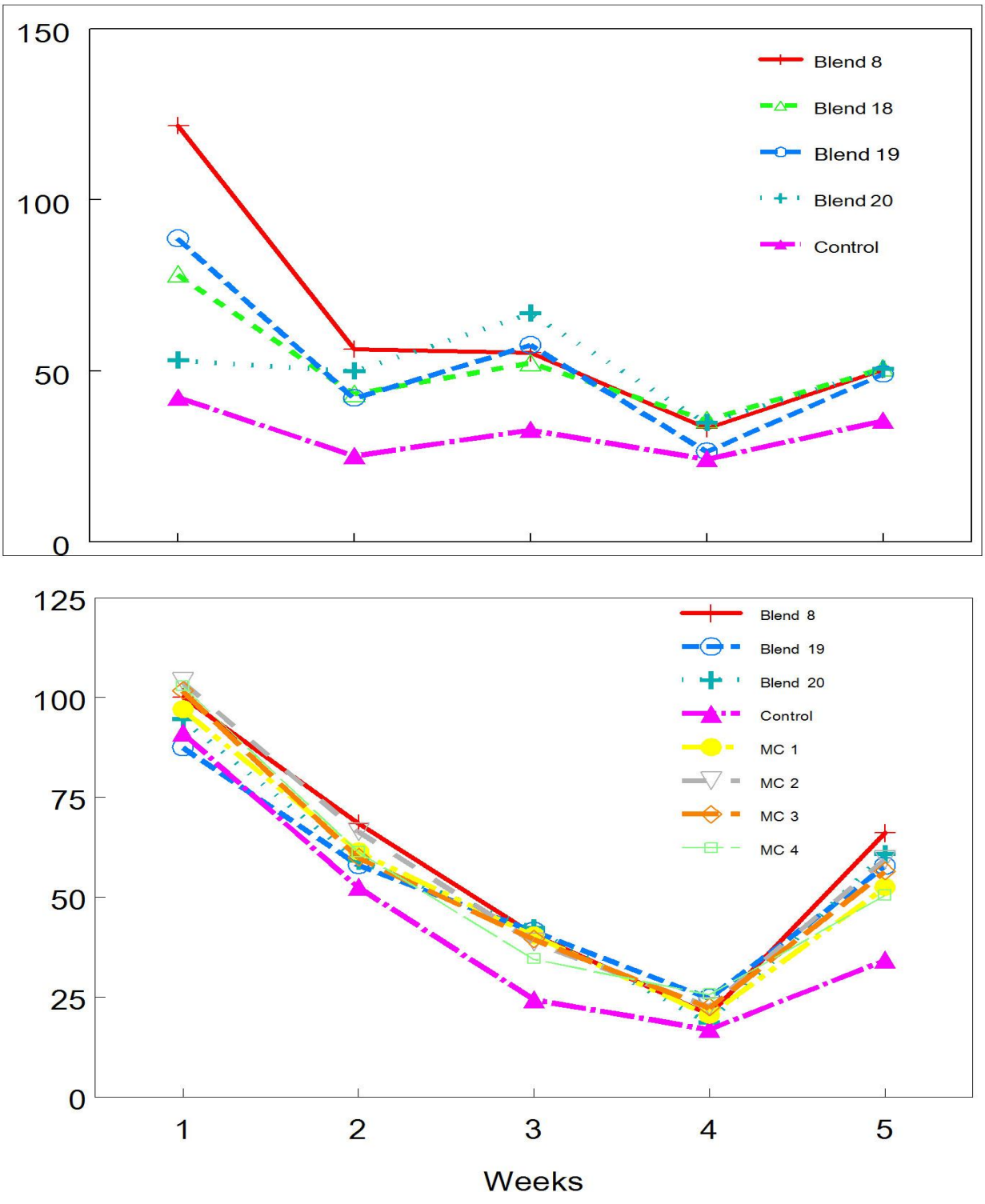


Figure 8: Weekly top growth dry mass (mg) from Greenhouse Trials 1 (top) and 2 (bottom).



Chapter 3

Effects of PGPR treatment of bermudagrass on the rearing, ovipositon, and natural enemies of the fall armyworm (*Spodoptera frugiperda)* (Smith) (Lepidoptera: Noctuidae)

Abstract

Non-pathogenic soil microbes can induce changes associated with phytohormones that may influence plant-insect interactions. Much of this work has focused on mycorrhizal fungi or certain rhizobacteria. These soil microbes may deter herbivore oviposition, influence performance of above ground herbivores, or attract natural enemies. Only a few studies have explored these interactions. Bacterial blends with commercial potential were screened for their ability to deter oviposition in no-choice greenhouse assays, negatively impact larval development in growth chamber conditions, and recruit natural enemies that would parasitize the fall armyworm (FAW) in field conditions. FAWs deposited most of their eggs on the grass in the control plants and ≤ 29% on the inoculated treated grass, suggesting that microbes can mediate interactions between females and oviposition hosts. Three blends negatively impacted larval weights, and two of these blends negatively impacted pupal weight and eclosion. Inoculants have shown to increase the attraction of natural enemies in laboratory settings. However we failed to find evidence supporting this under field conditions. These experiments were one of the first to examine parasitoid recruitment to plants treated with bacterial inoculants under field conditions and the first attempt to use microbial inoculants to manipulate natural enemies in turfgrass. Induced resistance to plant pathogens derived from microbe inoculation is well documented in the literature, but whether these interactions extend to herbivores, like insects, remains inconclusive.

Introduction

Non-pathogenic soil microbes can influence plant growth and induce changes associated with phytohormones that may influence plant-insect interactions. Much of this work has focused on mycorrhizal fungi or certain rhizobacteria in the genera *Pseuodomonas, Bacillus, Paenibacillus,* and *Lysinibacillus.* Plant growth-promoting rhizobacteria (PGPR) are non-pathogenic, free-living soil-and-root inhabiting bacteria that colonize seeds and roots (rhizosphere) (Kloepper and Schroth 1978, Kloepper 1993). Plant growth promotion mediated by these soil microbes may directly or indirectly influence performance of above ground herbivores, but only a few studies have explored these interactions (van Oosten et al. 2008, Pineda et al. 2012). *Pseudomonas fluorescens* positively affects weight gain of *Myzus periscae* feeding on treated *Arabidopsis thaliana* (Pineda et al. 2012), but the same microbe-plant combination negatively affects larval development of *Spodoptera exigua* (van Oosten et al. 2008).

Impacts of soil microbes on herbivores may also be indirect via ovipositional deterrence of females (Wilson and Faeth 2001, Nangle 2012) or by recruitment of natural enemies through changes in plant volatiles (van Oosten et al. 2008). The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are important in signaling pathways that mediate plant defenses against attacks from pathogens and herbivores (van Oosten et al. 2008). Typically, JA and ET are involved in plant defenses aginst herbivorous insect injuries (Bostock 2005). These defenses result in the production of chemicals that act as toxins or feeding deterrents to herbivores (Howe 2004). Indirectly, the plant is primed for defense from herbivory by emiting a blend of volatiles that may attract natural enemies (Turlings et al. 1995, Ngumbi 2011).

Building on our previous work with PGPR and bermudagrass (Chapter 2), we chose a grass-microbe system to evaluate the tri-trophic interaction with a foliage feeding herbivore. Our previous work identified blends of PGPR that, when applied to bermudagrass, result in growth

promotion relative to untreated plants. Grasses (including bermudagrass), corn, and sorghum are the primary hosts for the larvae of *Spodoptera frugiperda* (Smith), the fall armyworm (FAW), and females lay egg clusters on or near larval hosts. The FAW is a multivoltine noctuid moth that has three to four generations per year in the southern United States (Williamson et al. 2013). Larval development is about 2-3 wks and influenced by host quality. Females will discriminate during oviposition, laying eggs on plants that are of better quality for larval development (Lynch 1984). Using the FAW-bermudgrass system, we determined the extent bacterial inoculation on grass had on FAW development, oviposition, and natural enemies. Considering previous work, we expected that one or more PGPR blends would reduce oviposition by FAW females and retard larval development. We anticipated an increase in recruitment of natural enemies to treated bermudagrass and a subsequent increase in field parasitism relative to untreated grass plots. The objectives of these experiments were to screen blends with commercial potential that could deter oviposition and negatively impact larval development, while recruiting natural enemies with an emphasis on field parasitism.

Materials and Methods

Impact of PGPR-treated bermudagrass on oviposition. FAW pupae were obtained from a commercial insectary (Benzon Research, Carlise, PA) and held in the lab conditions until eclosion. Within 24 h of emergence, an adult male and two female moths were placed inside a plastic cup (11.4 x 8.9 cm) with a screen cut in the top and were allowed to mate overnight. Following the four day pre-oviposition period, the male moth was removed from the cup and the mated adult females were transferred to bermudagrass plugs in containers with the same plastic cup covering and allowed to oviposit. Adults were provided a diet of 50:50 mixture of honey and

water in 10 ml plastic vials with cotton wicks as a food source (Williamson and Shetlar 1995). The methods for growing and inoculating the Tifway hybrid bermudagrass plugs followed the methods described in the greenhouse bioassay trials. PGPR tested in this objective were Blends 8, 18, 19, and 20. Plants were cut to a height of 5 cm at the beginning of the experiment.

Experimental Design

The methods and protocols used for the no-choice ovipositional preference behavior of adult female FAW were modified from the methods described by Whitford et al. (1988). The oviposition assay was conducted inside a modified plastic cup cage in greenhouse settings (Figure 13). Seven plastic cup cages were established for each treatment, and seven replicates were completed. After the pre-oviposition period, two mated female moths were released in each cage and allowed to oviposit over a 2 day oviposition period.

Results of oviposition were recorded. Egg masses were collected and weighed after the 2 day oviposition period. A manual count of eggs was conducted. Egg masses that were laid on substrate that was not Tifway hybrid bermudagrass plant were counted and weighed. Egg masses were then transferred to a Petri dish (100 mm x 15 mm; VWR, Radnor, PA) and placed in a cool growth chamber for storage at 10° C, 50% relative humidity, and with a 14:10 (L/D) photoperiod.

Impact of PGPR-treated bermudagrass on larval development. 'Tifway' hybrid bermudagrass plots were established at the Auburn University Turfgrass Research Unit (TGRU). Plots 1 * 1 m with 1 m alleys were established on September 22, 2013. In the lab, PGPR blends were prepared from bacterial blend stock solutions made by the liter of equal parts of each bacterium to a final concentration of $1*10^7$ cfu/ml. PGPR that were tested in this objective are Blend 8, 18, 19, 20, MC 1, 2, 3, and 4.

Beginning on September 22, 2013, plots were treated with 500 ml/m^2 of PGPR or distilled water once weekly for 5 weeks. After 5 weeks, grass clippings were collected from each plot using a mower, bagged into separate labeled bags, and brought back to the lab and stored in a refrigerator. Fresh clippings were harvested each week with a Greensmaster 800 (Toro, Bloomington, MN) to maintain a fresh food source for the developing larvae.

Fall armyworm (Rice strain) eggs were purchased from Benzon Research (Carlise, PA). To induce hatching, eggs were placed in a growth chamber at 26.7° C, 50% relative humidity, and with a 14:10 (L/D) photoperiod (Lynch et al. 1984). Only newly hatched larvae <24 h old were used. Newly hatched larvae were weighed for their initial weight in groups of 50 to find an average weight and then transferred to a Petri dish (100 mm x 15 mm; VWR, Radnor, PA) with bermudagrass foliage. Each Petri dish received one FAW larva, and fresh clippings were provided daily (Figure 15). The larvae were reared in a growth chamber at 26.7° C, 50% relative humidity, and with a 14:10 (L/D) photoperiod. The larvae were weighed on Day 1, 3, 7, 10, 12, 14, and at pupation weights were recorded. Days until pupation were also determined for each larva. Each PGPR and control treatment had 22 replicates (caterpillars). At pupation, pupae were transferred to a new Petri dish with no clippings and placed in a growth chamber at 26.7° C, 50% relative humidity, and with a 14:10 (L/D) photoperiod. Pupae were monitored daily, and percentage adult eclosion was determined for each treatment.

Recruitment of natural enemies to PGPR-treated bermudagrass and parasitism of FAW. This experiment was conducted in two trials, a 5 wk trial in 2012 and a 6 wk trial in 2013. In 2012, field plots of Tifway bermudagrass were marked at the TGRU (Auburn, AL) and at the Robert Trent Jones (RTJ) Golf Course (Opelika, AL) for 5 weeks. Only the TGRU plots received daily irrigation. Field plots were circular and covered an area of .11 m were spaced by 2.5 m from the

paired plot. Bermudagrass was maintained at a height of 1.27 cm (cut 3 times weekly) at TGRU and 16 cm (cut once a week) at RTJ. A PVC ring (38.1 cm diam. by 20 cm tall; Figure 9) was placed over the plot when treatments were applied. Beginning on August 10, 2012, treated plots received weekly application of either Blend 9 or distilled water (control) for 5 wk. Blend 9 was selected because previous work (Ngumbi 2011) had shown attraction of parasitic wasps of lepidopteran pests in cotton under lab conditions. Applications were made with a plastic, trigger-type hand sprayer and each treatment (Blend 9 and Control) had its own sprayer. For each application, 20 ml of PGPR or water was applied. Following application of PGPR and control, treatments were watered in with 1.4 L to move the treatment to the root zone. Treated and control plots were replicated four times at each location. On the third and fifth week, Amdro Pro (Ambrands, Atlanta, GA) was applied at a rate of 4 oz / 5,000 ft^2 around the perimeter for fire ants, *Solenopsis* spp.

To evaluate the presence and abundance of natural enemies, yellow pan traps (diameter 15 cm, Party City Corporation, Rockaway, NJ; Figure 9) filled with a soapy dilution (Joy Lemon Scent, Procter & Gamble, Cincinnati, OH) were placed in the center of each plot immediately after post-treatment irrigation. After 8 h, traps were collected and contents emptied into plastic jars for transport to the lab. This procedure was used every week following application. In the lab, all insect predators and parasitoids were separated to family (order for spiders). After the 5^{th} week, PVC rings were hammered into the ground and used to confine 10 2^{nd} instar FAW larvae over the treated plot. Larvae (rice strain) were obtained from the USDA-ARS. Larvae were raised from eggs in the laboratory at Auburn University at 26.7° C, 50% relative humidity, and with a 14:10 (L/D) photoperiod on a pinto bean diet until they were 2^{nd} instars. To prevent escape, the top of the rings were coated with petroleum jelly (Vaseline, Unilever, London, UK)

and covered with a mesh screen with 1 cm spacing to prevent the escape of FAW larvae and predation from birds. The FAWs were placed into the ring for 7 d before being collected by a soap flush drench and returned to the lab. Each recovered larva was placed individually into a Petri dish (100 mm x 15 mm; VWR, Radnor, PA) with bermudagrass clippings from the respective plot and then placed in a growth chamber at 26.7° C, 50% relative humidity, with a 14:10 (L:D) photoperiod. Larvae were monitored daily to determine signs of parasitism.

The methodology for the 2013 trial was modified to evaluate more PGPR treatments. Field plots, 0.6 x 0.6 m with 0.6 m alleys (Figure 11), of Tifway bermudagrass were established at the TGRU in a randomized complete block design. The plots received daily irrigation. Bermudagrass on this site was maintained at a height of 1.27 cm (cut 3 times weekly). This trial was conducted for 6 wks and compared four PGPR Blends (8, 18, 19, and 20) along with a distilled water control, each with five replicates. Blends and distilled water were applied using a CO_2 powered, low volume sprayer (R&D Sprayers, Opelousas, LA) with a 0.5 m boom width equipped with 3 nozzles that delivered 100 ml per plot at normal walking speed. The 100 ml application volume is equivalent per unit area to the rate used in the 2012 trial. Applications were made weekly before 10 am CDT when winds were < 2 mph. Immediately following each application (control and PGPR), 4.7 L of water were applied to the plot from watering cans to wash the bacterial solution into the root zone. On the first, third, and fifth week, Amdro Pro (Ambrands, Atlanta, GA) was applied at a rate of 4 oz / 5,000 ft^2 around the perimeter to control fire ants, *Solenopsis spp.*

As in the 2012 trial, yellow pan traps (diameter 15 cm, Party City Corporation, Rockaway, NJ; Figure 9) filled with a soapy dilution (Joy Lemon Scent, Procter & Gamble, Cincinnati, OH) were used each week following treatment to monitor natural enemy presence

and abundance. Traps were deployed for 8 h, and insects recovered and sorted as previously described. After 6 wk, a PVC ring, used in 2012 trial, was inserted into the center of each plot to an approximate depth of 2.5 cm. In this trial, 12 3rd instar FAW larvae were introduced. Rice strain FAW eggs were purchased (Benzon Research, Carlisle, PA) and raised in the lab at 26.7° C, 50% relative humidity, with a 14:10 (L:D) on a pinto bean diet until they were 3rd instars. Larvae were transferred to field plots for a 72 h interval exposure to natural enemies inside PVC rings, as previously described, except the rings were not covered by mesh in this trial. After 72 h, the FAW were recovered by a soap flush drench and returned to the lab. Each recoved larva was placed individually into a Petri dish (100 mm x 15 mm; VWR, Radnor, PA) with bermudagrass clippings harvested from the respective plot using a Greensmaster 800 (Toro, Bloomington, MN) and then placed in a growth chamber at 26.7° C, 50% relative humidity, with a 14:10 (L:D) photoperiod. Larvae were monitored daily to determine signs of parasitism.

Statistical Analysis

In the no-choice oviposition study, the number of eggs laid on the grass for each treatment was analyzed using analysis of variance (ANOVA), Student's t-Test, ($P < 0.05$, JMP Version 10. SAS Institute Inc., Cary, NC. 1989-2007). Also, the mean number of eggs laid on the grass compared to the cup for each treatment was analyzed using a Log transformation + 0.50 analysis of variance (ANOVA), Student's t-Test, ($P < 0.05$, JMP Version 10. SAS Institute Inc., Cary, NC, 1989-2007). Analysis of variance (ANOVA), Student's t-Test, ($P < 0.05$, JMP Version 10. SAS Institute Inc., Cary, NC. 1989-2007) was used for analyses of larval weight, pupal weight, and days to pupation. Natural enemy recruitment (all parasitoids, FAW parasitoids, all predators, FAW predators, Hymenoptera, Diptera, and all natural enemies) was

analyzed for both the 2012 and 2013 trials using repeated measures multivariate analysis of difference (MANOVA), ($P < 0.05$, JMP Version 10. SAS Institute Inc., Cary, NC, 1989-2007).

Results

In the no-choice oviposition study, FAWs deposited most of their eggs (55%; Figure 14) on the grass in the control plants and ≤ 29% on the PGPR-treated grass with either Blend 8, 18, 19, and 20. The mean number of eggs laid on the grass was reduced by 25% with the use of Blend 20 (Figures 13 and 14). These data were used as a measure of preference for oviposition behavior of *Spodoptera frugiperda* relative to PGPR treatment.

Larvae were successfully reared to pupation (>90%) on PGPR and non-PGPR treated clippings in the growth chamber. Days 10 and 12 had larval weights that were negatively impacted from feeding on PGPR treated clippings compared to the control. Blend 18 and MC 4 larvae weighed 18% (149.7 mg vs. 171.6 mg; Table 5) less than the control on Day 10. MC 3 and MC 4 larvae weighed 20 and 41% less than the control. There was no observed negative impact on larval development for days to pupation, and larvae reared on Blend 20 developed and pupated significantly faster relative to the control pupae (15.14 vs. 15.86 days). MC 1 and Blends 18 and 20 produced pupae that were heavier than the control, while MC 2 and Blends 8 and 19 produced pupae similar to the control. Pupal weights and adult eclosion were also negatively affected with blends MC 3 and MC 4. Pupae of MC 3 and MC 4 weighed 11 and 21% less respectively, than the control (102.95 and 92.2 mg, respectively vs. 115.57). Adult eclosion was negatively impacted with MC 3 and MC 4 with only 66.6 and 78.9% of adults emerging compared to the >90% eclosion for the control. Eclosion was successful ≥85% of the time with all other blends.

In the 2012 parasitism study, only one instance (Blend 9; Figure 10) of parasitism was observed. Due to the lack of parasitism, no statistics were performed on parasitism. The data collected from the pan trap study demonstrated that there was no influence of PGPR (Blend 9) on recruitment of parasitoids, predators, or total natural enemies ($P \leq 0.09$) in general or specifically for *S. frugiperda.* Diptera and Hymenoptera were the most abundant of all natural enemies (Table 6) encountered.

In the 2013 parasitism study, no instances of parasitism were observed. Due to the lack of parasitism, no statistics were performed on parasitism. The data collected from the pan trap study (Figure 11) demonstrated that there was no influence of PGPR (Blend 8, 18, 19, and 20) on recruitment of parasitoids in general or specifically for *S. frugiperda,* total hymenoptera, and total diptera between PGPR-treated and non-treated plots (Figure 12). Statistical differences in predator abundance were observed with Blend 18 having significantly more predators ($P <$ 0.0431.

Discussion

Female FAW deposited eggs mostly on the grass in the control containers (55%) with the remaining eggs on the enclosure. Less than a third (≤ 29%) of eggs were deposited on the grass by female FAW in any of the PGPR-treated bermudagrass pots (Figure 14). There were no differences in total eggs deposited on grass between PGPR treatments. Number of eggs deposited on bermudagrass treated with Blend 20 was 25% of the average number deposited on untreated bermudagrass. These results suggest that microbes can mediate interactions between females and ovipositional hosts. Infection of woody plants (oak trees) with endophytic fungi reduced oviposition by female moths (Gracillariidae) (Wilson and Faeth 2001). There have only been a few examples in the literature that investigated the interactions of microbes with insect

interactions on which to draw precedents. Female FAWs prefer grasses with greater nitrogen content as ovipositional sites (Williamson et al. 2012). The plants in this study were fertilized before the application of the PGPR but not afterward. PGPR-treated plants typically have increased nitrogen uptake (Benizir et al. 2001), so arguably they would likely have had greater nitrogen content and more eggs than untreated plants. Data on nitrogen uptake in Tifway bermudagrass have been collected (Chapter 4). We also suspect PGPR may alter headspace volatiles and are currently collecting volatile organic compounds (VOCs) from PGPR-treated bermudagrasses that are mown and not mown, but those experiments are currently ongoing. Regardless of the mechanism, we do not expect PGPR treatment of grasses to significantly alter local populations of FAW in turfgrass stands. FAW females notoriously oviposit on non-host substrates and the larvae are mobile. Deterrence of oviposition may mean eggs are present in areas adjacent to managed turfgrass, yet larvae can still invade those stands.

Induced resistance in plants derived from microbe inoculation to plant pathogens is well documented in the literature, but whether these interactions are specific to plant pathogens or extends to herbivores, like insects, remains inconclusive. Investigations into the effect of microbe induced resistance to insects have varied in results as studies have used different host plants, insects, and microbe inoculants (van Oosten et al. 2008, Stout et al. 2006, Zehender et al. 2001). Inoculation of bermudagrass with five of our eight PGPR blends resulted in fresh larval weights that did not negatively impact *S. furgiperda* development; however, the use of Blend 18, MC 3 and MC 4 resulted in larvae weights that were negatively impacted on either Days 10, 12, or both. Blends MC 3 and MC 4 had the most detrimental impact on larval weights and pupal weights. Larvae reared on these blends were 80 and 59% the fresh weight of control larvae on Day 12. Pupal weights with the use of MC 3 and 4 were also negatively impacted as pupae were

89 and 79% the weight of control pupae. Even with reduced weight gain, all blends and control were successfully reared to pupation (>90%), and average days to pupation were not negatively impacted.

Adult eclosion was negatively impacted with MC 3 and MC 4 with only 67 and 79% of adults emerging compared to the >90% eclosion for the control (>85% all other blends). Our results both confirm and conflict with van Oosten et al. (2008) who found a negative impact on *S. exigua*, a related moth species reared on *Arabidopsis*, but did not see a negative impact on *P. rapae* in the same study with microbe inoculants. Our varying results suggest that the PGPR blend strain identity may play a role in defense signaling pathways that could impact larval development, as blends MC 3 and MC 4 had a negative impact on larval weight, and three of their four strains were the same as each other and blends MC 1 and MC 2. These results could suggest that the interaction of the different bacterial species in these blends with each other could have synergistic or antagonistic effects that impact larval performance and development in different blends. While negative impacts were observed, PGPR blends tested did not elicit toxic plant defenses that were able to control FAW populations.

A change in plant volatiles after inoculation of plants may be a mechanism to explain the effects of bacterial inoculants on plants. Plants emit volatile organic compounds (VOCs) that may vary quantitatively and qualitatively in response to herbivore feeding and plant treatment. The volatile compounds released by plants can be divided into constitutive compounds and herbivore induced plant volatiles (HIPVs). Constitutive compounds are constantly present and are released due to mechanical damage or immediately after herbivore feeding begins. HIPVs are delayed in their release by the plant due to herbivore feeding damage (Ngumbi et al. 2009). HIPVs are assumed to be detrimental to the herbivore through indirect defense mechanisms of

the plant because they attract natural enemies of herbivores (Carroll et al. 2006). The manipulation of VOCs by turfgrass managers could enhance the biological management of FAW with natural enemies by attraction of parasitoids.

In pan trap samples, Hymenoptera and Diptera were most represented of all natural enemy groups in both years (Table 4). Abundance in these groups as well as total natural enemies, parasitoids, or FAW parasitoids were not significantly different between PGPR-treated and control plots (Figure 12; $P < 0.09$). We were able to recover 70% and 77% of FAW larvae exposed to PGPR-treated or untreated bermudagrass plots for 7 d and 72 h, respectively. In 2012, there was one instance of parasitism with the use of Blend 9, but there were no observed parasitism events when larvae were reared to pupation in 2013. Treatment of *Arabidopsis* with *Pseudomonas syringae*, a PGPR strain, did not increase attraction to intact plants or plants fed upon by *S. exigua* (van Oosten et al. 2008). However, *P. syringae* is capable of influencing jasmonic acid and plant volatiles in greenhouse or laboratory tests (van Oosten et al. 2008, Pineda et al. 2012). The role of that finding in recruitment of natural enemies under field conditions was not found in the present studies and has yet to be confirmed. These experiments are one of the first to examine parasitoid recruitment to plants treated with PGPR under field conditions and the first attempt to use microbial inoculants to manipulate natural enemies in turfgrass.

Managed turf differs from other crops because its value determination is derived from its aesthetic qualities. Due to the demand for perfectly maintained turfgrass by consumers, there is zero or low pest tolerances which makes it difficult for turf managers to use action and economic thresholds. Therefore, turf managers use a myriad of tactics to manage their turf, often relying heavily on the use of chemical inputs and incorporating few sustainable management tactics into

their management practices. The heavy reliance on chemical inputs for turf management can disrupt the ecosystem leading to soil compaction, excessive thatch accumulation, pest resurgence, or secondary pest outbreaks (Held and Potter 2012). PGPR could offer a sustainable management tactic for turfgrass managers in a perennial system if a blend can successfully colonize the root system and provide plant defenses against insect herbivores by deterring oviposition, and larval feeding, demonstrating toxic effects, or recruiting natural enemies. Use of PGPR has been successful in other crop systems and has demonstrated persistence for up to 12 weeks post inoculation after only one inoculation in cotton (Durham 2013), but the demonstration of PGPR's ability for larval toxicity, natural enemy recruitment, and alterations of VOCs has yet to be observed in a turfgrass system.

Table 5. *S. frugiperda* larval rearing on Bermudagrass clippings after 5 wk exposure to bacterial inoculants

Mean (± SEM) values of fresh larval weights and % Eclosion (N=22)

Treatment	Day 10 weight (mg)	Day 12 weight (mg)	Pupal weight (mg)	Days to pupation	% Eclosion
Control	171.64 ± 5.13a	272.00 ± 9.79ab	115.57 ± 4.29cd	15.86 ± 0.19	95
Blend 8	162.09 ± 6.31ab	245.96 ± 12.42bcd	113.57 ± 2.48cd	15.67 ± 0.11	85
Blend 18	149.73 ± 7.82b	263.64 ± 14.21ab	120.20 ± 2.90bc	15.55 ± 0.11	90
Blend 19	164.00 ± 6.32ab	221.64 ± 12.42cd	114.14 ± 3.76cd	15.76 ± 0.17	85
Blend 20	159.73 ± 5.34ab	251.82 ± 11.13bc	124.62 ± 2.88ab	15.14 ± 0.48	87
MC 1	168.96 ± 5.23a	290.64 ± 10.50a	132.00 ± 3.45a	15.38 ± 0.13	90

MC 2	159.32 ± 6.42ab	214.32 ± 10.13de	108.50 ± 2.62de	15.60 ± 0.11	85
MC 3	159.86 ± 5.10ab	183.32 ± 7.07ef	102.95 ± 2.59e	15.75 ± 0.12	66.6
MC 4	149.05 ± 6.76b	160.82 ± 8.87f	92.20 ± 3.26f	16.10 ± 0.22	78.9
Statistics	F=1.37, P=0.018	F=13.48, P=0.002	F=13.85, P=0.005	F=1.65, P=0.019	--

Means presented are actual means. Within a column, means ± SEM followed by the same letter are not significantly different from each other ($P < 0.05$; JMP; ANOVA Student's t-Test; [SAS Institute Inc., Cary, NC. 1989-2007]).

Table 6. Natural Enemies Encountered

Araneae	Hymenoptera
Coleoptera	Braconidae
Carabidae	Chalcidiae
Coccinellidae	Chrysididae
Staphylinidae	Diapridae
Diptera	Encyrtidae
Asilidae	Figitidae
Phoridae	Formicidae
Sarcophagidae	Ichneumonidae
Syrphidae	Mymaridae
Tachinidae	Platygastridae
Hemiptera	Pompilidae
Nabidae	Pteromalidae
	Scoliidae
	Sphecidae
	Torymidae
	Vespidae

Figure 9: Experimental Design for Pan Trap Study in 2012

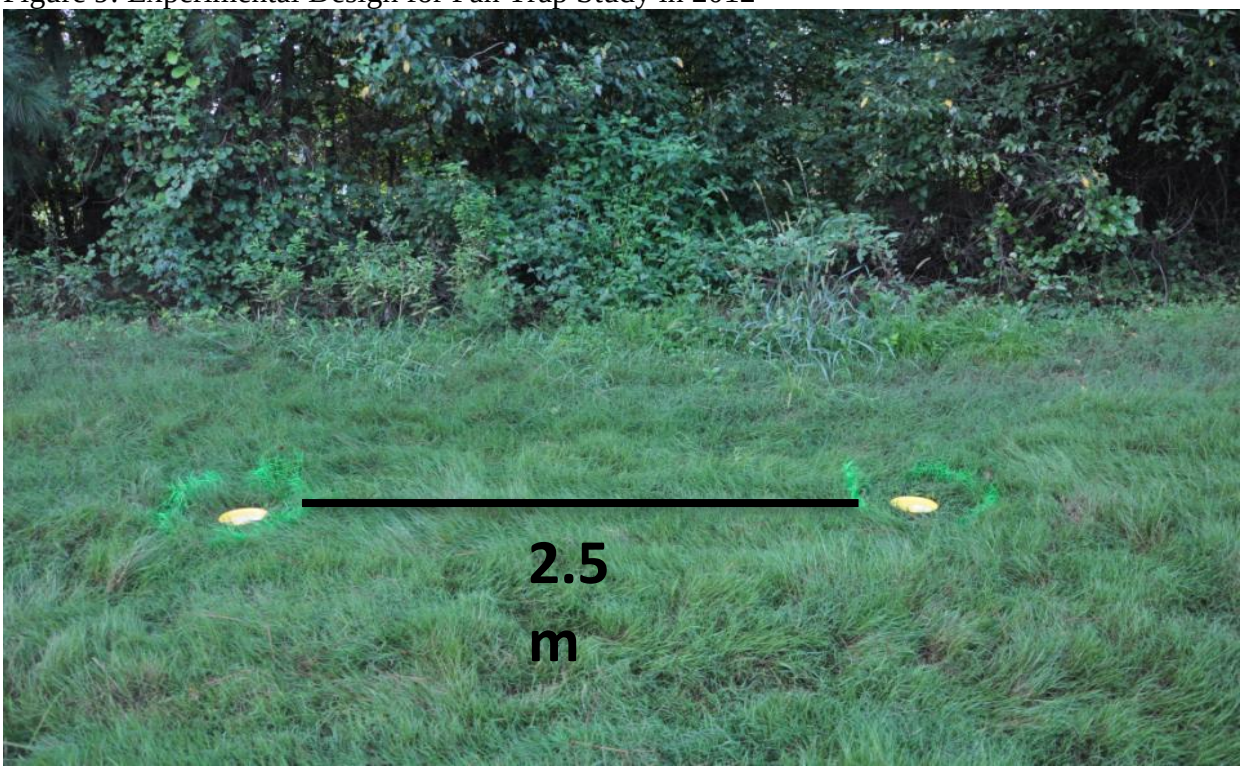


Figure 10: FAW parasitized by Tachindae fly

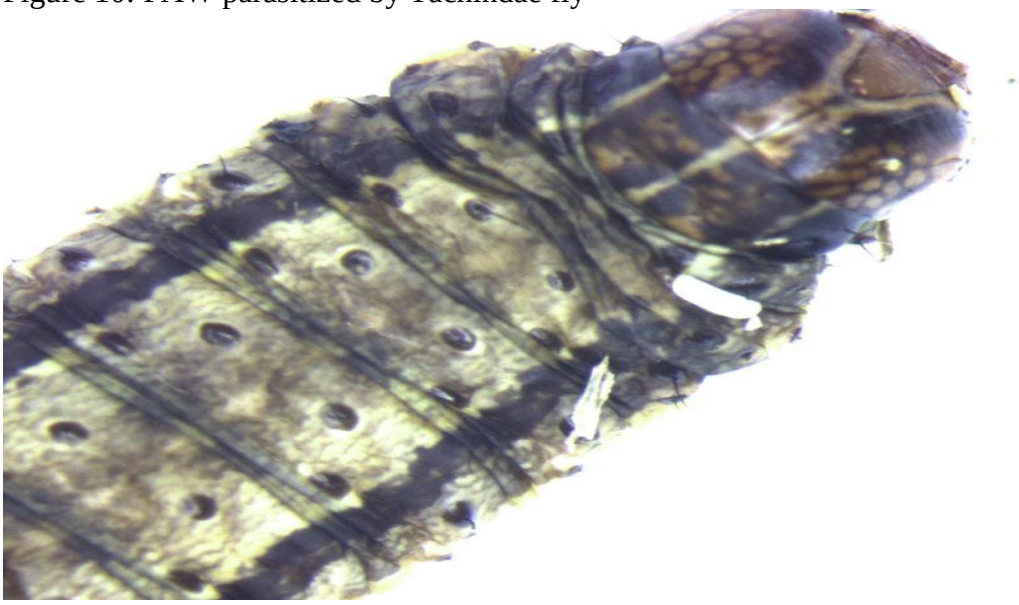


Figure 11: Experimental design for pan trap study 2013 and FAW enclosures for 2013

Figure 12: Parasitoids present in pan traps expressed as weekly means. MANOVA, Repeated measures, ($P < 0.05$, JMP Version 10, SAS Institute Inc., Cary NC. 1989-2007)

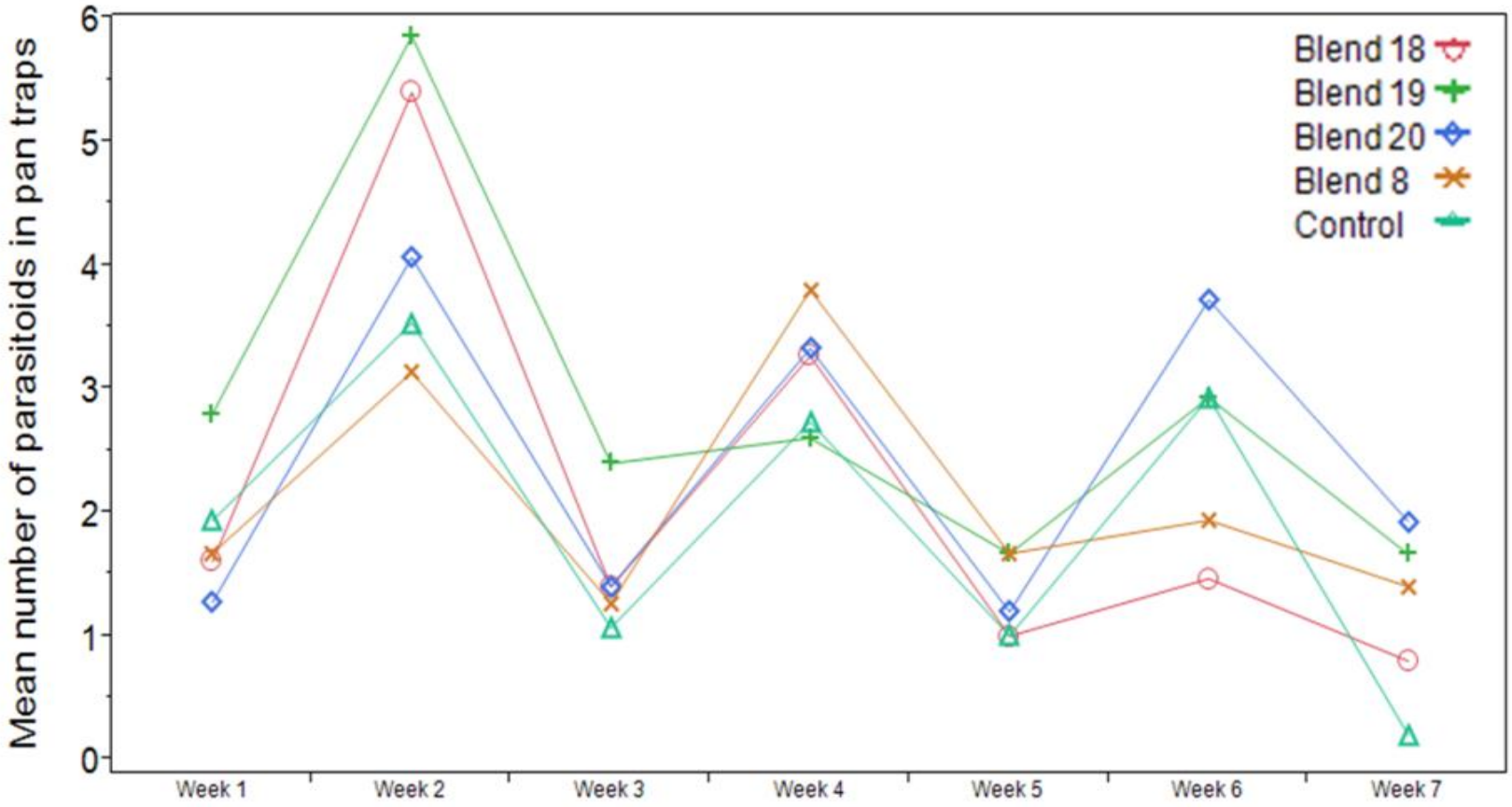


Figure 13: Experimental design for No-Choice ovipositional study and egg clusters (Top Right: Blend 20, Bottom: Control)

Figure 14: Oviposition of fall armyworm females expressed as mean number of eggs (left axis) and percentage of total eggs on grass. * indicates a treatment significantly different from the untreated control. ANOVA, Student's t-Test, ($P < 0.05$, JMP Version 10, SAS Institute Inc. Cary, NC. 1989-2007)

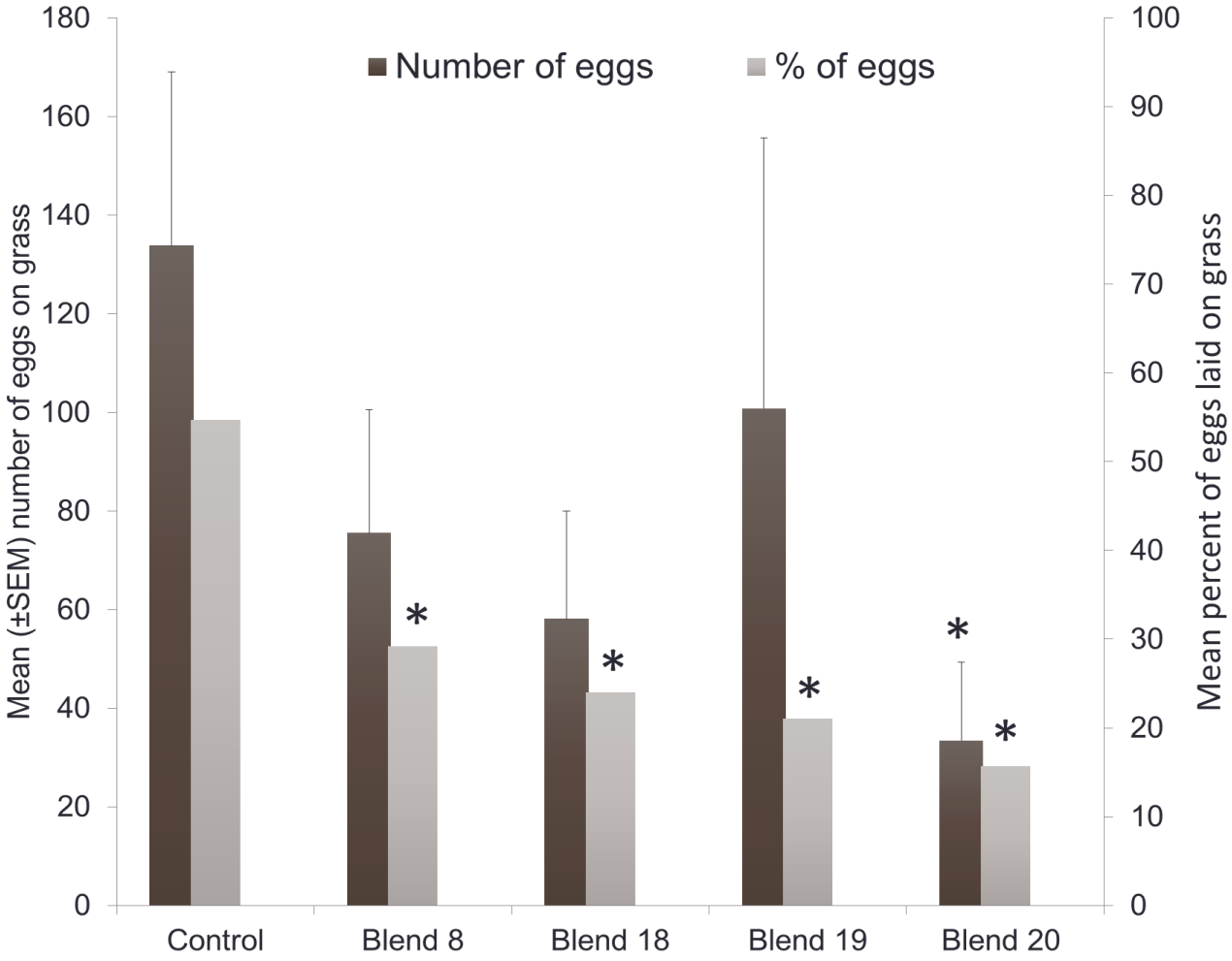


Figure 15: FAW reared on Tifway clippings

Chapter 4

Effect of PGPR treatment on Tifway bermudagrass on nitrogen rates

Abstract

Nitrogen (N) is the most important macronutrient for sustaining plant growth in turfgrass and is abundantly applied to amenity grass. Plant N use efficiency is estimated to be only 50%. Avenues of loss are leaching, immobilization, and denitrification which releases nitrous oxide, a greenhouse gas. Use of PGPR and other microbial inoculants could allow for a reduction of N rates if they can alter the soil microbe community by improving nutrient uptake and efficiency while reducing greenhouse gas emissions. Replicated field study experiments that evaluated varying N rates with and without a bacterial inoculant were conducted on a golf course. Parameters evaluated were foliar N content, visual turfgrass ratings, chlorophyll content, and tensile strength. We failed to demonstrate that the addition of PGPR to a N fertility management practice increased bermudagrass quality based on the parameters evaluated. Factors like site selection, weather, and N rates evaluated may have negatively impacted this study and may explain why differences were not observed. Differences may have been observed if the study had been conducted in a less managed turfgrass setting, like a home lawn or pasture.

Introduction

Amenity turfgrass is a high value, high input system. Temperature, soil moisture, and plant available nitrogen (N) are the most common limiting factors of growth. Of all the macronutrients required for turfgrass establishment and maintenance, nitrogen is the most crucial resource for sustaining plant growth; therefore, it is the most abundantly applied chemical to turfgrass (Frank and Guertal 2013). It is estimated that only 50% of applied N is used by plants

in temperate, humid climates where half of the applied N can be lost from leaching, denitrification, and immobilization (NRC 1993). In bermudagrass, nitrogen is commonly applied monthly at a rate of 0.45 kg / 93 m^2 to maintain the aesthetics of a dense, green stand that is tolerant of environmental and pest stressors during the growing season (Carrow et al. 2001). Urea, ammonium sulfate, and potassium nitrate are commonly used soluble nitrogen sources in turfgrass.

Dinitrogen (N_2) is the most abundant natural gas and makes up about 78% of the earth's troposphere; however, this form of nitrogen (N) cannot be utilized by plants. A series of complex reactions, referred to as the nitrogen cycle, transforms N_2 into a plant-available nutrient. Mineralization, atmospheric deposition of N by lightning, and N fixation by nonsymbiotic organisms create plant-available N. Immobilization, denitrification, leaching, and volatilization are reactions and processes in which N is lost. These chemical reactions and processes are impacted by environmental conditions pertaining to temperature, soil moisture, and soil microbe activity (Frank and Guertal 2013). Therefore, plant available nitrogen is often a limiting factor for plant uptake and growth (Bernhard 2012). Plant available N sources that are created during the N cycle are ammonium (NH_4^+) and nitrate (NO_3^-). Bermudagrass absorbs and utilizes both ammonium (NH_4^+) and nitrate (NO_3^-), which are the result of nitrification by nitrogen-fixing bacteria in the soil. During periods of rapid growth, N is supplied to the shoots but enters the roots and rhizomes otherwise, which accounts for variability in seasonal foliar nitrogen (Wherley et al. 2009).

Greenhouse gas emissions have increased during the past several decades. Nitrous oxide (N_2O) is a common greenhouse gas produced from denitrification in turfgrass with the use of fertilizers. According to the Environmental Protection Agency (EPA), N_2O accounts for 5% of

the world's greenhouse gas emissions and of that 5%, about 3% comes from non-agricultural fertilizer use (Terry and Kirby 1997). Loss of N by denitrification can range from 20-50% of applied N if the application is followed by heavy precipitation (Horgan et al. 2012, Barber 1995). Denitrification is favored by high temperatures with soil conditions being wet and oxygen poor (Frank and Guertal 2013). The use of PGPR and other soil microbial inoculants could alter the soil microbe community and improve the efficiency of nutrient uptake by the plant, allowing for a reduction or alternative to fertilizer use (Calvo-Velez 2013, Adesemoye and Kloepper 2009). Grass that is adequately fertilized has a dry N foliar content that is between 2-6% (Mills and Jones 1996). In bermudagrass, the use of N_2-fixing bacteria inoculants (*Azospirillum* and *Azotobacter*) resulted in a 21% increase of N in top growth and 13% in total harvested plant material when no N was applied, but had no effect when N was applied (Baltensperger et al 1978).

Materials and Methods

Impact of PGPR on N rates and use efficiency under field conditions. This study was conducted in an established Tifway bermudagrass at the Robert Trent Jones (RTJ) golf course (Opelika, AL). The bermudagrass was established in 1991, and the research was conducted in a Cecil sandy loam soil (Fine, kaolinitic, thermic Typic Kanhapludults) with 1-6% and 6-10% slopes (USDA Soil Survey 2014; Appendix 1). The site was irrigated daily, mowed once a week at a height of 7 cm, (grass clippings were left in the field) and fertilized with up to 3 lbs (1.36 kg) of a slow release product N product per 1,000 ft^2 (92.9 m^2) when possible. The site was not fertilized by RTJ in 2013. Bermudagrass was maintained at a height of 7 cm and cut once a week.

This trial was conducted for 15 wk and compared Blend 20 with a distilled water control and five nitrogen rates each with ten replicates. PGPR were applied weekly for the first two weeks of the study (July 3 and 10) in an attempt to thoroughly colonize the root system of the grass. After the first two initial treatments, the grass was treated every four weeks (August 7, September 4, and 25) for the remainder of the study. Blend 20 and distilled water were applied using a CO_2 powered, low volume sprayer (R&D Sprayers, Opelousas, LA) with a 0.5 m boom width equipped with 3 nozzles that delivered 250 ml per plot at normal walking speed. The 250 ml application volume is equivalent per unit area to the rate used in our previous greenhouse and field studies (Chapters 2, 3). The PGPR treatment was then immediately followed with 13 L water to move the treatment into the root zone and to prevent desiccation of the treatment. After the PGPR treatments were made, the control plots were then treated at the same rate of distilled water, and 13 L water were immediately applied.

The bermudagrass was fertilized on weeks 3, 7, and 11 (July 17, August 14, and September 11, 2013) at one of 5 rates (0, 25, 50, 75, 100%) of the recommended rate of ammonium sulfate fertilizer (PRO fertilizer, 21-0-0, Harrell's Inc., Lakewood, FL), or 0, 5.8, 11.6, 17.4, or 23.1 g/m^2, respectively. Foliar nutrient analyses were performed on weeks 1, 8, and 14 (July 3, August 21, and October 2, 2014). Grass clippings were taken from each plot and oven dried at 70C for 40 min. Samples were then sent to Waters Agricultural Laboratories (Camilla, GA) for basic nutrient analysis (N, P, K, Mg, Ca, S, B, Zn, Mn, Fe, and Cu). Change in foliar nitrogen (ΔN) was calculated by subtracting the final foliar N content from the initial foliar N content of each plot and then averaging the change in foliar N for each treatment.

Measurement of turf quality. Each week, visual turfgrass ratings (1-9 scale) were performed for turfgrass quality, color, density, uniformity, texture, and disease or environmental stress based on

the National Turfgrass Evaluation Program (NTEP). Additionally each week, five plant chlorophyll content measurements were recorded and averaged from each plot using a FieldScout CM 1000 Normalized Difference Vegetation Index (NDVI) (Spectrum Technologies, Inc., Aurora, IL). A stronger understanding of the strong relationship between leaf tissue N and plant chlorophyll content could allow future turf managers to make predictions on N fertility needs from estimations. Plant chlorophyll content can reach a plateau when the plant is adequately fertilized, but the plant continues N uptake. Due to this fact, estimates of relative plant N statuses have be compared within corn crop systems and cultivars (Schepers et al. 1992; Wood et al. 1992). This relationship is calculated by using an N Sufficiency Index (NSI) equation:

$NSI = \frac{Average\ meter\ reading\ from\ unknown\ area}{Average\ meter\ reading\ from\ comprable\ area\ with\ adequate\ N}$. An NSI value that is close or greater than 1.0 suggests that the unknown area has adequate fertilization. However, it does not detect excessive N. As values decrease from 1.0, it can be assumed that the area is not N sufficient (Schepers et al. 1998). This equation allows for comparisons between sampling dates, cultivars, and cropping systems and also offers an alternative to nutrient analyses that can be performed immediately. While few cropping systems have adopted this technique, it does allow turf managers a tool to be more efficient when determining fertility needs and fertilizer rates.

On October 9 (week 15), 0.46 m x 0.61 m strips of sod were harvested from each plot using a motorized sod cutter (Ryan, Johnson Creek, WI) and brought to the Auburn University Turfgrass Research Unit (TGRU) for sod strength evaluation. Sod was then ripped apart 3 times using a sod machine, and a tensile strength torque meter (Figure 19) was used to record the amount of force (torque pound) required to pull apart each strip of sod. The average torque pound was calculated for each piece of sod and treatment.

Statistical Analysis

The percentage of foliar nitrogen in each sample was transformed using an arcsine transformation (x + 0.5). The transformed data was analyzed using analysis of variance (ANOVA) and Student's t test ($P < 0.05$, JMP Version 10. SAS Institute Inc., Cary, NC 2007) for each sampling period. All the foliar nitrogen samples from the study were further analyzed using a repeated measures multivariate analysis of difference (MANOVA), ($P < 0.05$, JMP) and orthogonal contrasts ($P < 0.05$, JMP). Orthogonal contrasts were conducted if significance was detected in MANOVA analyses for all variables. The orthogonal contrasts were conducted to compare the main effect of PGPR + N-treated plants versus N-only-treated plants; All PGPR vs All N; PGPR + N vs PGPR; N vs 0N; PGPR vs 0N; N vs 0N; PGPR + N vs 0N; N vs PGPR; PGPR + .25N vs .25N; PGPR +.5N vs .5N; and PGPR +.75N vs .75N. Additional contrasts of .75N, PGPR +.25N, and .25N against all other treatments were conducted as these treatments had the highest N content at the study's conclusion. Change in foliar N content was also analyzed using an arcsine transformation (x + 0.5) analysis of variance (ANOVA) and Student's t test ($P < 0.05$, JMP).

The impact of PGPR and N rates on turfgrass quality (visual ratings) was analyzed using repeated measures multivariate analysis of difference (MANOVA; $P < 0.05$, JMP) and orthogonal contrasts ($P < 0.05$, JMP). The orthogonal contrasts were conducted to compare the main effect of PGPR + N-treated plants versus N-only-treated plants on turfgrass quality; All PGPR vs All N; PGPR +N vs N; PGPR +N vs N; N vs PGPR; PGPR vs 0N; PGPR +N vs 0N; N vs 0N; PGPR +.25N vs .25N; PGPR +.5N vs .5N; PGPR +.75N vs .75N; PGPR +N vs .25N; PGPR +N vs .5N; PGPR +.75N vs .75N; N vs PGPR +.25N; N vs PGPR +.5N; N vs PGPR +.75N; PGPR +.75N vs 0N; .75N vs 0N; PGPR +.75N vs PGPR; and .75N vs PGPR. The PGPR

+.75N and .75N contrasts were made because these treatments tended to have the highest visual rating following the treatments that received the full rate of N. Repeated measures multivariate analysis of difference (MANOVA; $P < 0.05$, JMP) was used to analyze chlorophyll content measurements. Tensile strength of turfgrass was analyzed using (ANOVA) Student's t-test ($P < 0.05$, JMP).

Results

July and August 2013 had greater than normal precipitation. The average amount of precipitation for July is 14.55 cm and 7.72 cm in August (22.7 cm total). In 2013, 15.88 cm was recorded in July and 13.5 cm in August (29.38 cm total; Auburn University Mesonet 2014); however only 16.47 cm of rain occurred after the initial N application on July 17. Between the first N application and the second N application, 8.97 cm of precipitation was recorded. Between the second N application and the third N application, 7.5 cm of precipitation was recorded. Rain occurred on five of six days after the second N application, totaling 7.49 cm of precipitation with 6.6 cm occurring the day after fertilization. September and the beginning of October were drier than normal conditions, recording 2.92 and 0.43 cm of precipitation (3.35 cm total), respectively.

Plant foliar N ranged from 2.94–3.84% (Table 7; Figure 16) during the study; all of which are considered to be adequate for normal growth. PGPR treated plants typically had a slightly greater mean foliar nitrogen concentration than the non-PGPR treated counterpart (ANOVA; Figure 16). When the initial N analysis was performed in July, no PGPR or N applications had been applied to the system. Foliar N ranged from 3.05–3.15% and no significant differences were detected. In August foliar nitrogen ranged from 2.9 % in non-fertilized controls to 3.4%. There was no evidence for a fertilizer rate effect with foliar nitrogen from PGPR-or

non-PGPR-treated plots. Furthermore, foliar nitrogen in August was not statistically different between full nitrogen rate compared to unfertilized grass, but the PGPR plus the full rate of nitrogen was (Student's t-test; Table 7). In October, foliar nitrogen ranged from 3.43–3.84% across treatments (Student's t-test; $P < 0.05$). PGPR + 25% rate of N treatment had the greatest foliar N content (3.84%) in October and was statistically equivalent to 25, 75, and 100% rates of N (with or without PGPR). Similar to August, contents in October were not statistically different between full nitrogen rate compared to unfertilized grass (Student's t-test; $P < 0.05$). Orthogonal contrasts were conducted to determine if the seasonal foliar N varied by treatments (Table 8). The 75% rate of N was significantly greater than the non-fertilized control ($F= 5.8241$, $P= 0.0178$). Contrasts of PGPR +N vs 0N; PGPR +.25 vs 0N; .75N vs 0N; .75N vs PGPR; and .25N vs 0N were marginally beyond our set level ($\alpha =0.05$). Seasonal changes in foliar N (ΔN) were not different between any of the nitrogen rates and nitrogen rates plus PGPR. The PGPR +.25N had the greatest increase in N, and this was a significantly greater increase in foliar nitrogen relative to several treatments (0N; .5N; PGPR; and PGPR +.5N; Table 8).

PGPR + N had the greatest visual turfgrass rating and PGPR only and 0N were typically the lowest. No significant differences were detected in visual turfgrass ratings when comparing corresponding treatments (MANOVA and Orthogonal Contrasts; Table 9, Figure 17). Significant differences were observed when comparing non-corresponding treatments (MANOVA, $F= 2.4097$, $P= 0.0169$; Figure 17). Additional orthogonal contrasts detected significant differences (Table 9) when comparing PGPR + N vs PGPR, 0N, .25N, .75N; N vs PGPR and 0N; and PGPR + .75N vs PGPR. No significant differences in weekly chlorophyll contents were found among treatments (MANOVA; $F= 0.0360$, $P= 0.951$; Figure 18). When evaluating bermudagrass tensile strength, PGPR + N was found to have the greatest tensile strength (72.84 torque pounds), and

PGPR-only-treated plants had the weakest tensile strength (52.41 torque pounds). No significant differences were observed between corresponding treatments, and the only significant difference was observed between PGPR + N vs PGPR-only-treated plants (ANOVA, Student's t-test, Table 7).

Discussion

In this study we failed to demonstrate that the addition of PGPR to a N fertility management practice increased bermudagrass quality based on the parameters evaluated (% foliar N, visual NTEP ratings, plant chlorophyll content, and plant tensile strength). Plant available N and water are the most common limiting factors for turfgrass growth and health. Measured foliar N and abundant rainfall and irrigation suggest that these two factors were not an issue. Several factors (site selection, weather, and N rates) why differences were not observed. Differences in these parameters may have been observed if the study had been conducted in a turfgrass setting that was a less-managed, low-input system, like a home lawn or pasture.

The management of the site may have contributed several secondary factors or legacy effects that could have impacted the study. Secondary factors include turfgrass age, soil type, soil pH, soil microbial community, irrigation, mowing, and past fertilization. RTJ's driving range has been established for over 20 years in a Cecil sandy loam soils. Only research plots at RTJ were fertilized in 2013. Sporadic fertilization of the site occurs when resources allow, and the site is fertilized at minimal recommended rates. The clippings are recycled, and the grass is regularly irrigated. The management practices may have increased soil carbon (C), N, and N mineralization rates, making the grass more productive (soil C sequestration, microbial biomass, nutrient use efficiency, plant growth, soil organic matter decomposition, nitrification, and CO_2

respiration). The productivity and soil C and N accumulation can be further increased from fertilization, recycling of grass clippings, and irrigation (Johannes et al. 2000, Shi et al. 2006, Yao et al. 2009).

Shi et al. (2006) evaluated the changes in microbial biomass, activity, and N transformations in Tifway grass stands that were 1, 6, 23, and 95 years old in a sandy loam soil. Turfgrass stands that were 6, 23, and 95 years old had soil C contents that were 3, 4, and 7.5 times greater than newly established turf. The same stands had soil N contents that were 3, 5, and 11 times greater than one year old turf. Potential C and N mineralization and use efficiency increased with turf age, suggesting that soil microbial communities are not harmed by turfgrass management. The site history likely would have allowed for C and N accumulation in the soil as well as increased N mineralization rates and would have increased the productivity of the grass similar to the 23 year old stand in Shi et al. (2006). The July nutrient analysis supports this assumption, as every sample had adequate foliar N content during the study (Mills and Jones 1996), suggesting that this is not a N-starved system. Considerations for future studies evaluating PGPR and N rates may be benefited by being conducted with grass seeding, sodding, newly established grasses, or less managed areas and could utilize NDVI chlorophyll content readings to determine the NSI of the site. Finding a more suitable site (N starved) might provide more insights and a better understanding of the benefits of the addition of PGPR to N fertilization.

The soil microbial community and biomass are influenced by soil types and long-term management practices, which include N fertilization (Bigelow et al. 2002, Elliot et al. 2004, Shi et al. 2006, Cheng et al. 2008). In the modified soil profiles (sand root zone),greens and tees soils have a finer texture and less surface area, which could be a limiting factor in microbial establishment and success, and this setting is atypical and would not be characteristic of driving

ranges, home lawns, and pastures (Bigelow et al. 2002). Our site, a Cecil sandy loam is well drained and has medium to rapid runoff, which is characteristic of the Southeast (USDA-NRCS Soil Survey Division 2014). This soil is sandy and well drained and could allow for leaching to occur by having applied N move through soil profile out of the root zone with heavy water (Frank and Guertal 2013). N fertilization impacts the microbial community by favoring bacterial rich soils that increase N mineralization potential and suppress soil fungi (Bardgett et al. 1996, Bardgett et. al 1999, Yeates et al. 1997, Shi et al. 2006). We suspect that the soil microbe community at this site was well established, as it had over 20 years to adapt to these conditions. Therefore, it is quite possible that the stability of the native microbial community was able to out compete the PGPR treatment and its ability to successfully colonize the root system.

The months of July and August 2013 had greater than normal rainfall, and when coupled with daily irrigation of the site, it could have allowed for N losses from leaching, denitrification, less so from runoff, and N immobilization. Plant foliar N had minimal changes between July and August, with some treatments having a reduction in foliar N content. This suggests that the applied N may have been lost. N fertilizer use efficiency is estimated to be 50% of applied N, and further losses of N can exceed 50% from denitrification (N_2O emissions) when the application is followed by heavy rain (NRC 1993, Bremer 2006). Leaching, denitrification, and runoff posed the largest threat as an avenue of N loss August 15-20, 2013. The second N application was made on August 14th and was followed by heavy rain (6.60 cm) on the 15th and a total of 7.49 cm by August 20th. Standing water was observed at the site from August 16-18, 2013, and these conditions could have lead to denitrification. Losses of N from denitrification are favored by frequent irrigation and excessively wet soils. When soils are excessively wet, they are oxygen poor, which influences soil microbe behaviors. Under these conditions, microbes use

nitrate (NO_3^-) as an electron acceptor and convert it to nitrous oxide (N_2O), which is the greenhouse gas released into the atmosphere (Steinke and Ervin 2013). Several studies have confirmed that increased N_2O emissions from denitrification occur in grass when fertilization is followed by heavy precipitation (Bremer 2006, Bijoor et al. 2008). Typical losses of N via denitrification can be up to 20% of applied N (Horgan et al. 2002). In addition to possible N loses, N may have been depleted from the system more readily than normal due to the growing conditions that favor rapid top growth and the rapid movement of N from plant roots to shoots (Wherley et al. 2009). However, the clippings from mowing were left in the field, which allows for N recycling by the grass. The October nutrient analysis showed changes in plant foliar N content and most likely reflects the uptake of N following the 3rd N application from September 11, 2013.

In summary, we failed to provide evidence that the addition of PGPR to an N fertility program could positively affect bermudagrass quality. However, several factors such as the site's age, soil properties, weather, and N rates may have influenced our results. Future studies investigating PGPR and N rates could have different results if conducted in low input grass systems or newly established grasses, whether at seeding or sodding. Research focusing on newly established grass could be conducted in new home lawns, sod farms, or recently renovated or established athletic turfgrass. This research would provide more insight into the interactions between PGPR and the soil microbial community. Under these conditions, the soil would have been recently disturbed, and the soil microbial community, soil C, and soil N would be less established than mature grass stands. PGPR may increase nutrient use efficiency and acquisition under these conditions. Further studies investigating PGPR and low N fertility rates would be beneficial for low input systems. Balternsperger et al. (1978) demonstrated that N_2-fixing

bacterial inoculants (*Azospirillum* and *Azotobacter*) increased plant foliar N, but these benefits were only observed with low N rates. Our results support this, as the PGPR + .25N had the greatest change in plant foliar N of any treatment during the study. Future research investigating PGPR and reduced N rates (0, 10, 15, and 20% of recommended N) may determine an optimal rate for influencing the soil microbe community and plant nutrient use efficiency in low input systems.

Table 7. Percent foliar nitrogen in Bermudagrass clippings and tensile strength

Treatment	Mean (± SEM) values of % foliar nitrogen and tensile strength (N=10)				
	July mg/kg Foliar N	August mg/kg Foliar N	October mg/kg Foliar N	Δ mg/kg Foliar N (October-July)	Tensile Strength (Torque Pound)
0 N	3.09 ± 0.55a	2.935 ± 0.426c	3.448 ± 0.419cd	0.357 ± 0.162c	67.66 ± 10.57ab
5.8 g/m^2	3.15 ± 0.57a	3.183 ± 0.378abc	3.777 ± 0.304a	0.623 ± 0.121abc	56.00 ± 4.79ab
11.6 g/m^2	3.064 ± 0.564a	3.353 ± 0.290a	3.431 ± 0.441d	0.367 ± 0.183c	73.06 ± 5.69ab
17.5 g/m^2	3.154 ± 0.662a	3.344 ± 0.383a	3.815 ± 0.286a	0.667 ± 0.134ab	71.09 ± 6.70ab
23.1 g/m^2	3.064 ± 0.574a	3.200 ± 0.362abc	3.683 ± 0.351abc	0.624 ± 0.150abc	72.07 ± 9.33ab

PGPR	3.094 ± 0.557a	2.968 ± 0.387bc	3.529 ± 0.300bcd	0.435 ± 0.175bc	52.41 ± 6.34b
PGPR + 5.8 g/m^2	3.048 ± 0.503a	3.287 ± 0.343a	3.835 ± 0.644a	0.787 ± 0.223a	70.86 ± 8.03ab
PGPR + 11.6 g/m^2	3.074 ± 0.528a	3.463 ± 0.385a	3.519 ± 0.256bcd	0.445 ± 0.115bc	71.50 ± 6.65ab
PGPR + 17.4 g/m^2	3.053 ± 0.527a	3.471 ± 0.322a	3.666 ± 0.367abcd	0.613 ± 0.151abc	67.08 ± 6.81ab
PGPR + 23.1 g/m^2	3.117 ± 0.573a	3.243 ± 0.351ab	3.717 ± 0.311ab	0.600 ± 0.114abc	74.84 ± 7.34a
Statistics	F=73.99, P >0.05	F=2.58, P=0.0453	F=11.67, P=0.0408	F= 21.04, P=0.0391	F=1.93, P=0.0454

Means presented are actual means. Within a column, means ± SEM followed by the same letter are not significantly different from each other (P < 0.05; JMP; ANOVA Student's t-Test; [SAS Institute Inc., Cary, NC. 1989-2007]).

Table 8 Orthogonal Contrasts of seasonal foliar nitrogen in Bermudagrass

Orthogonal Contrasts Compared		
		Statistics
All PGPR	All N	F= 0.4274, *P*= 0.5150
PGPR + N	PGPR	F= 1.1838, *P*= 0.3150
N	0N	F= 1.5317, *P*= 0.2191
PGPR	0N	F= 0.2379, *P*= 0.6269
PGPR + N	0N	F= 3.1463, *P* =0.0795
N	PGPR	F= 0.5623, *P*= 0.4553
PGPR + .25N	.25N	F= 0.0232, *P*= 0.8793
PGPR + .5N	.5N	F= 0.8240, *P*= 0.3613
PGPR + .75N	.75N	F= 0.1073, *P*= 0.7441
PGPR + N	N	F= 0.2875, *P*= 0.5932
PGPR + .25N	0N	F= 3.2878, *P*= 0.0731
	PGPR	F= 1.7569, *P*= 0.1884
.75N	0N	F= 5.8241, *P*= 0.0178*
	.5N	F= 2.0544, *P*= 0.1552
	PGPR	F= 3.7078, *P*= 0.0575
.25N	0N	F= 3.8632, *P*= 0.0524
	PGPR	F= 2.1837, *P*= 0.1430

* denotes significance between treatments from contrasts ($P < 0.05$; JMP; Orthogonal Contrasts; [SAS Institute Inc., Cary, NC. 1989-2007]).

Table 9 Orthogonal Contrasts of NTEP visual ratings

Orthogonal Contrasts Compared		
		Statistics
All PGPR	All N	F= 0.6479, *P*= 0.4230
PGPR + N	N	F= 2.1029, *P*= 0.1505
PGPR + N	PGPR	F= 13.6326, *P*= 0.0004*
N	PGPR	F= 5.0269, *P*= 0.0274*
PGPR	0N	F= 0.0512, *P*= 0.8215
PGPR + N	0N	F= 12.013, *P* =0.0008*
N	0N	F= 4.0635, *P*= 0.0468*
PGPR + .25N	.25N	F= 0.0232, *P*= 0.8793
PGPR + .5N	.5N	F= 0.824, *P*= 0.3613
PGPR + .75N	.75N	F= 0.1073, *P*= 0.7441
PGPR + N	.25N	F= 9.7113, *P*= 0.0025*
	.5N	F= 2.9500, *P*= 0.0893
	.75N	F= 4.7093, *P*= 0.0326*
N	PGPR +.25N	F= 5.0269, *P*= 0.2281
	PGPR + .5N	F= 0.4608, *P*= 0.1983
	PGPR + .75N	F= 0.0343, *P*= 0.8535
PGPR + .75N	0N	F= 3.3514, *P*= 0.0705
.75N	0N	F= 1.6793, *P*= 0.1983
PGPR + .75N	PGPR	F= 4.2311, *P*= 0.0426*
.75N	PGPR	F= 2.3169, *P*= 0.1315

* denotes significance between treatments from contrasts ($P < 0.05$; JMP; Orthogonal Contrasts; [SAS Institute Inc., Cary, NC. 1989-2007]).

Figure 16 Monthly Nutrient Analyses of % Foliar Nitrogen for each treatment ANOVA, Student's t-test, ($P < 0.05$, JMP Version 10, SAS Institute Inc., Cary NC. 1989-2007)

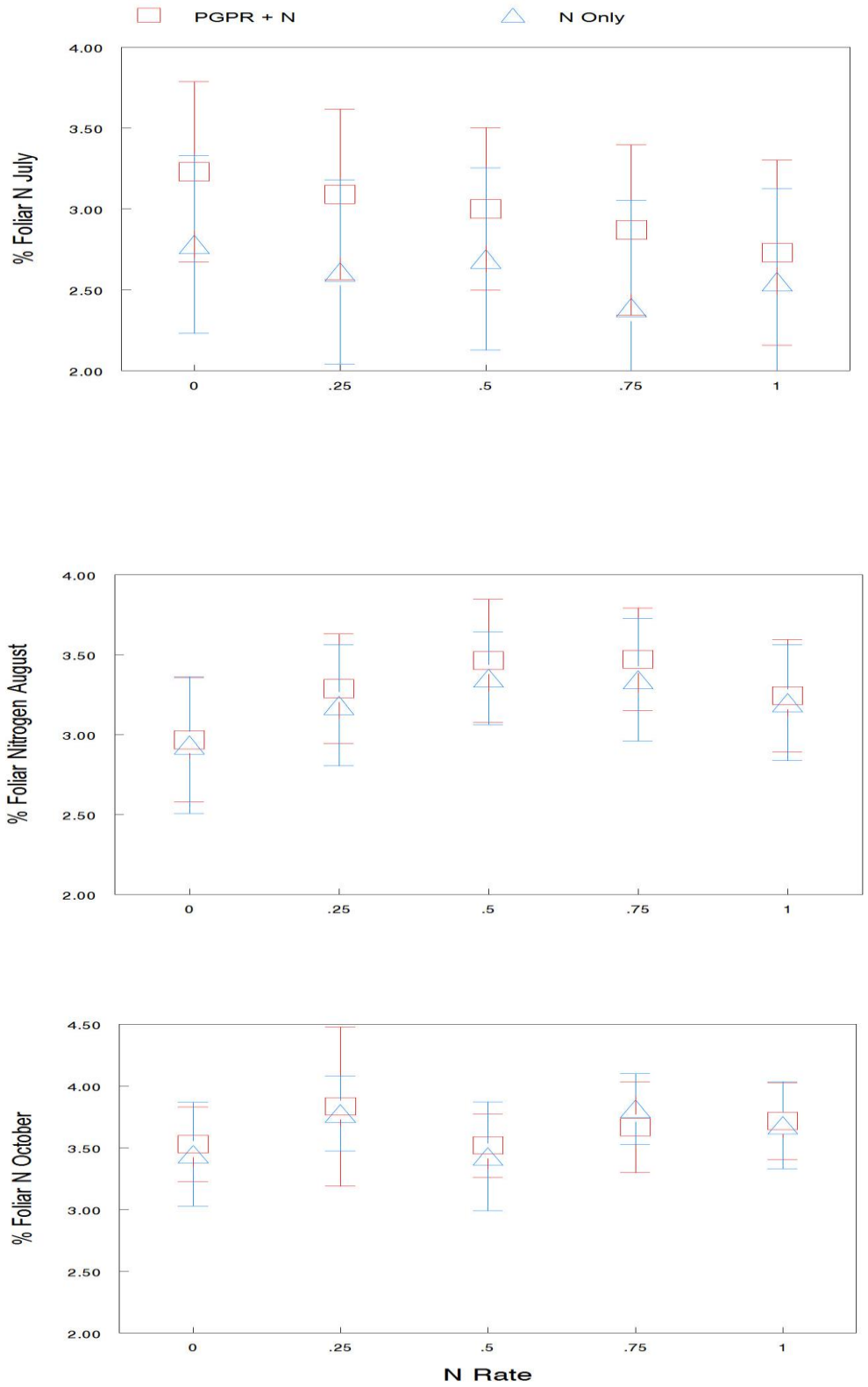


Figure 17 Weekly NTEP Visual Ratings for each treatment MANOVA, Repeated measures, (F= 2.4097; *P* = 0.0169, JMP Version 10, SAS Institute Inc., Cary NC. 1989-2007)

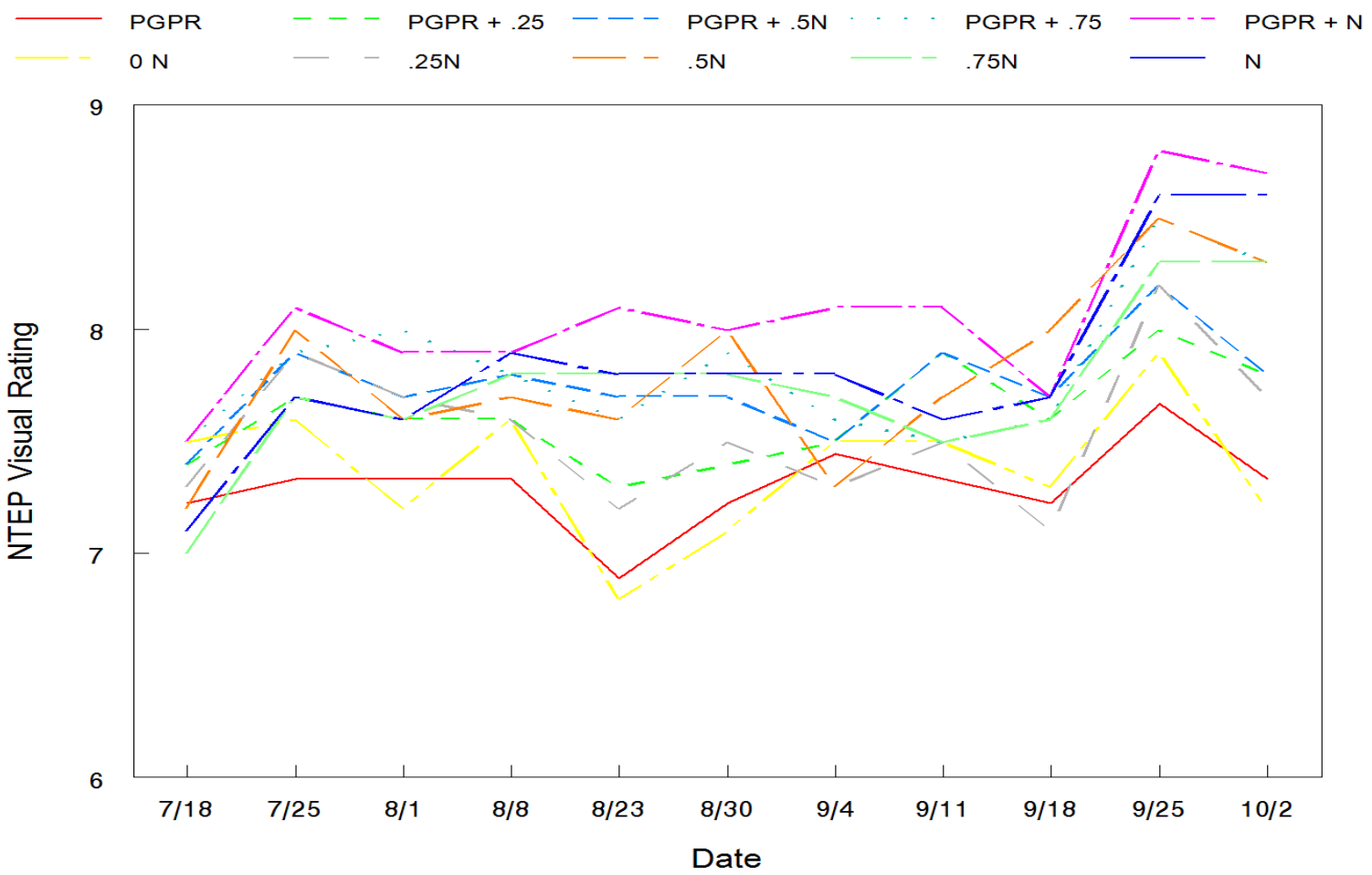


Figure 18 Weekly NDVI Plant Chlorophyll Contents for each Treatment MANOVA, Repeated measures, (F= 0.2889, *P* = 0.6233, JMP Version 10, SAS Institute Inc., Cary NC. 1989-2007)

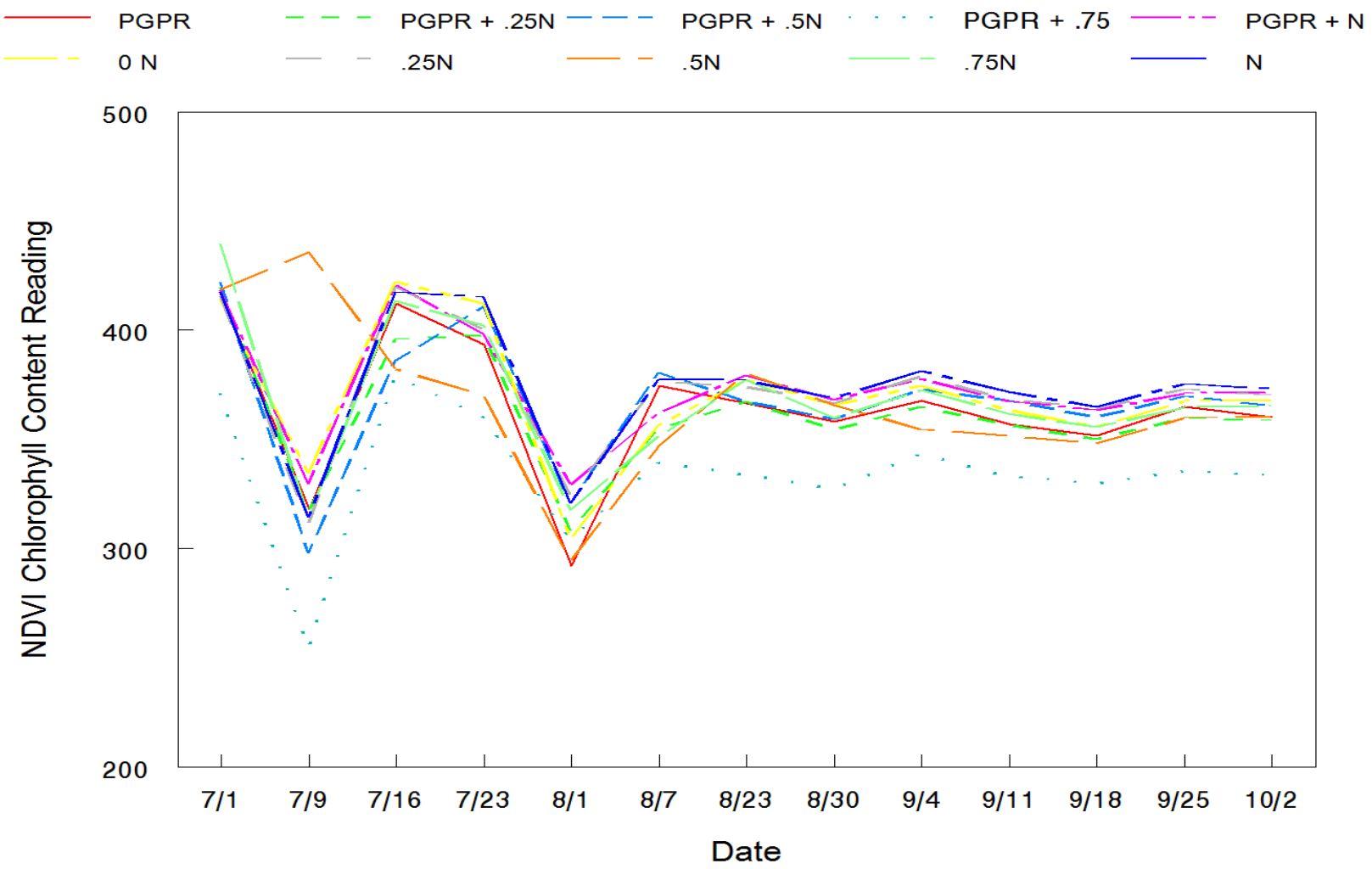


Figure 19: Sod machine and torque meter used to measure bermudagrass tensile strength

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Appendix 1. Official series description of the Cecil soil series described by the USDA-NRCS Soil Survey Division

CECIL SERIES

The Cecil series consists of very deep, well drained moderately permeable soils on ridges and side slopes of the Piedmont uplands. They are deep to saprolite and very deep to bedrock. They formed in residuum weathered from felsic, igneous and high-grade metamorphic rocks of the Piedmont uplands. Slopes range from 0 to 25 percent. Mean annual precipitation is 48 inches and mean annual temperature is 59 degrees F. near the type location.

TAXONOMIC CLASS: Fine, kaolinitic, thermic Typic Kanhapludults

TYPICAL PEDON: Cecil sandy loam--forested. (Colors are for moist soil unless otherwise stated.)

Ap--0 to 8 inches; dark yellowish brown (10YR 4/4) sandy loam; weak medium granular structure; very friable; slightly acid; abrupt smooth boundary. (2 to 8 inches thick)

Bt1--8 to 26 inches; red (10R 4/8) clay; moderate medium subangular blocky structure; firm; sticky, plastic; common clay films on faces of peds; few fine flakes of mica; strongly acid; gradual wavy boundary.

Bt2--26 to 42 inches; red (10R 4/8) clay; few fine prominent yellowish red (5YR 5/8) mottles; moderate medium subangular blocky structure; firm; sticky, plastic; common clay films on faces of peds; few fine flakes of mica; very strongly acid; gradual wavy boundary. (Combined thickness of the Bt horizon is 24 to 50 inches)

BC--42 to 50 inches; red (2.5YR 4/8) clay loam; few distinct yellowish red (5YR 5/8) mottles; weak medium subangular blocky structure; friable; few fine flakes of mica; very strongly acid; gradual wavy boundary. (0 to 10 inches thick)

C--50 to 80 inches; red (2.5YR 4/8) loam saprolite; common medium distinct pale yellow (2.5Y 7/4) and common distinct brown (7.5YR 5/4) mottles; massive; very friable; few fine flakes of mica; very strongly acid.

TYPE LOCATION: Franklin County, North Carolina; about 9.7 miles west of Louisburg on North Carolina Highway 56 to Franklinton, about 4.4 miles south on U.S. Highway 1, about 0.4 mile east on North Carolina Highway 96, about 500 feet north of the road, in a field; Franklinton USGS topographic quadrangle; lat. 36 degrees 02 minutes 24 seconds N. and long. 78 degrees 29 minutes 27 seconds W.

RANGE IN CHARACTERISTICS: The Bt horizon is at least 24 to 50 inches thick and extends to 40 inches or more. Depth to bedrock ranges from 6 to 10 feet or more. The soil ranges

from very strongly acid to moderately acid in the A horizons and is strongly acid or very strongly acid in the B and C horizons. Limed soils are typically moderately acid or slightly acid in the upper part. Content of coarse fragments range from 0 to 35 percent by volume in the A horizon and 0 to 10 percent by volume in the Bt horizon. Fragments are dominantly gravel or cobble in size. Most pedons have few to common flakes of mica in the Bt horizon and few to many flakes of mica in the BC and C horizons.

The A or Ap horizon has hue of 2.5YR to 10YR, value of 3 to 5, and chroma of 2 to 8. A horizons with value of 3 are less than 6 inches thick. The texture is sandy loam, fine sandy loam, or loam in the fine earth fraction. Eroded phases are sandy clay loam, or clay loam in the fine earth fraction.

The E horizon, where present, has hue of 7.5YR or 10YR, value of 4 to 6, and chroma of 3 to 8. It is sandy loam, fine sandy loam, or loam in the fine-earth fraction.

The BA or BE horizon, where present, has hue of 2.5YR to 10YR, value of 4 to 6, and chroma of 3 to 8. It is sandy clay loam, loam, or clay loam.

The Bt horizon averages 35 to 60 percent clay in the control section but may range to 70 percent in some subhorizons. It has hue of 10R or 2.5YR, value of 4 or 5, and chroma of 6 or 8. Hue also ranges to 5YR if evident patterns of mottling are lacking in the Bt and BC horizons. Mottles that are few and random are included. The Bt horizon is clay loam, clay, or sandy clay and contains less than 30 percent silt.

The BC horizon has hue of 10R to 5YR, value of 4 or 6, and chroma of 4 to 8. Mottles in shades of yellow or brown are few to common in some pedons. The texture is sandy clay loam, clay loam, or loam.

The C horizon is similar in color to the BC horizon or it is variegated. It is loamy saprolite weathered from felsic, igneous and high-grade metamorphic rocks.

COMPETING SERIES: These are the Appling, Bethlehem, Georgeville, Herndon, Madison, Nanford, Nankin, Pacolet, Saw, Tarrus, and Wedowee series in the same family. Those in closely related families are the Cataula, Chestatee, Cullen, Hulett, Lloyd, Mayodan, and Mecklenburg series. Appling soils have dominant hue of 7.5YR or yellower or where hue is 5YR it has evident patterns of mottling in a subhorizon of the Bt or BC horizon. Bethlehem soils have soft bedrock at depths of 20 to 40 inches. Cataula soils have a perched water table at 2 to 4 feet, Chestatee soils contain more than 15 percent, by volume, coarse fragments throughout. Cullen soils have more clay in the Bt horizon. Mayodan and Mecklenburg soils have mixed mineralogy and in addition, Mayodan soils formed in Triassic age sediments and Mecklenburg soils formed from basic diabase parent material. Georgeville, Herndon, Nanford, and Tarrus soils formed in Carolina slate and contain more than 30 percent silt. Hulett, Nankin, and Wedowee soils have a Bt horizon with hue of 5YR or yellower. In addition, Nankin soils formed from marine sediments. Lloyd soils have rhodic colors to depths of 40 inches or more. Madison, Pacolet, and Wedowee soils have thinner argillic horizons. Saw soils have hard bedrock at depths of 20 to 40 inches.

GEOGRAPHIC SETTING: Cecil soils are on nearly level to steep Piedmont uplands. Slope gradients are 0 to 25 percent, most commonly between 2 and 15 percent. These soils have developed in weathered felsic igneous and high-grade metamorphic rocks. Average annual precipitation is about 48 inches. Mean annual soil temperature is about 59 degrees F.

GEOGRAPHICALLY ASSOCIATED SOILS: In addition to the competing Appling, Bethlehem, Cataula, Chestatee, Cullen, Lloyd, Madison, Mecklenburg, Pacolet, Saw, and Wedowee series these are the Durham, Louisburg, Rion, and Worsham series. Durham, Louisburg, and Rion soils have less clay in the Bt horizon. Worsham soils are poorly drained and are around the heads of drains.

DRAINAGE AND PERMEABILITY: Well drained; medium to rapid runoff; moderate permeability.

USE AND VEGETATION: About half of the total acreage is in cultivation, with the remainder in pasture and forest. Common crops are small grains, corn, cotton, and tobacco.

DISTRIBUTION AND EXTENT: The Piedmont of Alabama, Georgia, North Carolina, South Carolina, and Virginia. The series is of large extent, with an area of more than 10 million acres.

MLRA SOIL SURVEY REGIONAL OFFICE (MO) RESPONSIBLE: Raleigh, North Carolina

SERIES ESTABLISHED: Cecil County, Maryland; 1899.

REMARKS: The June 1988 revision changed the classification to Typic Kanhapludults and recognized the low activity clay properties of this soil as defined in the Low Activity Clay Amendment to Soil Taxonomy, August 1986. The December 2005 revision changed the type location from Catawba County, North Carolina to a more representative location. The May 2006 revision changed language in competing series for Wedowee.

Diagnostic horizons and features recognized in this pedon are:
Ochric epipedon--the zone from the surface of the soil to a depth of 8 inches (Ap horizon)
Kandic horizon--the zone between 8 and 42 inches meets the low activity clay requirement in more than 50 percent of the horizon (Bt1 and Bt2 horizons)
Argillic horizon--the zone between 8 and 42 inches (Bt1 and Bt2 horizons)

ADDITIONAL DATA: McCracken, R. J., editor: Southern Cooperative Series Bulletin 61, issued January, 1959, Virginia Agricultural Experiment Station, Blacksburg, Virginia. Soil Survey of Catawba County, North Carolina, issued 1975. Soil Survey of Forsyth County, North Carolina, issued 1976.