BERMUDAGRASS SPECIES RESPONSE TO MICROBIAL INOCULANTS

by

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(Under the Direction of Gerald Henry)

ABSTRACT

The use of biostimulants, including microbial inoculants, have increased over the past decade due to potential benefits including increased nutrient uptake, enhanced growth, and improved stress tolerance. However, minimal research has been conducted on warm-season grasses and questions still exist regarding inoculant application timing, frequency, and need for supplemental nitrogen. Therefore, the objective of this research was to evaluate microbial inoculants in comparison to one another and synthetic fertilizer with respect to their impact on bermudagrass (*Cynodon* spp.) establishment, growth, quality, and function. Microbial inoculant applications in the field did not consistently increase bermudagrass normalized vegetation difference index, turfgrass color, nor turfgrass quality compared to the non-treated check. However, carbon efflux measurements increased following inoculation. In the greenhouse, greater bermudagrass root (R) and shoot (S) weights were observed in response to fertilizer treatments, while microbial inoculant treatments resulted in R and S weights similar or less than the non-treated check.

INDEX WORDS: Turfgrass, Nitrogen-producing, microbes, Symbiotic, Azospirillum

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Literature Review

N_2 Fixation Background

A growing demand for food in response to global population expansion has steadily increased the use of synthetic nitrogen (N) fertilizers in agriculture over the past century. Nitrogen is the most abundant element in the Earth's atmosphere, but often the most limiting nutrient for plant growth and production (Kox and Jetten, 2015; Bruijn, 2015). The amount of energy required to manufacture N fertilizers is almost 6 times the amount of energy it takes to manufacture phosphorous (P) or potassium (K) fertilizers due to the unreactive nature of N (Santi et al., 2013; Da Silva et al., 1978) (Frank et al., 2003; Shridhar, 2012). Industrial production of N fertilizers in the United States has an annual cost over $100 billion (Bruijn, 2015). Fossil fuel availability as a resource for fertilizer production will continue to decrease, making production more challenging and increasingly expensive over time (Bruijn, 2015; Burris and Roberts, 1993). Several negative environmental impacts are associated with continuous overuse of N fertilizers. Nitrate (NO_3^-) leaching, ammonia (NH_3) volatilization, or loss as nitrogen oxide (NO_x) or dinitrogen (N_2) gas may occur (Cameron et. al., 2013; Kox and Jetten, 2015). The potential solution to this problem may be in the form of biological nitrogen fixation (BNF). Biological fixation of atmospheric nitrogen is estimated to be about 175 million metric tons per year, accounting for approximately 70% of all nitrogen fixed per year (Shridhar, 2012).

Biological nitrogen fixation (BNF) is the reduction of inert N_2 gas from the atmosphere to biologically available NH_3 by a group of prokaryote microbes called diazotrophs (Bruijn, 2015;

Burris and Roberts, 1993; Kox and Jetten, 2015; Postgate, 1998; Raymond et al., 2003; Santi et al., 2013). Biological nitrogen fixation is carried out by symbiotic or free-living diazotrophs in nature, holding great promise for increasing agricultural sustainability (Shridhar, 2012). The BNF process is catalyzed by an enzyme. nitrogenase, which is an oxygen-sensitive complex, highly conserved in both free-living and symbiotic nitrogen-fixing bacteria (Franche et al., 2009). The nitrogenase enzyme is a complex of two multi-subunit proteins, the iron (Fe) protein and molybdenum-iron (MoFe) protein, together helping facilitate the reduction of atmospheric N to biologically available N (Kim and Rees, 1994). Some bacteria like *Azotobacter* along with several photosynthetic nitrogen fixers carry additional forms of nitrogenase, whose cofactor contains vanadium (V) or Fe making them more adaptable in situations where certain cofactors may not be available (Newton, 2007; Rubio and Ludden, 2005).

The term gene cluster refers to a set of homologous bacterial genes of a contiguous unit of the genome that share a generalized function. The genetic understanding of nitrogen fixation was initially investigated within a gene cluster of *Klebsiella oxytoca*, first identified as *K. pneumoniae* (Franche et al., 2009). A *nif* gene cluster located within the bacteria are responsible for encoding the proteins involved in the nitrogenase reaction (Zhimin et al., 2014). The specific genes responsible for encoding the molybdenum protein are *nifD* and *nifK*, while the *nifH* gene encodes the iron protein (Franche et al., 2009). Hydrolysis of 16 adenosine triphosphate (ATP) per molecule of fixed N_2 accompanied by the formation of one molecule of dihydrogen gas (H_2) by the nitrogenase system makes nitrogen fixation one of the most expensive biological processes (Raymond et. al., 2003; Simpson and Burris, 1984).

The sensitivity of Fe found in nitrogenase could be a potential limitation to the functioning of this enzyme (Georgiadis et al., 1992;Wang et al., 1985). Although anaerobic

respiration quickly produces large amounts of ATP required for BNF, the nitrogenase enzyme can be destroyed by oxygen, making it inactive (Bruijn, 2015; Postgate, 1998). Some diazotrophs function as obligate anaerobes (no oxygen) or facultative anaerobes (use oxygen but not required) and fix nitrogen anaerobically, thus avoiding the presence of oxygen altogether (Bruijn 2015; Postgate 1998). Symbiotic diazotrophs form nodules around host plant roots. Leghemoglobin, produced inside these nodules, has a high affinity for binding oxygen (Bruijn 2015; Postgate 1998).

Symbioses and Free-living Genera

Biological nitrogen fixation was first reported in leguminous plants in the 1830's by Boussingault, but the first significant evidence of BNF in leguminous plants was not published until 1888 by Hellriegel and Wilfarth (Burris and Roberts, 1993; Wilson, 1940). Initial research by Hellriegel and Wilfarth was conducted on pea (*Pisum sativum* L.) plants. Root nodules were observed in inoculated plants whereas non-inoculated plants did not have root nodules and experienced earlier death (Burris and Roberts, 1993). The practice of inoculation became widespread and the production of inoculum was commercialized, which still continues today (Burris and Roberts, 1993; Fred et al., 1932). Once entering the plant, the inoculum forms a symbiotic relationship with the host. *Rhizobium* spp., *Frankia* spp., *Azospirillum* spp., and some *Cyanobacteria* spp. are common bacteria responsible for the formation of these symbiotic relationships with plants (Bruijn, 2015; Mus et al., 2016).

Rhizobia spp., the most recognized of these bacteria, form symbiotic relationships with legumes. Establishment of the legume/*Rhizobia* spp. symbiosis first involves infection of the root tissue followed by the formation of nodular growth (Bauer, 1981). Initiation of nodule formation on compatible host plants results in a series of dialogues exchanged between the host and

bacteria (Schultze and Kondorosi, 1998). The nodulation process begins when the host plant sends out a signal called a flavonoid that is received by the *Rhizobia* spp. (Franche et al., 2009). These signals can be perceived by a specific bacterial receptor, *NodD*, which acts as an activator for the N-fixing genes encoded in the bacteria. (Franche et al., 2009) The *Rhizobia* spp. slowly starts to migrate towards the root hair of the plant which extends itself outward to intercept the bacteria. Once reaching the rhizosphere, the *Rhizobia* spp. forms an infection thread with the root hair closing around the bacteria, entrapping them to form the nodule. (Mylona et al., 1995; Van Spronsen et al., 1994). Next, the plant undergoes hydrolysis, dissolving the cell wall and allowing the Rhizobia spp. inside to form the infection thread (Van Spronsen et al., 1994). The root nodule then acts as both an N source as well as a carbon sink (Mylona et al., 1995). It has also been suggested that these root nodules evolved from previous carbon storage organs (Joshi et al., 1993). The legume-Rhizobia spp. symbiosis is among the most prevalent and important symbiotic associations in terms of BNF, which is capable of producing roughly 200 million tons of fixed N per year (Ferguson et al., 2010).

Frankia spp., a similar diazotroph as *Rhizobia* spp., fixes N through symbiotic formation of root nodules with actinorhizal plants (Franche et al., 2009). Actinorhizal plants represent about 200 species distributed among 24 genera that are considered important in terms of ecological succession since they usually colonize areas first and adapt to poor soil conditions (Franche et al., 2009; Huss-Danell, 1997). Unlike *Rhizobia* spp., culturing *Frankia* spp. was more challenging with the first successful culture not occurring until 1978 (Callaham et al., 1978). *Frankia* spp. can develop two different survival structures, vesicles and spores, which are unique to their species (Franche et al., 2009; Lechevlier, 1994). Spores are used as a means of reproduction, while vesicles are utilized for N fixation (Huss-Danell, 1997; Santi et al., 2013).

There are two modes of infection by *Frankia* spp. on actinorhizal plants, intracellular root hair infection and intercellular root invasion (Wall and Berry, 2008). Intracellular infection begins with the deformation of root hairs induced by signals from *Frankia* spp. that are currently unknown (Santi et al., 2013) Even though no root hair deformation is observed during intercellular root invasion, *Frankia* spp. hyphae penetrate root epidermis cells and progress into the apoplastic space between cortical cells (Wall and Berry, 2008). Compared to legume-*Rhizobia* symbiosis, actinorhizal symbiosis fixes approximately 240 to 350 kg N ha^{-1} yr^{-1} (Diagne et al., 2013; Wall, 2000). Unfortunately, *Frankia* spp. may also comprise other symbiotic and free-living bacteria (Wall, 2000).

Similar to the *Frankia* spp. bacteria, cyanobacteria can be free living as well as symbiotic with certain plant species. Cyanobacteria are a morphologically diverse group of prokaryotes that reside in the bacteria domain (Giavannoni et al., 1998; Meeks 1998). Cyanobacteria are the major N fixers in freshwater and marine systems, but also have the ability to fix N in diverse terrestrial ecosystems that range from rainforests to deserts (Peter et al., 2002). Chloroplasts are actually a cyanobacterium living within plant cells (Issa et al., 2014). Cyanobacteria are capable of forming symbiotic relationships with 4 major genera of plants: *Anthoceros*, *Blasia*, *Azolla*, and *Gunnera* spp. (Meeks, 1998; Rai, 1990) A difference between cyanobacteria plant associations and *Frankia*/*Rhizobia* symbioses is that those bacteria are hosted within the root nodule, while cyanobacterial infections develop separately from the symbiotic cyanobacteria (Santi et al., 2013).

While nitrogen fixation is found in unicellular and filamentous species of cyanobacteria, associations with plants are limited to heterocystous cyanobacteria Nostocales of the genus *Nostoc* and *Anabaena* (Franche et al., 2009). Heterocystous cyanobacteria are bacteria that are

able to differentiate specialized cells called heterocysts under limited N conditions (Rippka et al., 1979). Nitrogen fixed by heterocysts is exported to vegetative cells of filaments and in return those vegetative cells provide heterocysts with carbohydrates derived from photosynthesis (Franche et al., 2009). Many filamentous N-fixing cyanobacteria protect nitrogenase from oxygen within these specialized heterocysts (Golden and Yoon, 2003). Another unique function that involves differentiation of cells in the *Nostoc* genus is the development of hormogonia, filaments that provide a means of cyanobacteria motility important in the formation of symbiotic relationships (Rippka et al., 1979). Hormogonia also function as infection units of the cyanobacteria (Meeks, 1998). Cyanobacteria that make up the marine population of N fixers are known to supply upwards of 100 million metric tons of N yr^{-1} (Issa et al., 2014).

Azotobacter spp. is another free-living bacteria that fixes N in the rhizosphere without forming a symbiotic relationship with plants (Jimenez et al., 2011). The *Azotobacter* genus was discovered in 1901 by Dutch microbiologist and botanist Beijerinck. (Jnawali et al., 2015). There are around six species in the genus *Azotobacter*, some of which are motile by means of flagella, while others are not (Jnawali et al., 2015; Martyniuk and Martyniuk, 2003). The presence of *Azotobacter* spp. in the soil have beneficial effects on plants, but populations are often affected by soil physio–chemical and microbiological properties (Jnawali et al., 2015; Kizilkaya, 2009). *Azotobacter* spp. are non–symbiotic heterotrophic bacteria capable of fixing an average 20 kg N ha^{-1} yr^{-1} (Kizilkaya, 2009). *Azotobacter* is regarded as a plant growth promoting rhizobacteria (PGPR) which synthesize growth substances that enhance plant growth and development while also inhibiting phytopathogenic growth (Azcorn and Barea, 1975). Therefore, interest has recently increased utilizing *Azotobacter* as a biofertilizer or a seed coating inoculum (Jnawali et al., 2015).

Among other free-living N fixers is the genera *Beijerinckia*, which are characterized as non-symbiotic, aerobic bacteria with the ability to fix atmospheric N (Becking, 2006). *Beijerinckia* was originally isolated from a Malaysian quartzite soil in low pH conditions in 1936 (Alston, 1936; Becking, 2006). It was later found to be more widely distributed in acidic soils within tropical regions (Becking, 2006). One difference between *Beijerinckia* and other N fixers is that *Beijerinckia* use nitrate rather poorly (Becking, 1962). The efficiency of N_2 fixation in *Beijerinckia* strains is usually 10 to13 mg N g^{-1} glucose consumed. Furthermore, fast growing *Beijerinckia* strains tend to be poor N fixers while slow growing *Beijerinckia* strains are good N fixers (Becking, 2006).

Similar to *Beijerinckia*, the genera *Clostridium* was first isolated by S. Winogradsky in 1895 with *C. pasteurianum* as the first free-living N fixer isolated (Chen, 2004; Winogradsky, 1895). The genera *Clostridium* is a diverse collection of obligately anaerobic bacteria (Chen, 2004). Not much information exists regarding the amount of N fixed per year by *Clostridium* since a majority of these bacteria are not known to be N fixers.

Azospirillum and Relevant Research

The microbe that has shown the most promise in agriculture and is directly involved in our research is from the genera *Azospirillum*. First isolated by the Dutch scientist Martinus Beijerinck in 1925, *Azospirillum* did not become significant until the 1970's when it was reported to have close connections with grass roots while the shoots of associated plants never exhibited N deficiency symptoms (Beijerinck, 1925; Doebereiner and Day, 1976; Holguin et al., 1999). *Azospirillum* are categorized as microaerophilic, non-fermentative, nitrogen-fixing bacteria (Cassan et al., 2020). This genera has also been categorized as plant-growth-promoting-

bacteria due to their ability to secrete a series of phytohormones (Bashan, 1999; Holguin et al., 1999).

Bacteria in the genera *Azospirillum* are highly motile, an advantage that allows them to move towards more favorable nutrient conditions (Hall and Krieg, 1984; Steenhoudt and Vanderleyden, 2000). One polar flagellum is synthesized during growth for swimming, while lateral flagella growth occurs on solid media to induce bacterial swarming on that surface (Steenhoudt and Vanderleyden, 2000). The attachment of the bacteria to the root surface is a biphasic process mediated by the polar flagellum and the swarming flagella (Franche et al., 2009; Steenhoudt and Vanderleyden, 2000). After inoculation with *Azospirillum*, the host plant will begin to display numerous physiological and morphological changes (Franche et al., 2009). Among these changes are enhanced growth of roots and root hairs, increased water uptake, increased root respiration, delay in leaf senescence, and increased dry weight (Dobbelaere and Okon, 2007; Okon, 1985).

Azospirillum spp. display a wide range of physiological mechanisms that can help insure their survival in the rhizosphere. The possession of a versatile metabolism helps *Azospirillum* compete against other bacteria as well as survive harsh soil conditions (Holguin et al., 1999). One of the main survival mechanisms is the formation of cysts that help *Azospirillum* endure unfavorable soil conditions and prevent heat damage or desiccation (Bashan, 1999; Franche et al., 2009; Tapia-Hernandez at al., 1990). The level of adaptability of *Azospirillum* has led to a wide range of associations with many different genera of plants (Holguin et al., 1999). Hosts of *Azospirillum* are mostly comprised of annual plants, but they have also been shown to associate with some perennials (Bashan and Holguin, 1997). Although the mechanisms interaction

between *Azospirillum* and plants is not clear, potential for *Azospirillum* incorporation into agricultural production is promising (Bashan and Levanony, 1990; Cassan et al., 2020).

Previous research examining the use and persistence of *Azospirillum* in cropping systems is inconsistent. Harris et al. (1989) reported the survival of *Azospirillum* present within a Nigerian soil stored in a laboratory for 10 years, while Albrecht et al., (1983) noted the dissipation of *Azospirillum* from a highly inoculated forage crop trial within just 25 days. The soil residual activity of *Azospirillum* may be somewhat variable and survival could be linked to certain physiochemical properties of the soil (Stotzky, 1997). Survival of *Azospirillum* within the soil has been observed to increase with the addition of amendments like sugar that can be utilized as a food source (Mawdsley and Burns, 1994). However, the main factor that seems to determine survival and persistence of *Azospirillum* in the soil is competition from other microorganisms. *Azospirillum* populations remained high for 100 days when introduced to a sterilized soil, while populations were unable to be sustained and dropped dramatically over the same period of time when a non-sterilized soil was inoculated with *Azospirillum* (Christiansen-Weniger, 1992; Christiansen-Weniger and Van Veen, 1991). Furthermore, soil type and organic matter content may also play a big role in *Azospirillum* longevity and soil activity (Bashan, 1999).

Benefits and Disadvantages to NFB

As previously stated, the industrial production of N fertilizers costs the United States over $100 billion annually (Bruijn, 2015). Nitrogen fertilization is often seen as one of the most expensive inputs in agriculture. However, it is even more troublesome that approximately 65% of applied nitrogen fertilizer is lost through various environmental processes (Bhattacharjee et al., 2008). One of the greatest environmental concerns when examining nitrogen fate the pollution of groundwater from nitrates leached through the soil or lost through runoff

(Hornbaker, 1999). The use of NFB would reduce land application of fertilizers and therefore reduce potential groundwater pollution since NFB convert atmospheric N to NH_3, which is readily used by plants (Bhattacharjee et al., 2008). The NFB *Azotobacter* not only improves availability of N, but also enhances plant availability of P as well (Din et al., 2019; Velmourougane et al., 2019). *Azospirillum* often synthesize plant hormones and act as plant growth promoting bacteria (Fukami et al., 2018). Phytohormones that are released into the soil by *Azospirillum* can increase root growth which in turn enhances the ability of a plant to acquire moisture and nutrients from the soil (Ardakani and Mafakheri, 2011). Additionally, following inoculation with a *Rhizobium* bacteria, rice (*Oryza sativa* L.) stomatal conductance and photosynthetic activity increased by 12% while grain production increased by 16% (Bhattacharjee et al., 2008; Peng et al., 2002).

Negative impacts in response to NFB are limited thus far. It is theorized that NFB could potentially oversaturate the system with N and therefore contribute to groundwater contamination through leaching events (Field, 2004). Additionally, the introduction of too many NFB into the soil may create an increase in competition for limited carbon sources (Sylvia et al., 2004). Certain microorganisms may not be able to compete for limited resources, further changing the dynamics of the soil microbial community.

Use in Agronomic Crops

Nitrogen fixation has become a focus of several researchers in attempts to limit wasteful N applications and potential impacts on the environment (Aasfar et al., 2021). Nitrogen fixing bacteria are a principal component of many cropping systems including legumes, maize, wheat (*Triticum aestivum* L.), sugarcane (*Saccharum officinarum* L.), and rice (Bhattacharjee et al., 2008, Burris and Roberts 1993). Burris and Roberts (1993) estimate that almost 20% of food

production worldwide is derived from legumes; therefore, are large majority of NFB are associated with legumes. Research conducted on leguminous crops in Africa determined that these crops are highly dependent on NFB for N requirements (Bruijn, 2015; Dakora et al., 2015). Cowpeas (*Vigna unguiculata* L. Walp.) derive 30 to 96% of their N from NFB, while soybeans [*Glycine max* (L.) Merr.] obtain anywhere from 39 to 87% of their N from this interaction (Bruijn, 2015; Dakora et al., 2015). These symbiotic relationships have long been established since *Rhizobia* were discovered by Hellriegel and Wilfarth in 1888 (Burris and Roberts, 1993; Wilson, 1940).

More applicable to our own research are some of the experiments conducted with NFB and cereal crops. The addition of bacterial inoculants to the soil has led often led to an increase in plant yield (Gupta et al., 2012). Yield increases have been observed in maize of 36 to 48% in the greenhouse and 5.9 to 6.3% in the field in response to NFB (Gyaneshear et al., 2001). Sugarcane grown in the greenhouse with NFB exhibited 13 to 25% yield increases, while a 20% increase total plant biomass was observed in rice in response to NFB (Chaintruel et al., 2000; Riggs et al., 2001). However, application methodology may be the one factor that limits efficacy and use of NFB in agriculture.

Several different techniques have been adopted in order to inoculate plants (Bhattacharjee et al., 2008). Methods have included dipping seed or seedling roots in broth culture before sowing, application of bacterial suspensions to the soil, seed coating with strains of bacteria, and foliar sprays of inoculum suspensions (Baldani et al. 2000; Bhattacharjee et al., 2008 ; Biswas et al. 2000a, 2000b; Feng et al., 2006; Gutierrez-Zamora and Martinez-Romero 2001; Matthews et al., 2001;; Muthukumarasamy et al. 1999; Riggs et al. 2001; Yanni et al. 1997, 2001).

Unfortunately, none of these techniques have completely worked nor has one of them been singled out as a superior method for inoculation.

Challenges in Turfgrass Environments

Perennial cropping systems like turfgrass may create greater challenges for microbial inoculant use and performance. Bermudagrass (*Cynodon* spp.) is one of the most widely used turfgrass species in the southeastern region of the United States. Tolerance to drought and heat make bermudagrass a popular selection for home lawns, athletic fields, and golf courses (Christians et al., 2016; Duble, 2001; Emmons and Rossi, 2015; Hanna et al., 2013). Abundant production of aggressive stolons and rhizomes lead to the spread of a dense turfgrass canopy that tolerates excessive wear and traffic (McCarty and Miller, 2002). Subsequently, bermudagrass is one of the largest nitrogen consumers, requiring approximately 49 kg N ha^{-1} $month^{-1}$ during the summer in order to meet such high growth demand (Christians et al., 2016). Access to fertility is extremely important since nitrogen is a component of numerous plant biochemical constituents including chlorophyll, amino acids, and enzymes that are integral to turfgrass growth and metabolic function (Emmons and Rossi, 2015; Fry and Huang, 2004.)

Previous research involving the incorporation of microbial inoculants into turfgrass management has been limited and inconsistent. Peacock and Daniel (1992) did not observe improvements in tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] and hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt Davy] growth, nitrogen uptake, or disease suppression in response to a microbial inoculant (*Bacillus* spp.) when compared to urea fertilizer. Baltensperger et al. (1978) reported a 17% increase in bermudagrass shoot growth in the greenhouse when inoculated with *Azospirillum* and *Azotobacter* spp. under low nitrogen fertility, but no increase in rooting was detected. Acikgoz et al. (2016) reported

increased turfgrass color and clipping yield of perennial ryegrass (*Lolium perenne* L.) and tall fescue treated with rhizosphere-associated N_2-fixing bacteria (*Bacillus* spp.) while De Luca et al. (2020) noted an increase in perennial ryegrass turfgrass quality following plant exposure to nitrifying bacteria (*Azotobacter*, *Bacillus*, and *Pseudomonas* spp.), even when subjected to nutrient stress. Enhancements in 'Tifway 419' hybrid bermudagrass root and shoot growth were also observed by Coy et al. (2014) in the greenhouse in response to inoculant blends of *Bacillus*, *Paenibacillus*, and *Brevibacillus* spp.

Unfortunately, previous research evaluating microbial inoculants in turfgrass has primarily focused on cool-season turfgrass species with a majority of research conducted in controlled environments (Aamlid and Hanslin, 2009; Acikgoz et al., 2016; DeLuca et al., 2020). Endemic microbial populations that have adapted to survive and proliferate in turfgrass environments may outcompete non-endemic microorganisms recently introduced into the system. Furthermore, cultural practices (i.e. aerification, topdressing) and management inputs (i.e. fertilizer, irrigation, pesticides) specific to turfgrass growth and production may influence microbial activity, persistence, and species diversity through physical and chemical manipulation of the soil profile. Postemergence herbicides like triclopyr have been reported to inhibit soil bacteria that transform ammonia into nitrite, glyphosate has been shown to reduce the activity of free-living nitrogen-fixing bacteria in soil, and 2,4-D was shown to reduce nitrogen fixation by the bacteria that live on plant roots (Aktar et al., 2009; Arias and Fabra, 1993; Fabra et al., 1997; Pell *et al*., 1998; Santos and Flores, 1995). Some evidence has suggested that fungicides may have greater effects on soil organisms than insecticides or herbicides but minimal research has been conducted on this topic (Bünemann et al. 2006; Shi et al., 2007).

Most recently, nitrogenase activity and *Bacillus* spp. persistence in common bermudagrass root and shoot tissue was documented in the field by Coy et al. (2019) from single inoculation events. However, the bacterial strains utilized in the Coy et al. (2019) colonization field research were previously collected from native populations and isolated by Auburn University's Department of Entomology and Plant Pathology. Many commercially available inoculants that exist for use in turfgrass are not native to the locations they are applied; therefore, competition from indigenous microorganisms and intolerance to environmental conditions could reduce potential for colonization and nitrogen fixation. Furthermore, shelf-life of microbial inoculants may pose a challenge for maintaining viability and ensuring efficacy of products following application (Callaghan 2016).

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CHAPTER 2

BERMUDAGRASS ESTABLISHMENT, AESTHETICS, AND FUNCTION IN RESPONSE TO MICROBIAL INOCULANT AND FERTILIZER TIMING

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Abstract

The incorporation of biostimulants, including microbial inoculants, into turfgrass management programs has increased over the past decade due to potential benefits of their use including increased nutrient uptake, enhanced growth, and improved stress tolerance. However, minimal research has been conducted on warm-season grasses and questions still exist regarding microbial inoculant application timing, frequency of inoculation, and need for supplemental nitrogen. Therefore, the objective of our research was to investigate the influence of nitrogen fertilizer and microbial inoculant application timings on the establishment of common bermudagrass in the field and in a controlled environment. Treatments containing fertilizer (F) consistently had higher normalized difference vegetation index, turfgrass color, and turfgrass quality than treatments that only contained the microbial inoculant (P). Establishment of bermudagrass plots in response to treatments containing F were nearly at full cover at the conclusion of the study [8 weeks after seeding (WAS)], while treatments only containing P were ≤ 70% cover and similar to the non-treated check. However, the highest change in carbon efflux was often observed in the field in response to treatments that supplied P 3 WAS. In the greenhouse, the greatest root (R) and shoot (S) weights were typically observed in response to treatments containing F, while P alone treatments resulted in R and S weights either similar or less than the non-treated check.

Introduction

Nitrogen is the macronutrient not only required but also applied in the greatest amount in turfgrass environments (Bauer et al., 2012; Frank and Guertal, 2013; Mills and Jones, 1997; Turner and Hummel, 1992; Walker and Branham, 2020). Fertilization with nitrogen is important for both turfgrass growth and metabolism. Nitrogen fertility promotes the formation of a dense, resilient canopy that functions as an aesthetic landscape, a safe playing surface, and a productive ecosystem (Carrow et al., 2002; Christians et al., 2016; Frank and Guertal, 2013). As a component of numerous plant biochemical constituents that include chlorophyll, amino acids, and enzymes, accessibility to nitrogen will ultimately determine the persistence and health of a turfgrass system (Carrow et al., 2002; Christians et al., 2016; Frank and Guertal, 2013; Marschener, 2011). However, the application and production of synthetic nitrogen fertilizers can have a negative impact on the same environment they are intended to benefit.

Synthetic ammonium nitrate and urea fertilizers are created from ammonia that is generated during the artificial, industrial nitrogen fixation procedure known as the Haber-Bosch process (Kandemir et al., 2013; Kyriakou et al., 2020; Smith et al., 2020; Xu et al., 2019). This technique perpetuates the reaction of naturally abundant atmospheric nitrogen with hydrogen to directly synthesize ammonia (Amin et al., 2013; Jennings, 1991; Kandemir et al., 2013; Modak, 2002). The production of ammonia using the Haber-Bosch process consumes approximately 1 to 2% of the global energy while emitting 1.2% of CO_2 emissions (Kyriakou et al., 2020; Smith et al., 2020; Wand and Meyer, 2019). Furthermore, the high solubility of synthetic fertilizers can negatively impact the environment through nitrogen leaching/runoff and subsequent contamination of groundwater and eutrophication of lakes and streams (Frank and Guertal, 2013; Shuman, 2002; Walker and Branham, 2020).

Previous research has attempted to investigate alternatives to synthetic nitrogen fertilizer applications in an effort to reduce potential negative environmental impacts and enhance turfgrass sustainability. Microbial and non-microbial products intended to improve turfgrass establishment and growth, increase tolerance to environmental stress, and augment plant nutrition have collectively been categorized as biostimulants (Brown and Saa, 2015; Calvo et al., 2014; Du Jardin, 2015; Rouphael and Colla, 2020; Yahkin et al., 2017). However, minimal research has addressed the use of microbial inoculants in turfgrass systems and findings have been inconsistent to date. Increases in perennial ryegrass (*Lolium perenne* L.) and tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] color and clipping yield were observed by Acikgoz et al. (2016) in response to N_2-fixing bacteria (*Bacillus* spp.). De Luca et al. (2020) reported similar improvements in perennial ryegrass quality when it was treated with several nitrifying bacteria (*Azotobacter*, *Bacillus*, and *Pseudomonas* spp.). Contrarily, Peacock and Daniel (1992) could not discern any enhancements in growth, nitrogen uptake, or disease suppression of tall fescue and hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt Davy] following *Bacillus* spp. inoculation.

The production of a dense canopy and tolerance to an array of environmental stresses including heat, drought, and traffic make bermudagrass (*Cynodon* spp.) one of the most widely used turfgrass species in the southeastern region of the United States (Christians et al., 2016; Emmons and Rossi, 2015; Hanna et al., 2013; Taliaferro et al., 2004). Versatility and affordability of establishment whether from seed, sprigs, or sod, make common bermudagrass [*Cynodon dactylon* (L.) Pers.] a popular choice for home lawns and low-input areas (Chalmers et al., 2006; Rice et al., 2019; Taliaferro et al., 2004). Significant soil disturbance may occur prior to turfgrass establishment often resulting in the removal of topsoil. Therefore, nitrogen

applications may be necessary during establishment in these depleted soils, although starter fertilizers are typically low in nitrogen and high in phosphorus content.

Investigation into the use of nitrifying bacteria in turfgrass management has been limited, with most research conducted in the greenhouse and focusing on cool-season turfgrass species (Aamlid and Hanslin, 2009; Acikgoz et al. 2016; DeLuca et al., 2020). Uncertainties still exist regarding application timing and need for supplemental nitrogen or sequential inoculation applications in turfgrass environments. Therefore, the objective of our research was to investigate the influence of nitrogen fertilizer and microbial inoculant application timings on the establishment of common bermudagrass in the field and in a controlled environment.

Materials and Methods

Field Experiments

Trials were conducted at the Athens Turfgrass Research and Education Center (ATREC) in Athens, GA (33.54° N, 83.22° W) and at the University of Georgia Facilities Maintenance Division (FMD) complex in Athens, GA (33.92° N, 83.37° W). The soil at ATREC was a Cecil sandy clay loam (fine, kaolinitic, thermic Typic Kanhapludults) with a pH of 5.5 and organic matter (OM) content of 1.2% while the soil at FMD was a Cecil sandy clay loam with a pH of 5.9 and OM content of 1.7%. Seedbed preparation at ATREC consisted of cultivating the research site in two directions (perpendicular to one another) with a tractor mounted rototiller (Bush Hog, Selma, AL) to a depth of 10.2 cm and grading to provide a smooth planting bed of desired contour. A hand tiller (Stihl, Waiblingen, Germany) was employed to a depth of 10.2 cm at the FMD location prior to grading in order to prepare the seedbed for planting. Plots measured 1.5 x 1.5 m and were arranged in a randomized complete block design with four replications at both locations.

'Sahara II' common bermudagrass was seeded at 98 kg ha^{-1} on 28 July 2021 at both locations. A seeding box (1.5 x 1.5 m) was placed on the corners of each plot and seed was applied evenly across the soil surface using a shaker jar. Treatments were applied immediately following seeding and consisted of no treatment, fertilizer (F) ($5N\text{-}5P_2O_5\text{-}5K_2O$) (EarthWorks Natural Organic Products, Martins Creek, PA) at 24 kg N ha^{-1} at seeding, F at 24 kg N ha^{-1} at seeding + 3 weeks after seeding (WAS), ProveN (P) (*Klebsiella varriicola*) (Pivot Bio, Berkeley, CA) at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1} at seeding, P at an IR of 1.87 L ha^{-1} and CV of 3000 L ha^{-1} at seeding + 3 WAS, F at 24 kg N ha^{-1} at seeding + P at an IR of 1.87 L ha^{-1} and CV of 3000 L ha^{-1} 3 WAS, and F at 24 kg N ha^{-1} + P at an IR of 1.87 L ha^{-1} and CV of 3000 L ha^{-1} at seeding + P at an IR of 1.87 L ha^{-1} and CV of 3000 L ha^{-1} 3 WAS. Fertilizer was applied by hand using a shaker jar. Microbial inoculants were applied with distilled water using a watering can. Plots received approximately 0.3 cm of water through an overhead irrigation system following treatment application and were covered with germination cloth (A.M. Leonard, Inc., Piqua, OH) to promote seed germination and prevent desiccation. Plants were mowed weekly (starting at 4 WAS) to a height of 3.81 cm with a walk-behind rotary mower (American Honda Motor Company, Alpharetta, GA) with clippings collected and removed. Approximately 2.5 to 4 cm of water wk^{-1} were applied at ATREC and FMD through an overhead irrigation system (well water).

Turfgrass color (TC), turfgrass quality (TQ), and normalized difference vegetation index (NDVI) were recorded at trial initiation, 3, 5, and 8 WAS. Visual ratings of TC and TQ were recorded on a scale of 1 to 9 with a rating of 6 considered acceptable TC and TQ (Morris and Shearman, 2007). Normalized difference vegetation index was recorded with a Field Scout CM 1000 NDVI chlorophyll meter (Spectrum Technologies Inc., Aurora, IL). A vegetative index

[{NDVI = [(R770 – R 660) / (R770 + R 660)}] was calculated (0 to 1, where 1 is best) from the reflectance readings. An average of three readings were obtained per plot per rating date. Grid counts were conducted to assess bermudagrass cover 3, 5, and 8 WAS. A 0.3-m^2 grid with 2.5-cm x 2.5-cm intersect spacing was randomly placed within each plot. The following equation was used to convert grid counts to percent cover:

$$(a/b) \times 100 = c \quad [1]$$

where *a* is the number of intersects where bermudagrass was present, *b* is the total number of intersections (25), and *c* is % turfgrass cover (Richardson et al., 2001).

Measurements of carbon dioxide (CO_2) efflux (CE) (μmol m^{-2} s^{-1}) were recorded at trial initiation, 5, and 8 WAS with a LI-COR 8100 automated system (LI-COR, Inc., Lincoln, NE) to provide plant root and soil microbial respiration as well as determine the overall metabolic activity of each system. Measurement setup included an infrared gas analyzer (IRGA) connected to the LI-COR 8100 device via an RS-232 serial cable. The LI-COR 8100 was also connected to a laptop computer via ethernet cable to run the LI-8100A 4.0.0 software for sample collection. A 20-cm IRGA survey chamber was placed on top of a polyvinyl chloride (PVC) collar (20-cm diameter) that was randomly inserted into each plot to contain the sampling area (7.5-cm offset) for measurement. To prevent any irrelevant CO_2 buildup in the chamber, a 60 s pre-purge and 45 s post-purge was conducted before and after each measurement. After closing the chamber, a dead-band period of 40 s was utilized before measurements were initiated to obtain a constant rate of efflux. An observation length of 60 s was used for each measurement. Change over time (Δ) for CE was determined for 5 and 8 WAS by comparing back to initial measurements.

Greenhouse Experiments

Trials were conducted at the Athens Turfgrass Research and Education Center greenhouse complex (33.54°N, 83.22°W) in Athens, GA during the summer of 2021. On 27 July 2021, 'Sahara II' common bermudagrass [Cynodon dactylon (L.) Pers.] (non-coated/treated) was seeded at a rate of 98 kg ha^{-1} into circular pots (15.2 cm diameter) containing a 2:1 mixture of Cecil sandy clay loam (fine, kaolinitic, thermic Typic Kanhapludults) and Wakulla sand (siliceous, thermic Psammentic Hapludults). Seed was evenly applied to the soil surface and lightly topdressed to increase seed to soil contact and reduce desiccation. Two pots were established per treatment per trial replication in order to conduct a time-lapse destructive harvest at 3 and 6 WAS. Two experimental runs were conducted simultaneously in separate greenhouses using a randomized complete block design with five replications.

Treatments were applied on 27 July 2021 and were the same as previously described for field experiments. Fertilizer ($5N\text{-}5P_2O_5\text{-}5K_2O$) was applied by hand using a shaker jar at a rate of 24 kg N ha^{-1}. A stock solution of the microbial inoculant ProveN was prepared with distilled water and applied with a 50 ml syringe at an inoculation rate of 1.87 L ha^{-1} and carrier volume of 3000 L ha^{-1}. Treatments receiving sequential applications were made on 17 August 2021. Pots were immediately irrigated after inoculant and fertilizer applications with approximately 0.3 cm of water to move treatments into the soil profile. A non-treated check was included for comparison. The trial was watered using an overhead irrigation system calibrated to deliver 3.8 cm water wk^{-1}. Natural light was supplemented with artificial light (metal halide) in order to remain at 500 μmol m^{-2} s^{-1} photosynthetic photon flux (measured at the canopy) in a 12-h day to approximate summer light intensity and photoperiod. Conditions in the climate-controlled greenhouse were maintained at day/night temperatures of 32 C/26 C. Experimental blocks were arranged along a gradient created by the greenhouse cooling pads and associated fans.

Pots were destructively harvested at 3 and 6 WAS. Roots (R) and shoots (S) were separated from each other, washed of all soil, dried in an oven for 48 h at 110 C, and weighed to determine R and S biomass (g) at 3 WAS and 6 WAS.

Data Analysis

Analysis was conducted separately for TC, TQ, NDVI, and % turfgrass cover at 3, 5, and 8 WAS as well as CE and ΔCE at 5 and 8 WAS for field trials. Additionally, a separate analysis was also conducted for R and S weights at 3 and 6 WAS for greenhouse trials. ANOVA was performed using PROC MIXED with the appropriate expected mean square values described by McIntosh (1983) in SAS (SAS v. 9.2 for Windows; Statistical Analysis Systems Institute, Cary, NC). Means were separated according to Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$. Data were arcsine square-root transformed to stabilize variance as described by Bowley (2008). Transformed and non-transformed data were analyzed, and interpretations were not different; therefore, non-transformed means are presented for clarity.

Results and Discussion

Field experiments

Experimental run-by-treatment interactions for field trials were not significant ($P = 0.57$). Therefore, data from individual runs were pooled and presented accordingly. At 3 WAS, the highest % turfgrass cover was observed in response to F + P at seeding (64.5%) followed by (fb) F at seeding (48.5 to 56%) (Table 2.1). The microbial inoculant applied alone at seeding only resulted in 25.5 to 33.5%, which was slightly greater than the non-treated check 3 WAS (23%). Sequential applications of F, F + P at seeding + P 3 WAS, and F at seeding + P 3 WAS exhibited the highest % turfgrass cover (77, 76, and 67%, respectively) 5 WAS (Table 2.1). The single application of F at seeding resulted in slightly lower % turfgrass cover (63%) 5 WAS, while

sequential applications of P, P at seeding, and the non-treated check were statistically similar (47, 42, and 41%, respectively). At 8 WAS, all treatments that received at least one application of F resulted in similar % turfgrass cover (88.5 to 99%), while treatments only receiving P (59 to 68.5%) had similar cover as the non-treated check (65%) (Table 2.1).

At 3 WAS, the highest NDVI (0.52 to 0.58) and TQ (3.6 to 4.1) ratings were observed in response to treatments that supplied F at seeding (Table 2.2). Three of the four F at seeding treatments also resulted in the highest TC (5.1 to 5.6). The microbial inoculant applied alone at seeding resulted in similar NDVI (0.38 to 0.39), TC (4.1 to 4.3), and TQ (2.7 to 3.1) as the non-treated check (0.36, 0.39, and 2.6, respectively). Sequential F applications, F + P at seeding + P 3 WAS, F at seeding + P 3 WAS, and F at seeding treatments resulted in the highest NDVI (0.60 to 0.65) and TC (5.6 to 6.1) 5 WAS (Table 2.2). The highest TQ 5 WAS also observed in response to sequential F applications (6.1), F + P at seeding + P 3 WAS (6.1), and F at seeding + P 3 WAS (5.8). At 5 WAS, P alone treatments resulted in similar NDVI (0.51 to 0.54), TC (4.6 to 4.7), and TQ (3.6 to 3.9) as the non-treated check (0.50, 4.7, and 3.4, respectively). A similar trend was observed 8 WAS with the greatest NDVI, TC, and TQ in response to sequential F applications, F + P at seeding + P 3 WAS, F at seeding + P 3 WAS, and F at seeding (Table 2.2). Treatments receiving P alone still resulted in similar NDVI, TC, and TQ as the non-treated check 8 WAS.

Treatments receiving P 3 WAS resulted in the greatest ΔCE 5 WAS with F at seeding + P 3 WAS exhibiting the highest ΔCE (5.3 μmol m^{-2} s^{-1}) (Table 2.3). ProveN at seeding (3.4 μmol m^{-2} s^{-1}) and treatments only supplying F (3.1 μmol m^{-2} s^{-1}) were statistically similar to the ΔCE of the non-treated check (2.1 μmol m^{-2} s^{-1}). At 8 WAS, F at seeding + P 3 WAS and F + P at seeding + P 3 WAS resulted in the greatest ΔCE (4.5 to 4.7 μmol m^{-2} s^{-1}); however, the ΔCE of

the non-treated check (3.2 μmol m^{-2} s^{-1}) was statistically similar (Table 2.3). All other treatments exhibited $\Delta CE \leq 2.8$ μmol m^{-2} s^{-1}.

Greenhouse experiments

Experimental run-by-treatment interactions for greenhouse trials were significant (P = 0.03). Therefore, data were not pooled across experimental runs and results for each trial will be presented separately.

In experimental run 1, treatments that contained initial F applied alone exhibited the greatest R weights (0.43 to 0.56 g) 3 WAS (Table 2.4). Treatments that contained initial P applications resulted in the lowest R weights (0.32 to 0.41 g) and were statistically similar to the non-treated check (0.28 g) 3 WAS. The highest S weights (1.15 to 1.45 g) 3 WAS were observed in response to all treatments that contained initial F applications. Treatments that supplied initial P alone exhibited the lowest S weights (0.73 to 0.79 g), which were statistically similar to the non-treated check (0.68 g). At 6 WAS, the greatest R weight was observed in response to sequential F applications (5.32 g), F + P at seeding + P 3 WAS (3.84 g), and single applications of F (3.58 g) (Table 2.4). Treatments containing P alone resulted in the lowest R weights (2.16 to 2.45 g), which were less than the non-treated check (2.57 g) 6 WAS. Sequential applications of F resulted in the greatest S weight (9.15 g) 6 WAS fb F + P at seeding + P 3 WAS (7.68 g) and a single application of F at seeding (6.27 g). All other treatments resulted in S weight ≤ 4.95 g and were statistically similar to the non-treated check (4.82 g) 6 WAS.

The greatest R weight (0.57 to 0.71 g) 3 WAS in experimental run 2 was observed in response to treatments where F was applied at seeding (Table 2.4). Treatments that supplied P initially resulted in R weights of 0.24 to 0.36 g and were statistically comparable to the non-treated check (0.30 g) 3 WAS. A similar trend was observed with respect to S weight 3 WAS.

Single and sequential applications of F resulted in the greatest R weight (3.15 to 3.83 g) 6 WAS, while the other two treatments containing F resulted in similar R weight (2.44 to 2.57 g) as the non-treated check (1.99 g) (Table 2.4). The least R weight (1.59 to 1.64 g) was produced 6 WAS in response to treatments only supplying P. Single and sequential applications of F resulted in the greatest S weight (7.71 to 8.21 g) 6 WAS; however, F at seeding + P 3 WAS produced comparable R weight (5.42 g). All other treatments resulted in statistically similar or lower R weight 6 WAS as the non-treated check.

Treatments containing F consistently had higher NDVI, TC, and TQ than treatments that only contained P. Establishment of bermudagrass plots in response to treatments containing F were nearly at full cover at the conclusion of the study (8 WAS), while treatments only containing P were ≤ 70% cover and similar to the non-treated check. However, the highest ΔCE was often observed in the field in response to treatments that supplied P 3 WAS. In the greenhouse, the greatest R and S weights were typically observed in response to treatments containing F, while P alone treatments resulted in R and S weights either similar or less than the non-treated check. Similarly, Peacock and Daniel (1992) observed a significantly higher hybrid bermudagrass growth rate in the greenhouse in response to urea fertilizer (3187 mg m^{-2} d^{-1}) than inoculation with *Bacillus* spp. (1903 mg m^{-2} d^{-1}) 17 days after treatment (DAT). Only a 17% increase in bermudagrass shoot growth with no increase in root growth was observed by Baltensperger et al. (1978) in response to inoculation with *Azospirillum* and *Azotobacter* spp. under low nitrogen fertility in the greenhouse. Contrarily, Coy et al. (2014) reported enhancements in root and shoot growth of 'Tifway 419' hybrid bermudagrass following inoculation with blends of *Bacillus*, *Paenibacillus*, and *Brevibacillus* spp. However, although bermudagrass did not receive fertility during the trial, Coy et al. (2014) supplied bermudagrass

with fertility for three consecutive weeks prior to trial initiation. Furthermore, mature hybrid bermudagrass transplants utilized by Coy et al. (2014) may have been more conducive to colonization than the common bermudagrass established from seed in our research. Increases in root and shoot weight of other C-4 grasses [pearl millet, *Pennisetum glaucum* (L.) R. Br.; foxtail millet, *Setaria italica* (L.) P. Beauv.] in response to *Azospirillum* spp. have been documented; however, all of these grasses exhibit annual growth habits (Di Ciocco and Cáceres, 1994; Mane et al., 2000; Rafi et al., 2012).

Most turfgrass research documenting benefits from use of microbial inoculants has examined the response of cool-season grasses planted in sterile media and grown in controlled environments (Acikgoz et al., 2016; DeLuca et al., 2020). Therefore, microbial inoculants are often evaluated without competition from native microorganisms or under local environmental conditions. Coy et al. (2019) documented nitrogenase activity and microbial persistence in root and shoot tissue of common bermudagrass following field inoculation with *Bacillus* spp. However, bacterial strains utilized in their research were previously collected from native populations and isolated by Auburn University's Department of Entomology and Plant Pathology, ensuring some adaptation to indigenous environments and competition with endemic microorganisms. The *Klebsiella varriicola* bacteria within ProveN, the inoculant utilized in our research, has been gene-edited to continue nitrogen fixation even in the presence of high soil nitrogen (Bloch et al., 2020). Furthermore, it has also been gene-edited to decrease glutamine synthase production and activity; therefore, allowing bacteria to fix nitrogen in the presence of high soil inorganic nitrogen. Although these traits confer advantages, bacteria contained within this inoculant were originally isolated from the mucilage of aerial roots of an indigenous landrace of maize grown in Mexico (Van Deynze et al., 2018). Therefore, microorganisms

adapted to our soil and environmental conditions may pose enough competition to reduce potential for colonization and nitrogen fixation from ProveN.

Turfgrass is a perennial cropping system that requires large amounts of management inputs (i.e. fertilizer, irrigation, pesticides) and cultural practices (i.e. aerification, topdressing) in order to sustain adequate growth and functionality. However, those same inputs often influence microbial activity, persistence, and species diversity through the alteration of the physical and chemical characteristics of the soil. Additionally, turfgrass species may play a critical role in the potential for symbiotic relationships between plant roots and soil microorganisms. Research conducted within our group only examined one or two microbial inoculation events and monitored bermudagrass response over a short period of time. Coy et al. (2014) reported enhancement in root and shoot growth in response to weekly microbial inoculation over a five-week period. Future research should examine the impact of more frequent and numerous inoculation events on turfgrass performance over multiple growing seasons in order to determine the feasibility of colonization and potential adoption of more sustainable turfgrass nitrogen management.

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Table 2.1. Establishment (% turfgrass cover) of seeded 'Sahara II' common bermudagrass [Cynodon dactylon (L.) Pers.] in response to fertility and microbial inoculants applied during the summer of 2021 in Athens, GA.

	% Turfgrass Cover[z]		
Treatment[y]	3 WAS[x]	5 WAS	8 WAS
Non-treated check	23.0 d[w]	41.0 c	65.0 b
F at seeding	53.0 b	63.0 b	89.5 a
F at seeding + 3 WAS	48.5 b	77.0 a	99.0 a
P at seeding	25.5 cd	42.0 c	59.0 b
P at seeding + 3 WAS	33.5 c	47.0 c	68.5 b
F at seeding + P 3 WAS	56.0 ab	67.0 ab	88.5 a
F + P at seeding + P 3 WAS	64.5 a	76.0 a	95.0 a
$LSD_{(0.05)}$	10.2	11.1	13.6

[z]Percent turfgrass cover was assessed by randomly placing a 0.3-m^2 grid with 2.5-cm x 2.5-cm intersect spacing (25 intersections) in the middle of each plot.

[y]Fertilizer was applied by hand using a shaker jar at a rate of 24 kg N ha^{-1}. Microbial inoculants were applied with a watering can using distilled water at an inoculation rate of 1.87 L ha^{-1} and carrier volume of 3000 L ha^{-1}.

[x]Abbreviations: WAS, weeks after seeding; F, fertilizer (5N-5P_2O_5-5K_2O); P, ProveN (microbial inoculant containing *Klebsiella varriicola*); $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 2.2. Normalized difference vegetation index (NDVI), turfgrass color (TC), and turfgrass quality (TQ) of seeded 'Sahara II' common bermudagrass [Cynodon dactylon (L.) Pers.] in response to fertility and microbial inoculants applied during the summer of 2021 in Athens, GA.

	3 WAS[z]			5 WAS			8 WAS		
Treatment[y]	NDVI[x]	TC	TQ	NDVI	TC	TQ	NDVI	TC	TQ
Non-treated check	0.36 b[w]	3.9 d	2.6 c	0.50 c	4.7 b	3.4 c	0.56 c	4.9 c	4.4 c
F at seeding	0.52 a	4.9 bc	3.6 ab	0.60 ab	5.6 a	4.6 b	0.65 ab	6.0 b	5.6 b
F at seeding + 3 WAS	0.52 a	5.1 a	4.0 a	0.65 a	6.1 a	5.1 a	0.7 a	6.6 a	6.5 a
P at seeding	0.38 b	4.1 d	2.7 c	0.51 c	4.6 b	3.6 c	0.61 bc	5.1 c	4.3 c
P at seeding + 3 WAS	0.39 b	4.3 cd	3.1 bc	0.54 bc	4.7 b	3.9 c	0.61 bc	5.3 c	4.6 c
F at seeding + P 3 WAS	0.52 a	5.1 ab	4.0 a	0.63 a	5.8 a	4.9 ab	0.69 a	6.2 ab	5.8 ab
F + P at seeding + P 3 WAS	0.58 a	5.6 a	4.1 a	0.65 a	6.1 a	5.2 a	0.69 a	6.3 ab	6.0 ab
$LSD_{(0.05)}$	0.09	0.6	0.6	0.06	0.6	0.6	0.07	0.6	0.7

[z]Abbreviations: WAS, weeks after seeding; F, fertilizer ($5N\text{-}5P_2O_5\text{-}5K_2O$); P, ProveN (microbial inoculant containing *Klebsiella varriicola*); $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$.

[y]Fertilizer was applied by hand using a shaker jar at a rate of 24 kg N ha^{-1}. Microbial inoculants were applied with a watering can using distilled water at an inoculation rate of 1.87 L ha^{-1} and carrier volume of 3000 L ha^{-1}.

[x]NDVI was recorded with a Field Scout CM 1000 NDVI chlorophyll meter. A vegetative index [{NDVI = [(R770 – R 660) / (R770 + R 660)}] was calculated (0 to 1, where 1 is best) from the reflectance readings. An average of three readings were obtained per plot per rating date. Visual ratings of TC and TQ were recorded on a scale of 1 to 9 with a rating of 6 considered acceptable TC and TQ.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 2.3. Carbon efflux (μmol m^{-2} s^{-1}) of seeded 'Sahara II' common bermudagrass [Cynodon dactylon (L.) Pers.] in response to fertility and microbial inoculants applied during the summer of 2021 in Athens, GA.

	Carbon Efflux (μmol m^{-2} s^{-1})[z]			
Treatment[y]	5 WAS[x]	Δ5 WAS	8 WAS	Δ8 WAS
Non-treated check	4.7 c[w]	2.1 c	5.8 ab	3.2 ab
F at seeding	7.7 ab	3.1 bc	6.5 ab	1.9 b
F at seeding + 3 WAS	8.1 a	3.1 bc	6.8 ab	1.8 b
P at seeding	6.3 b	3.4 bc	5.8 ab	2.8 b
P at seeding + 3 WAS	8.1 a	4.7 ab	5.5 b	2.1 b
F at seeding + P 3 WAS	8.0 a	5.3 a	7.2 a	4.5 a
F + P at seeding + P 3 WAS	6.8 ab	4.4 ab	7.1 a	4.7 a
$LSD_{(0.05)}$	1.4	1.7	1.5	1.7

[z]Carbon efflux measurements were recorded with a LI-COR 8100A automated system at trial initiation as well as 5 and 8 WAS to provide insight into plant and soil microbial activity (respiration). Data recorded at 5 and 8 WAS were compared back to data recorded at trial initiation in order to calculate carbon efflux change over time (Δ).

[y]Fertilizer was applied by hand using a shaker jar at a rate of 24 kg N ha^{-1}. Microbial inoculants were applied with a watering can using distilled water at an inoculation rate of 1.87 L ha^{-1} and carrier volume of 3000 L ha^{-1}.

[x]Abbreviations: WAS, weeks after seeding; F, fertilizer (5N-5P_2O_5-5K_2O); P, ProveN (microbial inoculant containing *Klebsiella varriicola*); $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 2.4. Root and shoot weight (g) of seeded 'Sahara II' common bermudagrass [Cynodon dactylon (L.) Pers.] in response to fertility and microbial inoculants applied in the greenhouse during the spring of 2021 in Athens, GA.

	Experimental Run 1				Experimental Run 2			
	Harvest[z] – 3 WAS[y]		Harvest – 6 WAS		Harvest – 3 WAS		Harvest – 6 WAS	
Treatment[x]	R (g)	S (g)	R (g)	S (g)	R (g)	S (g)	R (g)	S (g)
Non-treated check	0.28 d[w]	0.68 b	2.57 b	4.82 bc	0.30 bc	0.64 b	1.99 bc	4.57 bc
F at seeding	0.43 abc	1.17 a	3.58 ab	6.27 abc	0.57 ab	1.18 a	3.15 ab	7.71 ab
F at seeding + 3 WAS	0.49 ab	1.45 a	5.32 a	9.15 a	0.69 a	1.2 a	3.83 a	8.21 a
P at seeding	0.32 cd	0.73 b	2.45 b	3.85 c	0.24 c	0.56 b	1.64 c	3.7 c
P at seeding + 3 WAS	0.33 cd	0.79 b	2.16 b	4.95 bc	0.26 c	0.51 b	1.59 c	3.61 c
F at seeding + P 3 WAS	0.56 a	1.45 a	3.36 b	4.84 bc	0.67 a	1.34 a	2.44 bc	5.42 abc
F + P at seeding + P 3 WAS	0.41 bcd	1.15 a	3.84 a	7.68 ab	0.71 a	1.45 a	2.57 bc	4.89 bc
$LSD_{(0.05)}$	0.13	0.33	1.88	3.05	0.28	0.39	1.19	3.03

[z]Destructive harvests were conducted 3 and 6 WAS. Roots and shoots were separated from each other, washed of all soil, dried in an oven for 48 h at 110 C, and weighed to determine root and shoot biomass (g).

[y]Abbreviations: WAS, weeks after seeding; R, roots; S, shoots; F, fertilizer ($5N\text{-}5P_2O_5\text{-}5K_2O$); P, ProveN (microbial inoculant containing *Klebsiella varriicola*); $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$.

[x]Fertilizer was applied by hand using a shaker jar at a rate of 24 kg N ha^{-1}. A stock solution of the microbial inoculant was prepared with distilled water and applied with a 50 ml syringe at an inoculation rate of 1.87 L ha^{-1} and carrier volume of 3000 L ha^{-1}.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

CHAPTER 3

THE IMPACT OF COMMERCIALLY AVAILABLE MICROBIAL INOCULANTS ON BERMUDAGRASS ESTABLISHMENT, AESTHETICS, AND FUNCTION

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Abstract

The production and application of fertilizers are not only costly but often harmful to the environment; therefore, sustainability research has been aimed at investigating alternatives to traditional nitrogen fertility applications. Biostimulants, including microbial inoculants, have been touted to enhance plant nutrition, growth, and stress tolerance, but research on warm-season turfgrass is limited. Therefore, the objective of our research was to compare bermudagrass establishment, quality, and function in response to commercially available microbial inoculants in controlled environment and field experiments. Microbial inoculant applications in the field did not consistently increase hybrid bermudagrass normalized vegetation difference index, turfgrass color, nor turfgrass quality compared to the non-treated check. However, although not always significant, inoculated bermudagrass often exhibited higher carbon efflux measurements than the non-treated check, but results were inoculant specific. No microbial inoculant resulted in greater common bermudagrass root or shoot production in the greenhouse. Perennial cropping systems like turfgrass may create greater challenges for microbial inoculant use and performance due to the presence of endemic microbial populations already adapted to survive and proliferate in turfgrass environments. Consequently, future research should examine inoculation frequency, soil and plant persistence, and use of microbial blends to determine best management practices leading to the greatest opportunity for colonization and subsequent turfgrass benefits.

Introduction

Bermudagrass (*Cynodon* spp.) is one of the most widely used turfgrass species in the southeastern region of the United States. Tolerance to drought and heat make bermudagrass a popular selection for home lawns, athletic fields, and golf courses (Christians et al., 2016; Duble, 2001; Emmons and Rossi, 2015; Hanna et al., 2013). Abundant production of aggressive stolons and rhizomes lead to the spread of a dense turfgrass canopy that tolerates excessive wear and traffic (McCarty and Miller, 2002; Pornaro et al., 2019) Subsequently, bermudagrass is one of the largest nitrogen consumers, requiring approximately 49 kg N ha^{-1} $month^{-1}$ during the summer in order to meet such high growth demand (Christians et al., 2016). Access to fertility is extremely important since nitrogen is a component of numerous plant biochemical constituents including chlorophyll, amino acids, and enzymes that are integral to turfgrass growth and metabolic function (Emmons and Rossi, 2015; Fry and Huang, 2004.)

Fertilizers are costly and may negatively influence the environment when applied at excessive rates or through improper techniques. The Haber-Bosch process is an artificial, industrial nitrogen fixation procedure that is mainly responsible for the current synthetic production of ammonia (Kandemir et al., 2013; Kyriakou et al., 2020; Smith et al., 2020; Xu et al., 2019). Ammonia generated from this reaction is predominantly used as nitrogen fertilizer in the form of ammonium nitrate and urea. However, environmental impacts resulting from this production process and subsequent fertilizer application include nitrogen leaching/runoff leading to groundwater contamination and eutrophication, atmospheric deposition of nitrates and ammonia, and increased greenhouse gas emissions including nitrous oxide (Mulvaney et al., 2009; Uduvardi et al., 2015; Xu et al., 2019).

Current turfgrass sustainability research is aimed at investigating alternatives to traditional nitrogen fertilizer applications in order to limit negative environmental impacts. Biostimulants include an assortment of microbial and non-microbial products that are intended to enhance plant nutrition, establishment/growth, and stress tolerance (Brown and Saa, 2015; Calvo et al., 2014; Du Jardin, 2015; Rouphael and Colla, 2020; Yahkin et al., 2017). Previous research involving the incorporation of biostimulants into turfgrass management has been limited and inconsistent. Peacock and Daniel (1992) did not observe improvements in tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] and hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt Davy] growth, nitrogen uptake, or disease suppression in response to a microbial inoculant (*Bacillus* spp.) when compared to urea fertilizer. Acikgoz et al. (2016) reported increased turfgrass color (TC) and clipping yield of perennial ryegrass (*Lolium perenne* L.) and tall fescue treated with rhizosphere-associated N_2-fixing bacteria (*Bacillus* spp.) while De Luca et al. (2020) noted an increase in perennial ryegrass turfgrass quality (TQ) following plant exposure to nitrifying bacteria (*Azotobacter, Bacillus*, and *Pseudomonas* spp.), even when subjected to nutrient stress.

Although microbial inoculants are more widely adopted for use in horticultural and agronomic crops, several challenges exist regarding their use and efficacy, with some more specific to turfgrass systems. Most commercial products contain foreign microorganisms that have difficulty competing with native populations acclimated to regional environmental conditions (Gómez-Godínez et al., 2021; Timmusk et al., 2017; Yahkin et al., 2017). Turfgrass management inputs such as fertilizer, pesticides, and irrigation as well as environmental issues like turfgrass species and soil profile composition can select for more adaptive microbial communities that quickly outcompete introduced organisms (Shi et al., 2007; Yao et al., 2006).

Previous research evaluating microbial inoculants in turfgrass has primarily focused on cool-season turfgrass species with a majority of research conducted in controlled environments (Aamlid and Hanslin, 2009; Acikgoz et al., 2016; DeLuca et al., 2020). Limited information still exists regarding application timing, carrier volume, water quality, inoculation size, and need for sequential inoculation applications in turfgrass environments. Therefore, the objective of our research was to compare bermudagrass establishment in a controlled environment as well as determine the quality and function of a mature bermudagrass stand in the field in response to commercially available microbial inoculants.

Materials and Methods

Field experiments

Trials were conducted at the Athens Turfgrass Research and Education Center (ATREC) in Athens, GA (33.54° N, 83.22° W) and at a residential lawn in Bogart, GA (BO) (33.92° N, 83.54° W). The soil at ATREC was a Cecil sandy clay loam (fine, kaolinitic, thermic Typic Kanhapludults) with a pH of 5.5 and organic matter (OM) content of 1.2% while the soil at BO was a Madison sandy clay loam (fine, kaolinitic, thermic Typic Kanhapludults) with a pH of 6.1 and OM content of 1.9%. Research at ATREC was performed on a mature (5-year-old) 'Tifway 419' hybrid bermudagrass mowed at 5.1 cm with a ride-on rotary mower (John Deere US, Moline, IL) while research at BO was conducted on a 2-year-old 'Tifway 419' hybrid bermudagrass mowed at 5.1 cm with a walk-behind rotary mower (American Honda Motor Company, Alpharetta, GA). Plots measured 1.5 x 1.5 m and were arranged in a randomized complete block design with four replications at both locations. All experimental areas were mowed just prior to treatment application and once weekly thereafter with turfgrass clippings returned to the canopy. Approximately 2.5 to 4 cm of water wk^{-1} were applied at ATREC

through an overhead irrigation system (well water) while the only source of water at BO was natural rainfall.

Treatments were applied on 5 July 2021 at ATREC and 21 July 2021 at BO and included single and sequential applications of ProveN (*Klebsiella varriicola*) (Pivot Bio, Berkeley, CA) at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1}, Return (*Klebsiella varriicola* – alternative formulation) (Pivot Bio, Berkeley, CA) at an IR of 1.87 L ha^{-1} and a CV of 3000 L ha^{-1}, Envita (*Gluconacetobacter diazotrophicus*) (Azotic North America, Raleigh, NC) at an IR of 0.95 L ha^{-1} and a CV of 189 L ha^{-1}, and Terramax Tazo-B liquid (*Azospirillum brasilense*) (Terramax, Inc., Bloomington, MN) at an IR of 2.55 L ha^{-1} and a CV of 990 L ha^{-1}. Sequential applications were made 3 weeks after initial treatment (WAIT). A non-treated check was included for comparison. Microbial inoculants were applied with distilled water using a watering can. Plots received approximately 0.3 cm of water through an overhead irrigation system following inoculant application in order to dislodge material from the turfgrass canopy and move it into the root zone. No fertility was applied to either site during the duration of each trial.

Turfgrass color, TQ, and normalized difference vegetation index (NDVI) were recorded at trial initiation, 2, 4, 6, and 8 WAIT. Visual ratings of TC and TQ were recorded on a scale of 1 to 9 with a rating of 6 considered acceptable TC and TQ (Morris and Shearman, 2007). Normalized difference vegetation index was recorded with a Field Scout CM 1000 NDVI chlorophyll meter (Spectrum Technologies Inc., Aurora, IL). A vegetative index [{NDVI = [(R770 – R 660) / (R770 + R 660)}] was calculated (0 to 1, where 1 is best) from the reflectance readings. An average of three readings were obtained per plot per rating date.

Measurements of carbon dioxide (CO_2) efflux (CE) (μmol m^{-2} s^{-1}) were recorded at trial initiation, 3, and 6 WAIT with a LI-COR 8100 automated system (LI-COR, Inc., Lincoln, NE) to provide plant root and soil microbial respiration as well as determine the overall metabolic activity of each system (Henry et al., 2021). Measurement setup included an infrared gas analyzer (IRGA) connected to the LI-COR 8100 device via an RS-232 serial cable. The LI-COR 8100 was also connected to a laptop computer via ethernet cable to run the LI-8100A 4.0.0 software for sample collection. A 20-cm IRGA survey chamber was placed on top of a polyvinyl chloride (PVC) collar (20-cm diameter) that was randomly inserted into each plot to contain the sampling area (7.5-cm offset) for measurement. To prevent any irrelevant CO_2 buildup in the chamber, a 60 s pre-purge and 45 s post-purge was conducted before and after each measurement. After closing the chamber, a dead-band period of 40 s was utilized before measurements were initiated to obtain a constant rate of efflux. An observation length of 60 s was used for each measurement. Change over time (Δ) for CE was determined for 4 and 8 WAIT by comparing back to initial measurements.

Greenhouse experiment

Trials were conducted at the Athens Turfgrass Research and Education Center greenhouse complex (33.54°N, 83.22°W) in Athens, GA during the summer of 2021. On 26 July 2021, 'Sahara II' common bermudagrass [*Cynodon dactylon* (L.) Pers.] (non-coated/treated) was seeded at a rate of 98 kg ha^{-1} into circular pots (15.2 cm diameter) containing a 2:1 mixture of a Cecil sandy clay loam (fine, kaolinitic, thermic Typic Kanhapludults) and a Wakulla sand (siliceous, thermic Psammentic Hapludults). Seed was evenly applied to the soil surface and lightly topdressed to increase seed to soil contact and reduce desiccation. Two pots were established per treatment per trial replication in order to conduct a time-lapse destructive harvest

at 3 and 6 weeks after seeding (WAS). Two experimental runs were conducted simultaneously in separate greenhouses using a randomized complete block design with five replications.

A stock solution of each microbial inoculant was prepared with distilled water in order to deliver the same eight treatments as described previously for the field experiments. Inoculants were applied evenly across the surface of each pot on 26 July 2021 using a 50 ml syringe. Treatments receiving sequential applications were made on 16 August 2021. Pots were immediately irrigated after inoculant application with approximately 0.3 cm of water to move treatments into the soil profile. A non-treated check was included for comparison. The trial was watered using an overhead irrigation system calibrated to deliver 3.8 cm water wk^{-1}. Natural light was supplemented with artificial light (metal halide) in order to remain at 500 μmol m^{-2} s^{-1} photosynthetic photon flux (measured at the canopy) in a 12-h day to approximate summer light intensity and photoperiod. Conditions in the climate-controlled greenhouse were maintained at day/night temperatures of 32 C/26 C. Experimental blocks were arranged along a gradient created by the greenhouse cooling pads and associated fans.

Pots were destructively harvested at 3 and 6 WAS. Roots (R) and shoots (S) were separated from each other, washed of all soil, dried in an oven for 48 h at 110 C, and weighed to determine R and S biomass (g) at 3 WAS and 6 WAS.

Data Analysis

Analysis was conducted separately for TC, TQ, and NDVI at 2, 6, and 8 WAIT as well as CE and Δ CE at 4 and 8 WAIT for field trials. Additionally, a separate analysis was also conducted for R and S weights at 3 and 6 WAS for greenhouse trials. ANOVA was performed using PROC MIXED with the appropriate expected mean square values described by McIntosh (1983) in SAS (SAS v. 9.2 for Windows; Statistical Analysis Systems Institute, Cary, NC). Means were

separated according to Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$. Data were arcsine square-root transformed to stabilize variance as described by Bowley (2008). Transformed and non-transformed data were analyzed, and interpretations were not different; therefore, non-transformed means are presented for clarity.

Results and Discussion

Field experiments

Experimental run-by-treatment interactions for field trials were significant ($F = 220.6$, $P < 0.0001$). Therefore, data were not pooled across experimental runs and results for each location will be presented separately.

ATREC location

Significant differences were observed between treatments for NDVI 2 WAIT; however, no microbial inoculant treatment resulted in statistically greater NDVI ratings than the non-treated check (Table 3.1). At 2 WAIT, plots only received single applications of microbial inoculants, regardless of treatment. ProveN resulted in the highest NDVI (0.76) followed by (fb) the non-treated check (0.75), Terramax (0.75), and Envita (0.74). Turfgrass color and TQ 2 WAIT ranged from 6.4 to 6.6 and 6.4 to 6.5, respectively; however, no significant differences were observed between treatments. No significant differences were observed in response to microbial inoculant treatments for NDVI (0.70 to 0.73) and TC (6.3 to 6.6) 6 WAIT, regardless of treatment (Table 3.1). Microbial inoculant treatments did not exhibit statistically greater TQ compared to the non-treated check (6.8) 6 WAIT; however, single and sequential applications of Terramax resulted in TQ of 6.8 to 7.0 while TQ in response to sequential applications of Envita were 6.8. At 8 WAIT, no significant differences were observed between treatments for TC (6.1 to 6.5) and TQ (6.3 to 6.5) ratings (Table 3.1). A single application of ProveN resulted in the highest NDVI rating

(0.72) fb single and sequential applications of Terramax (0.70 to 0.71), sequential applications of Return (0.71), and sequential applications of Envita (0.70).

Although no significant differences were observed between treatments for carbon efflux 4 WAIT (ΔCE = 3.4 to 6.5 μmol m^{-2} s^{-1}, regardless of treatment), respiration measurements increased when compared to readings at trial initiation (Table 3.2). Change in carbon efflux for the non-treated check (-2.1 μmol m^{-2} s^{-1}) was not only negative, but lowest among treatments 8 WAIT (Table 3.2). The highest ΔCE readings 8 WAIT were observed in response to single and sequential applications of Terramax (1.7 and 0.8 μmol m^{-2} s^{-1}, respectively) fb sequential applications of ProveN (0.5 μmol m^{-2} s^{-1}) and single applications of Return (0.5 μmol m^{-2} s^{-1}).

BO location

No statistical differences were observed between treatments with respect to NDVI (0.84 to 0.87), TC (7.0 to 7.3), and TQ (6.8 to 7.0) 2 WAIT (Table 3.3). Sequential applications of Terramax resulted in the highest NDVI (0.70) 6 WAIT, but no statistical differences were observed between microbial inoculant treatments and the non-treated check (0.68) (Table 3.3). Single applications of Return and sequential applications of Envita exhibited the highest TC (6.4) and TQ (6.3 and 6.4, respectively) 6 WAIT. However, TC and TQ for the non-treated check (6.3 and 6.1, respectively) 6 WAIT was statistically similar. No statistical differences were observed between treatments with respect to NDVI (0.75 to 0.79) 8 WAIT (Table 3.3). The non-treated check resulted in similar or greater TC (6.8) and TQ (6.6) ratings than all microbial inoculant treatments 8 WAIT.

The non-treated check resulted in the greatest reduction in carbon efflux (-5.6 μmol m^{-2} s^{-1}) 4 WAIT; however, a negative ΔCE was observed in response to all microbial inoculant treatments (Table 3.4). Change in CE 4 WAIT in response to single applications of Envita (-1.3

μmol m^{-2} s^{-1}), single and sequential applications of Terramax (-1.6 and -1.4 μmol m^{-2} s^{-1}, respectively), and single applications of Return (-2.1 μmol m^{-2} s^{-1}) were statistically greater than the non-treated check 4 WAIT. Carbon efflux measurements continued to decrease 8 WAIT, regardless of treatment; however, sequential applications of Return (-4.3 μmol m^{-2} s^{-1}), single applications of Envita and Terramax (-4.8 μmol m^{-2} s^{-1}), and single applications of Return (-5.7 μmol m^{-2} s^{-1}) resulted in the least reduction in carbon efflux (Table 3.4).

Greenhouse experiments

Experimental run-by-treatment interactions for greenhouse trials were significant ($P < 0.0001$). Therefore, data were not pooled across experimental runs and results for each trial will be presented separately.

In experimental run 1, significant differences were observed for R weight (0.23 to 0.36 g) 3 WAS; however, no microbial inoculant treatment resulted in statistically greater R weight than the non-treated check (0.32 g) (Table 3.5). No statistical differences were observed with respect to S weight (0.70 to 0.95 g) 3 WAS. The greatest amount of R weight 6 WAS observed in response to sequential applications of ProveN (3.43 g), the non-treated check (3.37 g), and single applications of Terramax (3.35 g) (Table 3.5). Single applications of ProveN resulted in the greatest amount of S weight (7.15 g) 6 WAS; however, S weight of the non-treated check (5.37 g) was statistically similar. Root and S weights 3 and 6 WAS in experimental run 2 were less than weights observed in experimental run 1, regardless of treatment. No significance was observed with respect to R and S weights in experimental run 2, regardless of harvest date.

Microbial inoculant applications did not consistently increase hybrid bermudagrass NDVI, TC, TQ, and CE in the field nor common bermudagrass R and S biomass in the greenhouse. Similarly, Peacock and Daniel (1992) did not observe improvements in turfgrass

growth in response to *Bacillus* spp. inoculants applied to hybrid bermudagrass established in sterile soil in the greenhouse. Contrarily, Baltensperger et al. (1978) reported a 17% increase in bermudagrass shoot growth in the greenhouse when inoculated with *Azospirillum* and *Azotobacter* spp. under low nitrogen fertility, but no increase in rooting was detected. Furthermore, enhancements in 'Tifway 419' hybrid bermudagrass root and shoot growth were observed by Coy et al. (2014) in the greenhouse in response to inoculant blends of *Bacillus*, *Paenibacillus*, and *Brevibacillus* spp. Differences in plant maturity and nitrogen applications prior to trial initiation may have created an environment more conducive to microbial colonization and nitrogenase activity. Common bermudagrass was established from seed in our research without fertility, while Coy et al. (2014) transplanted hybrid bermudagrass from the field into the greenhouse, acclimated it for 3 weeks, and supplied weekly nitrogen applications prior to trial initiation.

Although minimal research has evaluated the impact of microbial inoculants in turfgrass, most trials have either been conducted in controlled, sterile environments or on cool-season turfgrass species. For example, DeLuca et al. (2020) observed an increase in TQ when mature perennial ryegrass was inoculated with *Azotobacter*, *Bacillus*, and *Pseudomonas* spp. in the greenhouse, while Acikgoz et al. (2016) reported TC and clipping yield increases for tall fescue and perennial ryegrass inoculated with *Bacillus* spp. in the field. Recently, nitrogenase activity and *Bacillus* spp. persistence in common bermudagrass root and shoot tissue was documented in the field by Coy et al. (2019) from single inoculation events. However, the bacterial strains utilized in the Coy et al. (2019) colonization field research were previously collected from native populations and isolated by Auburn University's Department of Entomology and Plant Pathology. Many commercially available inoculants that exist for use in turfgrass are not native

to the locations they are applied; therefore, competition from indigenous microorganisms and intolerance to environmental conditions could reduce potential for colonization and nitrogen fixation.

Perennial cropping systems like turfgrass may create greater challenges for microbial inoculant use and performance. Endemic microbial populations that have adapted to survive and proliferate in turfgrass environments may outcompete non-endemic microorganisms recently introduced into the system. Furthermore, cultural practices (i.e. aerification, topdressing) and management inputs (i.e. fertilizer, irrigation, pesticides) specific to turfgrass growth and production may influence microbial activity, persistence, and species diversity through physical and chemical manipulation of the soil profile. Turfgrass species may also play a critical role in symbiotic relationships between plant roots and soil microorganisms. Relevance of previous field research may be limited since most research trials only lasted a few months. Future research should examine inoculation frequency, soil and plant persistence, and use of microbial blends to determine best management practices leading to the greatest opportunity for colonization and subsequent turfgrass benefits.

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Table 3.1. Normalized difference vegetation index (NDVI), turfgrass color (TC), and turfgrass quality (TQ) of mature 'Tifway 419' hybrid bermudagrass [Cynodon dactylon (L.) Pers. x *Cynodon transvaalensis* Burtt Davy] in response to microbial inoculants applied at the Athens Turfgrass Research and Education Center (ATREC) during the summer of 2021 in Athens, GA.

	2 WAIT[z]			6 WAIT			8 WAIT		
Treatment[y]	NDVI[x]	TC	TQ	NDVI	TC	TQ	NDVI	TC	TQ
Non-treated check	0.75 ab[w]	6.6	6.4	0.71	6.3	6.8 ab	0.69 bc	6.3	6.4
ProveN initial	0.76 a	6.4	6.5	0.73	6.5	6.6 b	0.72 a	6.5	6.5
ProveN initial + 3 WAIT	0.74 abc	6.4	6.5	0.70	6.4	6.6 b	0.69 bc	6.1	6.3
Terramax initial	0.75 ab	6.5	6.5	0.71	6.6	7.0 a	0.71 ab	6.1	6.4
Terramax initial + 3 WAIT	0.73 bcd	6.5	6.5	0.72	6.4	6.8 ab	0.70 abc	6.3	6.4
Return initial	0.72 d	6.5	6.5	0.71	6.3	6.5 b	0.68 c	6.4	6.4
Return initial + 3 WAIT	0.73 bcd	6.6	6.5	0.73	6.5	6.6 b	0.71 abc	6.3	6.3
Envita initial	0.72 cd	6.5	6.4	0.72	6.5	6.5 b	0.69 bc	6.1	6.4
Envita initial + 3 WAIT	0.74 abc	6.4	6.5	0.71	6.5	6.8 ab	0.70 abc	6.4	6.4
$LSD_{(0.05)}$	0.02	NS	NS	NS	NS	0.3	0.03	NS	NS

[z]Abbreviations: WAIT, weeks after initial treatment; $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$; NS, not significant.

[y]Microbial inoculants were applied with distilled water using a watering can. ProveN (*Klebsiella varriicola*) and Return (*Klebsiella variicola*) were applied at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1}; Terramax (*Azospirillum brasilense*) was applied at an IR of 2.55 L ha^{-1} and a CV of 990 L ha^{-1}; and Envita (*Gluconacetobacter diazotrophicus*) was applied at an IR of 0.95 L ha^{-1} and a CV of 189 L ha^{-1}.

[x]NDVI was recorded with a Field Scout CM 1000 NDVI chlorophyll meter. A vegetative index [{NDVI = [(R770 – R 660) / (R770 + R 660)}] was calculated (0 to 1, where 1 is best) from the reflectance readings. An average of three readings were obtained per plot per rating date. Visual ratings of TC and TQ were recorded on a scale of 1 to 9 with a rating of 6 considered acceptable TC and TQ.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 3.2. Carbon efflux (μmol m^{-2} s^{-1}) of mature 'Tifway 419' hybrid bermudagrass [Cynodon dactylon (L.) Pers. x *Cynodon transvaalensis* Burtt Davy] in response to microbial inoculants applied at the Athens Turfgrass Research and Education Center (ATREC) during the summer of 2021 in Athens, GA.

	Carbon Efflux (μmol m^{-2} s^{-1})[z]			
Treatment[y]	4 WAIT[x]	Δ4 WAIT	8 WAIT	Δ8 WAIT
Non-treated check	18.0 b[w]	3.4	12.5 b	-2.1 c
ProveN initial	18.7 ab	4.1	14.1 ab	-0.5 bc
ProveN initial + 3 WAIT	20.8 a	6.0	15.4 a	0.5 ab
Terramax initial	19.2 ab	5.9	15.0 a	1.7 a
Terramax initial + 3 WAIT	19.9 ab	6.5	14.2 ab	0.8 ab
Return initial	19.1 ab	5.2	14.3 ab	0.5 ab
Return initial + 3 WAIT	18.6 ab	4.0	14.0 ab	-0.7 bc
Envita initial	20.4 ab	6.2	13.6 ab	-0.6 bc
Envita initial + 3 WAIT	19.5 ab	3.6	14.8 a	-1.1 bc
$LSD_{(0.05)}$	2.8	NS	2.0	2.1

[z]Carbon efflux measurements were recorded with a LI-COR 8100A automated system at trial initiation as well as 4 and 8 WAIT to provide insight into plant and soil microbial activity (respiration). Data recorded at 4 and 8 WAIT were compared back to data recorded at trial initiation in order to calculate carbon efflux change over time (Δ).

[y]Microbial inoculants were applied with distilled water using a watering can. ProveN (*Klebsiella varriicola*) and Return (*Klebsiella variicola*) were applied at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1}; Terramax (*Azospirillum brasilense*) was applied at an IR of 2.55 L ha^{-1} and a CV of 990 L ha^{-1}; and Envita (*Gluconacetobacter diazotrophicus*) was applied at an IR of 0.95 L ha^{-1} and a CV of 189 L ha^{-1}.

[x]Abbreviations: WAIT, weeks after initial treatment; $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$; NS, not significant.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 3.3. Normalized difference vegetation index (NDVI), turfgrass color (TC), and turfgrass quality (TQ) of mature 'Tifway 419' hybrid bermudagrass [Cynodon dactylon (L.) Pers. x *Cynodon transvaalensis* Burtt Davy] in response to microbial inoculants applied at a home lawn in Bogart, GA (BO) during the summer of 2021.

	2 WAIT[z]			6 WAIT			8 WAIT		
Treatment[y]	NDVI[x]	TC	TQ	NDVI	TC	TQ	NDVI	TC	TQ
Non-treated check	0.87	7.1	7.0	0.68 ab[w]	6.3 ab	6.1 ab	0.79	6.8 a	6.6 a
ProveN initial	0.84	7.0	6.9	0.68 ab	6.0 b	6.1 ab	0.76	6.4 ab	6.5 b
ProveN initial + 3 WAIT	0.85	7.3	7.0	0.69 ab	6.0 b	6.0 b	0.77	6.5 ab	6.5 b
Terramax initial	0.84	7.1	6.9	0.69 ab	6.0 b	6.0 b	0.75	6.3 b	6.5 b
Terramax initial + 3 WAIT	0.84	7.3	6.8	0.70 a	6.1 ab	6.0 b	0.77	6.6 ab	6.5 b
Return initial	0.85	7.0	6.8	0.69 a	6.4 a	6.3 ab	0.79	6.8 a	6.5 b
Return initial + 3 WAIT	0.87	7.1	7.0	0.66 b	6.1 ab	6.1 ab	0.79	6.8 a	6.5 b
Envita initial	0.84	7.3	7.0	0.68 ab	6.3 ab	6.0 b	0.79	6.8 a	6.5 b
Envita initial + 3 WAIT	0.85	7.3	7.0	0.69 ab	6.4 a	6.4 a	0.79	6.8 a	6.5 b
$LSD_{(0.05)}$	NS	NS	NS	0.03	0.3	0.3	NS	0.4	0.1

[z]Abbreviations: WAIT, weeks after initial treatment; $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$; NS, not significant.

[y]Microbial inoculants were applied with distilled water using a watering can. ProveN (*Klebsiella varriicola*) and Return (*Klebsiella variicola*) were applied at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1}; Terramax (*Azospirillum brasilense*) was applied at an IR of 2.55 L ha^{-1} and a CV of 990 L ha^{-1}; and Envita (*Gluconacetobacter diazotrophicus*) was applied at an IR of 0.95 L ha^{-1} and a CV of 189 L ha^{-1}.

[x]NDVI was recorded with a Field Scout CM 1000 NDVI chlorophyll meter. A vegetative index [{NDVI = [(R770 – R 660) / (R770 + R 660)}] was calculated (0 to 1, where 1 is best) from the reflectance readings. An average of three readings were obtained per plot per rating date. Visual ratings of TC and TQ were recorded on a scale of 1 to 9 with a rating of 6 considered acceptable TC and TQ.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 3.4. Carbon efflux (μmol m^{-2} s^{-1}) of mature 'Tifway 419' hybrid bermudagrass [Cynodon dactylon (L.) Pers. x *Cynodon transvaalensis* Burtt Davy] in response to microbial inoculants applied at a home lawn in Bogart, GA (BO) during the summer of 2021.

	Carbon Efflux (μmol m^{-2} s^{-1})[z]			
Treatment[y]	4 WAIT[x]	Δ4 WAIT	8 WAIT	Δ8 WAIT
Non-treated check	14.1 ab[w]	-5.6 b	12.4	-7.4 cd
ProveN initial	15.2 ab	-3.7 ab	10.6	-8.3 d
ProveN initial + 3 WAIT	15.6 ab	-2.3 ab	10.8	-7.1 cd
Terramax initial	14.2 ab	-1.6 a	10.9	-4.8 ab
Terramax initial + 3 WAIT	16.9 a	-1.4 a	10.6	-7.7 d
Return initial	14.9 ab	-2.1 a	11.1	-5.7 abc
Return initial + 3 WAIT	12.5 b	-3.7 ab	11.8	-4.3 a
Envita initial	15.5 ab	-1.3 a	12.0	-4.8 a
Envita initial + 3 WAIT	15.5 ab	-2.7 ab	11.4	-6.8 bcd
$LSD_{(0.05)}$	3.8	3.3	NS	2.0

[z]Carbon efflux measurements were recorded with a LI-COR 8100A automated system at trial initiation as well as 4 and 8 WAIT to provide insight into plant and soil microbial activity (respiration). Data recorded at 4 and 8 WAIT were compared back to data recorded at trial initiation in order to calculate carbon efflux change over time (Δ).

[y]Microbial inoculants were applied with distilled water using a watering can. ProveN (*Klebsiella varriicola*) and Return (*Klebsiella variicola*) were applied at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1}; Terramax (*Azospirillum brasilense*) was applied at an IR of 2.55 L ha^{-1} and a CV of 990 L ha^{-1}; and Envita (*Gluconacetobacter diazotrophicus*) was applied at an IR of 0.95 L ha^{-1} and a CV of 189 L ha^{-1}.

[x]Abbreviations: WAIT, weeks after initial treatment; $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$; NS, not significant.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.

Table 3.5. Root and shoot weight (g) of seeded 'Sahara II' common bermudagrass [Cynodon dactylon (L.) Pers.] in response to microbial inoculants applied in the greenhouse during the spring of 2021 in Athens, GA.

	Experimental Run 1				Experimental Run 2			
	Harvest[z] – 3 WAS[y]		Harvest – 6 WAS		Harvest – 3 WAS		Harvest – 6 WAS	
Treatment[x]	R (g)	S (g)	R (g)	S (g)	R (g)	S (g)	R (g)	S (g)
Non-treated check	0.32 ab[w]	0.95	3.37 a	5.37 ab	0.11	0.36	1.18	1.93
ProveN initial	0.27 ab	0.86	3.20 ab	7.15 a	0.14	0.34	0.74	1.32
ProveN initial + 3 WAIT	0.36 a	0.77	3.43 a	5.26 ab	0.12	0.37	1.35	3.34
Terramax initial	0.33 ab	0.94	3.35 a	6.26 ab	0.16	0.44	1.24	2.24
Terramax initial + 3 WAIT	0.29 b	0.70	2.70 b	5.53 ab	0.10	0.44	1.67	2.91
Return initial	0.30 ab	0.81	2.62 b	4.11 b	0.10	0.25	1.33	2.44
Return initial + 3 WAIT	0.23 b	0.83	2.97 ab	4.87 ab	0.07	0.21	1.36	3.10
Envita initial	0.28 ab	0.84	2.59 b	4.55 b	0.07	0.25	1.27	2.83
Envita initial + 3 WAIT	0.32 ab	0.85	2.96 ab	5.28 ab	0.08	0.21	1.11	2.35
$LSD_{(0.05)}$	0.12	NS	0.60	2.56	NS	NS	NS	NS

[z]Destructive harvests were conducted 3 and 6 WAS. Roots and shoots were separated from each other, washed of all soil, dried in an oven for 48 h at 110 C, and weighed to determine root and shoot biomass (g).

[y]Abbreviations: WAS, weeks after seeding; R, roots; S, shoots; $LSD_{(0.05)}$, least significant difference at $P \leq 0.05$.; NS, not significant.

[x]Microbial inoculants were applied with distilled water using a watering can. ProveN (*Klebsiella varriicola*) and Return (*Klebsiella variicola*) were applied at an inoculant rate (IR) of 1.87 L ha^{-1} and a carrier volume (CV) of 3000 L ha^{-1}; Terramax (*Azospirillum brasilense*) was applied at an IR of 2.55 L ha^{-1} and a CV of 990 L ha^{-1}; and Envita (*Gluconacetobacter diazotrophicus*) was applied at an IR of 0.95 L ha^{-1} and a CV of 189 L ha^{-1}.

[w]Means within a column followed by the same lowercase letter are not significantly different at $P \leq 0.05$ according to Fisher's Protected LSD Test.