

Use of Plant Growing Promoting Bacteria as an Alternative Fertilizer for Bermudagrass

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Master of Science
Degree
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DEDICATION

I would like to dedicate this work to my parents, Romeu Antonio Nassar Neto and Andréa Fonseca Nassar, and my brother, Gabriel Fonseca Nassar, for their love and support along this journey.

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ABSTRACT

Greater demands for beef has been observed over the years; however, concerns related to its production efficiency and sustainability have been raised. To achieve desired production levels with less harm to the environment, newer technologies have been studied and adopted around the world. For the southeastern United States, bermudagrass (*Cynodon dactylon* (L.) Pers) is an important and predominant warm-season forage, highly responsive to nitrogen (N) fertilization. Nevertheless, higher N use has raised concerns regarding N leaching and greenhouse gas emissions, contaminating groundwater and damaging the atmosphere. Alternative strategies to enhance plant growth have been developed, such as various plant growth-promoting bacteria (PGPB), which have been adopted due to their beneficial characteristics associated with forage plants. Among PGPBs, *Azospirillum* spp., *Bacillus* spp., and *Paenibacillus* spp., have demonstrated beneficial interaction modulating phytohormones, fixing N, and solubilizing nutrients in the soil with the *Poaceae* family. Thus, this study aimed to evaluate the inclusion of *Azospirillum* Ab-V5 and Ab-V6, *Bacillus subtilis*, *Paenibacillus riograndensis*, and *Methylobacterium symbioticum* either in field or greenhouse conditions with or without varying N management strategies. Findings from this research may contribute to climate-smart agricultural practices, improving forage systems' sustainability while mitigating environmental impacts. The integration of PGPB in bermudagrass forage systems could serve as an alternative approach to increasing the system's efficiency, reducing inputs, and supporting long-term productivity in forage-based livestock systems.

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INTRODUCTION

The demand for food supplies (Ratnasiri, 2008) such as beef is increasing over time (Suryani et al., 2016). In addition, political strategies aiming for more sustainable or climate-smart strategies are being adopted (Chandra & Shmelev, 2017; Newell & Taylor, 2018). These strategies are not only exclusive to new technologies or products but also to increase overall system efficiency (Erekalo et al., 2024).

There are several ways to increase the efficiency and sustainability of grasslands (McCloud & Mott, 1953; Mott.,1960; Petersen et al., 1965), either by ensuring nutrients such as nitrogen (N) (De Almeida et al., 2024) are available in the system but also by incorporating different management practices such as rotational grazing (Crestani et al., 2022) or intercropping (Nave et al., 2025). In fact, research related to pasture management practices resulting in improved production systems goes back over 50 years.

The areas dedicated to grasslands in the US range around 267 million ha (Winters-Michaud, 2024), supporting 86.7 million head of cattle (USDA, 2025). These grasslands are generally classified as temperate pasture ecosystems, varying greatly worldwide (Sanderson et al., 2004). The variability of the systems along with increased climate variations is expected to lead to major environmental and economic impacts in systems of low diversity (Jaramillo et al., 2021).

As an example, the use of N in a low diversity system is essential for the intensification of the system (De Almeida et al., 2024), nevertheless, it has negative effects attached to it. The extraction of N from the atmosphere using the Haber-Bosch

system is highly energy-intensive, consuming significant fuel and releasing substantial greenhouse gases (GHGs) into the environment (Erisman et al., 2008).

The utilization of N as a mineral fertilizer can also lead to problems for the environment as it can leach (Di & Cameron, 2000), contaminating many aquifers in rural areas (Ibendahl & Fleming, 2007; Libutti & Monteleone, 2017; Puckett et al., 2011; Wang et al., 2019). However, agriculture has been evolving, showing an increase in nutrient use efficiency (Smith et al., 2018). Strategies such as a mixed forage sward by including different forage legumes have been developed as alternative approaches to increase efficiency (Corbin et al., 2024a). These strategies can also help overcome challenges in regions with highly variable and unstable climate conditions, enabling the growth of forage species with different seasonal growth (Yang et al., 2010).

Tennessee is located in the Humid Transition Zone. The region is characterized by its lower temperatures during winter and fall and warmer temperatures throughout summer (Oakes & Hancock, 2020). The region's broad temperature variability allows for the production of cool-season plants during mild temperatures and warm-season plants during warmer temperatures (Oakes & Hancock, 2020). However, cool-season grasses are the predominant type of forage in the region, such as tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort., nom. cons.), and like any other cool-season grass, is productive during the spring and fall when temperatures are moderate, but high summer temperatures and moisture deficits result in decreased growth and productivity (Hay, 1989). The low forage availability during summer often results in a general decrease in pasture condition, allowing for increased weed competition (Corbin et al., 2024b).

Frequent and severe droughts in this region have also resulted in major stand loss with a subsequent decrease in the productivity of tall fescue pastures (Sleper & West, 2015).

Interest has grown in the use of alternative species to be grown in temperate grasslands, especially species with high drought tolerance and nutritive value, as a practical biological solution to summer drought (Garay et al., 2004). These species can not only complement forage production in times of shortage, but also increase pasture competitiveness against noxious weeds (Garay et al., 2004).

Bermudagrass

In the Southeastern United States, bermudagrass [*Cynodon dactylon* (L.)] is the predominant warm-season perennial grass (Baxter et al., 2024). Most forages grown in the region, including bermudagrass (Weintraub, 1953), are introduced species. Originally from East Africa, bermudagrass has been widely adopted in tropical and sub-tropical production systems due to its adaptability.

Bermudagrass was likely introduced to the New World soon after Columbus's voyages, with its first recorded introduction to the contiguous U.S. attributed to Georgia Governor Henry Ellis in 1751. However, earlier introductions may have occurred through botanist Robert Miller in the 1730s. Thriving in southern Colonial America, it spread with settlers and agricultural expansion, becoming a key forage grass by the early 1800s (Taliaferro et al., 2016). By the mid-1800s, it had reached California and Arizona through multiple pathways, including seed contamination and cattle digestion. By the early 1900s, it was considered a major weed in irrigated alfalfa fields and was commercially harvested for seed in the southwestern U.S. Additionally, imported seed from Australia further

contributed to its spread. Over time, bermudagrass was planted across much of the country, becoming prevalent in the southern U.S. (Taliaferro et al., 2016).

Taliaferro et al., (2016) describe bermudagrass var. dactylon as using its underground stems (rhizomes) as asexual reproductive strategies. It also defines bermudagrass stolons as diverse, ranging from very fine to coarse, with linear-lanceolate leaves, following a lance shape, being usually longer and thinner.

Bermudagrass is highly drought tolerant, growing well in waterlogged conditions and intensive grazing, with its nutritive value maximized with proper management. Its adaptability, high biomass production, and resilience to biotic and abiotic stress conditions make it a valuable forage grass. Additionally, legumes and annual grasses can be integrated into bermudagrass pastures to improve forage quality and extend the grazing season (Ball et al., 2008).

Plant growth promoting bacteria (PGPB)

Bacteria are among the microorganisms that populate the soil, with some being beneficial to plant growth. These beneficial bacteria, which inhabit the root environment of plants, are known as plant growth-promoting rhizobacteria (PGPR) (Kloepper & Schroth, 1989). Later reports also described that these beneficial bacteria are not only present in the rhizosphere but also in the endosphere and phyllosphere (Dong et al, 2019), renaming Plant Growth Promoting Bacteria (PGPB).

Each microorganism is adapted to an environment and its variations, along the evolutionary process, as described by Darwin, the best-fitted organisms would prevail in a determined environment. This theory conveys to the presence of different species of

microorganisms in determined regions of a plant, such as the rhizosphere, endosphere, and phyllosphere (Dong et al., 2019).

For PGPBs to reach their full potential in enhancing crop productivity, an exchange of bio-signals between the bacteria and the plant must occur. These interactions are dependent on the specific range of bacteria present in the soil and the mutualistic relationship between both organisms (Tilak et al., 2006). These beneficial bacteria are capable of increasing plant growth through nitrogen fixation, phosphate solubilization, and improved assimilation of tryptophan, a precursor molecule for indole acetic acid (IAA), a phytohormone essential for root development and increased iron absorption (Kong & Liu, 2022).

As plants evolved to adapt to diverse terrains, their immune systems underwent mutations to enhance resistance against both biotic and abiotic stresses (Rennenberg & Simon, 2013). Abiotic stresses include environmental factors such as extreme temperatures, drought, flooding, high light intensity, altered salinity, and pollution of water, air, or soil. In contrast, biotic stresses arise primarily from competition with other plants for resources like nutrients and light, as well as challenges posed by pathogens and herbivores (Rennenberg & Simon, 2013).

These mutations influence not only plant morphology but also the production of costly metabolites that regulate activities, enabling plants to cope with environmental stress. At the individual level, plant defense mechanisms operate in both apoplastic and symplastic spaces, and depending on the environmental factor influencing the plant response, some compounds are translocated from distant regions of the plant through the

apoplastic spaces, where phloem flows, this translocation is primordially nonenergy expending (Farvardin et al., 2020). The symplastic, on the other hand, is the space surrounded by the membrane cells, resulting in an energy expense to move compounds among cells (Farvardin et al., 2020). The compound movement extends beyond the organ directly experiencing stress to encompass the entire plant and, in some cases, even influencing the surrounding ecosystem (Takahashi & Shinozaki, 2019).

According to Glick et al. (1997), PGPB contributes to plant growth primarily by lowering plant ethylene levels. This process starts with PGPB synthesizing IAA, the uptake of IAA by the plant can promote cell proliferation and elongation or stimulate ACC synthase to produce 1-aminocyclopropane-1-carboxylate (ACC) from S-adenosylmethionine (Glick et al., 1997). Some ACC is exuded by the plant and taken up by PGPB, where it is broken down by ACC deaminase into ammonia and α -ketobutyrate, reducing external ACC levels. To maintain equilibrium, the plant continuously exudes ACC, indirectly lowering ethylene production. This leads to reduced ethylene inhibition of root elongation, resulting in plants with longer roots and potentially longer shoots.

However, different species of bacteria may perform differently in the environment and in what is related to plant growth. For example, *Azospirillum spp.*, is able to provide N through biological nitrogen fixation (BNF) to enhance plant yield but also can enhance phytohormone production, such as IAA (Guimarães et al., 2022). Other species, including *Bacillus spp.*, can enhance nutrient solubilization or mobilization of nutrients in the soil, such as phosphorus, in addition to phytohormone production and modulation (Guimarães et al., 2022).

The *Paenibacillus spp.* is often cited as a PGPB capable of fixing nitrogen into environments through conventional and Fe-only nitrogenases (Ribeiro et al., 2024). According to Sullins et al. (2023), the inoculation of bermudagrass with *Paenibacillus spp.* can enhance positive plant characteristics, such as yield, decreasing the amount of N needed throughout the growth season.

The possibility of enhancing plant productivity by increasing the N availability on the system (Kong & Liu, 2022) or enhancing the stress response and subsequently increasing the underground biomass (roots) through pathways as described by Glick et al (1997), are foremost to greater development in what is related to sustainability in field products. Increasing N in the systems feasible greater productivity (De Almeida et al., 2024) without the industrialized process (Erisman et al., 2008), or the increment in plant underground material and plant resistance to stress (Hungria & Vargas, 2000) potentially increases systems efficiency and sustainability.

CHAPTER 1

***AZOSPIRILLUM* INOCULATION IN BERMUDAGRASS UNDER
GREENHOUSE CONDITIONS.**

Abstract

The incorporation of plant growth-promoting bacteria (PGPB) into fertility programs has gained increased attention for its potential to enhance nutrient uptake and contribute to more sustainable agricultural systems. Among these bacteria, *Azospirillum brasilense* – particularly the strains Ab-V5 and Ab-V6 – has shown promise. This study aimed to evaluate the effects of *Azospirillum* Ab-V5 and Ab-V6 on shoot and root weight, as well as shoot and root length of bermudagrass (*Cynodon dactylon*) under greenhouse conditions. Two experiments were conducted at the University of Tennessee, Knoxville, under greenhouse conditions from September to November of 2024. The first experiment was conducted using a completely randomized design (CRD) to evaluate the effects of two commercial PGPB products (MicroAZ-ST Dry and MicroAZ-ST Liquid) on bermudagrass development. The second experiment assessed the interaction between PGPB application and N fertilization rates, using a randomized complete block design (RCBD). Results from the first experiment showed greater ($P < 0.05$) shoot weight (16%), shoot length (9%), and number of tillers (11%) in inoculated plants compared to the control. In contrast, the second experiment showed no significant effects of inoculation on shoot or root weight nor on shoot and root length. These results suggested that *Azospirillum* inoculation may benefit bermudagrass during early developmental stages; however, N fertilization rates may impact the effectiveness of inoculation. Further research is needed to better understand plant-microorganism interactions across diverse environmental conditions.

Introduction

Interest has grown in exploring plant growth-promoting bacteria (PGPB) due to their benefits associated with plants (Kloepper et al., 1989). These benefits can be unraveled into many processes associated with enhancing plant growth, including biological nitrogen fixation, synthesis of hormones, and plant stress alleviation (Perrig et al., 2007). Due to earlier research, *Azospirillum* is considered one of the main PGPB related to improving plant growth (Bashan & Holguin, 1997).

However, not all reports have shown increased yield with the inoculation of different strains of PGPB (Okon & Labandera-Gonzalez, 1994). Potential reasons for non-response related to inoculation responses can be associated with environmental and biological conditions that negatively affect the inoculation efficacy (Okon & Labandera-Gonzalez, 1994).

In Poaceae such as maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.), Hungria et al. (2010) showed inoculation with *Azospirillum* strains increased crop yield from 16 to 30% when compared to non-inoculated control treatments. According to Silva et al. (2023), a trend associated with *Azospirillum* inoculation and nitrogen fertilization was observed, differing greatly from non-inoculated material under lower nitrogen rates.

As mineral fertilizer prices and environmental challenges are rising, technologies aiming for a more sustainable or climate-friendly solution are being encouraged. However, food security concerns are of great interest to upcoming technologies aiming to maintain yield but diminishing environmental problems. Therefore, the objective of this

experiment is to evaluate the inclusion of *Azospirillum* strains AB-V5 and AB-V6 in bermudagrass under greenhouse conditions.

Materials and Methods

Two experiments were conducted at the University of Tennessee in a controlled environment (greenhouse) in Knoxville, TN (35°56'39.6"N 83°56'13.4" W) from September to November of 2024. Both experiments were inoculated using two commercially available *Azospirillum* strains: AB-V5 and AB-V6, using MicroAZ-ST Dry (TerraMax, Eagan, MN, USA) and MicroAZ-ST Liquid (TerraMax, Eagan, MN, USA). The inoculation rates for the dry and liquid products were 4.5g kg⁻¹ and 0.2 mL of inoculant per gram of seed, respectively.

Experiment 1

The experiment was conducted using a completely randomized design (CRD) with 42 replications in six polystyrene trays, each containing 42 cells. The treatments were: control (no inoculation), POW (Powder) (inoculation with MicroAZ- ST dry), and LIQ (Liquid) (inoculation with MicroAZ- ST liq). The substrate used was a Miracle-Gro potting mix (Miracle-Gro, Marysville, OH, USA). Polystyrene trays were seeded with three seeds per cell, and thinning was performed, retaining the best-fitted plant in each cell. Throughout the experiment, the trays were placed in a water tank to float.

Thirty-five days after emergence (DAE), the plants were hand-plucked from each individual cell from the trays, the substrate was washed from the roots using tap water, and shoots and roots were separated were hand separated using a scissor cutting on the base of the plant stem. Roots and shoots were measured (length) on a flat surface and the

number of tillers was registered. The harvested samples from the greenhouse (shoots and roots) were put in an aluminum foil tray and placed inside an air-forced ventilation oven dryer, at 55°C to reach constant weight.

Experiment 2

The experiment was conducted using a randomized complete block design (RCBD) with six replicates per treatment. It was performed using a pot-in-pot system with black recycled plastic plant pots (9 cm x 9 cm x 10 cm), each containing approximately 415g of peat moss and perlite substrate (3 to 1 mixture, respectively). Pots were seeded with three seeds per cell, and thinning was performed, retaining the best-fitted plant in each cell.

The treatments were assigned as: control (no inoculation), POW (inoculated with MicroAZ- ST dry), and LIQ (inoculated with MicroAZ- ST liq), with two nitrogen rates in a nutritive solution of 100% (3 ml per 3.8 L water) and 75% (2.25 ml per 3.8 L of water) totaling 36 experimental units. The solution was applied to each individual pot every seven days (Mondays) at a rate of 200 ml per pot. Additionally, water was applied to the pots every three and five days (Wednesday and Friday) at a rate of 200 ml per pot, aiming to attend to plant needs.

Forty-two days after emergence, the plants were hand-plucked from the pots, and shoots and roots were separated. The root system was washed using tap water, roots and shoots were separated and measured (length) on a flat surface, and the number of tillers was registered for each plot. The harvested samples from the greenhouse (shoots and roots) of each replicate were put in paper bags and oven-dried at 55°C for 72 h until

reaching a constant weight. Dry material was then weighed on a precision scale, and weight values were further recorded for statistical analysis.

Statistical Analysis

For experiment one, data were statistically analyzed by one-way analysis of variance (ANOVA). Differences between means were evaluated for significance using the “LSMEANS” and the comparisons between them were made by the probability of the difference “PDIFF”, using Student's "t" test at the level of $\alpha = 0.05$ significance. All statistical procedures were done using the statistical program SAS[®] (Statistical Analyses System).

For experiment two, the data were analyzed using the MIXED procedure of the statistical program SAS[®] (Statistical Analyses System) (Littel et al., 2006). The Akaike Information Criterion (AIC) (Wolfinger, 1993) was used to choose the matrix of variance and covariance. The treatment effect was considered fixed, while the block effect was considered random. The means of treatment were assessed by the "LSMEANS" and the comparisons between them were made by the probability of the difference "PDIFF", using the Student's "t" test at the level of 5% significance.

Results

Experiment 1

Shoot weight

The shoot weight was affected by inoculation methods ($P = .0027$). Inoculation with POW or LIQ showed a 16% and 10% increase, respectively, in plant shoot weight compared to control treatments without inoculation (**Table 1.1**).

Root weight

Root weight was not affected by different inoculation methods containing AB-V5 and AB-V6 *Azospirillum* strains ($P = .798$) (**Table 1.1**).

Shoot length

The shoot length was affected by inoculation methods ($P = .0351$). Inoculation with POW or LIQ showed a 9% and 8.7% increase, respectively, in plant shoot length compared to control treatments without inoculation (**Table 1.1**).

Number of tillers

The number of tillers was affected by inoculation methods ($P = .0266$). Inoculation with POW or LIQ showed an 11% and 3.7% increase, respectively, in the number of tillers compared to control treatments without inoculation (**Table 1.1**).

Table 1.1 Variable response of shoot weight, root weight, shoot length, and number of tillers between inoculation of powder (MicroAZ-ST dry) and liquid (MicroAZ-ST liq) *Azospirillum* in bermudagrass in the greenhouse.

Treatments	Shoot Weight -- g plant ⁻¹ pot ⁻¹ --	Root Weight -- g plant ⁻¹ pot ⁻¹ --	Shoot Length -- cm plant ⁻¹ pot ⁻¹ --	Number of Tillers -- count plant ⁻¹ pot ⁻¹ --
Control	0.6 ^B	0.2	23.2 ^B	4.9 ^B
Powder	0.7 ^A	0.2	25.2 ^A	5.4 ^A
Liquid	0.7 ^A	0.2	25.1 ^A	5.1 ^{AB}
^a SEM	0.02	0.01	0.67	0.15
P-value	0.0027	0.7980	0.0351	0.0266

Means with a common uppercase letter are not significant at an $\alpha = 0.05$

^aSEM, Standard Error of the Mean

Note: Control = no inoculation, Powder = dry inoculation/MicroAZ-ST dry,
Liquid = liquid inoculation/MicroAZ-ST - liq

Experiment 2

Shoot weight

The values for shoot weight were not affected by different inoculation methods containing AB-V5 and AB-V6 *Azospirillum* strains and rates of nitrogen ($P = .3614$) (Table 1.2).

Root weight

The values for root weight were not affected by different inoculation methods containing AB-V5 and AB-V6 *Azospirillum* strains and rates of nitrogen ($P = .6943$) (Table 1.2).

Shoot length

The value for shoot length was not affected by the inoculation methods containing AB-V5 and AB-V6 *Azospirillum* strains and rates of nitrogen ($P = .3794$) (Table 1.2).

Root length

The value for root length was not affected by the inoculation methods containing AB-V5 and AB-V6 *Azospirillum* strains and rates of nitrogen ($P = .661$) (Table 1.2).

Number of tillers

The number of tillers was not affected by the inoculation methods containing AB-V5 and AB-V6 *Azospirillum* strains and rates of nitrogen ($P = .7676$) (Table 1.2).

Discussion

Experiment 1

The effect of positive inoculation was observed for the POW and LIQ treatments for shoot length, root length, and the number of tillers, indicating increased plant

Table 1.2 - Variable response of shoot weight, root weight, shoot length, and number tillers between inoculation of powder (MicroAZ-ST dry) and liquid (MicroAz-ST liq) *Azospirillum* and two nitrogen rates (75%, 100%) in bermudagrass in a greenhouse experiment.

Treatments	Shoot Weight -- g plant ⁻¹ pot ⁻¹ --	Root Weight -- g plant ⁻¹ pot ⁻¹ --	Shoot Length -- cm plant ⁻¹ pot ⁻¹ --	Root Length -- cm plant ⁻¹ pot ⁻¹ --	Number of Tillers -- count plant ⁻¹ pot ⁻¹ --
75 N + Control	0.2	0.03	14.4	4.1	5.5
75 N + Powder	0.2	0.02	12.5	3.8	4.7
75 N + Liquid	0.2	0.02	12.9	3.8	4.8
100 N + Control	0.2	0.02	14.9	4.3	5.1
100 N + Powder	0.2	0.03	14.6	4.4	5.7
100 N + Liquid	0.2	0.02	13.5	3.7	4.5
¹ SEM	0.0392	0.0052	0.9418	0.3748	0.6487
P-value	0.3614	0.6943	0.3794	0.6610	0.7676

Means with a common uppercase letter are not significant at an $\alpha = 0.05$

¹SEM, Standard Error of the Mean

Note: 100N+P corresponds to 100%N + Dry inoculation, 100N+C corresponds to 100%N + no inoculation, 75N+P corresponds to 75%N+Dry inoculation, 75N+C corresponds to 75%N + no inoculation, 100N+L corresponds to 100%N+Liquid inoculation, and 75N+L corresponds to 75%N+Liquid inoculation.

development in the greenhouse trial (**Table 1.1**). A similar positive effect was reported by Hungria et al. (2010), where inoculation with *Azospirillum* resulted in maize and wheat yield increases ranging from 9-18%.

The effect of the PGPB inoculation can promote both root and shoot development, contributing to improved environmental adaptability (Kong and Liu., 2022). According to Silva et al. (2023), Tifton 85 bermudagrass inoculated with *Azospirillum brasiliense* produced 1.4 to 1.9 Mg DM ha⁻¹ even without N applications.

These results support the idea that incorporating PGPBs into fertility programs can enhance overall plant growth, as suggested by Bashan & Holguin (1997). Additionally, these findings indicate that PGPBs may improve plant performance under low nitrogen conditions, aligning with findings from Hungria et al. (2016).

Coy et al. (2014) also demonstrated that introducing PGPBs into bermudagrass under controlled environment stimulated plant growth. In their study, shoot weight increased by 236 to 345% in inoculated treatments compared to the non-inoculated control, particularly with the application of diverse microbial blends.

Experiment 2

This study evaluates the effects of PGPB inoculation on bermudagrass under greenhouse conditions, aiming to reduce the reliance on mineral fertilization, particularly nitrogen, given the species' high responsiveness to N applications (Taliaferro et al., 2016). To determine the potential benefits of PGPB, the experiment incorporated both a full (100%) and reduced (75%) N application rates.

As shown on **Table 1.2**, no response to *Azospirillum* inoculation was observed across different N rates for shoot and root weight, or shoot and root length. Similar findings were reported by Silva et al. (2023), who noted that the benefits of PGPB inoculation in grasses may be negatively affected by greater N rates, with positive effects on shoot weight observed only occurring without N fertilization.

This implication may be attributed to passive nutrient uptake mechanisms in plants, such as diffusion or mass flow (Manzoor Alam, 1999), which require less net energy to acquire nutrients compared to the establishment and maintenance of mutualistic relationships with beneficial bacteria (Farvardin et al., 2020).

Conclusion

This study evaluated the effects of plant growth-promoting bacteria (PGPB) and nitrogen (N) fertilization on bermudagrass shoot and root weight, shoot and root length, and number of tillers in Knoxville, TN. The results indicated that N fertilization had no impact shoot and root weight, but PGPB inoculation alone did result in significant differences in shoot weight, shoot length, and number of tillers. Inoculation with Micro-AZ dry showed greater results overall. However, Further research on N rates and bacterial inoculation is needed to better understand bermudagrass responses under varying N rates.

CHAPTER 2

INCLUSION OF PLANT GROWTH-PROMOTING BACTERIA (PGPB) IN

BERMUDAGRASS FERTILITY PROGRAMS.

Abstract

The southern U.S. has greater importance in beef production. However, it relies on forages that require greater nutrient inputs, such as nitrogen (N). This study evaluated the inclusion of plant growth-promoting bacteria (PGPB) and N strategies in bermudagrass (*Cynodon dactylon*) on forage mass (FM), nutritive value (NV), and gas exchange measurements during two experimental periods (2023 and 2024) in Crossville, Tennessee. Forage mass was not affected by PGPB inoculation but differed among the N strategies ($P < .0001$). Greater N strategies increased FM with values of 1901 kg DM ha⁻¹ reported for A-67 Treatment. Treatments with 33 kg N ha⁻¹ showed no statistical difference among each other but differed from the control (no N application), showing a 37 % increase. Similar results were reported for the rising plate meter method, where FM mass had greater values when greater N strategies ($P < .0001$) were used. Crude protein also showed no differences among treatments for the interaction PGPB x N strategy ($P = .8031$), however, higher N strategies increased crude protein values ($P = .0030$). In vitro dry matter digestibility (IVDMD48) and neutral detergent fiber digestibility (NDFD48) were not affected by PGPB or N strategies ($P > .05$). Photosynthesis CO₂ exchange (A) and stomatal conductance (g_{sw}) were not impacted by the interaction treatments x N strategies ($P > .05$). Results suggest that FM and CP are affected by N strategies, however, PGPB inoculation may not positively affect variables. Further research is needed to better understand forage productivity under PGPB inoculation.

Introduction

The United States is the third largest country in the world, with 17% of its land used for cattle grazing. In 2021, the United States was the largest meat producer, accounting for approximately 20% of the world's production (USDA, 2021).

Bermudagrass (*Cynodon dactylon*) is a key perennial forage in the southeastern U.S. (Baxter et al., 2023), and it is cultivated on approximately 10–12 million hectares across the United States for forage production (Taliaferro et al., 2016).

The widespread use of bermudagrass in the southeastern U.S. has raised considerable research interest (Taliaferro et al., 2016). It is highly responsive to nitrogen (Garay et al., 2004; Osborne et al., 1999; Santos et al., 2021; Silveira et al., 2007), persistent under-grazing (Liu et al., 2011; Rouquette et al., 2011), and adapted to diverse regions around the world (Burton, 1948), making it suitable for a variety of forage systems (Barreta et al., 2023; Santos et al., 2021).

However, many systems have been shifting towards more environmentally conscious management strategies. The introduction of legumes in forage systems to supply nutrients such as N to the system has been widely studied (Corbin et al., 2024a, 2024b; McCloud & Mott, 1953; Santos et al., 2021), thus the increase in the system diversity not only impacts nutrients inputs but also the economic viability of the system. Nevertheless, for legumes to accomplish the desired role of nitrogen-fixing plants, a relationship between the plants and rhizobacteria needs to be realized. These bacteria infect the plant root, forming a colony that uses nitrogenase enzymes to convert atmospheric nitrogen into ammonia (Hungria & Vargas, 2000).

Subsequently, new technologies have been developed, focusing on replacing or building up N mineral fertilization (Griffin et al., 2020; Sullins et al., 2023). In the *Poaceae* family, plant growth-promoting bacteria (PGPB) is a relatively new technology that is used to reduce N mineral fertilization to increase the system's overall profitability. Nevertheless, for this functionality to be accomplished, there is to be a relationship between the bacteria and the plant (Tilak et al., 2006).

Therefore, the objective of this study was to identify and evaluate the effects of PGPB in Bermudagrass plants under field conditions, with varying N strategies.

Materials and Methods

Site description

This study was conducted at the University of Tennessee, Plateau AgResearch and Education Center (PREC) in Crossville, Tennessee (36°01' N, 85°12' W) from May 2023 to September 2024. The soil conditions on the location were Lily loam (fine-loamy residuum weathered from sandstone, 2-6% slopes, well-drained, 21 to 39 inches to lithic bedrock) (NRCS, 2018). Initial soil nutrient levels at the experiment site were pH = 5.88, P = 26 kg ha⁻¹, K = 74 kg ha⁻¹, Ca = 1180 kg ha⁻¹, and Mg = 90 kg ha⁻¹. On May 26th, 2021, the entire area was tilled, and N-P-K fertilizer was applied to the entire area at a rate of 67 kg ha⁻¹ and re-tilled to incorporate the fertilizer into the soil. On the same day, Bermudagrass cv. Cheyenne II was sown using a 3-meter-wide Gandy fertilizer (Gandy company, Owatonna, MN) at a rate of 16.8 kg ha⁻¹. Subsequently to sowing, a 3-meter-wide cultipacker was used to ensure a 3-millimeter depth. On April 25th of 2023, Shredder 2,4-D Amine (WinField United, Arden Hills, MN) and Prowl H2O (BASF,

Durham, NC) were applied following label recommendations. On May 1st of 2023, the entire experimental area was mowed with a 1.52-m wide zero-turn mower (TORO, Blomming, MN). On May 9th of 2023, P and K fertilization occurred using a 3-meter-wide fertilizer (Gandy company, Owatonna, MN) at a rate of 67 kg ha⁻¹ and 100 kg ha⁻¹, respectively.

The experimental design followed a randomized complete block design with a split-plot arrangement, consisting of 4 main plots, 5 N management strategies as subplots, replicated three times, for a total of 60 experimental units. The main plots (inoculation) consisted of PGPB sources, including *Bacillus subtilis* DH267 (DH-267), *Paenibacillus riograndensis* DH44 (DH-44), and UrtrishaN -*Methylobacterium symbioticum* (C-PGPB) (Corteva Agriscience, Indianapolis, IN), as well as a control with no PGPB (No-PGPB). Nitrogen (N) were applied in the subplots, including urea at 67 kg N ha⁻¹ (U-67) or 33 kg N ha⁻¹ (U-33); and ammonium sulfate at 67 kg N ha⁻¹ (A-67) or 33 kg N ha⁻¹ (A-33), along with a control receiving no nitrogen (NN). Each subplot measured 1.5 x 4.6 m.

The PGPB sources were applied on May 28th, June 23rd, and July 26th in 2023 and May 28th, and June 24th in 2024, at the beginning of the experiment and on each subsequent harvest via a manual backpack sprayer Husqvarna (Husqvarna AB, Charlotte, NC) at a rate of 0.72 L m⁻² containing 1.5x10⁸ cfu ml⁻¹ for DH267 and DH 44 and 3x10⁷ cfu ml⁻¹ for Urtrisha. On May 28th, June 23rd, and July 26th in 2023, and May 28th and June 24th in 2024, N strategies were applied, the total values applied throughout the year summed 67 kg ha⁻¹ or 33 kg ha⁻¹, respectively, to each treatment.

Harvest frequencies were done aiming an interval between 28 to 35 days from May to September of 2023 and 2024, according to weather conditions. Harvests occurred on June 23rd, July 26th, and September 7th in 2023, and June 20th, August 8th, and September 9th in 2024.

Response variables

For forage mass (FM) measurements, a self-propelled mower (Craftsman, Tupelo, MS, USA), 0.5 m wide, centrally placed on the plot, harvested a 4.6m strip. The harvested material was collected and weighed using a 121 L bucket from each experimental unit. A sub-sample was then collected from the bucket and dried in a forced air dryer at 60°C up to constant weight to determine the total dry matter (DM). These sub-samples were then ground in a Wiley Mill Grinder (Thomas-Wiley Laboratory Mill Model 4, Arthur H. Thomas Co., Philadelphia, PA) using a 2-mm screen, and then passed through a 1-mm cyclone mill (FOSS Cyclotec, Eden Prairie, MN) to reduce particle size prior forage nutritive value analyses (McIntosh et al., 2022). Samples were individually placed in foil tins and dried for 30 minutes in a forced air oven at 55°C to ensure consistent moisture content for scanning, reducing variability across results. These samples were scanned in small ring cups on near-infrared spectroscopy (NIRS) technology (Unity SpectraStar XL-R, Unity Scientific, Milford, MA) using a Foss DS2500 near-infrared spectrometer (Metrohm USA Inc., Riverview, FL) for determination of crude protein (CP) and neutral detergent fiber (NDF) by utilizing Grass Hay (GH) calibration equations provided and licensed by the NIRS Forage and Feed Consortium (NIRSC, Berea, KY). The global and neighborhood statistical tests were

monitored and analyzed for accuracy across all predictions with the entire data set fitting the calibrations within the ($H < 3.0$) limit of fit and reported accordingly by Murray and Cowe (2004). Units of measurement for nutritive analyses are presented at 100% dry matter (DM) across the entire data set.

In 2024, the total aboveground dry matter available forage was measured at each harvest in each plot with a calibrated rising-plate-meter (RPM). The RPM used consisted of a 0.1-m^2 ascendant disk, and the Measurements of the RPM was made by a mechanical counter that measured the partially compressed sward height in 5-mm increments (Nave & Corbin, 2019). Fifteen points were randomly measured across each plot, and at each sampling date, one 0.1m^2 quadrat was measured with the RPM, and after recording these individual measurements of the RPM, the forage under the plate was hand-clipped to ground level within a 0.1 m^2 and collected at each subplot, totaling 60 samples. After hand clipping, the samples were separated into botanical and morphological components (green leaves, stems, dead material, and weeds). Each sample was dried at 55°C until constant weight, then weighed. The calibration was developed using a regression equation to convert the MRPM to the total aboveground dry weight (Nave & Corbin, 2019).

Gas exchange measurements were conducted on June 23, 2023 and June 24, 2024, using the LI-6800 Portable Photosynthesis Systems and the attached Multiphase Flash Fluorometer (6800- 01A) (LI-COR, Biosciences, Lincoln, NE, USA). One reading per subplot was taken under steady-state conditions over a 5-minute period. Measurements were performed at midday and included leaf instantaneous photosynthetic CO_2

assimilation (A) and stomatal conductance (g_{sw}). During data collection, CO_2 concentration in the leaf chamber was maintained at $420 \mu\text{mol CO}_2 \text{ mol air}^{-1}$. The chamber was set to deliver $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ of photosynthetically active radiation (PAR). Air temperature was adjusted to match ambient conditions, and relative humidity was maintained at approximately 60%, in accordance with environmental fluctuations.

Statistical Analysis

The data was analyzed using the Linear model method (PROC MIXED), software SAS®, version 9.4; SAS® Institute Inc., Cary, NC, EUA). Block were considered random effects, while inoculation, N strategies, and year were considered fixed effects.

The Akaike Information Criterion (AIC) (Wolfinger, 1993) was used to choose the variance and covariance matrix. The means of treatment were estimated by the "LSMEANS" and the comparisons between them were made by the probability of the difference "PDIF", using the student's "t" test at the level of 5% significance.

Results and Discussion

Weather

Figure A.1 shows the monthly average temperatures for 2023 and 2024, and the 30-year average for Crossville, TN. Temperatures in 2023 and 2024 were generally consistent with the 30-year average (**Figure A.1**). The average temperature from April to October was 17.5°C in 2023 and 18.9°C in 2024, compared to the 30-year average of 18°C .

During the experimental period, 2024 experienced precipitation variations, especially in June and August, with a decrease of 73.2% and 80.5%, respectively, compared to the 30-year average (**Figure A.2**).

Average Forage mass

Average FM was not affected by the interaction between inoculation $\times$ N strategies ($P = .8504$) (**Table 2.1**). However, a significant interaction between N strategy $\times$ year was shown ($P < .0001$), indicating that greater N inputs increased average FM production in year 1 (**Table 2.2**). Specifically, treatment A-67 produced the greatest FM (2606 kg DM ha⁻¹), outperforming U-67 (2015 kg DM ha⁻¹) (**Table 2.2**). In contrast, A-33 (1777 kg DM ha⁻¹) did not differ from U-33 (1688 kg DM ha⁻¹). The control treatment consistently had the lowest FM, averaging 40% lower than A-33 and U-33 (**Table 2.2**).

Data shown on **Tables 2.1** and **2.3** indicate no differences in FM when accounting solely for the presence of PGPB. However, differences in FM due to N strategies were evident, particularly with the A-67 treatment, which yielded the greatest FM in year 1 (**Table 2.2**). This result aligns with the expected response of bermudagrass to increasing rates of N fertilization, as reported by Osborne et al., 1999.

Sullins et al, (2023) found that bermudagrass fertilized with 56 kg N ha⁻¹ plus the PGPB strain DH-44 produced yields comparable to treatments with 112 kg N ha⁻¹, both of which significantly outperformed the negative control treatment without N application. These findings suggest that variations in bacterial strain effectiveness could contribute to the inconsistent or modest results observed in PGPB studies.

Table 2.1 - Average forage mass (FM) of bermudagrass in response to different strains of PGPB (*inoculation*) (DH-44, C-PGPB, DH-267), and N strategies (33 kg, 67 kg), (Urea, Ammonium Sulfate) across two growing seasons 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	----- kg DM ha ⁻¹ -----				
NN	1053	1030	1128	1098	>0.05
U-33	1369	1574	1368	1618	>0.05
A-33	1466	1499	1526	1487	>0.05
U-67	1666	1566	1910	1709	>0.05
A-67	1901	2032	2043	1956	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($P < .05$).

Means within a row differ each N strategies into inoculation ($P < .05$).

SEM = 95.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

Table 2.2 - Average forage mass (FM) of Bermudagrass in response to N strategies (NN, U-33, A-33 kg, U-67, A-67), across two growing seasons (year) 2023 (1) and 2024 (2), in Crossville, Tennessee, U.S.

Year	NN	N strategies				P-Value
		U-33	A-33	U-67	A-67	
		----- kg DM ha ⁻¹ -----				
1	1050 ^D	1688 ^C	1777 ^C	2015 ^B	2606 ^A	<0.05
2	1105 ^C	1277 ^{AB}	1212 ^{B^C}	1411 ^A	1360 ^{AB}	<0.05
P-value	>0.05	<0.05	<0.05	<0.05	<0.05	

Means within a column differ each N strategies into year ($p < .05$).

Means within a row differ each year into N strategies ($p < .05$).

SEM = 63.

Note: U, urea; A, ammonium sulfate.

Table 2.3 - Average forage mass (FM) measured with rising plate method of bermudagrass in response to different strains of PGPB (*inoculation*) (DH-44, C-PGBR, DH-267), and N strategies (33 kg, 67 kg), (Urea, Ammonium Sulfate) across two growing seasons 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	----- kg DM ha ⁻¹ -----				
NN	839	765	815	795	>0.05
U-33	931	1007	939	1029	>0.05
A-33	1029	969	956	935	>0.05
U-67	1130	1089	1069	1142	>0.05
A-67	988	1085	1109	1066	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($P < .05$).

Means within a row differ each N strategies into inoculation ($P < .05$).

SEM = 95.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

Tilak et al. (2006) emphasized the importance of the specific interaction between bacterial strains and host plants, noting that such relationships can be either enhanced or limited depending on environmental conditions.

Griffin et al. (2020) described the strain DH-44 as being isolated from the rhizosphere of bermudagrass in Auburn, AL, suggesting that the geographic origin and host plant variety from which the microorganism is isolated may influence the overall inoculation response.

Similar average FM values have been reported in previous studies. Hendricks et al. (2020) documented a total FM of 7,877 kg DM ha⁻¹ for Tifton 85 bermudagrass monoculture in 2018. In Crossville, TN, Corbin et al. (2019) reported that bermudagrass fertilized with 67 kg N ha⁻¹ produced an average of 885 kg DM ha⁻¹ in 2016, with individual harvests ranging from 324 to 2153 kg DM ha⁻¹. In our study, average FM ranged from 1901 to 2043 kg DM ha⁻¹ (**Table 2.1**).

The FM recorded using the rising plate meter (RPM) was not affected by the interaction between inoculation × N strategies ($P = .3946$) (**Table 2.3**). However, there was a N strategy × year interaction ($P < 0.0001$) where greater N strategies increased average FM production. The greatest FM value of 2606 kg DM ha⁻¹ was observed in the A-67 treatment in year one, which differed from U-67 at 2015 kg DM ha⁻¹ (**Table 2.2**). The N strategy with 33 kg N ha⁻¹ did not differ from each other in year one but showed a 40% increase compared to the control without N application.

Average FM differed between years, with year 2 showing lesser average FM across treatments (**Table 2.2**). Comparable FM patterns were observed using both direct harvest measurements and RPM estimates, particularly during year 2 (**Table 2.2, 2.3**).

Forage accumulation

Forage accumulation (FA) was not affected by the interaction between inoculation $\times$ N strategies ($P = .04430$). However, a significant interaction between N strategy $\times$ year was observed ($P < .0001$), showing that increased N rates increased FA production in year 1 (**Table 2.4**). Forage accumulation values differed between year 1 from year 2 variations (**Table 2.4**) across all N strategies except for the control (NN), which showed no year-to-year variation.

FA was observed in A-67 treatment (7819 kg DM ha⁻¹), which significantly exceeded U-67 (6045 kg DM ha⁻¹) (**Table 2.4**). Meanwhile, A-33 (5332 kg DM ha⁻¹) did not differ from U-33 (5063 kg DM ha⁻¹). The control produced the lowest FM, averaging approximately 40% less than both A-33 and U33 (**Table 2.4**). These results align with FM trends, where similar differences were observed among N strategies and across years. The FA values for A-67 were comparable to those reported by Hendricks et al. (2020), who found Tifton 85 bermudagrass monoculture yielded up to 7,877 kg DM ha⁻¹, consistent with the results presented in our study (**Table 2.4**).

According to Sohm et al. (2014), bermudagrass can accumulate up to 18000 kg DM ha⁻¹ with an N rate of 672 kg N ha⁻¹, approaching its biological yield potential. Their study reported no differences in yield between N rates of 672 and 1344 kg N ha⁻¹ due to a quadratic response described by the equation $Y = 0.00002x^2 + 0.03x + 5.4$,

Table 2.4 - Forage accumulation (FA) of Bermudagrass in response to N strategies (NN, U-33, A-33 kg, U-67, A-67), across two growing seasons (year) 2023 (1) and 2024 (2), in Crossville, Tennessee, U.S.

Year	N strategies					P-Value
	NN	U-33	A-33	U-67	A-67	
	----- kg DM ha ⁻¹ -----					
1	3151 ^D	5063 ^C	5332 ^C	6045 ^B	7819 ^A	<0.05
2	3315 ^C	3832 ^{AB}	3636 ^B	4234 ^A	4082 ^{AB}	<0.05
P-value	>0.05	<0.05	<0.05	<0.05	<0.05	

Means within a column differ each N strategies into year ($p < .05$).

Means without within a row differ each year into N strategies ($p < .05$).

SEM = 189.

Note: U, urea; A, ammonium sulfate.

which closely matches the FA observed for A-67 in our study (**Table 2.4**).

Morphological and Botanical Composition

For dead material, stem, leaf, and weed values, there was no effect of the interaction of inoculation $\times$ N strategies ($P = .9963$), ($P = .7016$), ($P = .7132$), and ($P = .6157$). The mean values for each response variable were 81.2, 293.9, 384.7, and 240.2, respectively (**Figure 2.1**). According to Prine & Burton (1956), bermudagrass hay quality decreases with maturity; however, it is not possible to achieve accuracy in determining age and quality of bermudagrass. Furthermore, Prine & Burton demonstrated leaf percentual results ranging from 68.9 to 76.3% depending on varying rates of nitrogen, from 0 to 1000 kg N ha⁻¹.

Nutritive Value

Crude protein (CP) was not affected by the interaction of inoculation $\times$ N strategies ($P = .4363$) (**Table 2.5**). However, there was a N strategy $\times$ year effect ($P < .0001$) where greater N strategies increased the average CP percentage. The greatest CP value of 149 g kg⁻¹ was shown on treatment with A-67 in year 2, which differed from the other treatments. This represented an 18% increase compared to the control treatment (122 g kg⁻¹ CP). The standard error of the mean (SEM) for these values was 25.

Greater CP values are known to correlate with N strategies in bermudagrass. Similar CP content (136 g kg⁻¹) was reported by Sullins et al. (2023) with an N rate of 112 kg N ha⁻¹. Corbin et al. (2019) reported CP of 120 and 115 g kg⁻¹ for bermudagrass fertilized with 67.2 kg N ha⁻¹ at the Plateau AgResearch and Education Center in

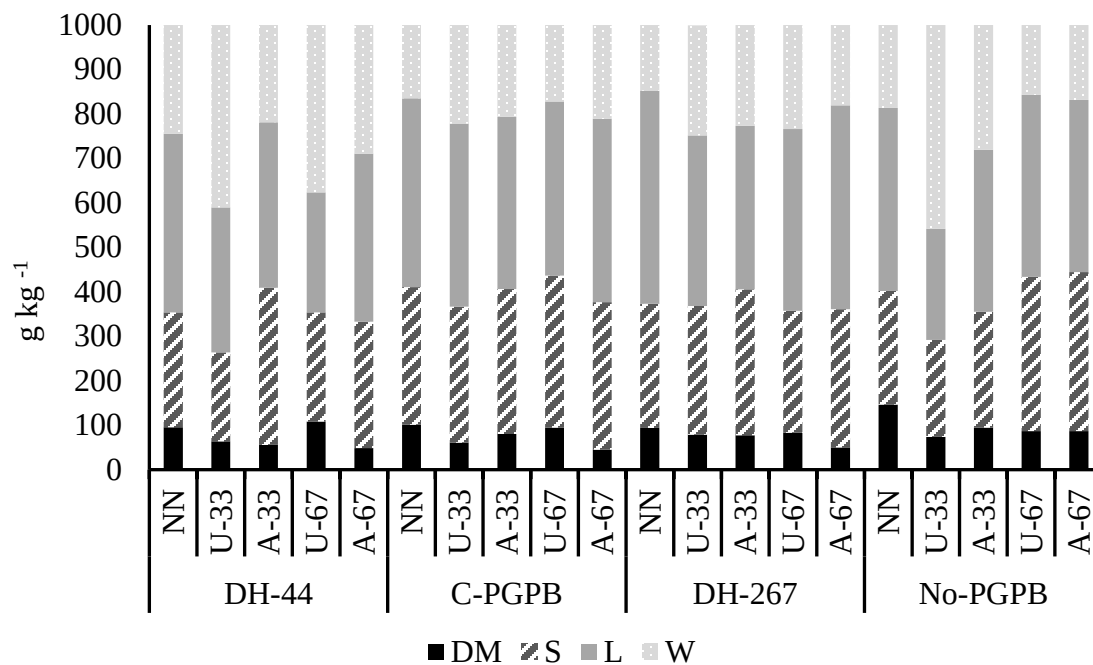


Figure 2.1 Illustrative figure showing percentages of each morphological and botanical component: dead material (DM), stem(S), leaf (L), and weed (W) throughout two harvest periods (June 20th, August 8th of 2024).

Crossville, TN. However, in Corbin et al.'s study, mineral fertilizer application did not produce a significant difference from the control treatment without N application, which contrasts with the current findings of this study (**Table 2.6**). In this study, A-67 showed a greater CP content in year 2, which may be related to the volatilization rate of ammonium sulfate when compared to urea. According to Aggarwal et al. (1987), when ammonium sulfate and urea were applied to row crops, ammonium sulfate not only had greater values for N use efficiency (32% and 15% for ammonium sulfate and urea, respectively) but also showed lower ammonia volatilization (12% and 16 % for ammonium sulfate and urea, respectively).

Nutritive Value

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Table 2.5 - Average crude protein (CP) of bermudagrass in response to different inoculation (DH-44, C-PGPB, DH-267), and N strategies (33 kg, 67 kg), (urea, ammonium Sulfate) across two growing seasons, 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	----- g kg ⁻¹ -----				
NN	124.8	128.8	124.3	125.5	>0.05
U-33	126.6	131.2	123.2	126.0	>0.05
A-33	137.4	126.1	126.0	126.6	>0.05
U-67	128.8	130.1	124.4	133.2	>0.05
A-67	137.7	136.3	139.9	137.7	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($P < .05$).

Means without within a row differ each N strategies into inoculation ($P < .05$).

SEM = 3.4.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

contrasts with the current findings of this study (**Table 2.6**). In this study, A-67 showed a greater CP content in year 2, which may be related to the volatilization rate of ammonium sulfate when compared to urea. According to Aggarwal et al. (1987), when ammonium sulfate and urea were applied to row crops, ammonium sulfate not only had greater values for N use efficiency (32% and 15% for ammonium sulfate and urea, respectively) but also showed lower ammonia volatilization (12% and 16 % for ammonium sulfate and urea, respectively).

The *in vitro* dry matter digestibility 48h (IVDMD) and neutral detergent fiber digestibility 48h (NDFD) were not affected by the interaction inoculation $\times$ N strategies ($P = .4240$) ($P = .1347$), respectively (**Table 2.7**) (**Table 2.8**). Additionally, there was a year effect ($P < 0.0001$) ($P < 0.0001$) for both variables reporting greater values for 2024 (year 2) for both variables (**Table 2.9**).

Pedreira et al. (1999), described a regression model for “Florakirk” bermudagrass based on grazing cycles, showing that forage mass decreased with longer grazing intervals. This decrease was associated with a significant reduction in CP and *in vitro* digestibility of organic matter, with CP content ranging from 96 to 133g kg⁻¹ over the experimental period.

The decline in forage nutritive value with plant maturity is well-known in forage systems, including effects on chemical composition, morphology, and animal performance. Earlier studies have shown that IVDMD decreases as bermudagrass matures (Akin et al., 1977). Similarly, warm-season grasses tend to show reduced CP

Table 2.6 - Crude protein (CP) of Bermudagrass in response to N strategies (NN, U-33, A-33 kg, U-67, A-67), across two growing seasons (year) 2023 (1) and 2024 (2), in Crossville, Tennessee, U.S.

Year	N strategies					P-Value
	NN	U-33	A-33	U-67	A-67	
	----- g kg ⁻¹ -----					
1	129 ^A	127 ^A	128 ^A	124 ^A	124 ^A	<0.05
2	122 ^D	126 ^{CD}	130 ^{BC}	134 ^B	149 ^A	<0.05
P-value	<0.05	>0.05	>0.05	<0.05	<0.05	

Means within a column differ each N strategies into year ($p < .05$).

Means without within a row differ each year into N strategies ($p < .05$).

SEM = 25.

Note: U, urea; A, ammonium sulfate.

Table 2.7 - Average in vitro dry matter digestibility 48 hours (IVTDMD48) of bermudagrass in response to inoculation (DH-44, C-PGPB, DH-267), and N strategies (33 kg, 67 kg), (Urea, Ammonium Sulfate) across two growing seasons 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	IVTDMD48 (g kg ⁻¹)				
NN	686.2	688.2	680.1	688.7	>0.05
U-33	693.3	682.1	686.8	690.0	>0.05
A-33	697.6	696.8	676.6	694.8	>0.05
U-67	695.2	682.6	683.0	688.7	>0.05
A-67	694.7	683.4	686.0	690.4	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($P < .05$).

Means without within a row differ each N strategies into inoculation ($P < .05$).

SEM = 5.6.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

Table 2.8 - Average neutral detergent fiber digestibility 48 hours (NDFD48) of bermudagrass in response to inoculation (DH-44, C-PGPB, DH-267), and N strategies (33 kg, 67 kg), (Urea, Ammonium Sulfate) across two growing seasons, 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	----- NDFD48 (g kg ⁻¹) -----				
NN	482.2	472.1	469.8	475.2	>0.05
U-33	472.1	463.9	479.0	488.0	>0.05
A-33	481.9	490.7	463.9	483.8	>0.05
U-67	482.3	478.6	473.5	474.8	>0.05
A-67	479.3	467.3	473.9	471.0	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($P < .05$).

Means without within a row differ each N strategies into inoculation ($P < .05$).

SEM = 6.8.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

Table 2.9 - *In vitro* dry matter digestibility 48 hours (IVTDMD) of Bermudagrass across two growing seasons, 2023 and 2024, in Crossville, Tennessee, U.S.

Year	IVTDMD ----- g kg ⁻¹ -----	NDFD
2023	669 ^B	455 ^B
2024	706 ^A	496 ^A
SEM	3	3.1
P-value	< 0.0001	< 0.0001

Means with a common uppercase letter are not significant at $\alpha = 0.05$

SEM, Standard Error of the Mean

Note: IVTDMD corresponds to in vitro dry matter digestibility and NDFD corresponds to neutral detergent fiber digestibility

content with increased plant maturity, Arthington & Brown, (2005) reported a 37.8% decrease in CP content 10-wk regrowth compared to 4-wk regrowth.

Forage nutritive value is an indicator of plant maturity when comparing regrowth periods (Arthington & Brown, 2005). The plants in this study likely had similar maturity levels (**Table 2.8**), which may explain the comparable NDFD values.

Leaf gas exchange

Stomatal conductance (g_{sw}) was not affected by the interaction inoculation $\times$ N strategies ($P = .9171$) (**Table 2.10**). Furthermore, no significant N strategies effect ($P = 0.6547$) or differences among inoculations ($P = .6579$) were observed, however, there was an effect by the year ($P = .0102$) (**Table 2.12**).

Photosynthesis CO_2 assimilation (A) was also not affected by the interaction inoculation $\times$ N strategies ($P = .9448$) (**Table 2.11**). Similarly, no significant N strategies effect ($P = .0972$) or differences among inoculation ($P = .8239$) were observed, however, there was a year effect on A ($P = .0133$) (**Table 2.12**).

Previous studies have suggested that plant variations in g_{sw} and A may contribute to drought tolerance, with values increasing or decreasing depending on drought conditions (Hu et al., 2009). However, no significant differences were observed in either A or g_{sw} in this study (**Tables 2.10; Table 2.11**). Moreover, PGPBs have the potential to enhance plant stress tolerance (Kong & Liu, 2022) by promoting root development through increased ACC deaminase activity (Glick et al., 1997). This positive relationship between plants and microorganisms (Tilak et al., 2006) may result from adaptive

Table 2.10 - Average stomatal conductance (gs(w)) of bermudagrass in response to different strains of PGPB (*inoculation*) (DH-44, C-PGPR, DH-267), and N strategies (33 kg, 67 kg), (Urea, Ammonium Sulfate) across two growing seasons 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	----- mol m ⁻² s ⁻¹ -----				
NN	0.2	0.3	0.2	0.2	>0.05
U-33	0.3	0.2	0.2	0.2	>0.05
A-33	0.1	0.3	0.7	0.2	>0.05
U-67	0.3	0.3	0.3	0.2	>0.05
A-67	0.3	0.3	0.3	0.2	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($p < .05$).

Means within a row differ each N strategies into inoculation ($p < .05$).

SEM = 0.16.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

Table 2.11 - Average photosynthesis CO₂ assimilation (A) of bermudagrass in response to different inoculation (DH-44, C-PGPB, DH-267), and N strategies (33 kg, 67 kg), (Urea, Ammonium Sulfate) across two growing seasons 2023 and 2024, in Crossville, Tennessee, U.S.

N strategies (kg N ha ⁻¹)	Inoculation				P-Value
	DH-44	C-PGPB	DH-267	No-PGPB	
	----- μ m ⁻² s ⁻¹ -----				
NN	15.5	18.6	16.8	15.7	>0.05
U-33	16.6	16.8	16.2	15.7	>0.05
A-33	18.3	17.0	17.8	17.9	>0.05
U-67	18.1	18.1	18.9	19.7	>0.05
A-67	17.6	19.7	19.8	17.1	>0.05
P-value	>0.05	>0.05	>0.05	>0.05	

Means within a column differ each inoculation into N strategies ($p < .05$).

Means within a row differ each N strategies into inoculation ($p < .05$).

SEM = 1.6.

Note: DH-44, *Paenibacillus riograndensis*; C-PGPB, UrtrishaN; DH-267, *Bacillus subtilis*; No-PGPB, control.

Note: U, urea; A, ammonium sulfate.

Table 2.12 - Stomatal conductance (gsw) and photosynthesis CO² assimilation (A) of Bermudagrass, across two growing seasons (year) 2023 (1) and 2024 (2), in Crossville, Tennessee, U.S.

Year	gsw	A
	----- mol m ⁻² s ⁻¹ -----	
2023	0.4 ^A	16.7 ^B
2024	0.2 ^B	18.4 ^A
SEM	0.06	3.1
P-value	< 0.05	< 0.05

Means with a common uppercase letter are not significant at $\alpha = 0.05$

SEM, Standard Error of the Mean

Note: gsw corresponds to Stomatal conductance, and A corresponds to photosynthesis CO² assimilation

mutations that improve defenses against biotic and abiotic stresses over time (Rennenberg & Simon, 2013).

Despite this potential, no evidence of stress alleviation due to PGPB inoculation was identified in bermudagrass in this study (**Tables 2.10; Table 2.11**).

Conclusion

This study evaluated the effects of plant growth-promoting bacteria (PGPB) and nitrogen (N) fertilization on bermudagrass forage mass (FM) and nutritive value over two growing seasons (2023 and 2024) at the Plateau AgResearch and Education Center in Crossville, TN. The results indicate that N fertilization significantly increased FM and crude protein (CP) content, but PGPB inoculation alone did not result in significant differences in average FM, FA, CP, or forage digestibility. Specifically, treatments with 67 kg N ha⁻¹ of ammonium sulfate produced the greatest FM (2606 kg DM ha⁻¹) and highest CP content (146 g kg⁻¹), consistent with the known response of bermudagrass to N application. Despite variations in FM and CP, in vitro dry matter digestibility (IVDMD48) and neutral detergent fiber digestibility (NDFD48) remained unaffected by both PGPB and N strategies, suggesting that the overall forage quality was maintained across treatments. The findings support the importance of N fertilization in improving bermudagrass yield and quality but highlight the need for further research to clarify the inconsistent responses to PGPB inoculation under different environmental and management conditions.

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APPENDIX

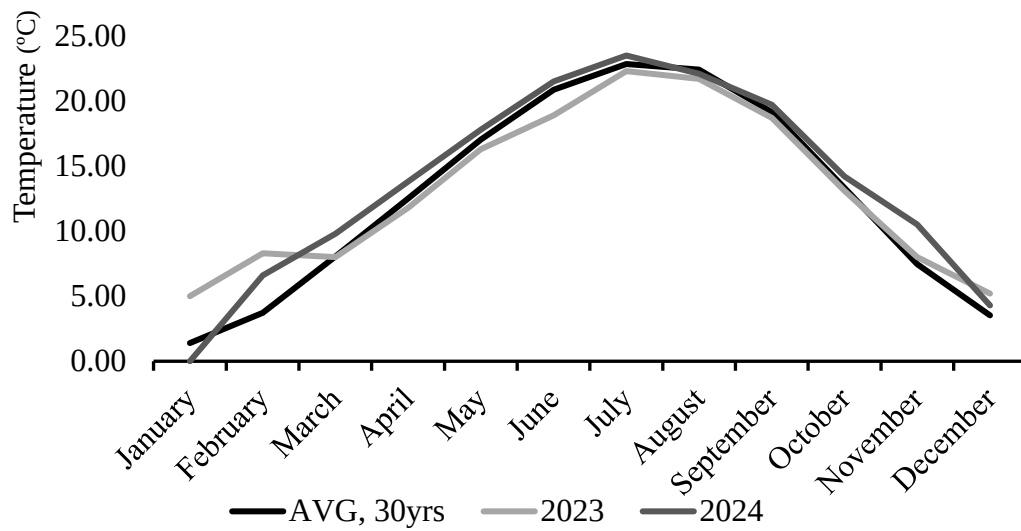


Figure A.1 Temperature for Plateau AgResearch and Education Center, Crossville, TN, including 2023 and 2024, including the 30-year average.

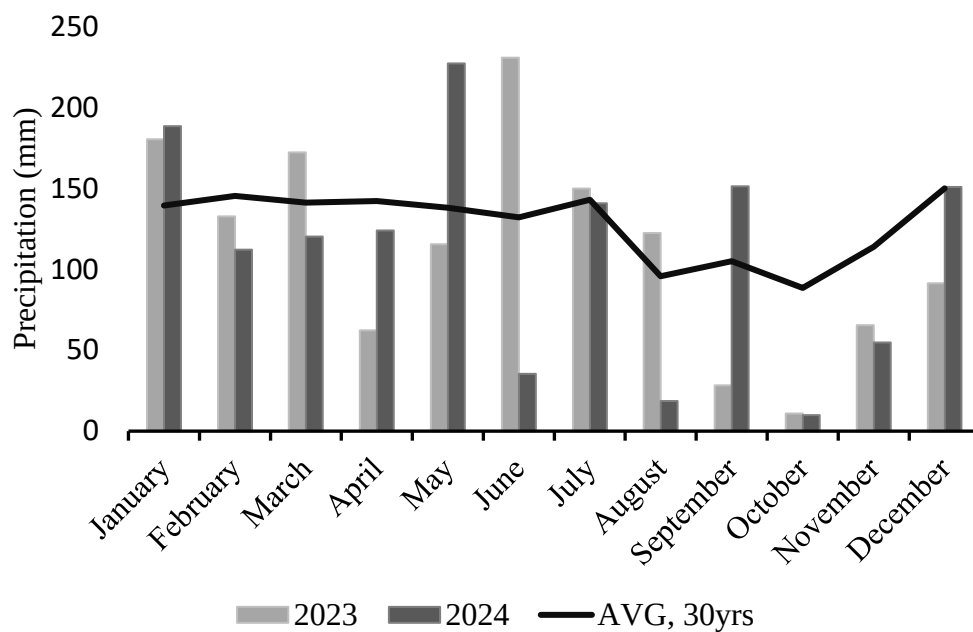


Figure A.2 Precipitation for Plateau AgResearch and Education Center, Crossville, TN, in 2023 and 2024, including the 30-year average.

CONCLUSION

This work evaluated the effects of plant growth-promoting bacteria (PGPB) and nitrogen (N) fertilization on bermudagrass shoot and root weight, shoot and root length, and number of tillers in Knoxville, TN and forage mass (FM) and nutritive value over two growing seasons (2023 and 2024) at the Plateau AgResearch and Education Center in Crossville, TN. The results indicate that N fertilization did not impact shoot and root weight, but PGPB inoculation alone did result in significant differences in shoot weight, shoot length, and number of tillers. Inoculation with Micro-AZ dry showed greater results overall. The present work also indicated that indicate that N fertilization significantly increased FM and crude protein (CP) content, but PGPB inoculation alone did not result in significant differences in FM, CP, or forage digestibility. Specifically, treatments with 67 kg N ha⁻¹ of ammonium sulfate produced the greatest FM (2606 kg DM ha⁻¹) and highest CP content (146 g kg⁻¹), consistent with the known response of bermudagrass to N application. Despite variations in FM and CP, in vitro dry matter digestibility (IVDMD48) and neutral detergent fiber digestibility (NDFD48) remained unaffected by both PGPB and N strategies, suggesting that the overall forage quality was maintained across treatments. The findings support the importance of N fertilization in improving bermudagrass yield and quality but highlight the need for further research to clarify the inconsistent responses to PGPB inoculation under different environmental and management conditions.

VITA

Felipe Fonseca Nassar was born in Catanduva, São Paulo, Brasil. He graduated in 2022 from the University of Sao Paulo (ESALQ) with a Bachelor of Science in Agronomic Engineering. After, Felipe worked for a year as a student intern under Dr. Oakes in the Middle Tennessee AgResearch and Education Center (MTREC), when he decided to pursue his master's degree at the University of Tennessee in 2023.