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CARLOS CRISTOBAL VELA GARCÍA

NITROGEN UTILIZATION AND CYCLING IN FORAGE PRODUCTION SYSTEMS

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CARLOS CRISTOBAL VELA GARCÍA

NITROGEN UTILIZATION AND CYCLING IN FORAGE PRODUCTION SYSTEMS

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Chair: Prof. PhD. Jose Carlos Batista Dubeux Jr.

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NITROGEN UTILIZATION AND CYCLING IN FORAGE PRODUCTION SYSTEMS

Dissertation conducted by

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1 Thessalonians 5:16-18

ABSTRACT

Nitrogen cycling is strongly affected by each factor of the pasture system, including the grazing animals, insects, the grazing intensity or clipping stubble height, harvest frequency, and forage varieties. The aim of this study was to evaluate nitrogen utilization and cycling in different forage production systems. Specific objectives included (i) to evaluate different species of dung beetles and their assemblages on GHG emission (N_2O), ammonia volatilization, and pearl millet [*Pennisetum americanum* (L.) Leeke] performance; (ii) to evaluate herbage accumulation (HA), crude protein (CP), nitrogen yield (Ny) and *in vitro* digestible organic matter (IVDOM) concentration of new Bermudagrass cultivars and (iii) to evaluate HA, Ny, biological nitrogen fixation (BNF) and nitrogen derived from atmosphere (%Ndfa), legume contribution (LC), dry matter (DM), CP, and IVDOM of Alfalfa-bermudagrass mixtures under contrasting harvesting regimes. All trials were allocated in a randomized complete block design (RCBD), each one with different treatments as (i) singles species as *Onthophagus taurus* (1), *Digitonthophagus gazella* (2) and *Phanaeus vindex* (3), and their assemblages combining species 1+2 and 1+2+3. In addition, two controls treatments were used; (ii) 10 bermudagrass genotypes, ‘Missouri’, ‘Tifton 85’, ‘Jiggs’, ‘FL44’, ‘322’, ‘323’, ‘276’, ‘282’, ‘283’, ‘286’and (iii) two alfalfa varieties which Bulldog 805 and UF2015-AP, clipped at 5, 10, 15 cm of stubble height each and subjected to three harvest frequencies as 2, 4, and 6 weeks. Overall results indicated that (i) there was an interaction period evaluation × treatment ($P<0.05$) for N_2O flux and ammonia (NH_3) volatilization due to contrasting gases emission initially but not at later measurements from livestock dung. Dung beetle species affected N_2O flux on dung, increasing the fluxes in the 6th day (80 g N_2O-N ha^{-1} day^{-1}) compared to treatment with just soil and dung (2. 6 g N_2O-N ha^{-1} day^{-1}). *D. gazella* as isolated species removed and buried more dung than other isolated species and other combining species assemblages. Dung application have affected the HA of pear millet regardless the presence of dung beetle, compared with treatments with just soil with average of 8 g DM $terraria^{-1}$ and 5 g DM $terraria^{-1}$, respectively. A PCA analysis was used to understand the variation and correlation of each variable, which showed a low principal component explanation (less than 80%) not enough to explain the variation of the results; (ii) there was genotype × evaluation interaction effects ($P<0.05$) for HA, CP, and Ny. The 10 bermudagrass genotypes showed significant variation for all characteristics, being 286 more productive (P<0.05) than Jiggs in the first evaluation with 4427 kg DM ha^{-1} and 3245 kg DM ha^{-1}, respectively and 323 had greater ($P<0.05$) CP in the fifth evaluation than 283, 286, 322 and Tifton 85 with average CP of 155, 128, 136 and 137 g kg^{-1} of DM, respectively.; (iii) there was effect of the treatments ($P<0.05$) on %Ndfa, HA, THA, Ny, TNy, BNF and CP. The genotype UF2015-AP produced 3525 kg DM ha^{-1} $harvest^{-1}$, which is greater ($P<0.05$) than Bulldog 805 harvested every 6-wk. Average LC, CP, and IVDOM were 36%, 175 g kg^{-1} of DM, and 540 g kg^{-1} of DM in the first evaluation, and these values were greater ($P<0.05$) than the ones observed in evaluation three. Overall results indicated that dung beetles are beneficial for N cycling increasing plant productivity. There are bermudagrass genotypes that are more efficient in N utilization; and finally, harvesting management affect productivity responses of alfalfa-bermudagrass mixtures, but overall, this grass-legume combination did not persist for more than one year in North Florida.

Keywords: genotypes, dung removal, legume-grass mixture, nutrient cycling, coprophagous insect.

SUMMARY

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LIST OF ABBREVIATIONS

BNF – Biological nitrogen fixation

CH_4 – Methane

CO_2 – Carbon dioxide

CP – Crude protein

DM – Dry matter

GHG – Greenhouse gas

HA – Herbage accumulation

IVDOM – *In vitro* digestible organic matter

N_2O – Nitrous oxide

N_2O-N – Nitrous oxide nitrogen

Ndfa – Nitrogen derived from atmosphere

NH_3-N – Ammonia nitrogen

Ny – Nitrogen yield

TNy – Total nitrogen yield

RCBD – Randomized complete block design

THA – Total herbage accumulation

INTRODUCTION

The air contains more than 78% gaseous dinitrogen (N_2); however, most living organisms are unable to use it in that state. There are free-living or symbiotic microorganisms that are capable of converting N_2 into NH_4^+, a form easily absorbed by plants. Of these two groups of fixing organisms, the most important are those that do so in symbiosis with legumes and belong to the *Rhizobium* or *Bradyrhizobium* genera (Kuypers et al., 2018). Symbiosis is a relationship between living beings in which both organisms' benefit. Rhizobium invades root hairs and cortical cells, inducing the formation of nodules, in which the bacteria reproduce and settle (Shimoda et al., 2020). The legume provides carbohydrates (energy source) to the bacteria, which in turn provides the host plant with nitrogenous compounds, synthesized from fixed atmospheric N_2.

Nitrogen has an important role as fertilizer to the plant productivity and quality (Sete et al., 2019), however, the incorrect management of nitrogen fertilization increase the N losses as nitrate by leaching, polluting the groundwater and destroying the ecological balance (Mencio et al., 2016); as well emitting nitrous oxide (N_2O) by denitrification process, contributing to global warming (Cui et al., 2020). Over time many strategies were created to reduce the negative impacts caused by improper N management (Bryant et al., 2019). For example, using forages as cover crops could be used to manage efficiently the N entries in the soil (Box et al., 2017 and Martin et al., 2017) avoiding losses by leaching and its negative impact to groundwater.

Pasture fertilization is usually an effective tool to replenish nutrient losses and extracted via animal products. Fertilizer application affects production of forage biomass in the short time (Bernal 1984), and helps to maintain soil fertility over time; however, for the proper use of fertilizers and for the nutrients applied through them to be properly absorbed by the plant, there must be an adequate level of moisture in the soil and fertilization levels must be used according to the demands and potential plant uptake. Production in any agricultural system, such as animal production based on the use of fodder, is centered on three factors as soil, plant, and weather. The key is how to make an integral and rational management of these components in decision making, regarding different interventions, one of which may be the fertilization of pastures (Pezo-Quevedo, 2018).

As much as N fertilization have a great impact in the plant performance, the grazing livestock have a large influence on it as well (e.g., stocking rate, grazing intensity, forage fouling). Some forage species are better adapted to grazing than others and, thus, the dynamics of plant community are often altered significantly under grazing. Besides providing nutrients for livestock production, forages confer many other positive benefits to the environments where they grow (Baron and Bélanger, 2020; Moore et al., 2020). Furthermore, grazing activity strongly modifies the vegetation structure (Jugovic et al., 2018). Hence, incorrect grazing management is often seen to have a negative impact on biodiversity (Jugovic et al., 2017).

The excreta produced by grazing animals increase the diversity of insects in the ecosystem (Silva et al., 2010). Invertebrates are major contributors during dung decomposition. They are attracted by dung odor (e.g., dung beetles; Coleoptera: Scarabaeidae), and they depend on livestock feces as a main source of nutrients (Verdu and Galante, 2004; Zamora et al., 2007). Dung beetles bury dung and, in the process, deliver significant benefits to both agriculture and the environment. Establishment of dung beetles in agricultural systems, therefore, provides a rare opportunity to support and enhance land management practices that preserve the health of soil and water while, may reduce the greenhouse gas emission also improving the sustainability and economic return of farming (Doube and Marshall 2014).

It is important to emphasize that forages constitute an important part of ruminant feed worldwide and the grasslands used for livestock production represent 77% (40 million km^2; Richie and Roser, 2020) of livestock intake in USA. The rest of the livestock feeds are in the form of concentrated grain feeds and supplemented with protein (Sundstøl, 1993). Forages consist of herbaceous plants, annual or multiannual, grasses or legumes, whose livestock use can be made directly through grazing, or deriving production through the agricultural practice of mowing (Allen et al., 2011). This fresh fodder can be supplied to cattle as is (green) or preserved by reducing its humidity to levels that allow it to be preserved in time and space (e.g., hay). Anaerobic fermentation using silos is another traditional conservation practice.

Management strategies are important to reduce N losses in forage production systems and keep a balance between their components, as soil, plant, animal, environment, macro e microfauna. Management items include soil fertility, planting method, forages species, harvest frequency, intensity, and timing, grazing method. All of these are essential to avoid losses in pasture productivity and nutrient losses to the environment; however, they are essential to bring benefits to the system.

Thus, this work evaluated nitrogen utilization and cycling in forage production systems in three contrasting studies: (i) evaluation of different assemblages of dung beetles and their effect on greenhouse gas emission and nutrient cycling (ii) agronomic characteristics and nutritional value of bermudagrass cultivars during the growing season, and (iii) stubble height and harvest frequency on agronomic characteristics and nutritive values of alfalfa-bermudagrass mixtures in North Florida, United Stated.

CHAPTER 1

Literature Review

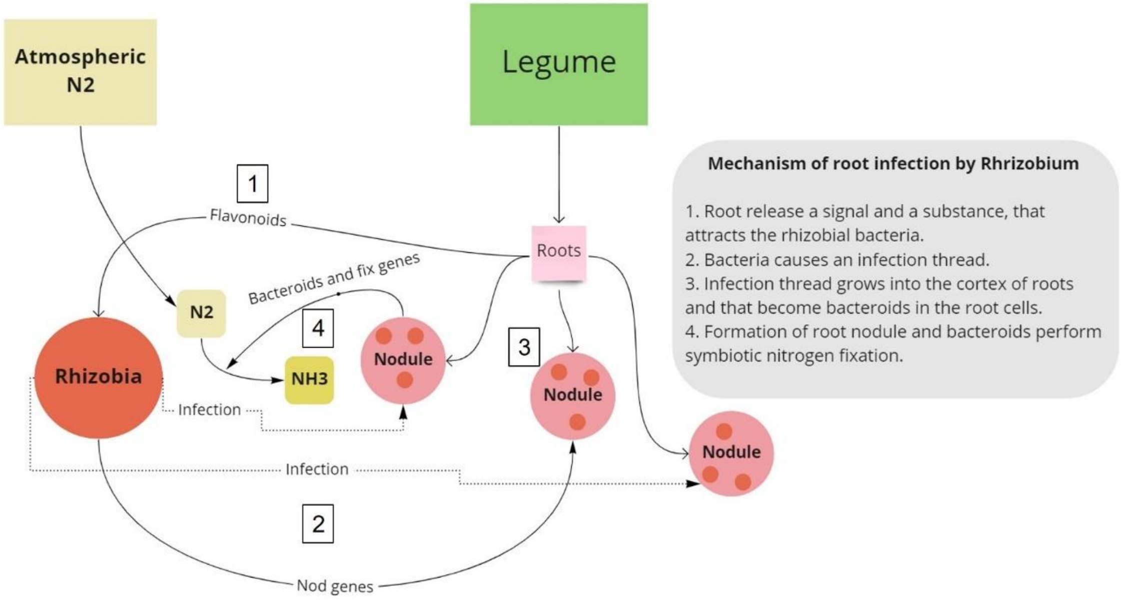
1 Nitrogen entry and cycling in grassland ecosystems

Grassland ecosystems have many biotic and abiotic components and all of them have a strong interaction. In most forage production systems for example, litter, livestock excreta, soil organic matter, and geochemically-bound mineral forms produce nutrients needed for plant growth, which are released to plant-available forms by the microbially-mediated breakdown (Wedin and Russelle, 2020). Thus, nutrient coming from the system interaction is a network of pools of a particular element, joined by fluxes (transfers) connecting those pools (Chapin et al., 2011). Despite most of those elements have a great pool in the atmosphere as C and N (Sitters et al., 2020) or geologic as P and K (Manning, 2018), the fluxes or transfer rate of elements from those pools into organic forms are usually low (Simard et al., 2015).

Nitrogen is the dominant nutrient constraint on primary production in most forage systems, though a study replicated across several continents suggests that N and P collectively constrain productivity in many grasslands (Vitousek, 2015). Nitrogen is fixed and incorporated into the soil in grassland ecosystems through different ways as the action of electrical discharges from lightning, the symbiotic action of fixing bacteria, application of N fertilizer, and nitrogen recycling through the decomposition of organic material such as litter from dead plant tissue and animals (Vieira, 2017). Nitrogen is a structural element of most organic components in the soil, which accounts for up to 98% of soil N (i.e., N in organic form). The total N content of grassland soils in the surface layer (10 to 20 cm) ranges from 6390 to 8200 kg N ha^{-1}(Eliziario, 2018).

1.1 Legume and nitrogen fixation

Biological nitrogen fixation (BNF) is commanded by different phylogenetic groups of prokaryotic microorganisms, known as diazotrophic bacteria (Reis et al., 2006), which perform a symbiotic association with most plants of the legume family. Among the most common diazotrophic bacteria is the genus *Rhizobium*, with species that are active in soil biomass (rhizosphere). Rhizobia and legumes begin to interact due to both releasing secretions that attract or influence certain chemical processes between them. In the rhizosphere, flavonoids derived from plants are perceived by rhizobia, which induces them to produce a chemical signal, called "lipo-chitooligosaccharides", also known as Nod factor (NF) (Shimoda et al., 2020). The NF is sensed by the host legume, resulting in the activation of subsequent symbiotic reactions, which lead to rhizobial infection and formation of nodules in the roots, where the bacteria will remain (Figure 1).



Mechanism of root infection by Rhrizobium

1. Root release a signal and a substance, that attracts the rhizobial bacteria.
2. Bacteria causes an infection thread.
3. Infection thread grows into the cortex of roots and that become bacteroids in the root cells.
4. Formation of root nodule and bacteroids perform symbiotic nitrogen fixation.

Figure 1. Biological nitrogen fixation summarized model.
Note: Adapted from Lindström and Mousavi (2020), and Raza et al., (2020).

BNF by legumes depends on many factors including host species and genotype (Akter et al., 2018), rhizobial strain and population size (Nabintu et al., 2019), developmental stage of the host, inorganic N (mainly NO_3^-) supply, toxic element level (Jaiswal et al., 2018), and abiotic growing conditions (Suter et al., 2015). In general terms, BNF by forage legumes usually ranges from 50 to 200 kg N ha^{-1} year^{-1}. In white clover-perennial ryegrass mixtures, BNF ranges from 0 to more than 300 kg N ha^{-1} year^{-1} (Russelle, 2008) and BNF in alfalfa-bermudagrass mixtures range from 80 to 222 kg N ha^{-1} year^{-1} (Haby et al., 2006). BNF in pastures tends to be less than in mown forages because of N recycling through excreta, causing a feedback mechanism that reduces the nitrogen fixing by symbiosis (Tonn et al., 2019).

The efficiency of the nitrogen fixing activity of the symbiotic association between bacteria and plants varies among legume species, and common bean is often characterized as good nitrogen fixer (Hardarson, 2004; Peoples et al., 2009). Each legume species or varieties fix different N amount (Vasconcelos et al., 2020); for example, some beans could have a mean value of 39% nitrogen derived from atmosphere (%Ndfa), in different environments (Peoples et al., 2009). In general, climbing legumes (e.g., beans) have better nodulation and higher BNF compared to other legumes (Wilker et al., 2019). The same author found considerable variation in nitrogen fixing capacity (measured as %Ndfa), ranging from 21 to 76%, in a collection of Mesoamerican and Andean bean genotypes.

1.2 Nitrogen fertilization in pastures

Nitrogen fertilization is important to ensure not only the cell division process, but also to sustain the photosynthetic activity of the leaves for a longer period, delaying N internal remobilization (Costa et al., 2019). The reserve nitrogen compounds are important for the supply of N and C to the plant growth zones after defoliation, due to temporary reduction in N acquisition capacity by defoliated plants (Iqbal et al., 2012). Nitrogen uptake and translocation by roots, as well as the mobilization of N from storage nitrogen compounds, are critical processes for the nutrition of growing grass leaves (Qubain et al., 2021). These studies clearly demonstrate that grass regrowth after severe defoliation is more dependent, in magnitude and duration, on nitrogen reserves than on reserve carbohydrates (Sollenberger et al., 2020).

The plant can obtain nitrogen in two chemical forms, as NO_3^- and/or ammonium (NH_4^+) (Coutinho et al., 2014). Mismanagement of N fertilizers (e.g., level of application, fertilizer type) can cause major environmental problems. Furthermore, high doses of N fertilizer, increase nitrite (NO_2^-) accumulation into the leaf of forage grass. Extreme conditions where NO_3^- concentration in forage achieve 4500 mg kg^{-1} could poison the grazing animals and even cause death (Jonck et al., 2013).

Plant growth depends on N availability (Leghari et al., 2016). Nitrogen fertilization is recommended when applied to forages with high production potential and these are properly managed (Costa et al., 2019). Despite N being one of the most important nutrients to plant development, it is necessary to know the right dose of this nutrient to avoid losses and maximize nitrogen use efficiency in animal production (Tei et al., 2020) and economic return. The response of tropical forage plants to N is highly variable. For example, mombaça guinea grass (*Megathyrsus maximus* cv. Mombaça) in a rainy tropical savanna from Brazil, under three nitrogen rates (100, 200, and 300 kg N ha^{-1}) and under grazing has a variation of productive and nutritive value performance, which at 300 kg N ha^{-1}, resulted in greater post-grazing herbage mass with 2800 kg DM ha^{-1} and crude protein (CP) of 115 g kg^{-1} of DM (Euclides et al., 2022) as long as other factors are not limiting growth.

Application of N fertilizer according to plant needs result in greater nutritive value and faster tissue turnover (Delevatti et al., 2019; Paiva et al., 2015). In general, young tillers of grass may have leaf blades with a greater proportion of tissues of greater digestibility (Batistoti et al., 2012). Mesophyll cells in greater proportions are essential for the qualitative characteristics of forage grass (Moore et al., 2020). Thus, forages with older tillers present lower forage digestibility, that probably is associated with the increase in the cell-wall constituents (Ramírez et al., 2014).

Forage crude protein (CP) and other bromatological variables could be affected by N supply in grass pasture fertilized with nitrogen, compared with grasses without fertilizer (Homem, 2021); however, this response is variable and interacts with the environment

1.3 Nitrogen losses in pastures

Nitrogen losses as ammonia (NH_3) emitted in the atmosphere are increased when urine spots deposited by grazing ruminants combine with high soil pH from urea hydrolysis and high NH_4^+ concentration. However, this process slows nitrification (Wedin and Russelle, 2020). Nitrogen losses via NH_3 volatilization is an important pathway of N loss in grazed semiarid grassland. Under subhumid and humid pasture conditions, NH_3 losses account for between 2 and 25% of urinary N (Mulvaney et al., 2008). Higher NH_3 loss rates from urine and manure occur for concentrated or confined animals (Powell and Rots, 2015). Gaseous N loss by denitrification can be significant when soils become waterlogged and anoxic (Robertson and Groffman, 2015), but generally accounts for only a few percent of urinary-N loss (Ball et al., 2019).

Some sources of nitrogen applied to the soil are already available to plants (nitrogen fertilizers in the form of NH_4^+ and NO_3^-), however, organic sources (e.g., feces, organic matter, litter) must be mineralized to inorganic N (NH_3 and NH_4^+). For example, organic N from ruminant dung can be mineralized in NH_4^+ and NO_3^-, and be readily available to plants. Mineralization is a process that is carried out under aerobic or anaerobic conditions, which is completed by the action of microorganisms that act under these types of conditions. This action is affected by several factors including temperature, soil moisture, oxygen availability, and pH (Johnson et al., 2005; Osterholz et al., 2017).

By incorporating urea into the soil, the urease enzyme comes into action, reducing the compound into ammonium, hydroxyls, carbon groups and ammonia in aqueous conditions (Vieira, 2017). The nitrogen fertilizers and sources of organic nitrogen (feces, urine, decomposition materials) are subject to N losses in the form of ammonia (Qi et al., 2020). Ammonia, already in the atmosphere, is transformed into ammonium through chemical reactions, and can be displaced thousands of kilometers before being deposited, mainly by precipitation. Ammonia can also react in the atmosphere with oxides of sulfur to form ammonium sulfate, which reaches the ground through rain and causes acidification. Although NH_3 has no direct effect on global warming (Good and Beatty, 2011), it can be oxidized in the atmosphere to N_2O, and contribute to the deterioration of the ozone layer.

Synthetic organic sources that consist of N in the form of NH_4^+ and NO_3^- when applied to the soil can be volatilized by up to 78% due to edaphic, climatic, plant canopy factors and the action of soil organisms (Tasca et al., 2011). Work carried out by Zhou et al. (2009) determined the volatilization of ammonia in rice fields (for silage) in Japan under fertilization with liquid bovine excreta (residue C/N ratio was 14.3:1) and chemical fertilizers. Before the application of organic residues, the fluxes of volatilized ammonia went from 0.4 to 3.1 mg of N m^{2-1}, and after the consecutive application of residues (short application intervals) the fluxes increased quickly. Nitrogen losses from liquid bovine excreta ranged from 4.9 to 8.2% of the total applied (206 and 996 mg of N L^{-1}). The authors explained that nitrogen from organic residues is immediately available (90% of N in the form of NH_4^+) to plants, but high availability in short periods of application can accelerate losses to the atmosphere.

The high concentrations of N in the form of nitrates are subject to losses (leaching when large flows of water e.g., high rainfall, poor irrigation management) due to the high mobility of nitrate (Chintala et al., 2013), as they are not retained by the cation exchange capacity (CEC) of the soil colloids (Austin, 2006). Soils in tropical environments are generally clayey in the surface layer, and some clays are characterized by having negative charges. Nitrate, because it has negative charges, is not absorbed when the base saturation of the soil is above 80% and remains in the soil solution.

Each nitrogen cycle process depends on a substrate to be carried out. For example, ammonification relies on organic sources for the formation of ammonium and ammonia; nitrification needs ammonium sources for nitrate transformation; and denitrification uses nitrate as a substrate to release N_2 to the atmosphere (Mosier et al., 2002). In denitrification, the respiratory reduction of nitrate to nitrite occurs through the action of the reductase enzyme, for each compound produced, generating during this process NO, N_2O, and N_2 (Lazcano et al., 2021). Microorganisms use nitrate as an electron acceptor (anaerobic respiration) and bacteria need a source of C and electrons, using oxidizable organic matter to obtain it (Vieira, 2017).

As already mentioned in previous topics, the alteration of the natural nitrogen cycle is due to the mismanagement of anthropogenic activities and the waste generated by each activity (e.g., agriculture, livestock, industry). The compounds generated contribute to global warming and according to the US EPA (2018) inventory of greenhouse gas emissions, nitrous oxide contributes 7% to global warming, which together with other greenhouse gases (GHG) comes from 10% of agriculture, 12% of wastewater and solid waste, 22% from industry, 28% from transport of natural gas, coal, oil and 27% from electricity (Pearson et al., 2017).

Nowadays there has been an emphasis on looking for ways to mitigate N_2O emissions. Some instances include the evaluation of soil and water systems and their responses under different pH conditions, humidity, temperature, salinity, animal grazing, and use of cover crops, agroforestry, silvopastoral systems, and use of biological inhibitors. There are few studies assessing how soil pH affects the denitrification process, especially related to the type of microorganisms. Ammonia oxidizing archaea can act at an acidic pH of 4.44 and nitrite oxidizing bacteria act in high limestone soil (Huang et al., 2012). The rapid N mineralization of organic compounds increases the potential N losses to the environment; however, in studies comparing leaching of N from organic manures with that from inorganic fertilizers applied at the same total N amount showed that N leaching was lower from organic manure than mineral fertilizer under grazing (Jarvis et al., 1987; Vendramini et al., 2007).

1.4 Effect of livestock excreta on forage systems

A pasture with a stocking density of 700 cow ha^{-1} day^{-1} results in 6.4% of the total grazed area covered by dung pats, which provide a carbon (C) recycling equivalent to 22.5 Mg C ha^{-1} (Bol et al., 2000; Rumpel et al., 2015). Large grazing herbivores remove carbon from the soil and nutrients by grazing and return them by excretion, leading to cycling and redistribution, which in turn influences the structure and functioning of grassland systems (Haynes and Williams, 1992) and the organic matter (OM) dynamics through carbon dioxide release and incorporation into soil. Dung is a source of labile C, N, and phosphorus (P), which may increase microbial biomass (Lovell and Jarvis, 1996; Hatch et al., 2000) and induce priming of native organic matter (Fontaine et al., 2003). Molecular studies have shown that carbohydrates constitute part of the dung-derived C in soil (Dungait et al., 2009).

Most of the N, P, and potassium (K) in livestock diets is excreted in dung and urine (Rueda et al., 2020). Dung contains useful amounts of these plant available nutrients, as well as the other major nutrients such as sulphur (S), magnesium (Mg), and trace elements. For example, in UK, based on some prices for N, P, and K fertilizers, the slurry produced by 100 dairy cows over the winter housing period has a potential value of almost $2748. With opportunity to apply to grassland on several occasions during the growing season, this could provide up to 20, 100, and 80%, respectively, of the N, P and K fertilizer required by grassland used for hay or silage production (Rumpel et al., 2015).

Soil macrofauna are pivotal for optimal crop yield attainment as they play a role in improving soil structure, infiltration, and facilitating decomposition to supply nutrients (Sofo et al., 2020). Given that the quality of organic resources changes over time during the growing season, macrofauna diversity is critical in the decomposition and mineralization processes as food availability and quality changes (Moore and Ruiter, 2012). Thus, high diversity will allow for a steady supply of nutrients to the growing crop. Diverse macrofauna also supply nutrients from their excretory products and decomposition after death. In addition, those excretory products can house beneficial microbes to interact with the crop, facilitate nutrient solubilization and decomposition.

Soil macrofauna (e.g., Isoptera, Elateridae, Holotrichia serrata) can affect the growth of the crops as pest, or the duration of mulch, such that monitoring their dynamics can help to control the potential damage (Crowther et al., 2011). The soil macrofauna involve many interactions on the dung and could affect positively the plant during the growing season. Soil mesofauna may also improve nutrient cycling efficiency as well. Dung beetles (Scarabaeidae family) and earthworms (Lumbricidae family) increase the rate of mineralization and may reduce NH_3 volatilization by incorporating feces into the soil, reducing denitrification through elimination of anaerobic zones within fecal deposits (Mathews et al., 1996; Mathews et al., 2001; Vendramini et al., 2007).

1.5 The role of dung beetle in grassland ecosystems

1.5.1 Ecosystem services

Perhaps the best ecosystem service provided by insect is pollination (Losey and Vaughan, 2006). McGregor (1976) estimates that around 15 to 30% of the human diet in the United States is a result, either directly or indirectly, of animal-mediated pollination. Recent studies show that dung beetles develop this function as well. For example, Moretto et al. (2019) did a study in Tropical Africa evaluating different pollinator in a Bagana plant (*Amorphophallus abyssinicus* [A. Rich]). They observed in the inflorescence of Bagana, four different dung beetle species: *Onthophagus liberianus* (Lansberge), *Cleptocaccobius dorbignyi* (Cambefort), *Cleptocaccobius uniseries* (d'Orbigny); *Trichaphodius copulatus* (Schmidt), these species belong to Onthophagini tribe and subfamily Aphodiidae respectively.

Sakai and Inoue (1999) evaluated pollination service in a perennial herb named *Orchidantha inouei* in a park localize in Sarawak from Malaysia. The authors observed the most common presence of dung beetles belonged to genus *Onthophagus* spp. and *Paragymnopleurus* spp. The authors explained this fact due to *Orchidantha inouei* produces a mucilaginous secretion on the ventral surface of the stigma, that works as a glue to hold deposited pollen on the pollinator. In this case, the dung beetles were presumably deceived by the plant due to the secretion odor being similar to dung- or carrion-like odor.

The second important ecosystem service from dung beetles is seed dispersal of plants, due to animal-dispersed that include birds, primates, carnivorous mammals, and insects (Schupp et al., 2010). The dung beetles are considered to be important secondary dispersers of seeds in the tropics (Andresen and Feer, 2005) due to seed burial. This process facilitates the germination and reducing seedbed predation (Andresen, 2001). This occurs because incidentally dung beetles move seeds defecated by mammals vertically (seed burial) and/or horizontally as they process and relocate dung (Urrea-Galeano et al., 2019).

An environmentally controlled study developed in Tokyo by Koike et al. (2011) showed that *Phelotrupes auratus* (Motschulsky), *Prunus jamasakura* (Siebold ex Koidz), *Prunus verecunda* (Koidz) and *Prunus grayana* (Maxim), *Vitis. Coignetiae* (Pulliat ex Planch) have the capacity to bury seeds into the soil with the burial rate range from 27 to 51% and depths range from 1 to 27 mm. The author concluded that the behavior of dung beetles during dung decomposition, especially large tunnellers like *Phaneus auratus*, can lead to secondary dispersal via burial of seeds in the dung, as well seed burial facilitates germination by covering seeds with soil and enables seeds to avoid predators.

In another study, Urrea-Galeano et al. (2019) evaluated the effects of secondary seed dispersal by dung beetles, with emphasis on the horizontal movement of seeds and the spatial distribution of seeds and seedlings in Veracruz state, Mexico. The mean seed dispersal distance by dung beetles was 6 cm for two seed species (*Bursera sinaruba* [L.] and *Poulsenia armata* [Miq.]) that represented >97% moved horizontally and >55% moved vertically by dung beetles. This likely happened because around the site existed dung from mammals. Therefore, dung beetle of the roller group made more balls and brought seeds together, helping seed dispersal and distribution.

Santos-Heredia et al. (2011) said that dung beetles *Aphodiinae* spp. *Onthophagus haematopus* Harold, 1875 could bury 44% of *Pseudolmedia* and *Clarisia* seed in places with monkey dung around the soil. Moreover, the presence of dung beetles improved ecosystem functioning in terms of dung removal and secondary seed dispersal. For example, dung beetle of dwellers in 10 countries in the Western Palaearctic realm, being the most abundant functional group in the northern regions, did not contribute very much to dung removal in these regions (Dortel et al., 2013). However, in the Iberian highlands dung beetle removed a significant amount of dung, although the functional group was underrepresented in the sampled dung beetle assemblage (Milotić et al., 2018).

Exist a strong positive relationship between secondary seed dispersal and dung removal because dung beetles cannot distinguish or separate between seed-containing dung and seed-free dung (Andresen, 2002; Slade et al. 2016), although the number of buried seeds increased with dung pat size and dung beetle size (Andresen and Feer, 2005). Moreover, the relationship might switch from positive to negative, if the proportion of seeds present in the dung is higher (Shepherd and Chapman, 1998; Milotić et al., 2018).

Besides contributing to plant pollination, dung removal, and secondary seed dispersal, dung beetles can be a biological pest controller. For example, the dung from cattle presents gastrointestinal parasites and various undesirable flies, such as the common fly (*Musca domestica*) and the horn fly (*Haematobia irritans*) (González-Chang and Reyes, 2016). Given the abundant presence of common flies on Easter Island from Chile because of the introduction of livestock, Ripa et al. (1995) carried out an introduction program of the dung beetles *Digitonthofagus gazella* and *Onitis Vanderkellenis*. The authors reported a significant reduction in fly density attributable to the successful introduction of dung beetles (González-Chang and Reyes, 2016).

1.5.2 Nutrient cycling

The abundance and diversity of soil macrofauna are involved in the biogeochemical cycles of multiples elements, and all of them could modify the soil structure and soil fertility (Maldonado et al., 2019). Bertone et al. (2006) said that the presence of *Onthophagus taurus* (Schreber) and

Onthophagus gazella (Fabricius) in cattle pasture from North Carolina increased soil P, K, Ca, Mg, and micronutrients levels over the sites with dung only (i.e., no dung beetles), more so in clayey than sandy soils in the coastal plains. The author suggest that dung beetle could change soil characteristics including pH, exchangeable acidity, cation exchange capacity, base saturation, and humic matter content.

Girón-Vanderhuck et al. (2010) said that the presence of dung beetle *Dichotomius satanas* (Harold, 1867) in soils with dung from cattle and pig increased the levels of P from 6 to 120 ppm. On the other hand, 85 to 95% of nitrogen consumed by cattle returns to the soil via livestock excreta (dung and urine). This nitrogen could be buried and mineralized in short period by dung beetle activity and breakdown nitrogen and phosphorus in available forms to the plants (González-Chang and Reyes, 2016).

Maldonado et al. (2019) did an experiment at cattle field in Mendoza Province from Argentine, to assess the effect of four dung beetle species (*Sulcophanaeus imperator*, *Eucranium arachnoides*, *Digitonthophagus gazella*, and *Malagoniella puncticollis*) on nutrient cycling. According to the author, all dung beetle species of their study increased soil fertility, but *Digitonthophagus gazella* was more efficient, increasing soil organic matter by 159 g in 600 m^2 (equivalent to 2647 kg ha^{-1}).

Yamada et al. (2007) used dung beetle species *Aphodius quadratus* (Reiche), *Caccobius jessoensis* (Harold), *Onthophagus bivertex* (Heyden), and *Onthophagus ater* (Waterhouse) collected in orchardgrass (*Dactylis glomerata* [L.]) pastures from Miyota, Nagano Japan, to evaluate their effect on nutrient cycling. The authors concluded that inorganic N, available P, and exchangeable K in the soil showed great values with dung beetles with average values of 18 g N

kg^{-1}, 49 g P kg^{-1} and 400 g K kg^{-1}, respectively, but this occurred over time after the action of the beetles. In other words, dung beetle accelerates the nitrogen transfer from the dung to the soil.

Furthermore, the dung beetles modify the soil propriety when it buries the dung to build their burrow (Fincher, 1981). Brown et al. (2010) did a study at Potshini catchment in Kwazulu Natal, South Africa, about the effect of dung beetles on soil bulk density and soil moisture. Dung beetle decreased soil bulk density from 1.34 g/cm^3 (soil with dung and without beetles) to 1.23 g/cm^3 (soil with dung and with beetles), but increased porosity in the A horizon. In addition, soil moisture increased from 151.91 to 202.09 g/kg in a period of five days with decreasing bulk density.

In mixed pastures of *Eragrotis tef* (Trotter), *Chloris gayana* (Kunth), and *Digitaria eriantha* (Steud) with cattle dung and dung beetles, plant biomass productivity, soil macronutrients, and rate of water infiltration was greater over time when compared with the sites without dung beetles, and penetration resistance was reduced in soils with dung beetles (Chan and Barchia, 2007). Bang et al. (2005) said that in the top 10 cm of soil occurs most of the dung beetle activity, whereby their burrowing activity loosens the top layer of soil. The loosening of the top layer of the soil may further increase water infiltration rate by creating a more porous soil structure. High soil strength hinders the root growth of plants, resulting in a decrease in nutrient and water uptake as well as poor herbaceous plant cover (Badenhorst et al., 2018).

Kaleri et al. (2020) confirm that dung beetles and their interaction with dung from cattle pastures ecosystems improved soil macro and micronutrient status, and drastically decreased soil density, pH, and electrical conductivity compared with places with just cow dung and control groups. In addition, dung beetles increased total leaf sugar, vitamin C, polyphenols, total protein, and amino acids compared with control plants of field mustard (*Brassica rapa* [L.]). Finally, dung beetles significantly increased net photosynthetic rate, stomatal conductance, and chlorophyll content of the plant.

Soil ammonium concentration increased with dung beetle activity in the first 10 cm of the soil compared to sites with just dung and sites without dung. In the presence of dung beetles (both tunnellers and dwellers), the nitrite concentrations were greater than in sites without beetle activity. The nitrogen coming from beetle activity contributed significantly to the total nitrite pool in the upper soil layers (67% and 45% of total nitrite at 0–5 and 5–10 cm, respectively) after 1 month to release dung and dung beetles (Nervo et al., 2017).

1.5.3 Effect on Greenhouse gas emission (GHGs)

The excreta produced by livestock is a significant source of GHG as nitrous oxide and methane (Piccini et al., 2017). The GHG are directly dependent on microbiological processes occurring in decomposition of organic materials by micro-organism (Kool et al., 2010). Piccini et al. (2017) suggests that GHG fluxes from dung pats could be affected by biotic interactions involving dung beetles. These insects create aerobic environment in the soil due to aeration, and it might reduce nitrous oxide flux. However, Fowler et al. (2020) reported that dung beetles generally decrease methane but increase nitrous oxide.

The excreta of livestock, especially dung, is a major source of GHG emission. Some studies about the effect of dung beetle on greenhouse emission reveal over time that the gas emissions are increased (Penttila et al., 2013). For example, some studies showed that methane, carbon dioxide, and carbon dioxide equivalent decreased but nitrous oxide emission increased 1.93 times (Yokoyama et al., 1991). Penttila et al. (2013) reported that methane decreased 1.65 times and carbon dioxide and carbon dioxide equivalent did not present any effect, and nitrous oxide increased 27.2 times. Iwasa et al. (2015) showed that methane reduced 2.61 times, carbon dioxide increased 7.87 times, and nitrous oxide 10.81 times. Piccini et al. (2017), Slade et al. (2016) and Fowler et al. (2020a) showed no effect of dung beetle activity on these same greenhouse gases. Evans et al. (2019) showed increase of three times to methane and nitrous oxide and no effect to carbon dioxide and CO_2 equivalent. The study most recently published by Fowler et al. (2020b) with dung beetles indicated that carbon dioxide and equivalent is not affected, methane decreased 1.4 times, and nitrous oxide increased 1.56 times by dung beetle activity.

The carbon dioxide increase indicates that dung beetle activity mainly occurs within the first seven days after dung application. During these initial days, dung beetles feed on dung liquid nutrients; therefore, significant evaporation of dung moisture content resulted in short-lived dung beetle abundance (Evans et al., 2019). Other processes affected by dung beetle activity include soil microbial respiration and the respiration from dung beetle eggs (Iwasa et al., 2015).

The age of the dung pat affects its decay, which physically alters the dung by leaching/evaporating water and loosening the dung structure, a process aided by the disturbance and disassembly of dung pats by dung beetles (Fowler et al., 2020b). Presumably transitioning from an anaerobic to an aerobic dung pat by mixing or aging should predictably increase carbon dioxide emissions via environmental respiration or enhanced gas transport, though not dung beetle respiration (Iwasa et al., 2015; Fowler et al., 2020b).

The higher nitrous oxide flux showed in almost all the studies evaluating the effect of the dung beetle activity on GHGs suggest that it is a result from incomplete byproducts of denitrification (Sylvia et al., 2005), nitrate reduction (Penttilä et al., 2013; Slade et al., 2016, Piccini et al., 2017), and nitrification (Iwasa et al., 2015) from increased microbial abundance and activity (Yokoyama et al., 1991), and/or increased gas transport such as when dung beetles microtunnel into wet dung (Evans et al., 2019). In addition, dung beetle activity provides nitrate pools by enhancing ammonification and nitrification through aerobic soil activity (Yokoyama et al. 1991). Collectively this suggests that cow dung is an obvious moisture and fertilizer source, and that dung beetles may increase the denitrification rate (Fowler et al., 2020b).

2 Bermuda and Alfalfa utilization in tropical forage systems

2.1 Bermuda

Warm-season perennial grasses predominate in subtropical and tropical climates and are the primary feed source for many livestock. They utilize C_4 carbon fixation pathway, associated with high-growth rates, high water and N-use efficiencies, and relatively low-nutritive value compared with C_3 grasses. Conditions of high temperature, water stress, and saline soils generally favor C_4 over C_3 species, but C_4 grasses may even dominate temperate ecosystems up to 50ºN latitude (Sollenberger et al., 2020).

In southern states of US, there are approximately 12 million ha of growing Bermudagrass (*Cynodon dactylon*), and it is the most popular and used perennial forage (Redfearn and Nelson, 2003). It is grown widely in tropical and subtropical regions of Africa, Asia, Australia, and the Americas. That forage has deep roots into the soil and is tolerant to dry environments, and produce rhizomes and it is more persistent under grazing than stargrass (*Cynodon nefluencis*; Hanna, 1992). Bermudagrass have greater growth rate when the daily temperature average is above 24ºC and pH above 5.5; however, there is a privately released bermudagrass ecotype Jiggs that has shown greater tolerance to poorly drained soils (Aguiar et al., 2014). In the peak of winter, freezing temperatures kill the leaves, but the rhizomes survive, getting the dormant stage over the winter, depending on the severity of cold, and beginning new growth in the following spring.

Breeding programs are important because they can generate new varieties that are adapted to different environment conditions. Natural selection occurring biotypes provided bermudagrass cultivars for agricultural purpose until 1940s. The genetic diversity within bermudagrass in the USA provided a broad array of genotypes differing in adaptation and performance features. There are many examples of bermudagrass biotype selection and used clonal cultivars provided by breeding programs. Bermudagrass is typically planted using vegetative propagules called sprigs, which are tillers, rhizomes, stolons, and root portions. The planting rate of bermudagrass sprigs varies greatly, but it usually ranges from about 17 to 52 hL ha^{-1} (Sollenberger et al., 2020).

Almost all bermudagrass varieties are planted during the late winter or early spring with dormant sprigs or sprigs harvested in very early growth stages. Soil temperatures from 26 to 33ºC appear to be optimal for sprig germination and subsequent growth (Keeley and Thullen, 1989). Some bermudagrass cultivars, such as “Alicia”, “Tifton 78”, and “Tifton 85” usually are established by planting freshly harvested aboveground biomass commonly referred to as stem cuttings. Bermudagrass stem cuttings are usually planted in spring or early summer, although later plantings can be successful if soil moisture is adequate. On the other hand, bermudagrass requires relatively high soil nutrient availability to maintain good production performance. Plant response to fertilizer is determined by many factors including defoliation regime and method (clipping vs. grazing), climate, native soil nutrient status, source and rate of applied nutrient, season application, cultivar, and harvest frequency (Sollenberger et al., 2020).

Experiments initiated in 2000 by Vendramini and Moriel (2020) demonstrated that different bermudagrass cultivars have different responses. For example, they studied Mislevy, Jiggs, World Feeder, Florona, Tifton 85 and others bermuda entries at four grazing/ clipping frequencies with 30 kg N ha^{-1} application. Mislevy, Jiggs, and Florona had the greatest overall herbage accumulation (HA); however, Mislevy had greater HA at late maturity (7 weeks) with 2100 kg DM ha^{-1}. In the clipping trials, they used another cultivar (Jiggs, Tifton 85, Tifton 44, Coastal, Alicia, and Russell) and compared with Mislevy. Mislevy and Jiggs had greater spring HA and total HA than the other cultivars/entries in South Florida and North Florida, with 14800 and 14400 kg total HA ha^{-1}, respectively

Several variations of *C. dactylon* are commercially used in southern North America including Suwannee, Callie, and Coastal. One of the most recently released cultivars, Florakirk, appeared commercially in 1995 (Mislevy et al., 1995). Like Tifton 78, Florakirk is also a sterile F_1 hybrid, between Tifton 44 and Callie (Mislevy et al., 1999), being evaluated in Florida since 1978, in the experimental condition, having been named Tifton 35-3 (Mislevy et al., 1995). In an evaluation under continuous stocking for three years in Florida, a drastic drop in the persistence of Florakirk was reported under this type of management, reducing the area coverage from 89% to 35% after three years of grazing (Pedreira et al., 2016).

Periodically, new commercial Cynodon forages appear on the market, coming from breeding programs aimed at specific purposes or from casual obtaining of new plants that, after selection and evaluation under cutting and grazing, are made available to producers and ranchers. Recent examples include the cultivars Russell (Ball et al., 1996), released in 1994 and registered as a cultivar in 1996, and Quickstand (Phillips et al., 1997), released in 1993 and registered as a cultivar in 1997. These new selections can be attributed to the great genetic variability within the genus, which is probably related to the geographic diversity of the centers of origin and dispersion, giving the plants of *Cynodon* spp. high flexibility of edaphoclimatic adaptation and, consequently, considerable potential for use in the tropics and subtropics.

According to Zhang et al. (2020), the management can affect the bermudagrass performance and nutritive characteristics as well, for example mowing frequency (2, 4, 6, and 12 weeks) could influence the yield and nutritive value of bermudagrass. An appropriate mowing frequency may contribute to increasing the yields and nutritive value of bermudagrass. In particular, the frequencies of 4 and 6 weeks maximized the yield; however, these frequencies were not optimal for nutritive value and future research is required. According to the authors, the N concentration did not exhibit any difference at various mowing frequencies. The highest concentration of crude protein was attained at the 2-weeks mowing frequency, although the 4-weeks mowing frequency resulted in a relatively higher shoot fresh weight, crude fat content, water content, P concentration, and plant height.

2.2 Alfalfa

Alfalfa is the oldest crop grown solely for forage, having been cultivated for about 9000 years (Russelle, 2001). It was used as livestock feed by Middle Eastern civilizations and later spread through the Old World by traders and armies. Despite alfalfa being the most cultivated forage in temperate regions, it is from Asia Minor (Iran, Iraq, Syria, Turkey, and Afghanistan) where exist desert environments. Alfalfa roots are mostly found in the top 0.5 m of soil, but taproots can penetrate from 7 to 9 m deep (Sheaffer et al., 1988). Alfalfa grows best on soils that are well drained, neutral in pH, and have high fertility. It is poorly adapted to wet or saline soils and will not tolerate flooding. Cold tolerance is a major factor influencing cultivar adaptation. Very winter hardy cultivars can survive air temperatures as low as -25ºC. Water use by alfalfa exceeds that of many annual row crops that have a shorter period of vegetative growth (Sheaffer et al., 2020).

Alfalfa has deep taproot, it contributes to resistance and great production in areas with low or poor seasonal distribution of rainfall (Andrzejewska et al., 2020). The highest dry matter (DM) yield of leaves, rich in protein and carotenoids, is achieved at the early developmental stages of alfalfa, and then later a slight decrease is noted due to senescence and leaf loss from the lower, shaded parts of the plant (Albrecht et al., 1987). The digestibility of alfalfa stems decreases markedly with plant development due to the increasing concentration of cell walls and lignin, while the digestibility of leaves changes only slightly as the plant matures. Crude protein concentration in alfalfa leaves is 2 to 3 times greater than in stems, while fiber and lignin concentration in stems is 2 to 3 times greater than in leaves (Albrecht et al., 1987). Moreover, stems have higher concentrations of non-protein and indigestible nitrogen than leaves (Hakl et al., 2018; Andrzejewska et al., 2020).

During regrowth following dormancy or harvesting, herbage DM yield increases until flowering and then declines due to leaf loss (Sheaffer et al., 1988). Depending on length of the growing season, climate, and maturity at harvest, alfalfa will have from 2 to 10 regrowth cycles each year. The forage yield and quality of alfalfa are greatly influenced by the maturity at harvest for hay and silage. Producers who value high-quality forage harvest more frequently, often at bud stage, and sacrifice some yield and persistence. Alfalfa provides high-quality forage but should be mixed with grasses to reduce incidence of bloat (Catalano et al., 2019). Grazing-tolerant cultivars have been developed (Sheaffer et al., 2020).

Persistence is an important parameter for producers when considering use of different forages in their system. Alfalfa persistence depends on parameters such as chosen variety, environmental conditions, and management on the field (Smith et al., 1992; Brumer and Bouton, 1991; Beck et al., 2016). Harvesting frequency also plays an important role in alfalfa persistence. Alfalfa is a plant with a taproot system, and it relies on its root system to regrow after each harvest. According to Rimi et al. (2014), harvesting alfalfa at early flowering stage allows an increase in its taproot when compared to alfalfa harvested at early bud; intensive harvesting frequencies decrease the number of plants per m^2, therefore decreasing stand density and persistence of alfalfa.

Persistence and productivity of alfalfa as much as other forages, is strongly related to management, being the plant regrowth depend on root carbohydrate reserves, besides the apical bud. The first alfalfa clipping or grazing must happen in the peak of the flowering, around 80% of flowering plants. The aim of this recommendation is to get great carbohydrate accumulation and develop the root system. The clipping recommendations are among 8 and 10 cm of stubble height to preserve the basal bud. It is recommended a daily grazing with rest period of 34 days to winter climates, and 28 days to summer and another seasons. The grazing rotation provide the necessary rest for recompositing reserves in the roots, resulting in vigorous regrowth and long-lasting and productive pastures (Pedreira et al.,2020).

Alfalfa re-sprouting is affected by reserve carbohydrates constituted in greater proportion by starch and to a lesser extent by glucose, fructose, and sucrose. Depending on the type of exploitation of the plant (cutting or grazing), this accumulation of reserves is continuous, since it is not interrupted in each production period of the plant (Rassini et al., 2008). On the other hand, the harvest method has a strong effect on plant performance.

2.3 Alfalfa and Bermuda mixtures

Legume integration into grass pasture might have several benefits, such as increased nutritive value and increased total forage mass of the stand. Alfalfa, such as another several legumes, has the ability of atmospheric dinitrogen (N_2) fixation, which is a process where N_2 is transformed into ammonia by rhizobium bacteria that infects the plant roots (Rodrigues et al., 2017; Vymazal, 2007). The rhizobium bacteria make a symbiotic process with the plant roots, colonizing the nodules. This association will provide all the nutrients and energy for the bacteria; and, in exchange, the bacteria provide N for the plant in an efficient way (Bauer, 2003; Atkins et al., 1984).

Grass-legume mixtures present benefits compared with grass monoculture because they often increase the total yields of herbage and protein and offer balanced nutrition (Albayrak and Ekiz, 2005). In addition, mixtures tend to provide a superior nutrient balance and produce greater forage yields. However, grass-legume mixtures are more difficult to manage than monoculture pastures because of competition among the mixture components for light, water, and nutrients (Berdahl et al., 2001; Albayrak and Ekiz, 2005). Mixtures have many advantages as control of soil erosion, weed control, and prolonged stand longevity (Casler, 1988). Among grass-legume mixtures, alfalfa is one of the most commonly used legumes for bermudagrass mixture to hay and pasture in tropical regions because of its high yield, high nutritional value, ability to fix nitrogen, and vigorous fall regrowth (Acikgoz, 2001).

The great dinitrogen fixation potential of alfalfa reduces the need for N fertilizers as urea or organic source as animal excreta. Consequently, it will decrease the cost of production and reduces environmental concerns, such as nitrogen losses through nitrate leaching, NH_3 volatilization or nitrous oxide emission (N_2O; Massey et al., 2011; Rech et al., 2017). Another benefit for legume-grass mixture utilization is because increase forage mass and contribute to a uniform distribution of mass production throughout the season by a complementary effect among species, especially N sharing (Waldron et al., 2017).

Another advantage to grass-legume mixture is when root and shoot turnover or bacterial decomposition occurs, N will be available for uptake by non-legume plants or non-nitrogen fixing plants that otherwise would not be able to access the atmospheric N pool (Pirhofer-Walzl et al., 2012), ensuring the benefits of these mixed systems. Besides BNF from alfalfa, their roots will be occupying different niches throughout the soil. These roots are deeper than grass roots and can better exploit the soil resources. Its roots can reach a depth of 5-6 feet, and up to 20 feet or more depending on the age of the plants (Weaver, 1926), reducing nutrient competition among species.

A study by Cinar and Hatipoglu (2014) arranged a duo (alfalfa + one grass) and trio (alfalfa + two grasses) mixtures of some warm season perennial grass species such as dallisgrass (*Paspalum dilatatum* Poir.), rhodes grass (*Chloris gayana* L.), and bermudagrass with alfalfa as well as their pure sowings under irrigated conditions of Mediterranean region of Turkey. According to the results reported by the author, the mixtures gave greater dry matter yield than pure sowing of the species. The greatest green (fresh) herbage yield (68.4 t ha^{-1}) was obtained from the mixture of alfalfa + rhodesgrass. The greatest DM yield (15.40 t ha^{-1}) was obtained from the mixture of alfalfa + bermudagrass + rhodesgrass. The greatest alfalfa proportion (50.0%) was obtained from the mixture of alfalfa + bermudagrass duo mixture of legume and dallisgrass or trio mixture of alfalfa. Thus, bermudagrass and rhodesgrass could be recommended to mix with alfalfa in the summer season.

Cinar and Hatipoglu (2015) tested a combination legume-grass mixture as alfalfa and bermudagrass, rhodesgrass, and dallisgrass. This study reported significant differences among mixtures and pure sowings in the forage quality characteristics. The highest crude protein yield and crude protein ratio was obtained from the mixture of dallisgrass + alfalfa, with average values of 2.32 t ha^{-1} and 206 g kg^{-1} of DM, respectively. The highest acid detergent fiber (ADF; 402 g kg^{-1}) and neutral detergent fiber (NDF; 709 g kg^{-1}) was obtained from the pure dallisgrass. The second great combination was alfalfa-bermudagrass with crude protein yield average of 2.24 t ha^{-1}.

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CHAPTER 2

Dung beetle assemblage affects greenhouse gas emission and nutrient cycling

ABSTRACT

Dung beetles are beneficial for the environment because they modify the soil ecosystem and may affect greenhouse gas (GHG) emissions, especially nitrous oxide (N_2O) from livestock excreta in grassland ecosystems. This research evaluated different species of dung beetles and their assemblages on GHG emission (N_2O), ammonia volatilization, and Pearl millet [*Pennisetum americanum* (L.) Leeke] performance. Three species of common dung beetles were used: *Onthophagus taurus* [Schreber] (1), *Digitonthophagus gazella* [Fabricius] (2), and *Phanaeus vindex* [MacLeay] (3). Treatments included isolated species and assemblages combining species 1+2 and 1+2+3. In addition, there were two controls: soil only and soil + dung without beetles. Experimental units were PVC buckets (terraria) containing 10 kg soil from grazing systems. Treatments were replicated three times in a randomized complete block design. Pearl millet was seeded to measure growth and nitrogen content following dung application and activity of dung beetles. There was an evaluation × treatment interaction ($P<0.05$) for N_2O flux, due to contrasting GHG emission initially but not at later measurements. Dung beetle species affected N_2O flux on dung increasing the fluxes in the 6th day (80 g N_2O-N ha^{-1} day^{-1}) compared to treatment with just soil and dung (2.6 g N_2O-N ha^{-1} day^{-1}). Nonetheless from day 12 to 24 there was a N_2O flux reduction, except to treatment with *P. vindex*, with the highest ($P<0.05$) fluxes in day 12 and 24 (117 g and 30 g N_2O-N ha^{-1} day^{-1}, respectively). Ammonia emissions from dung without and under dung beetle activity were different ($P<0.05$), and the treatment with *D. gazella* presented lesser NH_3-N since day 1, 6, and 12 with average of 2061, 1526, and 1048 g of NH_3-N ha^{-1} day^{-1}, respectively. *D. gazella* showed lesser ($P<0.05$) total nitrogen emission compared with the second control treatment (just dung). Assemblages combining species 1+2 were inefficient to remove dung comparing with *D. gazella* as single species. Dung application affected Pearl millet DM herbage accumulation (HA) regardless the presence of dung beetle, compared with treatments with just soil, with average of 8 g of DM $terraria^{-1}$ and 5 g of DM $terraria^{-1}$, respectively. A PCA analysis was used to understand the variation and correlation of each variable, which showed a low principal component explanation (less than 80%) not enough to explain the variation of the results. Dung beetles showed to have a beneficial effect on primary plant productivity by improving N cycling; however, some beetle assemblages increased N losses to the environment by denitrification.

Keywords: dung removal, nitrogen losses, plant growth, soil macrofauna.

1 INTRODUCTION

Several factors contribute to the intensification of the greenhouse effect, including some agriculture and livestock practices. Major agriculture gas emissions include methane (CH_4) from livestock enteric fermentation (Henry et al., 2015), fresh dung (Yamulki et al., 1999), and rice production (Lassey, 2007); nitrous oxide (N_2O) from N fertilizers (Foley et al., 2011) and livestock excreta (urine and dung). Jensen et al. (2012) mentioned that yearly GHG emissions from N fertilizer production and use are estimated to reach 300 Tg CO_2.

Dung beetles can reduce GHG emissions and this effect might be due to their activity aerating the soil, reducing organic matter, and relocating dung. These activities alter the interaction between deposited excreta and soil microbial communities (Slade et al., 2015). Dung beetles are from the *Scarabaeidae* family and exert a key role in nutrient cycling in both temperate and tropical agricultural grasslands (Gittings and Pokhrel et a., 2021). They may help mitigate GHG emissions (Piccini et al., 2017) and aid carbon sequestration, improving grass growth and soil fertility (Nichols, et al., 2008).

Different dung beetle taxa can modify gas fluxes to different extents (Fowler et al., 2020). Dung beetle taxa vary in their nesting strategies, and can be divided in dwellers, tunnelers, and rollers (Tonelli, 2021). These different nesting strategies may significantly affect ecological function, such as dung removal efficiency (Kaartinen et al., 2013). Beetle species have different forms to dig the soil, with contrasting diameter and sizes, creating different micro-environments that may affect GHG fluxes (Slade et al., 2016; Iwasa et al., 2015). Dung beetles may also contribute to improve nutrient cycling by increasing soil carbon transfer (Menéndez et al., 2016) and bacteria soil diversity (Kaleri et al., 2021).

Thus, the goal of this experiment was to quantify GHG emissions including ammonia volatilization and nitrous oxide from manure over time, and to assess forage productivity and N concentration following dung application and the activity of different dung beetle species assemblage in North Florida.

2 MATERIALS AND METHODS

2.1 Site description

This study occurred at the North Florida Research and Education Center, in Marianna, FL (30°46′35″N 85°14′17″W, 51 m above sea level). The trial was performed in 2019 and repeated in 2020.

The experiment was a greenhouse study, and the soil used was collected from pastures (rhizoma peanut and Argentine bahiagrass as main forages) and taken to the buckets (terraria). Soils at the experimental site were classified as Orangeburg loamy sand (fine-loamy-kaolinitic, thermic Typic Kandiudults), with a $pH_{water}$of 6.7, Mehlich-1-extratable P, K, Mg and Ca concentrations of 41, 59, 63, 368 mg kg^{-1}, respectively. Average of minimum and maximum daily temperature and relative humidity in the greenhouse for August and November of 2019 and 2020 were 11 and 33ºC, 81%; 10 and 35ºC, 77%, respectively.

2.2 Biological material

Three species most abundantly (Figure 1) caught, according previous research by Conover et al. (2019), classified as nesting tunnellers were used: *Onthophagus taurus* (Schreber, 1759), *Digitonthophagus gazella* (Fabricius, 1787) and *Phanaeus vindex* (Macleay, 1819). The dung beetles were collected on August 28th 2019 and August 24th 2020, using the standard cattle-dung-baited pitfall traps described in Bertone et al. (2005), located in grassland with grazing animals. A total of 18 traps were randomized in nine paddocks (two traps per paddock) and installed inside metal cages to avoid losing samples by cattle trampling, and the beetles were collected after 24 hours (see procedures in Appendix, Figure 1). The dung beetle number and total mass per treatment and mass average per specie are described in Table 1.

Table 1. Dung beetle treatments description

Treatment	***Ot***	***Dg***	***Pv***	**Total mass (g)**
1	-	-	-	-
2	-	-	-	-
3	43	-	-	1.72
4	-	30	-	1.75
5	-	-	7	1.82
6	25	13	-	1.76
7	9	6	4	1.75

Dung beetle specie	**Average mass (mg)**
Phaneus vindex (Pv)	261.15
Digitonthophagus gazella (Dg)	58.57
Onthophagus taurus (Ot)	40.12

The beetle collection happened 24 hours before starting the experiment in the greenhouse. After retrieving the beetles from the field traps, they were classified using a net, separated, and stored in a small glass bottle provided with a stopper linked to a mesh to keep the ventilation and maintain the beetle alive.

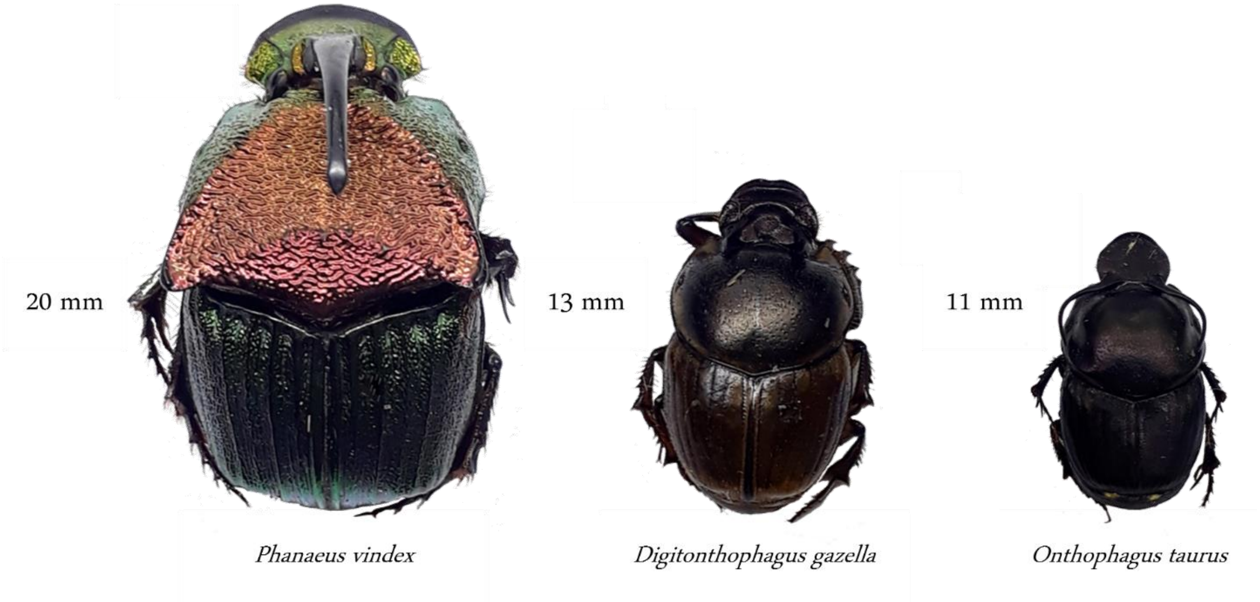

Figure 1. Most common dung beetle species in Marianna, FL, used in the experiment.

2.3 Treatments and Experimental design

Three species of common communal dung beetles were used: *Onthophagus taurus* [Shreber] (1), *Digitonthophagus gazella* [Fabricius] (2), and *Phanaeus vindex* [Macleay] (3). Treatments included isolated species and assemblages combining species 1+2 and 1+2+3. In addition, there were two controls: soil only and soil + dung without beetles. Therefore, a total of seven treatments were allocated in a randomized complete block design (RCBD), with three replications. To keep uniformity across treatments we kept beetle biomass constant across species at roughly 1.7 to 1.8 g per assemblage (Table 1). Treatment identification for the single species 1, 2, and 3 were named as T3, T4, and T5, combining species (1+2 and 1+2+3) as T6 and T7; and controls (soil and soil + dung) as T1 and T2, respectively. More details about the treatment distribution are in Appendix, Figure 2.

2.4 Terraria building

The soil used to build the terraria was from a grazing trial and collected in August (2019 and 2020) across three paddocks (0.9 ha each) with same management history. The terraria chamber area used in this experiment was 0.034 m^2. To build the terraria, 21 plastic buckets were used, each recipient receiving 10 kg of soil. At the bottom of the recipient, three holes were made for water drainage using a metallic mesh with 1 mm diameter above the surface of the holes to prevent dung beetles from escaping (more details in Appendix, Figure 3). The natural soil conditions were kept every four days using the field (i.e., bucket) capacity (60%) of soil adding water (measured with the soil weight and water holding capacity of the soil). Because soil from the three paddocks had a slightly different texture (sandy clay and sandy clay loam), we use them as blocking criterion.

The fresh dung amount used in the trial was determined using the average area covered by dung and the average dung weight (0.05 to 0.09 m^2 and 1.5 to 2.7 kg) from cattle in grazing systems, according to the method suggested by Carpinelli et al. (2020). Angus steers from grass pasture were brought to chute to fresh dung collection. Fresh dung was collected and stored in fridge 24 h prior to start the experiment. A total of 16.2 kg of fresh dung was collected, with 0.9 kg placed on each bucket, with the dung beetles were released thereafter. To prevent dung beetles from escaping by flying, a mobile plastic mesh with 0.5 mm diameter was used, removed, and placed before and after each evaluation. The experiment lasted for 24 days, with average temperature 28ºC and relative humidity of 79%.

2.5 Chamber measurements

The gas fluxes from terraria were evaluated using a closed static chamber. The chambers were circular, with a radius of 10.5 cm (0.034 m^2). Chamber bases and tops were made with polyvinyl chloride (PVC), and the tops were lined with an acrylic sheet to avoid any reactions of gases of interest with chamber material (Parkin and Venterea, 2010). The chamber tops were covered with reflective tape to provide insulation, and they were equipped with a rubber septum for sampling (Clough et al., 2012). The top was fitted with a 6-mm diameter, 10-cm length copper venting tube to ensure adequate air pressure inside the chamber during measurements, considering an average wind speed of 1.7 m s^{-1} (Hutchinson and Mosier, 1981; Hutchinson and Livingston, 2001). During measurements, chamber tops and bases were kept sealed by fitting bicycle tire inner tubes tightly over the area separating top and base. Bases of chambers were installed in the terraria's top to an 8-cm depth, with 5 cm extending above ground level. Bases were removed in the last evaluation day (more details in Appendix, Figure 4).

2.6 Gas flux measurement

The gas fluxes were measured at 10:00 am following sampling recommendations by Parkin and Venterea (2010), on seven occasions from August 28th to September 22nd 2019 and 2020 (days 0, 1, 2, 3, 6, 12, and 24). On each specific day, gas fluxes were measured in three simultaneously blocks, with each block encompassing one replicate of each treatment (T1, T2, T3, T4, T5, T6, and T7). The three blocks were initiated simultaneously. Samples were taken with a syringe of 60-mL capacity, per chamber, separated by 15-min intervals (t0, t15, and t30). The syringe content was immediately flushed into 30-mL glass vials equipped with a butyl rubber stopper sealed with an aluminium septum (this procedure was made twice per vial and per collection time). Time zero (t0) represented the gas collected out of the terraria (before closing the terraria with the chamber). After t0 collection, the top of the chamber was placed on the terraria with two more gas samples collected every 15 min (t15 and t30). The contents of N_2O were analysed by the Dumas dry combustion method (Vario Micro Cube; Elementar, Hanau, Germany) coupled to an isotope ratio mass spectrometer (IsoPrime 100, Manchester, UK).

Flux of N_2O-N (g ha^{-1} day^{-1}) was calculated as described in Equation 1:

$$F=A*dC/dt \tag{1}$$

where F is flux of N_2O (g ha^{-1} day^{-1}), A is the area of the chamber, and dC/dt is the change of concentration in time calculated using a linear method of integration by Venterea et al. (2009).

2.7 Ammonia volatilization measurement

Ammonia volatilization was measured with open chamber, based on work reported by Araújo et al. (2009). This chamber was assembled with a polyethylene terephthalate (PET) soda bottle 2-L capacity. The bottom of the bottle was removed and used as a cap above the top opening just to keep the environment controlled, without rain and insects. Fastened to the top of the wire support was a strip of polyfoam (250 mm in length, 25 mm wide, and 3 mm thick) that had been soaked in 20 ml of acid solution (H_2SO_4 1 mol dm^{-3} + glicerina 2% v/v), with the bottom end of the foam remaining inside the plastic jar. Plastic jars were used to carry the acid immersed foam strips from the experimental building to storage in the freezer until starting NH_3 sampling procedures. Inside each chamber there was a 250-mm long wire designed with a hook to support it from the top of the bottle, and wire basket at the bottom end to support a plastic jar (25 mL) that contained the acid solution to keep the foam strip moist during sampling periods (more details in Appendix, Figure 5). The chambers were installed in the surface middle bucket after the last flux gas measurement of the day and removed before to start the next evaluation day. The 86 (21 bottles per evaluation) total samples were collected.

2.8 Nutrient cycling

In the last day of gas flux measurement (one day after day 24 of gas measurements), seeds of Pearl millet forage (*Pennisetum glaucum* cv. 'Tifleaf III') were planted in each treatment and replication. After five days of seed germination, the plants were thinned, leaving four plants per bucket. Plant height evaluations started in September 24th to October 23th (first cut) and lasted until November 24th (second cut) of 2019 and 2020 (five weeks). In the last week of plant height measurements (day 30), all plants were harvested above 10 cm of ground level (see procedures in Appendix, Figure 6). Samples were dried at 55°C until constant weight then ball milled using a Mixer Mill MM400 (Retsch) at 25 Hz for 9 min, thereafter to start N and ^{15}N sampling procedures. The total N from the samples were measured by dry combustion method (Vario Micro Cube, Elementar) coupled to an isotope ratio mass spectrometer (IsoPrime 100, IsoPrime).

2.9 Statistical analysis

Data were analyzed using Mixed Procedure of SAS (SAS Inst., Cary, NC) and LSMEANS compared using PDIFF adjusted by T Student ($P < 0.05$), with period of evaluation and harvest as repeated measures. Block and year were considered as random effect and treatments fixed effect. Orthogonal contrasts were used to test the effect of dung absence (contrast Soil *vs.* Soil + Dung), the effect of dung beetle (contrast Soil + Dung *vs.* Soil + Dung + Dung beetle species) and the effect of each species and their combinations (contrast Species 1 *vs.* 2, 3, 1+2, 1+2+3) on greenhouse gases and nutrient cycling. A principal component analysis (PCA) was used to achieve a better understanding on the effect of dung beetle treatments.

3 RESULTS AND DISCUSSION

3.1 Nitrous oxide flux

There was an evaluation × treatment interaction on fluxes of N_2O ($P < 0.05$), with average emissions ranging from 2 g N_2O-N ha^{-1}day^{-1} to all treatments in day 0 (after feces and dung beetle application) and significantly greater peak with 46 g N_2O-N ha^{-1}day^{-1} and 80 g N_2O-N ha^{-1}day^{-1} on day 6 to terraria with just dung and terraria with dung + dung beetle species, respectively (Figure 2). The fluxes of N_2O from terraria with dung beetle species were the greatest and differed significantly to terraria with dung and terraria with just soil over time ($P<0.001$), except in day 2, when N_2O flux was greater to just dung than dung + beetles.

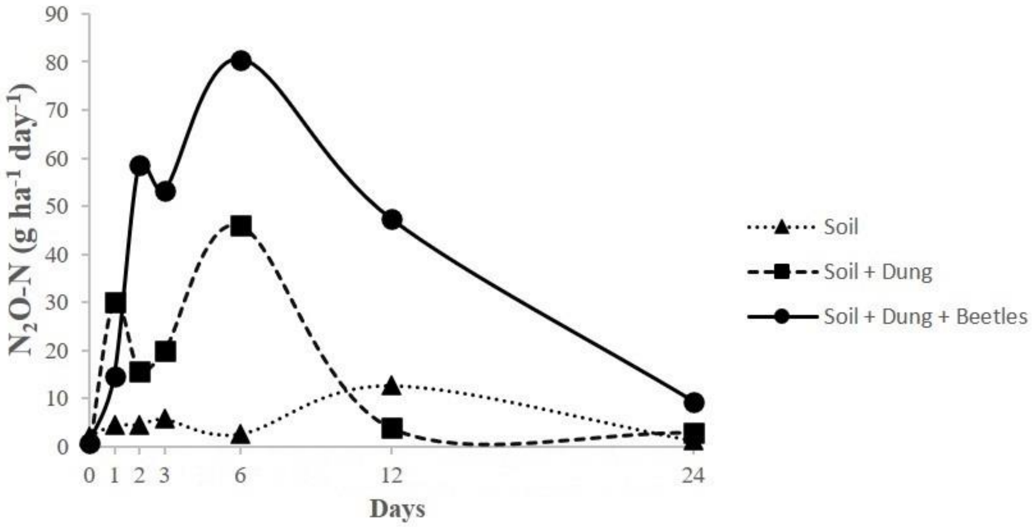

Figure 2. Nitrous oxide flux comparing the two control treatments and dung beetle treatments.

Fluxes of N_2O were relatively high over time to terraria with dung + beetles and were greater than the ones observed for soil and soil + dung ($P<0.0001$). However, *O. taurus* and *D. gazella* had the least N_2O flux compared with other beetle treatments over time (Figure 3). The N_2O flux from the terraria with dung (T2) increased over time, however, since day 6 the fluxes decreased considerably until day 24, and the values ranged from 45 g N_2O-N ha^{-1}day^{-1} to 2.9 g N_2O-N ha^{-1}day^{-1}, respectively. The treatment 3 (*O. taurus*) and treatment 4 (*D.* gazella) showed the most depleted N_2O-N among the dung beetle treatments in days 0, 1, and 12, with averages of -3, 12.3, 25.8 g N_2O-N ha^{-1}day^{-1} and -1, 17.5, 23.5 g N_2O-N ha^{-1}day^{-1}, respectively. Treatment 7 (*O. taurus + D. gazella + P. vindex*) presented the greatest pick of N_2O-N in day 6 with average of 145.7 g N_2O-N ha^{-1}day^{-1}; however, in day 12 decreased considerable, not differing ($P>0.05$) from T3, and T4 Figure 3). Treatment 1 (control terraria with just soil) had the least N_2O flux and didn't show significant variation over time. Treatment 5 showed a progressive increase over time, with the greatest pick of N_2O-N in day 12 and day 24.

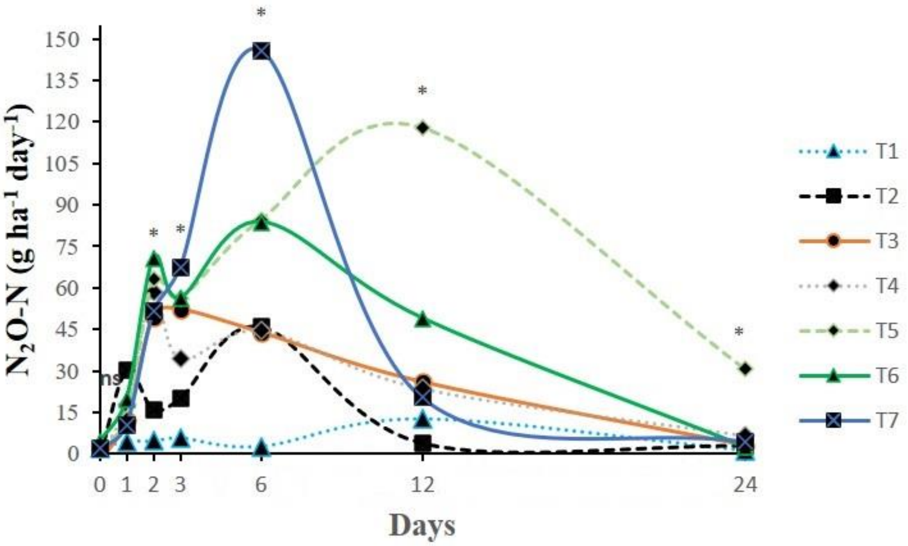

Figure 3. Nitrous oxide flux over time from livestock dung under contrasting dung beetle assemblage. T1: just soil, T2: soil + dung, T3: soil + dung + *O. taurus* (OT), T4: soil + dung + *D. gazella* (DG), T5: soil + dung + *P. vindex* (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV. (*): Indicates significant difference at the 0.05 probability level among treatments in the same month, according to orthogonal contrast test.

Our results indicate that dung beetle presence on dung from livestock increased the N_2O-N fluxes over time, despite some beetles as *O. taurus and D. gazella*, showed low N fluxes in day 0, 1, and 12 (Figure 3) regarding to *P. vindex* and other beetle assemblage. Previous studies have revealed an increase of dung beetles on fluxes of nitrous oxide from cow dung. Penttila et al. (2013) observed episodic high flux on days 15, 20, and 30, with 17, 110, and 40 g N_2O-N ha^{-1}day^{-1} in dung pats with dung beetles, respectively, differing with our results, in the days of the emission events, and lower values than the ones found in this study (Figure 3). This might have occurred because of the N_2O dynamics during denitrification, is particularly related to soil depth, labile organic carbon © and microbial biomass C (Hu et al., 2020). Dung beetles have been suggested to increase NO_3^- levels by aerating the substrate, a process leading to more N_2O being released from denitrification (Maldonado et al., 2019), possibly by providing optimal conditions for denitrifying bacteria to function in their gut as the earthworms (Lubbers et al., 2013). In our study, dung beetle activity increased N_2O-N flux by 71% and 79% when compared to terraria with dung and terraria with just soil in day 2, respectively. The increase occurred mainly on days 1, 2, and 6 (Figures 2 and 3).

Even though our experimental design was based on the same total beetle biomass in each experimental unit, the single species *P. vindex* had a tendency ($P<0.05$) to release more N_2O-N than the other single species (Figure 3), even when this species was included in other assemblages as in treatment 7. A possible explanation for this response is the fact that the species *P. vindex* has its life time longer (can live over a year; Paris et al., 2013) than the other species. Furthermore, *P. vindex* presents in its gut 24% of bacteria that belong to Enterecoccace72amilyliy (Winfrey and Sheldon, 2021). Some bacteria belonging to this family could contribute to denitrifying process as *Enterococcus casseliflavus* (Heylen et al., 2006). These are the likely reasons why the N_2O fluxes were greater in terrarias with *P. vindex*. Evans et al. (2019) indicated that dung beetle affects N_2O flux during the late summer season by modification of the moisture-dependent gas transport processes. The least value of N_2O from all treatments with dung in the first day of evaluation (Figure 3) might be related to the fact that organic N needs to go through several processes before producing N_2O (Lazicki et al., 2019).

3.2 Ammonia volatilization

There was an evaluation × treatment interaction ($P<0.05$) on ammonia emission, which varied from a maximum of 6431 g NH_3-N ha^{-1} for T6 in day 2, decreasing over time, showing the least value in day 24, with average of 241 g NH_3-N ha^{-1}. The treatment T3 and T4 presented the least values with 1536 and 1575 g of NH_3-N ha^{-1}, respectively, when compared to other beetle treatments and T2 (Figure 4). The T4 presented the most depleted NH_3-N emission from day 6, 12, and 24 with average of 1526, 1048, and 245 g of NH_3-N ha^{-1} when compared to T2 and to other beetle treatments. The T5 showed a peak on day 6, which was greater ($P<0.05$) than T1, T2, T3, and T4. The T1 presented the least NH_3-N emission and it did not significantly vary over time (Figure 4).

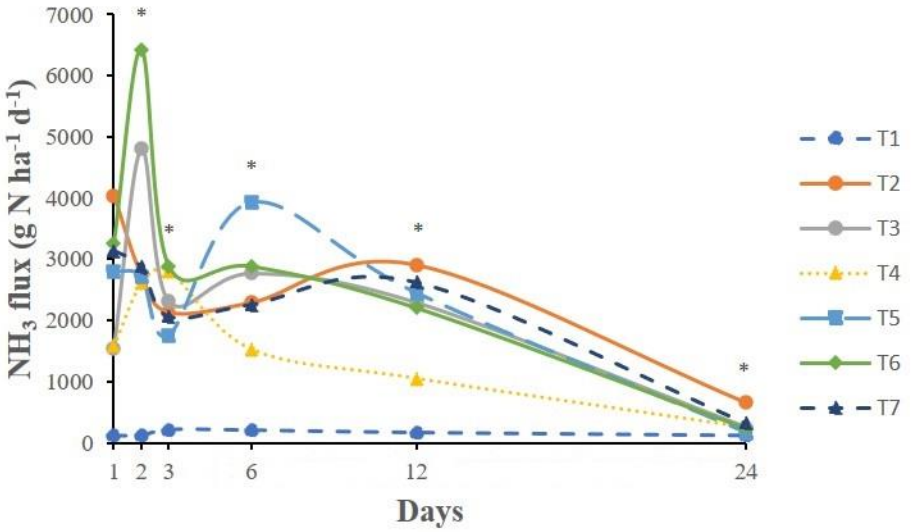

Figure 4. Ammonia flux over time from livestock dung under dung beetle assemblage effect.
T1: just soil, T2: soil + dung, T3: soil + dung + *O. taurus* (OT), T4: soil + dung + *D. gazella* (DG), T5: soil + dung + *P. vindex* (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV. (*): Indicates significant difference at the 0.05 probability level among treatments in the same month, according to orthogonal contrast test.

The NH_3-N emission is dependent on soil pH, moisture, texture, CEC, and soil temperature, as well as on the wind speed and air temperature (Bolan et al., 2004). In this experiment, N_2O-N and NH_3-N showed the highest peak with the presence of dung beetles. Soil temperature and humidity averaged 27ºC, 37% in terraria without dung nor beetles, and 26ºC, 78% to terraria with dung + dung plus beetles, respectively (See Appendix, Table 1). An explanation for the lesser NH_3 emission in terraria with just dung as compared to terraria with dung + beetle is the formation of a superficial crust on the dung. This crust can act as a physical barrier to the wind, preventing NH_3 emission. Furthermore, NH_3 tends to diffuse between fecal matter making soil surface contact, in which it will be converted into NH_4^+, making NH_3 emission even more difficult (Mulvaney et al., 2008). Dung beetle buried the dung into the soil in the terraria (Figure 6), modifying the soil and soil surface structure, accelerating the nitrogen mineralization process (González-Chang & Reyes, 2016).

3.3 Nutrient cycling

At the end of the experiment the dung removal efficiency wasn't statistically analyzed. However, according to a visual observation, the terraria with just dung didn't show any changes over time, losing humidity and getting a superficial crust (Figure 5). On the other hand, the dung beetle species on the terraria surface buried almost all the dung into the soil as much as the single and assemblage species. Furthermore, according to de Castro-Arrazola et al. (2020), smaller dung beetles remove more dung than bigger beetles, due to shorter legs and head that helps to bury and make holes. That fact makes sense with our study due to the dung removal observed in terrarias with D. gazella (DG), (Figure 5) it was able to remove and bury all dung on the soil surface.

O. taurus, despite being the smallest beetle in the trial, did not remove dung as *D. gazella*. Furthermore, when it grouped with *D. gazella*, the dung removal apparently was reduced considerably (Figure 5). Because *D. gazella* is a beetle species with strong invasion capacity, it might have impacted negatively the abundance and nesting behavior of other beetle species. As a result, there was inefficient dung removal in this group for intense intraspecific competition for dung (Filho et al., 2017).

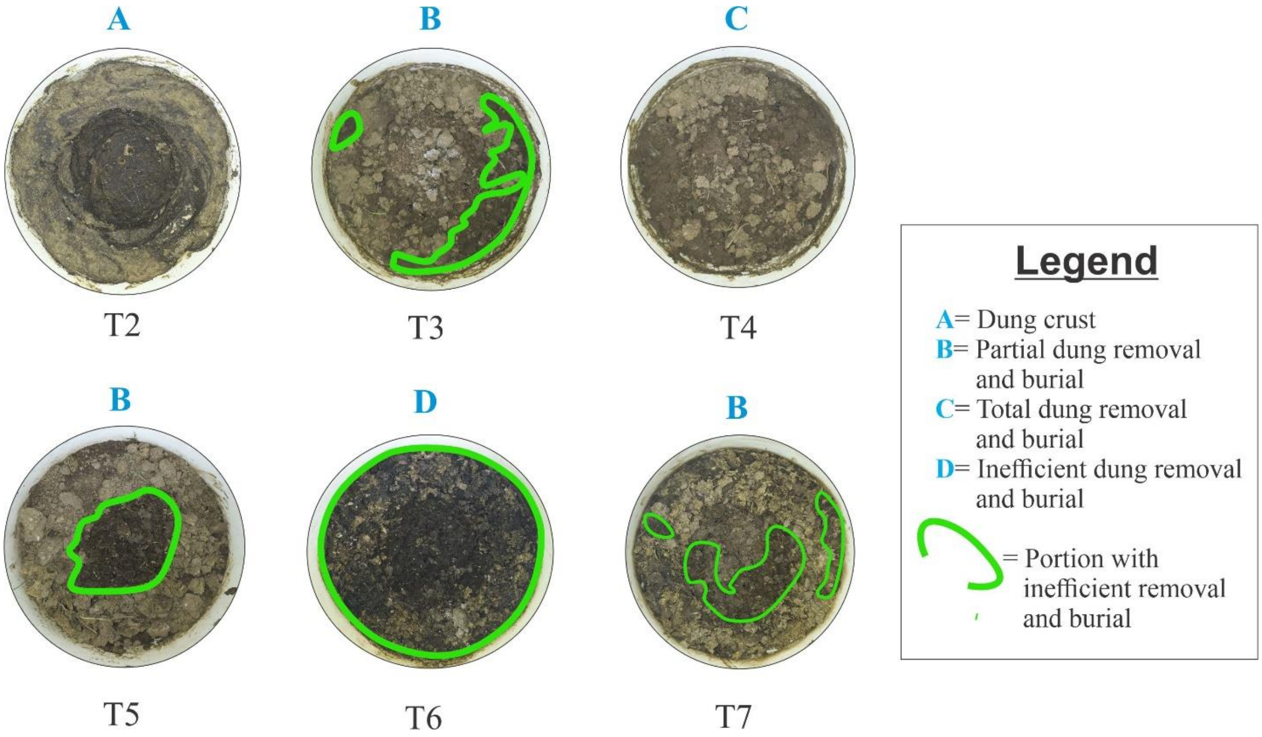

Figure 5. Dung burial without the presence of dung beetles and with different dung beetle assemblage.
T2: soil + dung, T3: soil + dung + *O. taurus* (OT), T4: soil + dung + *D. gazella* (DG), T5: soil + dung + *P. vindex* (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV.

There was a treatment effect ($P<0.05$) on soil nitrogen content. The two control treatments (soil and soil + dung) didn't differ ($P>0.05$) among them. The soil from terraria with dung + beetle application (T3, T4, T5, T6 and T7) presented greater nitrogen content compared with T1 and T2 (Figure 6). This result can be explained to the fact that 85 to 95% of nitrogen consumed by cattle returns to the soil via excreta (dung and urine; Ledgard, 1991). This nitrogen could be buried and mineralized in short period by dung beetle activity and break nitrogen and phosphorus available to the plant (González-Chang & Reyes, 2016). Maldonado et al. (2019) did an experiment at a cattle field in Mendoza Province, Argentine, with four dung beetle species (*Sulcophanaeus imperator*, *Eucranium arachnoides*, *Digitonthophagus gazella* and *Malagoniella puncticollis*) and their effect on nutrient cycling. All dung beetle species increased soil fertility, but *Digitonthophagus gazella* was more efficient, increasing soil organic matter by 159 g in 600 m^2 (equivalent to 2647 kg ha^{-1}).

Perhaps a similar effect occurred in our experiment in the terrarias with beetles. Although we did not measure organic matter content, dung beetles removed and buried the dung (Figure 5) from the soil surface. This could promote the action of soil microbial respiration and affect the decomposition rate of soil organic matter (Cheng et al., 2022).

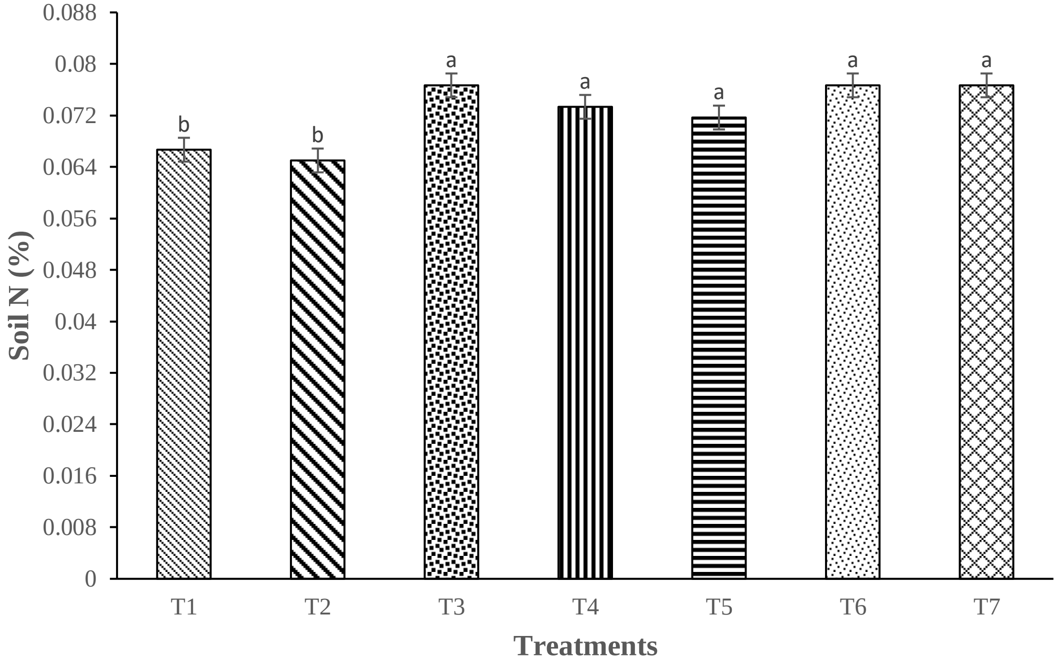

Figure 6. Effect of feces and dung beetle assemblage on soil nitrogen content.
T1: just soil, T2: soil + dung, T3: soil + dung + O. taurus (OT), T4: soil + dung + D. gazella (DG), T5: soil + dung + P. vindex (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV. Different letters in lowercase indicate statistically significant differences among treatments, according to student test.

All treatments with dung beetle species resulted in taller Pear millet than just dung on the terraria surface, with both groups differing significantly (p<0.10). Millet plants under dung beetle effect had 41.8 cm, significantly greater than millet plants with just dung and no beetle, showing 39.9 cm (Figure 7). Greater soil nitrogen availability (Figure 6) in the presence of dung beetles affect nitrogen mineralization, producing nitrate from the dung applied, and making it available for plant uptake and growth.

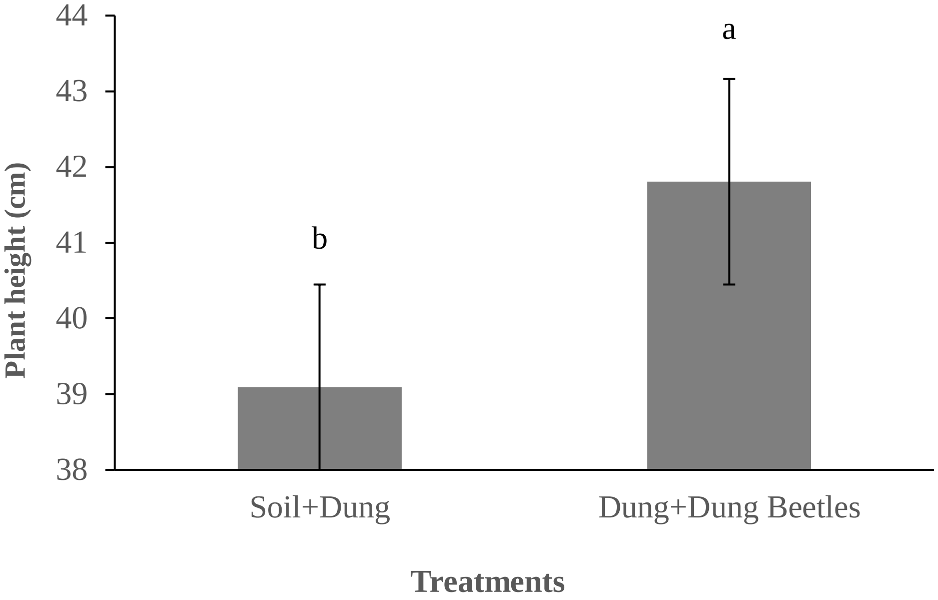

Figure 7. Pearl millet height in the presence or not of dung beetles.
*Different letters in lowercase indicate statistically significant differences at the 0.05 probability level among treatments in the same month, according to orthogonal contrast test.

There was a harvest × treatment interaction ($P<0.05$) on herbage accumulation (HA) of Pear millet. Dung application had a positive effect on the HA of Pear millet. The greatest HA was observed in all treatments with dung application in the first harvest with average of 8 g of DM terraria^{-1} (terraria area of 0.32 m^{2}), greater ($P<0.05$) than T1 (control with just soil), which averaged 5 g of DM terraria^{-1}. In the second harvest, treatments did not differ among them, presenting the least DM values (Figure 8). Dung has N and other nutrients required for plant development, increasing tillering and forage mass (Silva et al., 2005; Castro et al., 2016). Badenhorst et al. (2018) in a study with mixed pastures (*Eragrotis tef* Trotter, *Chloris gayana* Kunth, and *Digitaria eriantha* Steud) with cattle dung and dung beetles. They observed that plant average biomass yield was 120 g DM m^{-2} and 200 g DM m^{-2} in the first and six months of evaluation to treatments with dung beetles [(*Euoniticellus intermedius* (Reiche, 1849), *Digitonthophagus gazella* (Fabricius, 1787), and *Onitis alexis* (Klug, 1835)] respectively, greater ($P<0.05$) than the treatment without beetles with 50 g DM m^{-2} and 110 g DM m^{-2} in the first and sixth month of evaluation, respectively, which differs from the results of this current experiment when treatment with just dung and dung + beetles didn’t differ from each other. The low HA in the second harvest for all treatments might have happened because of the low regrowth capacity of Pear millet due to the frequent harvest (each 30 days) and for this cultivar is recommend each 45 days or 90 days (Guretzky et al., 2020).

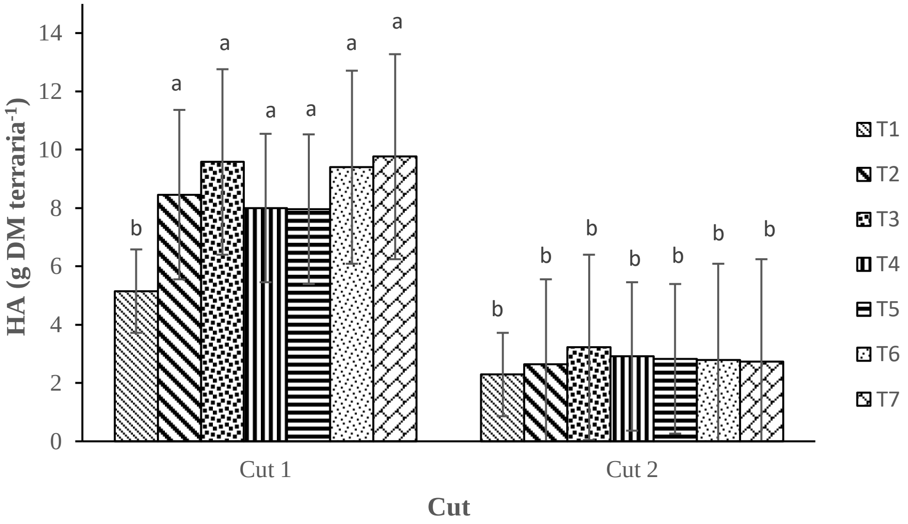

Figure 8. Pearl millet herbage accumulation (HA) comparing the two controls and different dung beetle effect. T1: just soil, T2: soil + dung, T3: soil + dung + O. taurus (OT), T4: soil + dung + D. gazella (DG), T5: soil + dung + P. vindex (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV. Different letters in lowercase indicate statistically significant differences among treatments, according to orthogonal contrast.

There was a treatment effect ($P<0.05$) on nitrogen yield (Figure 9). Treatments T3, T6, and T7 were greater than T1, T2, T4, and T5. The T3, T6, and T7 also had more soil N available, with average of 0.34, 0.30, and 0.31 g N terraria^{-1}, respectively. These results indicate that dung beetle activity as *O. taurus* and their mixture with other beetles might accelerate the nitrogen mineralization coming from the applied dung. According to Badenhorst et al. (2018), nutrient concentration in the vegetation increases significantly when dung beetles were active on a site. In this experiment, the activity of dung beetles did not have a significant effect ($P>0.05$) on millet nitrogen content for any of the treatments, with average of 1.65%. However, the N yield (Ny) was strongly related with dry matter accumulation (DMA), which means that Ny in this experiment corresponded to timing when the harvest occurred. An increasing trend (but not statistically significant; *P*=0.2706) was seen to *O. taurus*, OT+DG, and OT+DG+PV in the first harvest on the HA of Pearl millet (Figure 8). This could be explained by the relationship between N uptake and soil pH, whereby N needs to be mineralized to inorganic N for plant uptake and this process will be reduced if the pH of the soil is low (< 5.5; Mengel and Kirkby, 2001).

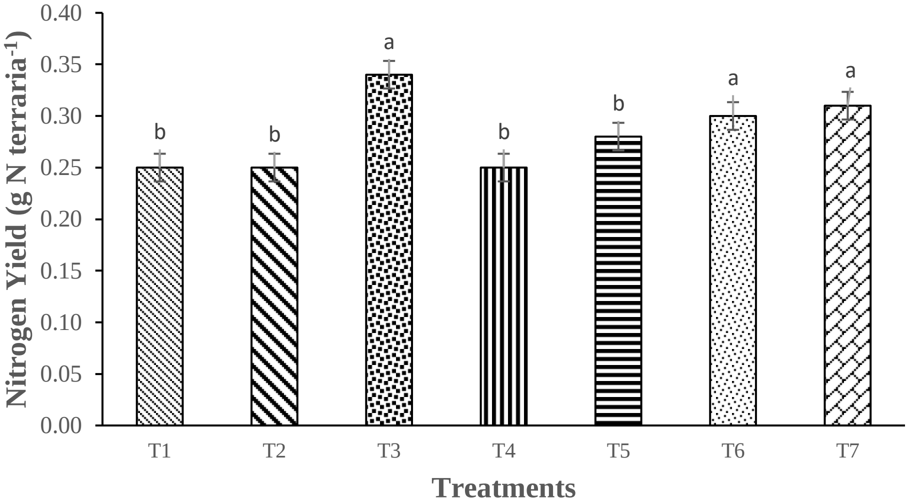

Figure 9. Pearl millet nitrogen yield comparing the dung and dung beetle effect.
T1: just soil, T2: soil + dung, T3: soil + dung + O. taurus (OT), T4: soil + dung + D. gazella (DG), T5: soil + dung + P. vindex (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV. Different letters in lowercase indicate statistically significant differences among treatments, according to orthogonal contrast test.

In this experiment, we hypothesized that dung beetle increased the N_2O-N and NH_3-N volatilization because of faster dung N mineralization in the soil as a result of dung incorporation and breakdown of dung pat crust, enhancing availability of oxygen for nitrification to occur (height and HA; Figure 7 and 8).

On the other hand, to simplify the response of the treatments on this experiment regarding gas emissions and nutrient cycling variables, a principal component analysis was used (Figure 10). The first and second principal components (PC1, PC2) explained 29.5% and 21.4% of the variability in the data set, respectively. The PC1 provided the highest variation, whereas herbage accumulation (HA) had a positive correlation with plant height (PH). Treatment 3 (*O. taurus*) showed to relate more with soil nitrogen (SN) and nitrogen yield (NY). Treatment 5 (*P. vindex*) had a positive association with nitrous oxide emission (N_2O), as well as Treatment 2 and other beetle treatments, but with a wider variation, which was confirmed in the higher fluxes of N_2O when *P. vindex* was present (Figure 3). Treatment 1 have no dung nor beetles, which may be the reason why it had a negative correlation with all the variables. From all beetle treatments, Treatment 4 is least associated with N_2O when compared with the other ones. Both PC explained 50.9% of the variability in the data set, which means that the percentage value is not enough to provide a better description, since, according to Cruz et al. (2012), the accumulated of both PC must be higher than 80% to account for the variability manifested among the characteristics, in order to get a better interpretation of the response of each variable.

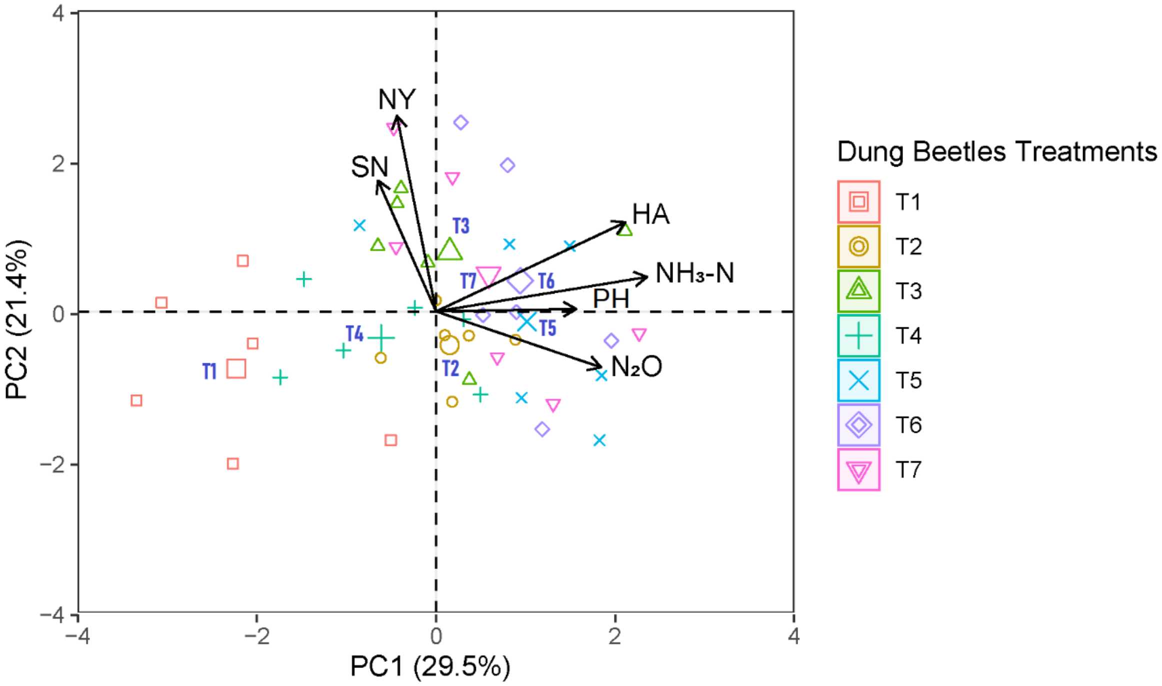

Figure 10. Principal component analysis of nitrogen emissions and nutrient cycling variables of dung beetle experiment in NFREC, Marianna, FL. T1: just soil, T2: soil + dung, T3: soil + dung + *O. taurus* (OT), T4: soil + dung + *D. gazella* (DG), T5: soil + dung + *P. vindex* (PV), T6: soil + dung + OT+DG, T7: soil + dung + OT+DG+PV. NY: nitrogen yield, SN: soil nitrogen, HA: herbage accumulation of pear millet, NH_3-N: ammonia volatilization, PH: plant height, N_2O: nitrous oxide emission.

4 CONCLUSIONS

The dung beetle presence in cattle dung from grassland systems tested in this experiment provided some ecosystem services, such as an improvement on the nitrogen concentration in the soil, giving the plants more nitrogen to grow and accumulate as forage mass, whereas dung removal, in contrast, provided some disservices as well, such as an increase in the nitrogen nitrous oxide emission and ammonia volatilization from cattle dung. Among the single dung beetle combination, the species *D. gazella* tended to reduce the total nitrogen lost of N_2O and NH_3 from cattle dung and was more efficient in removing and burying dung from the soil surface, standing out as a recommended selection in future nutrient cycling and environment changes research. On the other hand, the biggest one, *P. vindex* and the species combined to it, needs to be further studied to get a better understanding regarding its contribution on greenhouse gases.

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CHAPTER 3

Agronomic characteristics, nitrogen yield, and nutritional value of bermudagrass cultivars

ABSTRACT

Bermudagrass [*Cynodon dactylon* (L.) Pers.] is a major feed source for livestock in the southeastern USA; however, there is limited information about the characterization on new genotypes. The aim of this study was to measure productive and nutritive traits of new bermudagrass genotypes in the growing season. Treatments were 10 bermudagrass genotypes (‘Missouri’, ‘Tifton 85’, ‘Jiggs’, ‘FL44’, ‘322’, ‘323’, ‘276’, ‘282’, ‘283’, and ‘286’), and they were grown in North Florida, in 2018 and 2019. Treatments were distributed in a randomized complete block design with four replicates. Plots were harvested every five weeks (five harvests per year) and evaluated for herbage accumulation (HA), total herbage accumulation (THA), crude protein (CP), nitrogen yield (Ny), total Ny (TNy) and *in vitro* digestible organic matter (IVDOM) concentration. There was genotype × evaluation interaction ($P<0.05$) for HA, CP, and Ny. The 10 bermudagrass genotypes showed significant variation for all characteristics, being 286 more productive ($P<0.05$) than Jiggs in the first evaluation with 4427 kg DM ha^{-1} and 3245 kg DM ha^{-1}, respectively. 323 had greater ($P<0.05$) CP in the fifth evaluation than 283, 286, 322 and Tifton 85 with average CP of 155, 128, 136, and 137 g kg^{-1} of DM, respectively. All genotypes had greater Ny in the first evaluation than in the last, with 323 and 286 having greater Ny than Missouri and Jiggs 93 and 92 kg N ha^{-1}, 61 and 62 kg N ha^{-1}, respectively. Average IVDOM for 323 (450 g kg^{-1} of DM) was greater than Missouri with 393 g kg^{-1} of DM ($P<0.05$), however, 323 was similar to Tifton 85 and 322. Several cultivars in this study showed different responses for all traits and different evaluation dates. Missouri was the genotype that presented lower association with the productive and nutritive traits than other genotypes, according to the principal component analysis (PCA). Genotype 286 tended to produce more forage, with great protein, and great Ny. Therefore, 286 should be considered for public cultivar release in the United States as forage alternative and nitrogen use efficiency.

Keywords: genotype, nitrogen removal, production, selection, warm-season grass.

1 INTRODUCTION

Cynodon species are the most important perennial warm-season grasses for livestock production and has been adopted as the preferred forage for many livestock and hay producers in the southeastern USA (Anderson et al., 2021) and are geographically widely distributed around the world (Iqbal et al., 2022). Bermudagrass species can be used for grazing (Sanchez et al., 2018), hay (Neres et al., 2021), or silage (Coblentz and Akins, 2018). There is no historical record pinpointing the date when *Cynodon* was introduced to the new world, but it may have occurred soon after discovery by Columbus in 1942 (Taliaferro et al.,2004). *Cynodon dactylon* and *C. nlemfuensis* are one of the C_4 perennial grasses used for grazing and hay production in warm climates (Mahomar, 2020). In the USA, bermudagrass covers approximately 15 million ha in southern states, and it is a major forage in Central and South America, tropical Africa, and the Caribbean (Sollenberger, 2008).

Most bermudagrass cultivars are well suited for livestock production, but the majority are not adapted to poorly drained soils and extreme environmental conditions (Aguiar et al., 2015). Most hybrid bermudagrass cultivars and genotypes are propagated by vegetative material, roots, and rhizomes (sprigs) or mature tops (Boeri et al., 2018). Many breeding programs developed Bermuda cultivars that were more productive than the southern common strains and capable of supplying highly nutritious and palatable forage during a greater portion of the year (Baxter et al., 2018). Bermuda cultivar evaluations are very important to get new alternatives to produce forage under different conditions and management. Vendramini et al. (2010) compared four bermudagrass genotypes including Jiggs, ‘Coastcross-2’, ‘Tifton-85’ (*Cynodon* spp.), and ‘Florakirk’, and reported that Jiggs had the greatest herbage accumulation among the bermudagrass cultivars during the summer in Florida. Mislevy et al. (2008) evaluated herbage accumulation and nutritive value of Jiggs and Tifton 85 using the mob stocking technique and reported greater herbage accumulation for Jiggs than Tifton 85 (13900 kg DM ha^{-1} and 11900 kg DM ha^{-1} respectively).

Other studies evaluating productive and nutritive value of Bermudagrass cultivars provided useful information about new forage alternatives, as 322 and 242 cultivar that present a great total herbage accumulation (THA), crude protein (CP), phosphorus (P), potassium (K), and *in vitro* true digestible dry matter (IVTDDM) with 34000 and 32000 kg DM ha^{-1}; 127 and 114 g kg^{-1}; 3 and 2.9 g kg^{-1}; 20 and 21 g kg^{-1}; 713 and 713 g kg^{-1} of DM, respectively (Mahomar, 2020). It has been observed that hybrid bermudagrass cultivars are usually more productive than seeded cultivars (Gomes, et al., 2019).

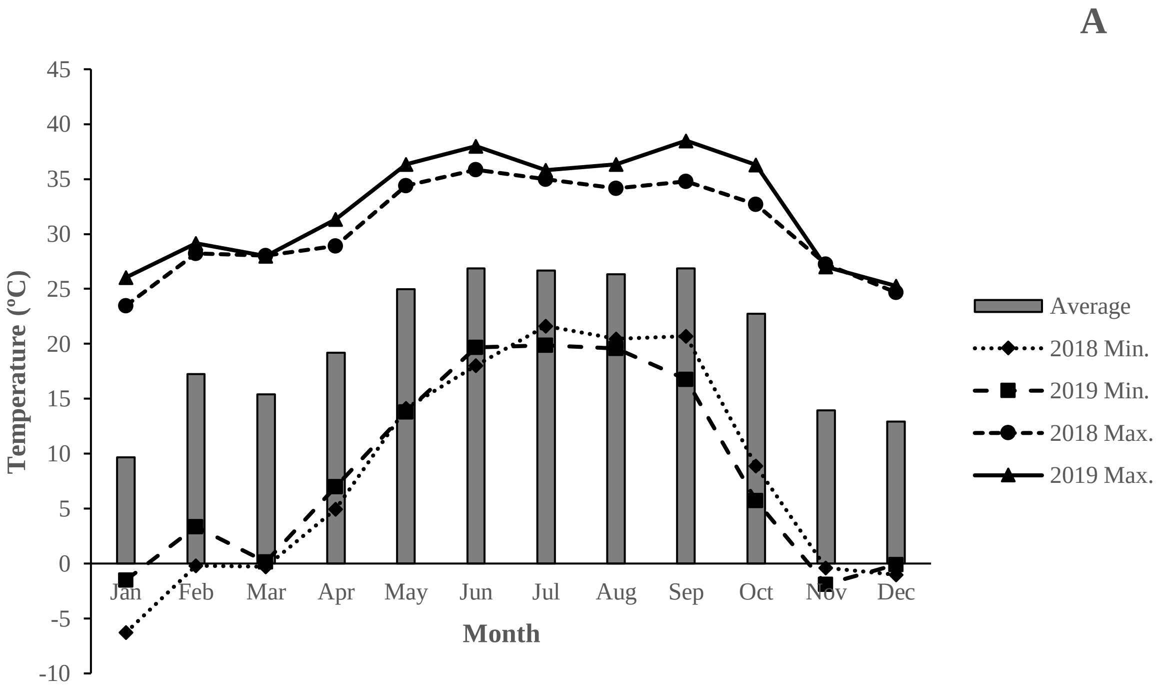
On the other hand, forages are used as a strategy to reduce over 50% the nitrogen losses by nitrate leaching to groundwater (Bryant et al., 2019). Grasses and legumes use or remove part of the nitrogen available in the soil and use it to produce forage mass and replace the nutrients used in their metabolism. That N removed by the plant from the soil and accumulated in the aboveground plant biomass is known as Nitrogen yield (Ny). This is an important characteristic to choose to keep the N balance in the forage production system and to reduce environmental N losses. For example, bermudagrass “Tifton 85” as monoculture could remove 25 kg N ha^{-1} or more per harvest (Santos et al., 2021).

There are few reports in the literature evaluating the bermudagrass genotypes as 322, 242, Tifton 85, Jiggs, and other entries. This research was conducted to evaluate the forage responses in the summer growing season in North Florida. Specific objectives were to evaluate the productive and nutritive value as well as N use efficiency of 10 bermudagrass genotypes from University of Florida breeding program.

2 MATERIALS AND METHODS

2.1 Site description

The experiment was conducted during the growing seasons of 2018 and 2019 at the University of Florida, Institute of Food and Agricultural Sciences, North Florida Research and Education Center, Marianna, FL (30°46′35″N and 85°14′17″W; 51 m above sea level). The soil at the experimental site was an Orangeburg loamy sand (fine-loamy, kaolinitic, thermic Typic Kandiudults) (USDA Soil Survey Staff, 2021). Bermudagrass varieties stolons were planted onsite using a drill planter (HEGE Equipment, 1000 Series) in May 2017. Minimum and maximum temperature, rainfall, and relative humidity during the growing seasons of 2018 and 2019 are given on Figure 1.



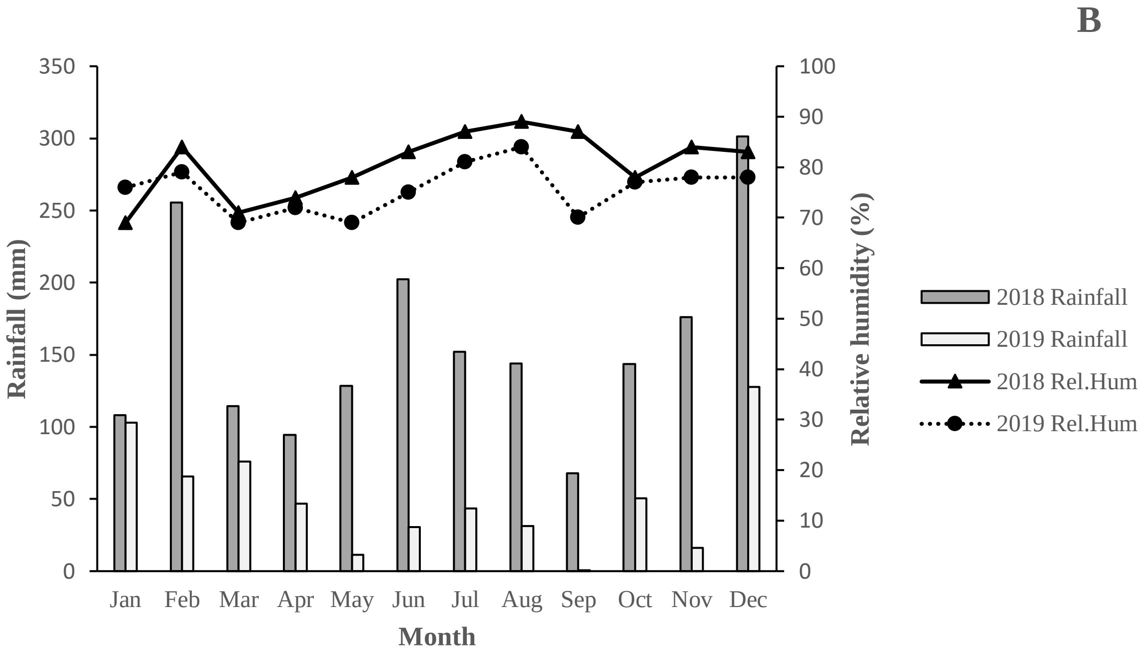


Figure 1. Historical monthly weather conditions at North Florida Research and Education Center (NFREC), Marianna, FL. A: minimum, maximum and average temperature, B: rainfall and relative humidity in 2018 and 2019.

2.2 Treatments and Management

Treatments consisted of 10 bermudagrass [*Cynodon dactylon* (L)] cultivars: FL44, Jiggs, Tifton 85, 322, 276, 282, 286, 283, 323, and Missouri. The experiment was conducted as a randomized complete block design with 10 treatments and four replicates (*n*=40). Each plot (experimental unit) measured 1 x 4.5 m with 2 m alleys between plots (see map in Appendix, Figure 7).

Planting material was obtained from the University of Florida forage breeding program (genotypes originated from different places; Table 1). The planting of seedlings was on 26 May 2017. Pendimethalin ($C_{13}H_{19}N_3O_4$), commercial product as Prowl H_2O, BASF Corporation, was applied 7 June 2017, 2 March 2018, 25 April 2019, and 14 April 2020, at 2.82 L ha^{-1}, 4.25 L ha^{-1}, 4.25 L ha^{-1}, and 3.52 L ha^{-1}, respectively, providing pre-emergent weed control early in each growing season. On 8 June 2017, 17 kg N ha^{-1}, 22 kg P ha^{-1} and 84 kg K ha^{-1} were applied 1 year before the first harvest (340 kg ha^{-1} of the commercial formula 5–15–30). The fertilization dates during the following years were 8 June, 13 August, and 18 September 2018; 29 April, 4 June, and 19 September 2019, 30 kg N ha^{-1}, 6 kg P ha^{-1} and 49 kg K ha^{-1} were applied (300 kg ha^{-1} of the commercial formula 20-5–20: see in Appendix, Figure 8).

Table 1. Bermudagrass genotypes origin and breeding information.

Genotype		Field code	Ploidy*	Origin
PI nº	Cultivar name			
-	-	Missouri	-	Missouri, United States
Jiggs	Jiggs	Jiggs	4	Texas, United States
Tifton 85	Tifton 85	Tifton 85	5	Georgia, United States
294467	-	276	4	Taiwan
295114	-	282	4	Zimbabwe, Africa
-	Callie	283	4	-
290813	-	286	4	Lesotho, South Africa
316510	Newell	322	4	Germany
316507	-	323	4	-
FL44	Florida 44	FL44	3	Florida, United States

Genotype: PI, plant introduction from the USDA Nation Plant Germplasm System, Germplasm Resources Information Network (NPGS-GRIN) database (Mahomar, 2020). (*) Ploidy Levels from Grossman et al. 2021. (-) Information no registered.

2.3 Sampling and measurements

Plots were harvested approximately every 5 weeks or five times each year. In 2018, plots were harvested on 1 June, 5 July, 9 August, 16 September, and 23 October. In 2019, harvests occurred on 30 May, 3 July, 7 August, 11 September, and 16 October. Since harvests did not happen in the same month in each year, we will name each harvest as first, second, third, fourth, and fifth evaluation in the Results and Discussion section. The harvest occurred at the central portion of the plot and the sample removed from the plot using a flail-style mower (Carter Manufacturing Company); plots were staged thereafter to a 15-cm stubble height and fertilized to start a new regrowth cycle (see procedures and timeline in Appendix, Figure 8 and 9).

2.4 Herbage Accumulation and Nutritive Value

At harvest, total fresh weight was determined, and two subsamples taken for determination of DM concentration and nutritive value. After harvesting, the first subsamples were dried in a forced-air oven at 55ºC for 72 h. After that, the dried samples were grounded to pass a 2-mm screen using a Wiley Mill (Model 4, Thomas-Wiley Laboratory Mill, Thomas Scientific). The dry mass was recorded to determine the herbage accumulation (HA) from each harvest. The total herbage accumulation (THA) was obtained by summing HA across all regrowth periods each year. To determine the *in vitro* digestible organic matter (IVDOM), the ground samples were used according to the procedure described by Moore and Mott (1974). A second subsample was taken from the 2-mm ground sample and ball milled in a Mixer Mill (MM 400, Retsch) at 25 Hz for 9 min. Ball milled samples were analyzed for N by dry combustion using an elemental analyzer (Vario Micro cube, Elementar). Crude protein (CP) was obtained by multiplying the N concentration by 6.25. The Nitrogen yield (Ny) was estimated by multiplying the nitrogen concentration of each bermudagrass cultivar times the HA, divided by 100, according he follow equation (1):

$$Ny\ (kg\ ha^{-1}) = \frac{Nitrogen\ concentration\ x\ Herbage\ accumulation}{100} \qquad (1)$$

2.5 Statistical analysis

Data were analyzed using Mixed Procedure of SAS (SAS Inst., Cary, NC) and LSMEANS were compared using PDIFF adjusted by Tukey ($P< 0.05$), with evaluation as repeated measure. Data was tested for normality for all response variables. Block and year were considered random effect; treatment was considered fixed effects. To simplify the results and get a better understanding about bermudagrass genotypes correlation in respect to their productive and nutritive value characteristics, we used a multivariate statistical procedure, principal component analysis (PCA).

Hierarchical clustering dendrograms were built using the unweighted pair group method with arithmetic mean (upgma) method (Rholf and Sokal, 1981). The PCA and dendrograms graphic were analyzed using R studio software (R Core Team, 2018).

3 RESULTS AND DISCUSSION

There were significant interactions between genotype × evaluation date for HA, CP, and Ny (Table 4). The cultivar and evaluations significantly affected bermudagrass performance. All the treatments did not differ among first- and second-year harvest.

3.1 Herbage accumulation

Missouri genotype was the least productive bermudagrass, with average HA ranging from 3561 kg ha^{-1} to 45 kg DM ha^{-1} in the first and last evaluation, respectively (Table 2). On average, cultivar 286 was more productive ($p<0.05$) than Jiggs in the first evaluation with 4427 kg DM ha^{-1} and 3245 kg DM ha^{-1}, respectively. From June to October, the genotypes 282, 276, 286, and 322 were more productive than other entries with 5673 kg DM ha^{-1}, 4697 kg DM ha^{-1}, 3829 kg DM ha^{-1}, and 1567 kg DM ha^{-1}, respectively. Genotype 286 was more productive in the first and fourth evaluations ($p<0.05$), but produced similar HA to other varieties, except Jiggs and Missouri in the first and fourth evaluations, respectively ($p<0.05$). In general, bermudagrass HA tended to decrease during the late warm-season period of the year (Table 2) due to decreasing of temperature and daylength (Figure 1).

Table 2. Herbage accumulation of bermudagrass genotypes over five evaluations at UF IFAS NFREC, Marianna, FL.

Genotype	**Evaluation**				
	Jun	Jul	Aug	Sep	Oct
	kg DM ha^{-1}				

FL44	4085Aab	3881 Ac	3436 Ac	2439 Bc	793 Cab
Jiggs	3245 Ab	4004 Ac	3945 Aabc	3317 Aabc	1013 Bab
Tifton85	3967 Aab	4472 Abc	4423 Aab	2752 Bbc	880 Cab
322	3749 Aab	4662 Abc	4308 Aabc	3772 Aa	1567 Ba
276	3699 BCab	5336 Aab	4697 ABa	3347 Cabc	1429 Da
282	3978 Bab	5673 Aa	3948 Babc	3493 Bab	1075 Cab
286	4428 ABa	5104 Aab	4263 ABabc	3829 Ba	1400 Ca
283	3900 ABab	4447 Abc	3446 BCbc	2500 Cc	849 Dab
323	4039 Aab	4418 Abc	3612 Abc	3359 Aabc	1529 Ba
Missouri	3562 Aab	1770 Bd	-	264 Cd	45 Db
Standard Error of the Mean 358					

Values followed by a different small letter within a column the treatments are significantly different at 5% probability level and values followed by a different upper letter within a line the evaluations are significantly different at 5% probability level by Tukey test.

Jiggs and genotype 322 had greater HA the first up to the fourth evaluation than the other genotypes. HA during summer and fall are impacted by variety, moisture, and timing of precipitation, temperature, available soil N, timing of N, and interaction of these factors (Habermann et al., 2018). All genotypes showed the least HA in the last evaluation of each year (October) due to low rainfall (Figure 1), reduced daylength, and changes in temperature. However, some cultivars including 322, 282, and 286 produced greater HA than Missouri in the fall. Cultivar 322 kept great HA during the fall, indicating extended production to lower temperature and shorter daylength than Missouri. This suggests that 322, 282, and 286 could be utilized to fill the forage gap during fall.

There was no difference among all genotypes, except for 322 and Missouri. Genotype 286 had greater Total HA - THA (18815 kg DM ha^{-1} yr^{-1}) than Missouri and 322, with 10458 kg DM ha^{-1}

yr^{-1} and 13939 kg DM ha^{-1} yr^{-1}, respectively (Figure 2). Recently 322 was released as cultivar named "Newell bermudagrass", reporting a predicted THA average of 10333 kg DM ha^{-1} yr^{-1} from three Florida locations (Rios et al., 2022), similar to THA of 322 in this study. Different environmental conditions (temperature, photoperiod, season, rainfall) and nutrient availability affected growth among these genotypes (Andrade et al., 2015). On average across 10 harvests, cultivar 286 reached 18815 kg DM ha^{-1} yr^{-1} while Missouri produced the least value with 10458 kg of DM ha^{-1} yr^{-1}. Greater HA were reported by Mahomar (2020) who evaluated different Bermudagrass cultivar under different management and fertility, as Tifton 85 with 27200 kg DM ha^{-1} yr^{-1} while 322 produced over 29300 kg of DM ha^{-1} yr^{-1}.

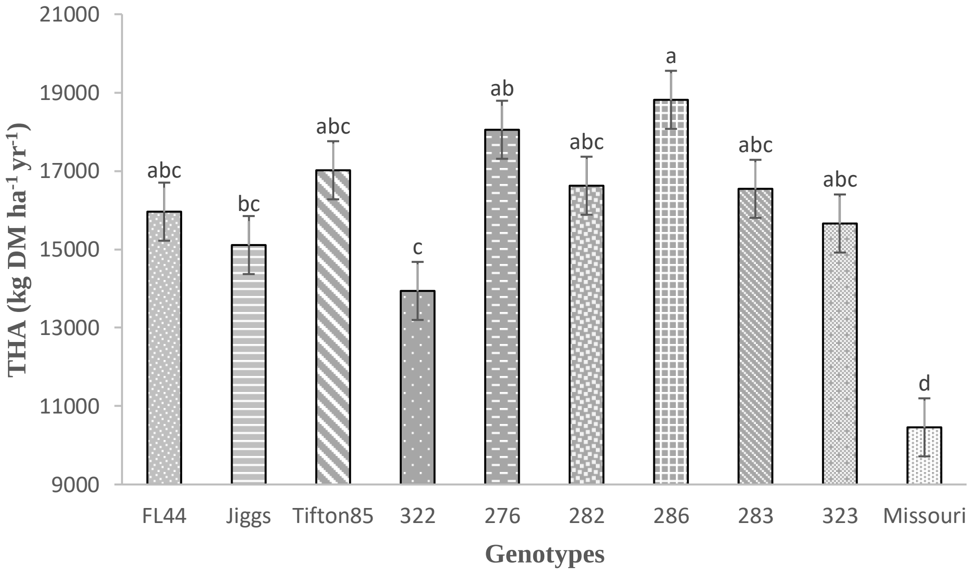


Figure 2. Total herbage accumulation (THA) of bermudagrass cultivars in NFREC, Marianna, FL.
Values followed by a different small letter between a column the treatments are significantly different at 5% probability level by Tukey test.

According to Peppers et al. (2021), bermudagrass growth may decline in low temperatures and reduced daylength and that is related to storage of reserve carbohydrates, which in winter season is important for survival and regrowth in the following summer. In our experiments, noticeable high HA was seen in summer for all bermudagrass cultivars, except for Jiggs in the first evaluation. Greater HA in the summer likely happened because of longer days (photosynthesis is extended; Mastalerczuk and Borawska-Jarmulowicz, 2021), mean temperature above 25°C (benefits transpiration and consequently nutrient absorption; Tubeileh et al., 2016), and rainfall (mass flow of nutrients and water; Quigley et al., 2020) ranging from 94 to 150 mm per month (Figure 1). These conditions seem to be adequate for bermudagrass productivity.

3.2 Nutritive value

There was genotype × evaluation interaction ($P<0.05$) for CP. The interaction occurs because all genotypes presented large variation of CP in each evaluation, with 323 having greater ($P<0.05$) CP than other genotypes in June, however, CP did not differ among cultivars in July and September, except for 286 being lower than other ones. Furthermore, 323 had greater ($P<0.05$) CP in the last evaluation than 283, 286, 322 and Tifton 85 with average CP of 155, 128, 136, and 137 g kg^{-1} of DM, respectively (Table 3). Missouri had greater CP ($P<0.05$) than 286, 282, 276 and Jiggs in August with average values of 125, 105, 108, 98, and 106 g kg^{-1} of DM, respectively. Tifton 85, 283, Jiggs, FL44, and Missouri had greater ($P<0.05$) CP than 286 and did not differ from 323, 282, 276, and 322 (Table 3). In general, all genotypes presented greater CP in June ($P<0.05$) and October.

Gomes et al. (2019) studied how the interaction season ×bermudagrass cultivars on CP, in FL. The results showed that Tifton 85 CP concentration decreased from spring to summer for all cultivars and it did not differ from summer to fall, with average CP of 214, 160, and 140 g kg^{-1} of DM, respectively. Those results differ from the CP values observed in this study, where Tifton 85 CP increased from September to October, with values of 122 and 134 g kg^{-1} of DM, respectively. Typically tropical grasses CP concentration increases after N fertilization and declines as growth progresses (Sigua et al., 2012), resulting in lesser CP with increasing regrowth period (Table 3).

Similar results were reported by Silva et al. (2015), who evaluated effects of three harvest frequencies (14, 28, and 42 d) on Tifton 85, Jiggs, and Vaquero, with 120, 123, and 145 g kg^{-1} of DM, respectively.

Silva et al. (2020) reported lower CP concentration of Jiggs and Tifton 85 when the harvest frequency increased from 3 to 6 weeks with average CP values of 140 and 115 g kg^{-1} of DM and 140 and 110 g kg^{-1} of DM, respectively for Jiggs and Tifton 85. These results indicate that CP concentration has been associated with harvest frequency or time of harvest. Arthington and Brown (2005) suggested that bermudagrass harvested over one month, it increase the maturity, causing a reduction of CP concentration. The CP values reported by the author were similar to this study with average of 115 g kg^{-1} of DM.

Table 3. Crude protein (CP) concentration of bermudagrass varieties at different evaluation dates in NFREC, Marianna, FL.

Genotype	**Evaluation**				
	Jun	Jul	Aug	Sep	Oct
	g kg^{-1} of DM				
FL44	118 Bbcd	116 Ba	111 Babc	124 Ba	147 Aab
Jiggs	119 BCbcd	121 Ba	106 Cbc	121 Ba	146 Aab
Tifton85	112 Bcd	116 Ba	113 Babc	122 ABa	134 Abc
322	128 ABb	118 Ba	117 Bab	114 Bab	137 Abc
276	124 Bbcd	112 BCa	98 Cc	112 BCab	141 Aabc
282	124 Bbcd	110 BCa	108 Cbc	113 BCab	141 Aabc
286	127 ABbc	111 BCa	105 Cbc	103 Cb	136 Abc
283	120 Abcd	116 Aa	113 Aabc	121 Aa	128 Ac

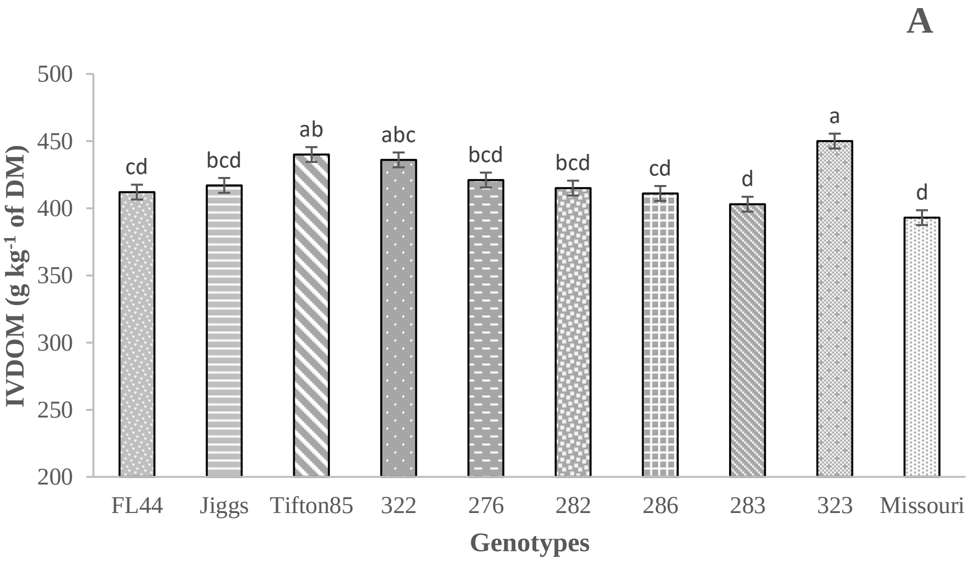
323	149 Aa	122 Ba	114 Babc	113 Bab	155 Aa
Missouri	109 Cd	125 Ba	125 Ba	128 Ba	149 Aab
Standard Error of the Mean 5.8					

Values followed by a different small letter within a column the treatments are significantly different at 5% probability level and values followed by a different upper letter within a line the evaluations are significantly different at 5% probability level by Tukey test.

There was effect ($P<0.05$) of the genotypes and evaluations on IVDOM (Figure 3). Genotype 323 had greater ($P<0.05$) IVDOM (450 g kg^{-1} of DM) than Jiggs, FL44, 276, 282, 286, 283, and Missouri, (417, 412, 421, 415, 411, 403, and 393 g kg^{-1} of DM, respectively). Tifton 85 and 322 did not differ from other cultivars (Figure 4). Missouri had the least ($P<0.05$) IVDOM compared with Tifton 85, 322, and 323 with values of 393, 440, and 450 g kg^{-1} of DM, respectively. The least ($P<0.05$) IVDOM was observed in October compared with August and September, with average of 392, 455, and 426 g kg^{-1} of DM, respectively (Figure 3). Similar IVDOM values were reported by Grossman et al. (2021) in phenotypic characterization of bermudagrass germplasm in North Florida, USA, where Tifton 85, Jiggs, 282, 286, 322, and 323 had IVDOM values of 674, 496, 424, 525, and 523 g kg^{-1} of DM, respectively.

In general, the IVDOM of bermudagrass cultivars decreased over time due to an increasing in fiber concentration (specially lignin). Greater nutritive value is likely due to the greater proportion of younger tissue with lesser cell wall concentration. Greatest bermudagrass IVDOM might happen if forages are harvested more frequent (Silva et al., 2015). In this study, the lesser IVDOM values occurred in the summer (Figure 1), likely due to greater lignin deposition and decrease in forage nutritive value (Wilson et al., 1976; Vendramini et al., 2021). Brandstetter et al. (2019) indicated that factors as temperature, moisture, and general cimate conditions affect the protein fraction A in the spring and summer, and the protein fraction C in bermudagrass increases in winter (protein fraction A refered to soluble and digestible protein and fraction C to non-digestible protein; Sunahara et al., 2018). Although we did not measure protein fractions, that afirmation could explain the variation of IVDOM among genotypes and over the five evaluations.

Sanchez et al. (2018) reported IVDOM values similar to the ones found in this experiment, when they evaluated the effect of overseeding Amarillo pintoi peanut (*Arachis pintoi* cv. Amarillo) into Jiggs bermudagrass pastures managed at different grazing intensities. The author indicated that Jiggs monoculture had IVDOM of 437 g kg^{-1} of DM, similar to our results, where Jiggs was 417 g kg^{-1} of DM. The literature suggests that Tifton 85 and Jiggs bermudagrass are similar in their physical makeup. They both contain large stems with big leaves (Dore, 2006). According to Hill et al. (2001), Tifton 85 has lesser concentrations of ether-linked ferulic acid in the cell wall compared to other bermudagrass cultivars, which explains the greater IVDOM. Vendramini et al. (2010) observed IVDOM values to Jiggs, Tifton 85, and Florakirk with average of 584, 639, and 580 g kg^{-1} of DM to, respectively, greater than genotypes of this study.



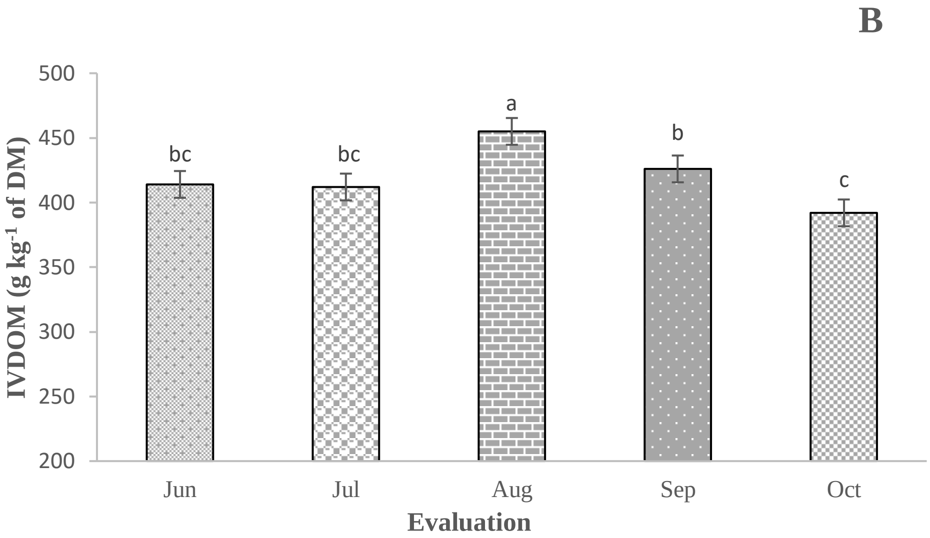


Figure 3. *In vitro* digestible organic matter (IVDOM) of bermudagrass varieties among genotypes and over five evaluations in NFREC, Marianna, FL. Values followed by a different small letter are significantly different at 5% probability level by Tukey test. Means are average of 10 bermudagrass and four blocks. A: IVDOM for genotypes, B: IVDOM for evaluation.

3.3 Nitrogen response

There was genotype × evaluation interaction ($P<0.05$) for Ny (Table 4). The interaction occurred because the degree of the response of genotypes in each evaluation was different for Ny (Table 2). Genotype 323 had greater ($P<0.05$) Ny than Jiggs, Tifton 85, 276, and Missouri, with values of 93, 62, 72, 73, and 61 kg N ha^{-1}, respectively. Missouri was the genotype that showed the least Ny values. In July, 282 had greater ($P<0.05$) Ny than Jiggs, FL44, and Missouri with average of 101, 78, 73, and 35 kg N ha^{-1}, respectively, and 282 did not differ from other genotypes in this period. From August to October there was a reduction in Ny for all genotypes (Table 4).

Even though the lesser Ny values started from August to October, in August, Tifton 85, 322 and Jiggs had greater ($P<0.05$) Ny than Missouri, with 80, 79, and 63 kg N ha^{-1}. Each genotype had different performance over evaluations; 322 had greater Ny ($P<0.05$) than FL44 and Missouri, and similar to other genotypes in September (Table 4). The least Ny values were observed in

October for all genotypes, with 323 having greater (*P*<0.05) Ny than FL44, 283, and Missouri, respectively. Total nitrogen yield (TNy) was estimated to assess how much N was removed from the system. Cultivar 322 had greater TNy (*P*<0.05) than 283, Jiggs, FL44, and Missouri, with values of 348, 286, 292, 273, and 104 kg N ha^{-1}, respectively, and Missouri had the least TNy (Table 4).

Table 4. Nitrogen yield per harvest (Ny) and Total nitrogen yield (TNy) of bermudagrass varieties x evaluations in NFREC, Marianna, FL.

Genotype	**Evaluation**					
	Jun	Jul	Aug	Sep	Oct	TNy
	kg N ha^{-1}					
FL44	76 Aabc	73 Ac	59 ABb	48 Bb	18 Cb	273 d
Jiggs	62 Ac	78 Abc	63 Aa	64 Aab	24 Bab	292 bcd
Tifton85	72 ABc	83 Aabc	80 Aa	54 Bab	19 Cab	307 abcd
322	78 Aabc	87 Aabc	79 Aa	68 Aa	35 Bab	348 a
276	73 Bbc	96 Aab	73 Aab	60 Bab	32 Cab	334 abc
282	79 Babc	101 Aa	67 Bab	63 Bab	23 Cab	333 abc
286	92 Aab	91 Aabc	68 Bab	55 Bab	31 Cab	337 abc
283	76 ABabc	83 Aabc	61 BCab	48 Cab	17 Dbc	286 cd
323	93 Aa	87 Aabc	64 Bab	61 Bab	37 Ca	343 ab
Missouri	61 Ac	35 Bd	4 Cc	5 Cc	2 Cc	104 e
Standard Error of the Mean 2.8						13.4

Values followed by a different small letter within a column the treatments are significantly different at 5% probability level and values followed by a different upper letter within a line the evaluations are significantly different at 5% probability level by Tukey test.

Nitrogen yield “Ny” represents the nitrogen recovery or removed by the plant from the soil when applied nitrogen sources as fertilizer or animal excreta (Eriksen et al., 2015). The genotypes of this study received different nitrogen fertilization events (see in Appendix, Figure 8). In Florida, traditional agricultural activities require large nitrogen applications, leading to leaching events that can pollute water bodies (Dubeux et al., 2021).

Each genotype of this study removed different amount of N from the soil. On average for all evaluations, 323 and 322 ($P<0.05$) removed more N than Missouri and FL44 (Table 4). One possible explanation is that all bermudagrass cultivars allocated more biomass and accumulated N towards the green tissues (Li et al. 2018) at low N conditions, which is associated with perennial bermudagrass re-growth after clipping and spring green-up in the next year after dormancy (happening in June at summer; Table 4).

According Schneider-Canny et al., (2019) bermudagrass germplasm present high variability for Ny, that make sense with the results of this study, due all genotypes had variation among them and over time, as well it can be check in principal component analysis (PCA; Figure 4), where all genotypes had high dispersion.

3.4 Principal component analysis (PCA)

The variation of all genotypes for productive (HA, THA, Ny, TNy) and nutritive value (DM, CP, and IVDOM) traits is described in the Figure 4. The HA, TNy, and Ny had a positive correlation ($P<0.05$) because they were bundled together in a different positive quadrant, however, there were a high dispersion of the genotypes with these variables. Genotypes 276, 282, 286, and Tifton 85 showed higher and moderate association with THA and DM, respectively. Missouri presented a high dispersion and was the genotype with least THA and HA (Figure 2 and Table 2).

Jiggs had a strong and moderate association with IVDOM and CP, respectively, although presented low values of IVDOM and CP (Figure 3 and Table 3). The first principal component (PC) explained 47.2% of the variability in the data set, with DM, NY, TNY, HA, and THA traits providing the higher variation. The second PC explained 19.7%, where IDVOM and CP had the higher contribution in the variation. Both PC explained 66.9% of the variability in the data set.

The traits THA and DM were also highly correlated, and both traits were negatively correlated with IVDOM and CP. These results reflect the relationship between forage nutritive value and phenological stage (Wangchuk et al., 2015), which IVDOM decreased in the last period (Figure 3, A and B). According to Garcia et al. (2021), in tropical forages some nutritional parameters (e.g., DM, NDF) have a negative association with IVDOM and CP. Genotypic variability for all traits could be explained by the large phenotypic variation of morphological and productive traits within the Cynodon genus, and that might be related with multiple ploidy levels (Rios et al., 2015). In this study, the genotypes are triploid, tetraploid, and pentaploid (Table 1). According to Grossman et al. (2021), pentaploid genotypes as Tifton 85 is highly associated with leaf width and IVDOM traits; however, different response was observed in this study, where Tifton 85 was just associated with DM and THA.

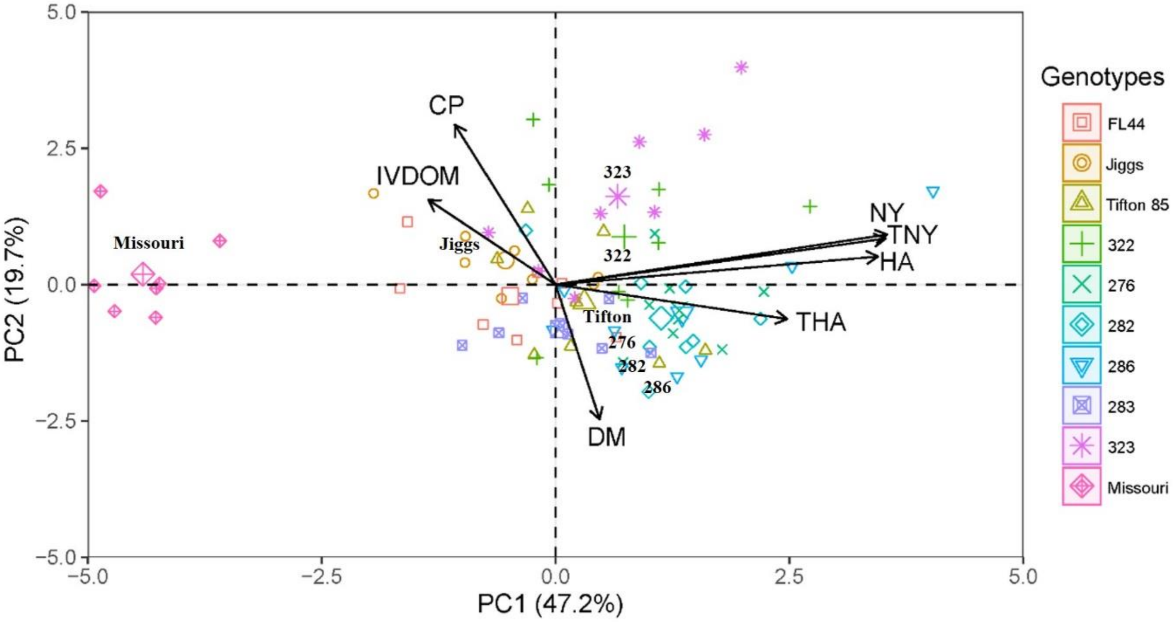
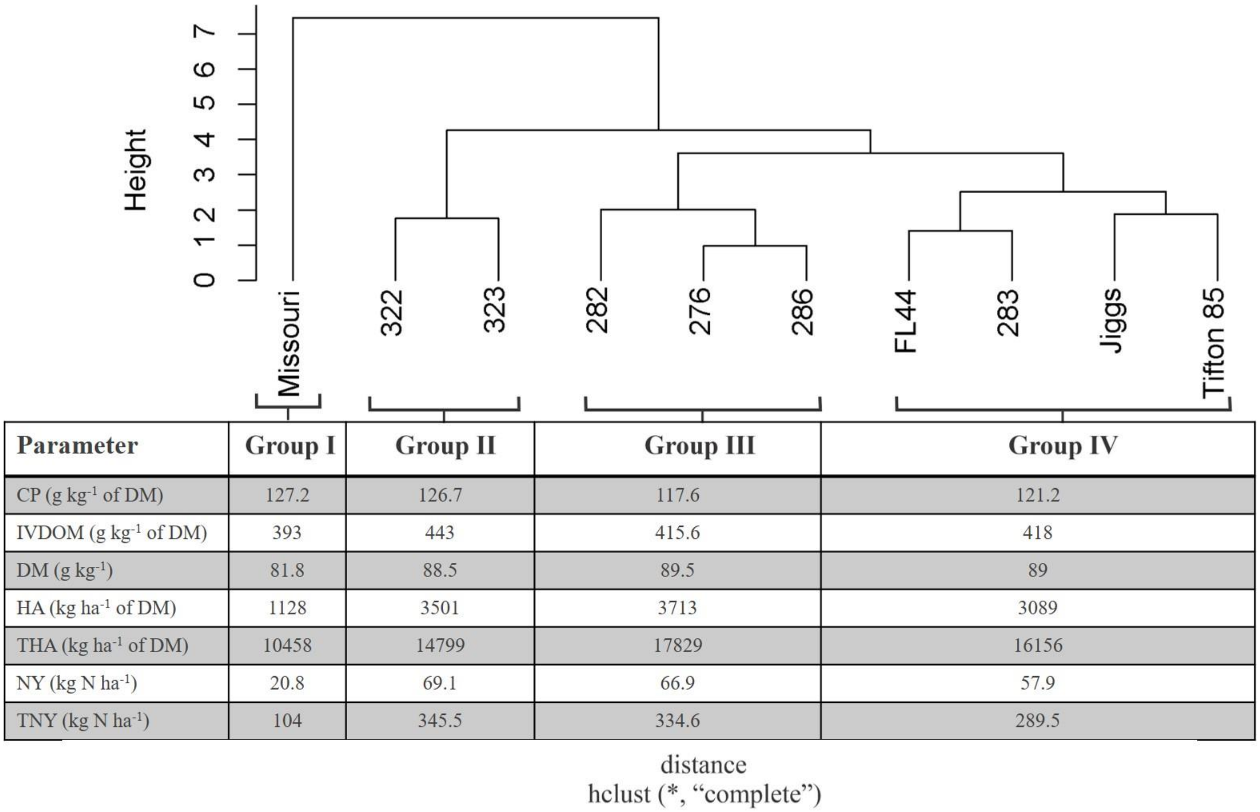


Figure 4. Principal component analysis of productive and nutritive value of bermudagrass genotypes in NFREC, Marianna, FL. CP: crude protein, IVDOM: *in-vitro* digestible organic matter, DM: dry matter, NY: nitrogen yield, TNY: total nitrogen yield, HA: herbage accumulation, THA: total herbage accumulation.

The Euclidean distances study the correlations between productive and nutritive value traits and help to identify genotypic variability. The dendrogram by cluster analysis divided the genotypes in four groups (Figure 5) with similar distribution observed in the PCA, where each genotype is closely related to each other, forming a group and showing their closer association with productive or nutritive value traits. The Figure 6 shows that Missouri genotype (Group 1) had the longest Euclidean distance, confirming the PCA where Missouri formed the only group away from the productive and nutritive value traits. This mean that Missouri had lower (P<0.05) productive (HA, THA. Ny, TNy) and nutritive (CP, IVDOM except for DM) value than other genotypes (Figure 2, 3, 4 and Table 2, 3, 4). Beyond that, the Group I had the lowest productive values compared to other groups (Figure 5).



Parameter	Group I	Group II	Group III	Group IV
CP (g kg^{-1} of DM)	127.2	126.7	117.6	121.2
IVDOM (g kg^{-1} of DM)	393	443	415.6	418
DM (g kg^{-1})	81.8	88.5	89.5	89
HA (kg ha^{-1} of DM)	1128	3501	3713	3089
THA (kg ha^{-1} of DM)	10458	14799	17829	16156
NY (kg N ha^{-1})	20.8	69.1	66.9	57.9
TNY (kg N ha^{-1})	104	345.5	334.6	289.5

Figure 5. Cluster analysis dendrogram of 10 bermudagrass genotypes in NFREC, Marianna, FL.
The group contain an average of each genotype parameter.
CP: crude protein, IVDOM: in-vitro digestible organic matter, DM: dry matter, NY: nitrogen yield, TNY: total nitrogen yield, HA: herbage accumulation, THA: total herbage accumulation.

4 CONCLUSIONS

Through the results provided in this study, it is possible to observe a wide diversity of bermudagrass genotypes with a significance variation among them, where genotypes such as 322 (recently new bermudagrass cultivar named as Newell) and 323 exhibited great nitrogen removal, which could represent an option to remove excess N in intensive systems, enhancing nitrogen use efficiency and cycling. In addition, 286 genotype tended to produce more forage, presenting great protein and nitrogen yields. Missouri showed the least values to all characteristics compared to other genotypes and had low association and high dispersion in the PC analysis. The significance of the cultivars evaluated showed on most traits that differences among genotypes should be considered when making recommendations for forage management practices in bermudagrass, particularly due to the significant interaction between genotypes and environmental and management factors, including fertilizer rates and harvest management, or even alternatives to nitrogen leaching reduction.

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CHAPTER 4

Harvest management affects agronomic characteristics and nutritive value of alfalfa-bermudagrass mixtures

ABSTRACT

Grass-legume mixtures are one alternative to reduce nitrogen fertilizer in grassland systems as well as to improve their nutritional value to grazing animals. Considering recent increases in N fertilizer prices, alfalfa (*Medicago sativa* L) and bermudagrass (*Cynodon dactylon* L.) mixtures are becoming more popular in SE USA. However, studies evaluating the performance of both forages in a mixture are still limited. The aim of this study was to measure the productive and nutritive value responses of two alfalfa varieties and their mixture with bermudagrass under different stubble heights and harvest frequencies. Treatments consisted of the factorial arrangement of two alfalfa varieties (Bulldog 805 and UF2015-AP) mixed with Tifton 85 bermudagrass, clipped at 5, 10, or 15-cm stubble height and subjected to three harvest frequencies (2, 4, or 6 weeks). The 18 treatments were distributed in a randomized complete block design with four replicates. Response variables included herbage accumulation rate (HA rate), total herbage accumulation (THA), nitrogen yield (Ny), biological nitrogen fixation (BNF), nitrogen derived from the atmosphere (%Ndfa), legume contribution (LC), dry matter (DM), crude protein (CP), and in vitro digestible organic matter (IVDOM). There was evaluation × stubble height significant interactions ($P<0.05$) on HA rate. HA was greater (P 0.05) in April regardless of the clipping height (ranging from 44 to 46 kg DM ha^{-1} day^{-1}) than in September under the same stubble height (2 to 9 kg DM ha^{-1} day^{-1}). There was frequency × stubble height interactions on THA, LC, Ny, and BNF, where the greatest values were obtained when both alfalfa varieties were harvested every 6-wk. clipped either at 5 or 15 cm. The UF2015-AP had greater Ny and BNF (32 and 23 kg N ha^{-1}, respectively) compared with Bulldog 805 (26 and 18 kg N ha^{-1}, respectively) in April. At 2-wk and 6-wk harvest intervals, there was interaction with the alfalfa varieties on THA, being UF2015-AP greater for than Bulldog 805. Triple interaction occurred for %Ndfa, with variety, frequency, and stubble height affecting this response. UF2015-AP had greater %Ndfa (P 0.05) when harvested every 6-wk at 15 cm of stubble height (77.2%) than Bulldog 805, clipped at 5 cm every 2-wk (65.5%). LC decreased over evaluation, with a lesser value in September (1.9%). DM concentration was greater ($P < 0.05$) in June (907 g kg^{-1}) than in April (894 g kg^{-1}) and September (900 g kg^{-1}). CP and IVDOM were affected by evaluation and had the greatest and least values in April (167 to 178; 523 to 527 g kg^{-1} of DM) and September (122 to 136; 421 to 457 g kg^{-1} of DM). Alfalfa mixed with grass tends to not persist over time, but great performance in some productive and nutritive value traits has been shown when cutting every 6-wk at 5 and 15 cm of height. UF2015-AP could be a candidate genotype to be released as a cultivar in North Florida and be used as an alternative to nitrogen use efficiency in grassland systems.

Keywords: grass-legume mixture, harvest interval, nitrogen response.

1 INTRODUCTION

Grasses of the genus *Cynodon* are frequently recommended as forages for feeding cattle and horses all over the world, and they are considered one of the most important warm-season forages for livestock production in the southeastern USA (Hill et al., 2001). Tifton 85 bermudagrass is the most popular hybrid of this genus and it is highly digestible, makes an excellent hay, and could be used for grazing or silage (Taliaferro et al., 2004). In addition, another important forage source worldwide is alfalfa, known as the “Queen of the forages”, because of its high nutritive value and productive potential (Hancock et al., 2015; McDonald et al., 2021). Alfalfa is the fourth most important crop after corn, soybean, and wheat in the United States (Zhang et al., 2021), and ranks first in cultivated grassland in China (Fang et al., 2019; Chen et al. 2020; Du et al. 2020). It adapts well to temperate and Mediterranean climates. In tropical climates with high temperatures and high humidity, alfalfa is vulnerable to various diseases that do not present a challenge in cold weather. Some alfalfa cultivars tolerate extremely cold winters, hot summers, and droughts due to their deep root system. However, they do not tolerate acid soils (Clements, 2019; Srisaikham and Rupitak 2020).

One of the key roles of forage legumes is the nitrogen contribution to the soil. Legumes have the ability to associate with several species of nitrogen-fixing microorganism as bacteria (Mahmud et al., 2020) and archaea (Soumare et al., 2020), a symbiotic relationship that benefits both organisms. The bacteria uses the energy of the legume photosynthetic metabolism, and the host plant benefits from the fixed nitrogen to increase its productive performance, which is often limited by the lack of N (Shimoda et al., 2020). Currently there is a pressing need to develop sustainable livestock systems using grass-legume mixtures, developing sustainable grazing systems with reduced levels of off-farm inputs (Jaramillo et al., 2021).

Alfalfa and grass as a mixture improve the nutritive value when compared with grass monoculture and oftentimes improves animal performance (Doblado et al., 2022). If the alfalfa is integrated into a grass sod, it increases the fiber digestibility of the mixture compared with the alfalfa alone (McDonald et al., 2021). Veira et al. (2010) reported significantly less bloating when an alfalfa–orchardgrass (*Dactylis glomerata* L.) mixture was fed to ruminants compared with alfalfa hay. This was likely due to the higher neutral detergent fiber digestibility in grasses than in alfalfa, which helps regulate the rumen pH. The Tifton 85-Alfalfa binary mixture increased the seasonal HA from 11788 to 14755 kg DM ha^{-1} from March to September, crude protein from 11 to 21% in three years of evaluation, indicating that overseeding alfalfa into a bermudagrass system improves forage mass and nutritive value (Hendricks et al., 2020).

Despite the productive potential and nutritive value of alfalfa, this legume is considered the fragile species in the system (Ren et al., 2021). Constant harvesting of legumes like alfalfa affects the persistence due to leaf losses. Therefore, cutting at the vegetative stages will decrease stand persistence compared with harvesting at the reproductive stages. Frequent harvests lead to depletion of reserve carbohydrates to support regrowth or cold tolerance (Atis et al., 2019). Quinby et al. (2020) reported 90% fewer stems of alfalfa in a mixture with bermudagrass under less frequent harvesting regime, resulting in less persistence of the alfalfa.

Stubble height is another important factor in the management of alfalfa production systems because it has a significant impact on regrowth rate, yield, and forage nutritive value (Tudsri et al. 2002; Wadi et al. 2004; Yolcu et al. 2006). Kim et al. (2021) reported an increase in the total dry matter accumulation from 3600 to 4218 kg DM ha^{-1} (in four harvest) in alfalfa as monoculture when subjected to 5-cm stubble height rather than 25-cm stubble height. However, the shorter stubble height could have a visible decline in forage nutritive value. Beyond that, when alfalfa monoculture is clipped at 10-cm stubble height, the CP and IVDOM reported is from 16 to 18% and 63 to 64.2% (Seid et al. 2005). Yolcu et al. (2006) also suggested that biomass productivity as DM and nitrogen yield was greater in alfalfa alone subjected to a lower cutting height from 6670 to 8140 kg DM ha^{-1} and 150 to 225 kg N ha^{-1}.

Currently there are limited information about productivity and nutritive value of alfalfa in a mixture with Tifton 85 bermudagrass. Furthermore, there are another alfalfa varieties as Bulldog 805 and UF2015-AP that still present few data in binary mixture with other grasses about productive, nutritive value, and nitrogen responses under different harvesting management. Therefore, this study evaluated the response of two alfalfa varieties under three stubble heights and three harvest frequencies in North Florida.

2 MATERIALS AND METHODS

2.1 Site description

This one-year study was conducted in 2019 at the University of Florida, Institute of Food and Agricultural Sciences, North Florida Research and Education Center, Marianna, FL (30°46′35″N 85°14′17″W, 51 m above sea level). Soil in the experimental site were classified as an Orangeburg loamy sand (fine-loamy, kaolinitic, thermic Typic Kandiudults) with Mehlich-1 extractable P, K, Mg, and Ca concentrations of 33.5, 46, 66, and 262 mg kg^{-1} respectively. Weather data were collected from Florida Automated Weather Network (FAWN) for minimum and maximum temperature, rainfall, and relative humidity during 2017, 2018, and 2019. Total annual rainfall was 937, 1109, and 229 mm in 2017, 2018, and 2019 respectively (Figure 1).

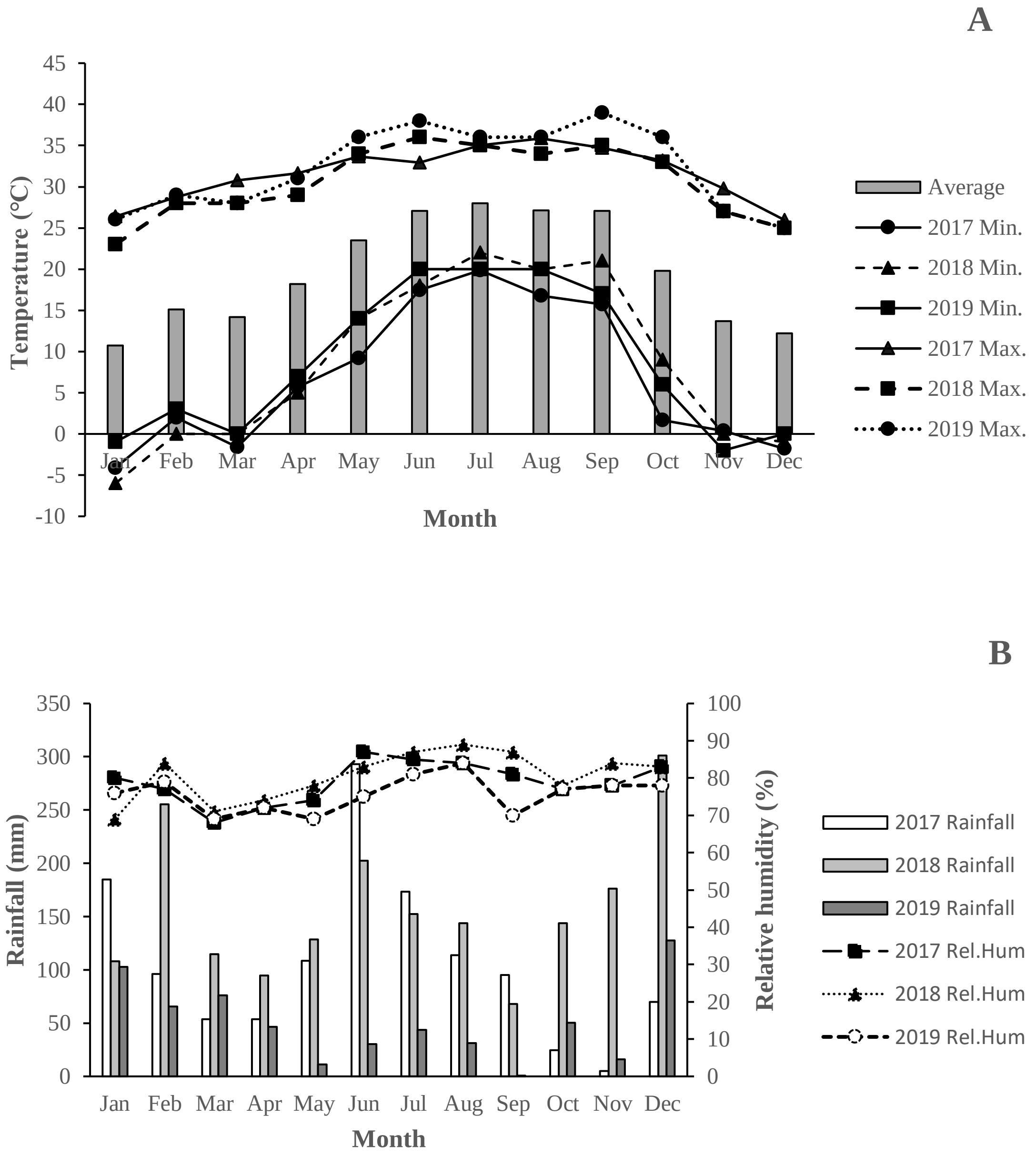

Figure 1. Historical monthly weather conditions at North Florida Research and Education Center (NFREC), Marianna, FL. A: minimum, maximum and average temperature, B: rainfall and relative humidity in 2017, 2018, and 2019.

2.2 Initial Management

In August 2017, the soil at the experimental site was prepared and limed at a rate of 1550 kg dolomitic lime ha^{-1}. One week after lime application, Tifton-85 bermudagrass was established using approximately 1683 kg ha^{-1}. Weed management included the use of herbicide Diuron [1,1-

dimethyl, 3-(3',4'-dichlorophenyl) urea], 2.2 L ha^{-1} of Basagran® (contain 480 g of Bentanzon L^{-1}), and 0.8 L of glyphosate [glyphosate-4 Plus, Alligare, LLC; (N-(phosphonomethyl) glycine)]. Insecticide MustangMax (zeta-cypermethrin) was applied at a rate of 0.5 L per hectare (Table 1). Four months after bermudagrass establishment, plots were limed at a rate of 1683 kg dolomitic lime ha^{-1}.

Table 1. Herbicide application for weeds control in stands of bermudagrass and alfalfa NFREC, Marianna, FL.

Period of application	Herbicide	Commercial product rate L ha^{-1}
8-Aug-17	Diuron 4L	1.3
26-Sep-17	Basagran®	2.2
11-Oct-17	Basagran®	3.2
13-Nov-17	Glyphosate	0.8
18-Dec-17	MustangMax	0.5
2-Mar-18	Prowl® H2O	3.4
7-Jan-19	MustangMax	0.5
14-Feb-19	Prowl® H2O	1.1
19-Feb-19	MustangMax	0.5
10-Apr-19	Roundup WheatherMAX® 4.5S	6
28-Jun-19	Roundup WheatherMAX® 4.5S	6
27-Sep-19	Roundup WheatherMAX® 4.5S	6
7-Oct-19	Prowl® H2O	1.1

2.3 Treatments and Management

The treatments consisted of the factorial arrangement of two alfalfa varieties (UF2015-AP and Bulldog 805 hybrid), three harvest frequencies (2, 4, and 6 weeks), and three stubble heights (5, 10, and 15 cm). Planting material was obtained from the University of Florida forage breading program and drilled into the previously established Tifton 85 bermudagrass. The experiment was conducted as a randomized complete block design with 18 treatments (factorial arrangements) and four replicates (*n*=72 plots). Each plot (experimental unit) measured 1.5 x 4.6 m with 2 m alleys between plots (see in Appendix, Figure 10).

November 2018 alfalfa seeds were drilled using a no-till drill (1-cm depth) and four rows were 35-cm apart, adding 1 g of inoculant (*Rhizobium meliloti*) in alfalfa seeds per plot. Plots were irrigated (3 mm/event) twice after planting (once a week). Inorganic fertilizers were applied separated into 10 applications as displayed in Table 2.

Table 2. Fertilization and amendment rates applied in the establishment and evaluation of the experimental plants at UF IFAS NFREC, Marianna, FL.

Period of application	Nutrients										
	dolomite	N	P	K	S	Mg	B	Cu	Fe	Mn	Zn
	kg ha^{-1}										
1-Aug-17	1550	-	-	-	-	-	-	-	-	-	-
31-Aug-17		67	16	67	-	-	-	-	-	-	-
23-Dec-17		32	39	-	89	-	-	-	-	-	-
15-Feb-18	1683	-	-	-	-	-	-	-	-	-	-
20-Feb-18		44	51	116	-	-	4	-	-	-	-
17-Dec-18		32	39	89	-	-	3	-	-	-	-
31-Jan-19		-	28.1	112	-	-	-	-	-	-	-
4-Mar-19		-	-	12	12	5	1	1	7	3	3
10-Apr-19*		-	28	112	-	-	-	-	-	-	-
28-Jun-19*		-	28	112	-	-	-	-	-	-	-
27-Sep-19*		-	28	112	-	-		-	-	-	-

* Fertilization occurs 1-2 days after harvest.

2.4 Sampling and measurements

Plots were harvested every 2, 4, or 6 weeks to 5, 10, or 15 cm stubble height, resulting in 18 individual evaluation dates. However, we reported just the three evaluations containing all the treatments (72 samples), corresponding to 4 April, 27 June, and 19 September 2019. The experiment lasted one year, due to the low persistence of alfalfa in the mix with bermudagrass over the evaluations.

The plot was harvested at the central portion and all forage was removed from the plot using a flail-style mower (Carter Manufacturing Company), staging the plots (treatments) to a 5, 10 and 15-cm stubble height (see procedures in Appendix, Figure 11). After all samples were taken, plots were staged to the target stubble height, and days later fertilized to start a new regrowth cycle. Unfertilized Tifton-85 bermudagrass (*Cynodon* spp.) samples (four per harvest) were collected in an adjacent field within each sampling evaluation and used as a reference to estimate Biological Nitrogen Fixation - BNF (using the natural abundance technique by Freitas et al., 2010). The %Ndfa was estimated using the equation 1 described by Shearer and Kohl (1986):

$$\%Ndfa = \frac{(\delta^{15}N_{reference} - \delta^{15}N_{N_2-fixing\ legume})}{\delta^{15}N_{reference} - B}\ x\ 100 \qquad (1)$$

where $\delta^{15}N_{reference}$ is the $\delta^{15}N$ value for the non N_2–fixing reference plant, $\delta^{15}N_{N_2-fixing\ legume}$ is the $\delta^{15}N$ value for N_2–fixing alfalfa varieties in this study, and B is the $\delta^{15}N$ value for the N_2–fixing plant grown in the absence of inorganic N. The B value used in this study was reported by Unkovich et al. (2008) for *Lupinus* sp. ($\delta^{15}N$ = –0.16). Plant BNF was estimated by multiplying plant N harvested by %Ndfa.

FBN=%Ndfa*Nyield/100

Legume contribution (LC) percentage was estimated using the % carbon derived from the legume in a grass + legume which was estimated as described by Vitorello et al. (1989) using the equation 2 and 3:

$$\%LC_1 = \frac{(\delta^{13}C_{mixture} - \delta^{13}C_{legume\ alone})}{(\delta^{13}C_{grass\ alone} - \delta^{13}C_{legume\ alone})}\ x\ 100 \qquad (2)$$

$$\%LC_2 = 100 - \%LC_1 \qquad (3)$$

where $\delta^{13}C_{mixture}$ is $\delta^{13}C$ value for alfalfa and bermudagrass mixture, $\delta^{13}C_{legume\ alone}$ is $\delta^{13}C$ value for alfalfa alone and $\delta^{13}C_{grass\ alone}$ is $\delta^{13}C$ value for bermudagrass alone, $\delta^{13}C_{legume\ alone}$ is $\delta^{13}C$ value for alfalfa alone.

2.5 Herbage Accumulation and Nutritive Value (mixture)

At harvest, total fresh weight was determined and two subsamples taken for determination of DM concentration and nutritive value. After harvesting, the first subsamples were dried in a forced-air oven at 55ºC until constant weight. After that, the dried samples were grounded to pass a 2-mm screen using a Wiley Mill (Model 4, Thomas-Wiley Laboratory Mill, Thomas Scientific). The dry mass was recorded to determine the herbage accumulation (HA) from each harvest, and the value was used to calculate herbage accumulation rate (HA rate) dividing the HA by 14, 28, or 42 days (2-, 4-, and 6-wk, respectively). The total herbage accumulation (THA) was obtained by summing HA across all regrowth periods each year. Beyond that, to contrast harvest frequencies, the sum of three harvest of 2-wk (THA) was obtained to compare to 6-wk THA.

To determine the *in vitro* digestible organic matter (IVDOM) the ground samples were used according to the procedure described by Moore and Mott (1974). A second subsample was taken from the 2-mm ground sample and ball milled in a Mixer Mill (MM 400, Retsch) at 25 Hz for 9 min. Ball milled samples were analyzed for N by dry combustion using an elemental analyzer (Vario Micro cube, Elementar) coupled to an isotope ratio mass spectrometer (IsoPrime 100, IsoPrime). Crude protein (CP) was obtained by multiplying the N concentration by 6.25. The Nitrogen yield (Ny) was estimated by multiplying the nitrogen concentration of each bermudagrass cultivar times the HA, divided by 100% (Wagger, 1989; Hesterman et al. 1992), according to the following equation 4:

$$Ny\ (kg\ ha^{-1}) = \frac{Nitrogen\ concentration\ x\ Herbage\ accumulation}{100\%} \qquad (4)$$

2.6 Statistical analysis

The data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) and LSMEANS were compared using PDIFF adjusted by Tukey ($p < 0.05$) with stubble height, harvest frequency, alfalfa varieties, and their interactions as fixed effects. Evaluation was analyzed as a repeated measure. Total HA was analyzed with treatment as fixed effects and block and its interactions were random effects. To contrast the shorter and the longest frequencies, the sum of three 2-wk and 6-wk harvest were analyzed separately, with harvest frequency, alfalfa varieties and their interactions as fixed effects.

3 RESULTS AND DISCUSSION

3.1 Herbage accumulation and legume contribution

There was evaluation × stubble height and evaluation × harvest frequency interaction on herbage accumulation rate (HA rate). Overall, the HA rate showed greater (P<0.05) values clipped at 5, 10, and 15 cm in April and in June (except for 15 cm) than September clipped at same stubble height (Figure 2, A). The least HA rate were in September and didn't differ for 5 and 10 cm stubble height. The HA rate values ranged from 2 to 47 kg DM ha^{-1} day^{-1} clipped at 15 cm and 10 cm in September and April, respectively (Figure 2, A). Regarding the evaluation × harvest frequency interaction, HA rate was greater (P<0.05) in April clipped every 2-wk than clipped every 4 and 6-wk in June and in September, even in April. The greatest HA rate was 75 kg DM ha^{-1} day^{-1} and that occurred when forages were harvested every 2-wk in April. HA rate in June clipped every 4 and 6-wk were greater than September clipped with the same frequency, showing values of 42, 46, 8 and 11 kg DM ha^{-1} day^{-1}, respectively (Figure 2, B).

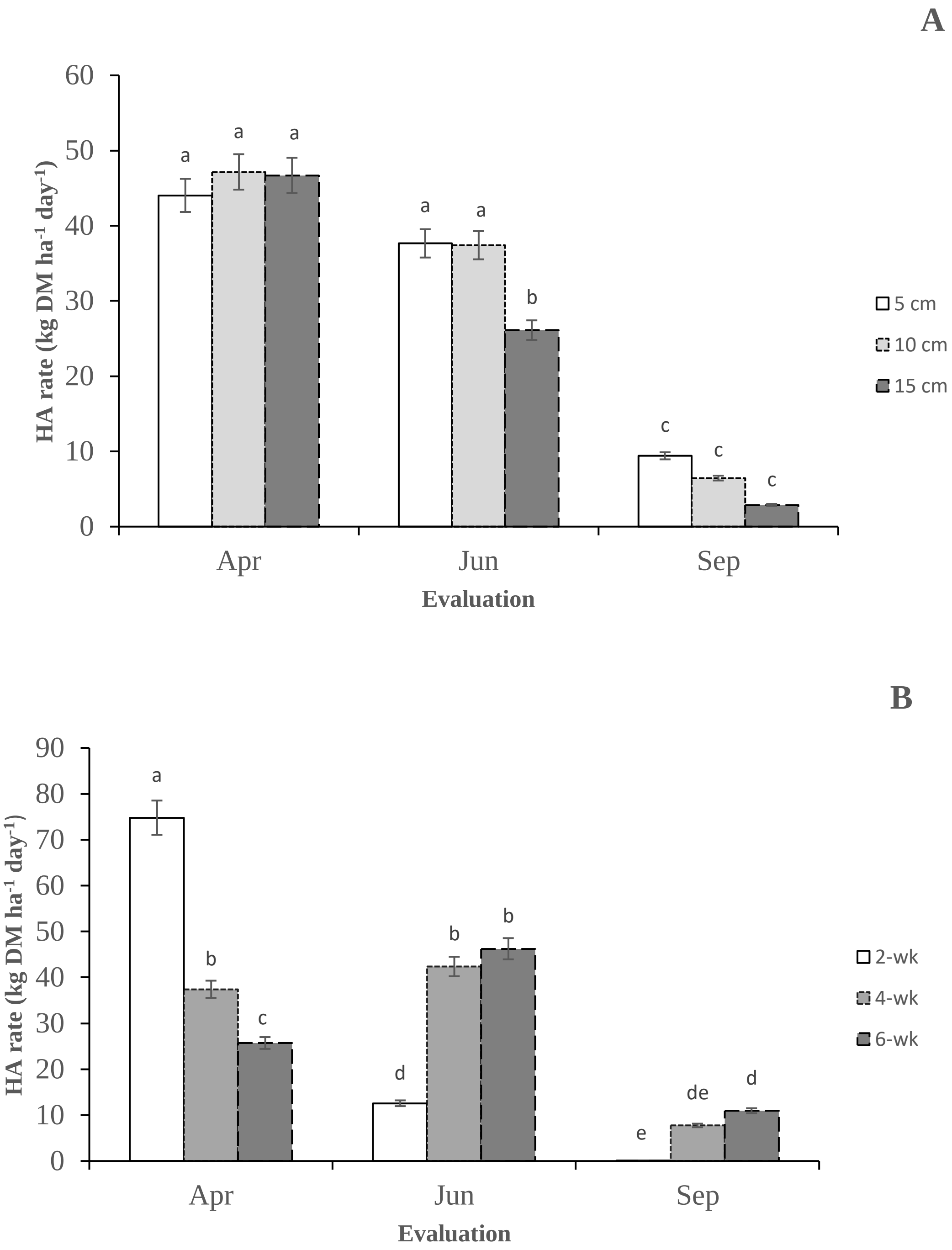

Figure 2. Evaluation × stubble height (A) and evaluation × harvest frequency (B) interaction on herbage accumulation rate (HA rate) in NFREC, Marianna, FL. Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

The likely reason why the greatest and the least HA rates (Figure 2, A and B) could be explained by many factors including frequency and stubble heights, which have a significant effect on HA rate because it directly affects the physiological and maturity stage (Kolberg et al., 2018). In addition, forage growth is largely determined by leaf area (Venter et al., 2020), so it is important to consider the residual stubble height to assure the plant regrows and accumulate more dry matter. The decrease in HA rate from June to September was likely due to less favorable temperature and rainfall (Figure 1). Similar trends were observed in other bermudagrass-legume mixtures as showed by Sanchez et al. (2018), who reported a decrease from July to September in the HA rate of Jiggs-Pintoi mixtures. The authors justified that lesser HA rate likely occurred due to the excessive herbage mass, which may result in self-shading, accumulation of senescent and non-photosynthetic residue, and reduced photosynthesis, especially from young basal tillers.

Total herbage accumulation (THA) had a stubble height × frequency interaction. The least THA values ($P<0.05$) occurred when forages were harvested every 2-wk, regardless of the stubble height ($P>0.05$). Great THA was obtained when the grass-legume mixture was clipped at 5 and 10 cm every 6-wk ($P<0.05$), being greater than when clipped at 10 and 15 cm every 4-wk. The THA values of the mixture ranged from 1161 to 3414 kg DM ha^{-1} clipped at 5 cm every 2 and 6-wk, respectively (Figure 3, A). There was alfalfa variety × frequency interaction on THA values ($P<0.05$). The THA of variety UF2015-AP was greater than Bulldog 805 when it was clipped every 2 and 6-wk, with average values of 3595 and 2920 kg DM ha^{-1}. The least THA values of the alfalfa-Bermuda mixture occurred when the mixture was clipped every 2-wk, regardless of the alfalfa variety (Figure 3, B).

The stubble height × frequency interaction likely occurred because a different stubble height and harvesting frequency modify the canopy structure, reserve carbohydrates, and therefore plant responses. Similar values (1020 and 3800 kg DM ha^{-1}) were reported by Quinby et al. (2021) in a study with bermudagrass and alfalfa mixture (Wrangler and Ameristand 403T varieties) harvested every 6-wk at 8 cm stubble height in the spring and summer seasons, respectively. Acharya et al. (2020) in a study assessing the performance of alfalfa germplasm, as UF2015, Bulldog 805, and FL99, reported that UF2015 produced greater HA with 2800 kg DM ha^{-1} than other genotypes with averages of 2000 kg DM ha^{-1}. Similar result was obtained in this study, with UF2015-AP being greater than Bulldog 805.

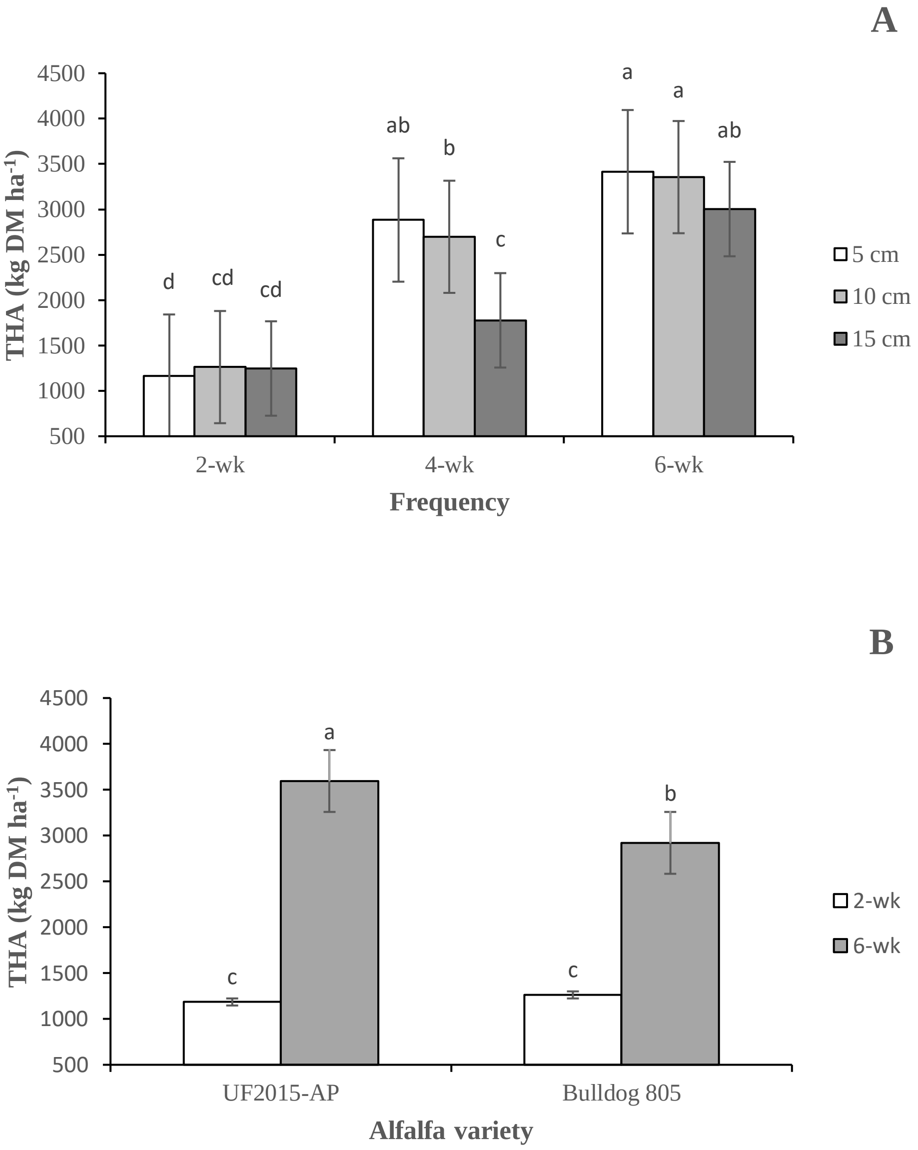

Figure 3. Stubble height × harvest frequency and variety × harvest frequency interaction on total herbage accumulation (THA) of alfalfa-bermudagrass mixtures in NFREC, Marianna, FL. A: frequency × stubble height interaction, B: variety × frequency interaction. Column followed by a different small letter are significantly different at 5% probability

level by Tukey test. Bars refer to the standard error of mean.

Legume contribution (LC) showed an evaluation × frequency, alfalfa variety × evaluation, alfalfa variety × stubble height and frequency × stubble height interactions ($P<0.05$). The least LC were 0.2 and 0.7% for the 2-wk and 4-wk interval in September, being lesser ($P<0.05$) than LC obtained in April with 33.7, 34.6 and 33.6% harvested every 2, 4 and 6-wk, respectively (Figure 4, A). The LC decreased from June to September for all frequencies, however being harvested every 6-wk greater than September in 2 and 4. Furthermore, LC was affected ($P<0.05$) when clipped at different stubble height in different harvest frequency. LC was greater harvested every 6-wk at 15 cm of stubble height than harvested every 2-wk at 5, 10 and 15 cm, with means of 19.2, 11.9, 12.6 and 10.7%, respectively (Figure 4, B). Alfalfa UF2015-AP clipped at 5 cm had greater ($P<0.05$) LC than Bulldog 805 when clipped at 5 and 10 cm of stubble height, showing means of 17.6 and 12.8 respectively; however, the LC of UF2015-AP didn't differ to Bulldog 805 when clipped at 5 and 10 cm of stubble height (Figure 4, C). On the other hand, the least LC was obtained in April, but UF2015-AP was greater ($P<0.05$) than Bulldog 805 with average of 36.5 and 31.3%, respectively. The LC means showed in June and in September did not differ between alfalfa varieties (Figure 4, D).

Yuksel and Balabanli (2021) in a study with alfalfa and temperate grasses mixture in south-western Turkey, reported that in general alfalfa contribute in the mixture by 40% and grasses 60%. In this study the alfalfa contribution was lower than 40% in the mixture, however the greatest contribution occurred in April being UF2015-AP greater than Bulldog 805 with 36.5 and 31.3%, respectively (Figure 4, C and D). Aponte et al. (2019) tested alfalfa in mixture with different warm-season grasses. Their 2-yr study indicated that the contribution of alfalfa to the mixture fluctuated between 48 and 69% in the first and between 51 and 65% in the second year. The reason why the results reported by Aponte et al. (2019) are different might be due to more favorable weather conditions in their trial. They reported max and min temperature of 25 and -5°C and total rainfall means of 100 mm over their experiment, respectively, while in this study the temperature over the periods was over 25°C and low rainfall (Figure 1, A and B).

Many legumes and grasses could be used as a mixture, but the ability of the shoot meristem to respond with increased growth after cutting is essential to sustain continued regrowth. Furthermore, the importance of N supply for re-growth after cutting grass is important and has been demonstrated (Ghosh et al., 2018). In this study the results of frequency × stubble height interaction (Figure 4, B) suggests that when the legume-grass mixture is clipped more frequently, it decreases the LC regardless the stubble height. Yixin et al. (2013) in a study with alfalfa cutting at 5 and 10 cm of stubble height every 6-wk in temperate weather of China, reported that cutting closer to the soil surface (5 cm) increased forage yield of alfalfa every 6-wk by increasing the weight of individual shoots harvested. This fact likely increases the LC in a legume-grass mixture like happened in this study (Figure 4, A and B). Another important aspect to mention is that alfalfa contribution likely decreased from June to September due to lack of persistence in a grass mixture in tropical regions when compared its persistence as monoculture, representing 100% and 98% in relation to weeds in April and in September, as reported by Santos et al. (2021). That fact may explain the reason why the linear decrease of LC happened over the three evaluations.

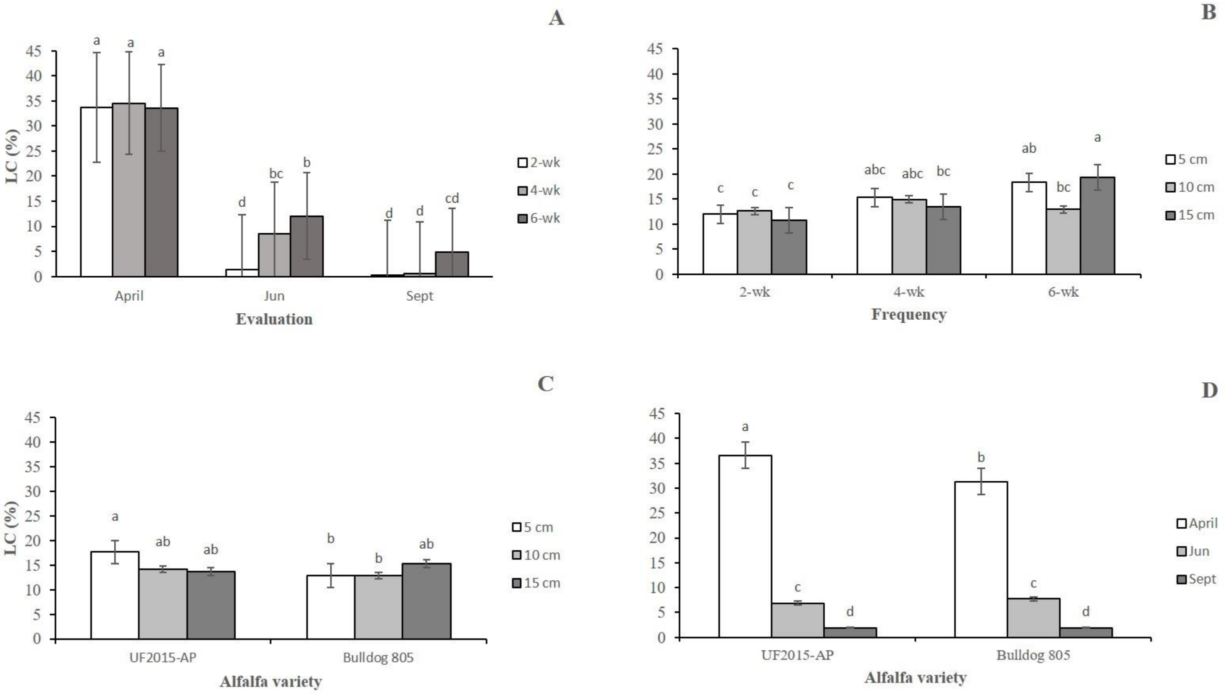

Figure 4. Evaluation × frequency (A), frequency × stubble height (B), alfalfa varieties × stubble height (C) and alfalfa varieties × evaluation (D) interactions on legume contribution percentage (LC). Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

3.3 Nitrogen responses

There was a frequency × stubble height, alfalfa variety × evaluation, and evaluation × frequency interactions affecting ($P<0.05$) nitrogen yield (Ny). The Ny of both alfalfa varieties increased ($P<0.05$) when these were clipped at 5 cm of stubble height every 6-wk, nevertheless cutting every 2-wk the Ny decreased regardless the stubble height with averages around 26.4 and 10.1 kg N ha^{-1}, respectively (Figure 5, A). UF2015-AP and Bulldog 805 did not differ in June and in September, however they were different ($P<0.05$) in April, being UF2015-AP greater than Bulldog 805 with average of 32 and 26 kg N ha^{-1}, respectively (Figure 5, B). The Ny values ranged from 0.01 kg N ha^{-1} to 29.1 kg N ha^{-1} harvested every 2-wk in April, both differing probability, respectively, however, the greatest Ny was obtained in June harvest every 6-wk with average of 35.4 kg N ha^{-1} (Figure 5, C).

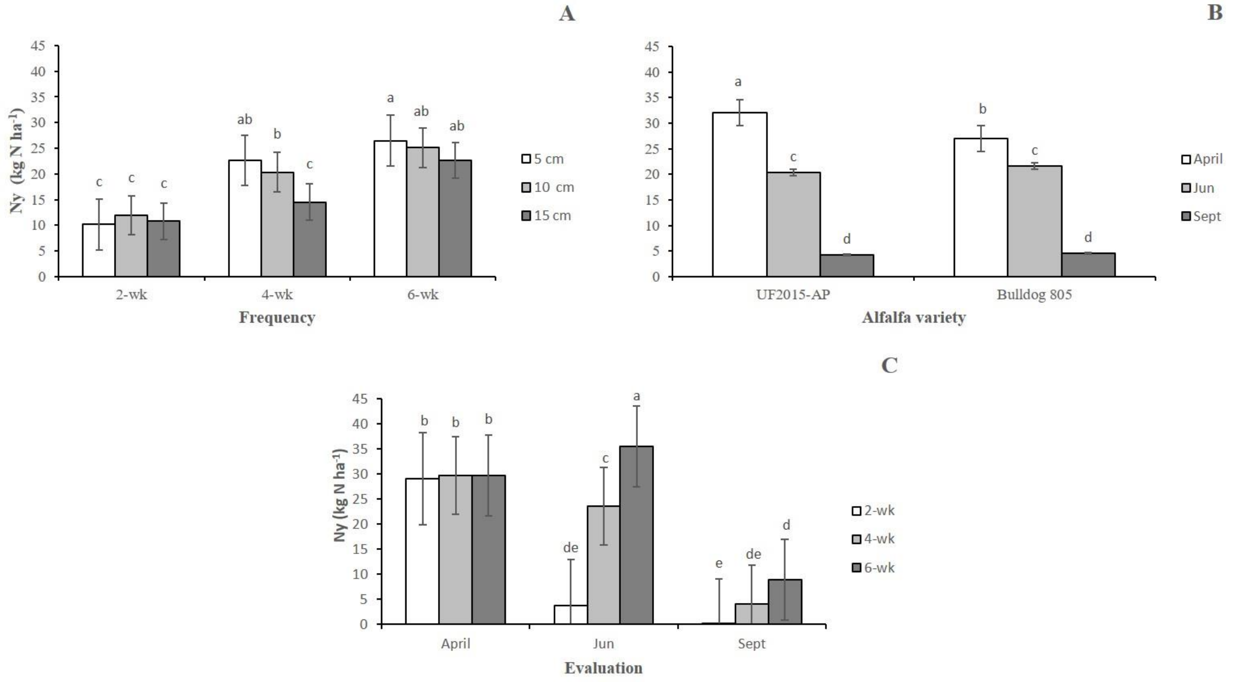

Figure 5. Frequency × stubble height (A), alfalfa varieties × evaluation (B) and evaluation × frequency (C) interactions on nitrogen yield (Ny). Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

There was a frequency × stubble height interaction affecting (P<0.05) Total nitrogen yield (TNy). Both alfalfa TNy had the greatest values when harvested every 6-wk regardless the stubble height (5, 10, and 15 cm; 79.4, 75.2 and 67.9 kg N ha^{-1} respectively), followed by 4-wk clipped at 5 cm (67.8 kg N ha^{-1}) greater than 2-wk regardless the cutting height (average of 32.8 kg N ha^{-1}). The least TNy values was obtained every 2-wk, followed by 4-wk cutting at 15 cm of stubble height (Figure 6).

The Ny and TNy is related to HA, thus, when the forage production increases the N yield increases as well (Figure 3, 5 and 6) rather than changes in N concentration. Forage species that do not elevate apical meristems within vegetative tillers or that produce lower proportions (as bermudagrass) of reproductive tillers generally can be grazed more frequently without reducing herbage production (Calvano et al., 2011), being less affected by harvesting regime. This confirms that HA is the overriding factor influencing Ny, rather than N concentration (Jeranyama et al. 1998). Santos et al. (2021) evaluated the TNy of bermudagrass + 90 kg N ha^{-1}, bermudagrass-alfalfa mixture + 45 kg N ha^{-1} (Tifton-85 and Alfagraze 600 RR respectively), and another grass-legume mixture (Tifton-85 and rhizoma peanut [*Arachis glabrata* Benth]) clipped at 7.5 cm stubble height every 5-wk. The results indicated greater TNy value for bermuda + N with 90 kg N ha^{-1} harvest^{-1} (93 kg N ha^{-1}) than bermuda-alfalfa (53 kg N ha^{-1}). The authors justified their results explaining that the main factor contributing TNy was HA and not nitrogen concentration. The TNy of alfalfa-bermuda reported in this study was superior (ranging from 30 to 79 kg N ha^{-1}; Figure 6) than reported by Santos et al., (2021).

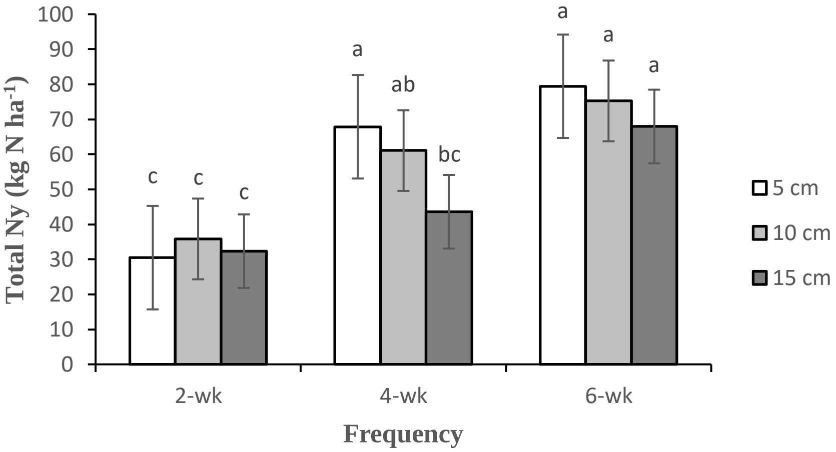

Figure 6. Frequency × stubble height interaction on total nitrogen yield (TNy). Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

Nitrogen derived from the atmosphere (%Ndfa) was affected by a frequency × height × alfalfa variety interaction ($P < 0.05$). Proportions of Ndfa in both alfalfa varieties ranged from 64.9 (Bulldog 805 clipped every 6-wk at 5 cm of height) to 77.2 (UF2015-AP clipped every 6-wk at 15 cm of stubble height), being different ($P<0.05$) among them. UF2015-AP had greater ($P<0.05$) %Ndfa when harvested every 6-wk at 15 cm of stubble height than Bulldog 805 cutting with 2- and 4-wk interval at 5 cm of height; nevertheless, it didn't differ when harvested every 2, 4, and 6-

wk at 5 and 10 cm and 5, 10, and 15 cm of stubble height respectively to same alfalfa variety (Table 3).

The estimation of %Ndfa was determined using ^{15}N isotopes from the alfalfa samples and it varied with alfalfa varieties (Table 3). In a study reported by Louarn et al. (2015) evaluating the effect of alfalfa-fescue mixture (Orca and Noria cultivars, respectively) clipped at 5 cm stubble height, reported that %Ndfa exceeded 90% in the first cut. Therefore, the authors justified that the amount of BNF are directly related to the growth and N yield. Other study with legume-grass mixture as reported by Jaramillo et al. (2018), using different rhizoma peanut cultivars (Ecoturf, Amarillo, Florigraze and TUFRunner 727) mixture with bermudagrass Tifton 85 in North Florida-USA, showed proportions of Ndfa ranging from 0 to 91%. The author justified their results by the fact that a possible issue related to the rhizobium used to inoculate the seeds, as well that presence of peanut diseases that likely impacted N_2–fixation pathways. In this study, alfalfa seeds were inoculated and they did not present disease over evaluations, contributing to have great Ndfa percentage (Table 3).

Table 3. Alfalfa variety × frequency × stubble height interaction on proportion of nitrogen derived from the atmosphere (%Ndfa).

Variety	**Frequency**	**Height**	**Ndfa** (%)	
UF2015-AP	2-wk	5 cm	69.2	abc
		10 cm	76.2	ab
		15 cm	66.2	bc
	4-wk	5 cm	69.9	abc
		10 cm	70.8	abc
		15 cm	74.5	abc
	6-wk	5 cm	66.9	abc
		10 cm	68.4	abc
		15 cm	77.2	a
Bulldog 805	2-wk	5 cm	65.5	c
		10 cm	67.7	abc

	15 cm	66.6	abc
	5 cm	76.8	ab
4-wk	10 cm	69.4	abc
	15 cm	69.3	abc
	5 cm	64.9	c
6-wk	10 cm	72.9	abc
	15 cm	71.4	abc
Standard error of mean 2.1			

Values followed by a different letter in the column are significantly different at 5% probability level by Tukey test.

Biological Nitrogen Fixation (BNF) was affected by frequency × stubble height, variety × evaluation, and evaluation × frequency interactions. The BNF was greater ($p<0.05$) when the mixture was harvested every 6-wk, regardless the stubble height. The least BNF occurred when harvesting occurred every 2-wk at 5, 10, and 15 cm stubble height with 6.9, 8.5, and 7.2 kg N ha^{-1}, respectively (Figure 7, A). Alfalfa varieties differed in April, being UF2015-AP (23 kg N ha^{-1}) greater ($P<0.05$) than Bulldog 805 (18 kg N ha^{-1}), however, they did not differ in June and in September (Figure 7, B). BNF did not differ among cutting frequencies in April; there were differences in the second evaluation just when harvesting every 4- and 6-wk. The BNF ranged from 0.006 kg N ha^{-1} to 24.5 kg N ha^{-1}. The greater ($P<0.05$) BNF value was 26.4 kg of N ha^{-1} per harvest when cutting every 6-wk in the second evaluation, and in all frequencies in the third evaluation (Figure 7, C).

The literature reports BNF in grass-legume pastures ranging from 13 to 682 kg N ha^{-1} yr^{-1} (Ledgard and Steele, 1992; Li et al., 2015; Yu et al., 2018; Li et al., 2019), with about 80% transferred from legumes to grasses depending on the donor and recipient plant species (Pirhofer-Walzl et al. 2012). However, Chen et al. (2004) reported that under field conditions, a wide range (40–153 kg N ha^{-1} yr^{-1}) of BNF is expected in legume pastures. Alfalfa as a monoculture can fix from 4 to 650 kg N ha^{-1} yr^{-1} across different environments under different managements (Issah et al., 2020). Nonetheless, in this study the BNF values are lower (Figure 7, A, B and C) than those reported by Chen et al. (2004). Issah et al. (2020) suggested that BNF in alfalfa depends on the harvest frequency. For example, in this experiment the BNF values were greater when forages were clipped every 6 weeks at 15-cm and every 4 weeks at 5 and 10 cm of stubble height (Figure 7, A). However, this BNF is still lesser than the one reported by Ruselle et al. (2004) with 152 kg N ha^{-1} for alfalfa monoculture harvested at 0.5 cm stubble height.

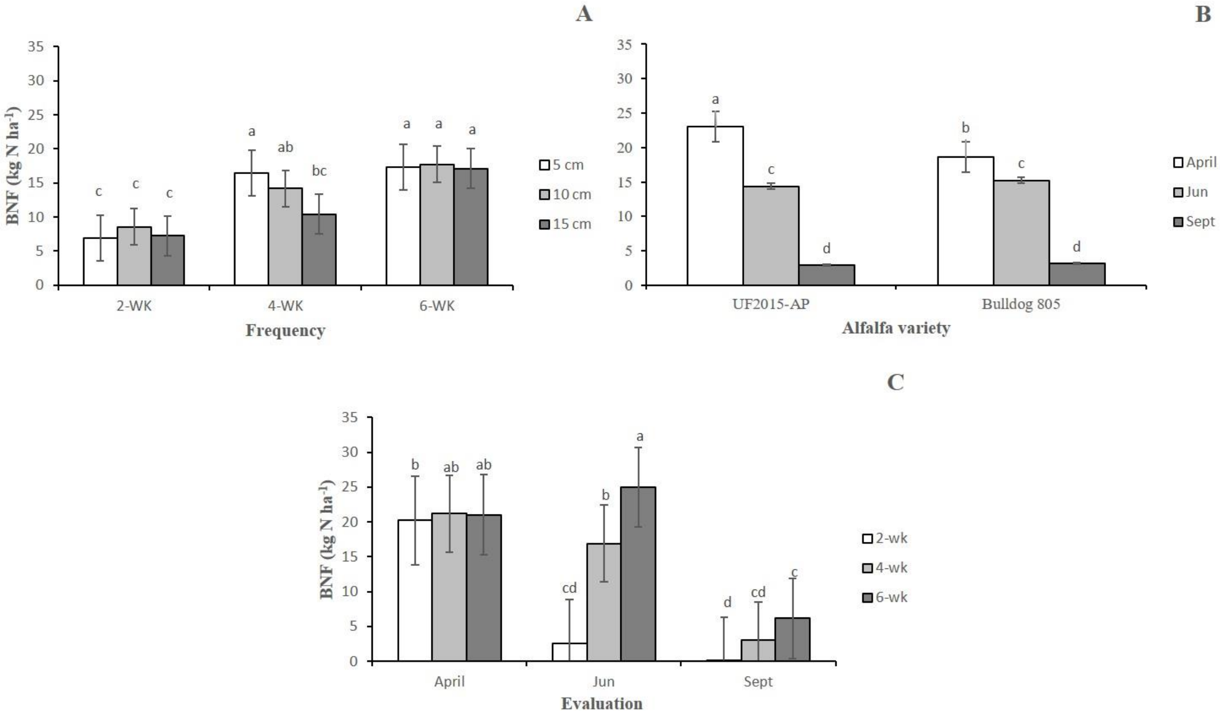

Figure 7. Frequency × stubble height (A), alfalfa variety × evaluation (B), evaluation × frequency (C) interactions on total biological nitrogen fixation (BNF). Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

3.4 Nutritive value

Crude protein (CP) was affected by evaluation × stubble height, evaluation × frequency, frequency × stubble height interactions. Over the evaluations (April, June, and September), the greatest CP (178, 177, and 167 g kg^{-1}, respectively) values were obtained when clipped at 5 cm of stubble height, differing ($P<0.05$) with CP values clipped at 10 and 15 cm (CP ranged from 122 to 129 g kg^{-1}) harvested in the same period (Figure 8, A). Over the evaluations, the CP values decreased from 175 to 115 g kg^{-1} and then to 122 g kg^{-1} in different frequencies, differing at 5% of probability (Figure 8, B). Stubble height × frequency interaction affected the CP values that ranged from 135 to 154 g kg^{-1}. The greater CP value ($P<0.05$) was at 10 cm stubble height, cutting every 2-wk (154 g kg^{-1}), however, low CP values occurred at 5 cm and 10 cm stubble height, cutting every 4- and 6-wk (142, 140; 141, 136 g kg^{-1}, respectively), respectively (Figure 8, C).

The greatest CP values occurred in the shortest harvest intervals and lower stubble heights (5 cm and 10 cm, 2-wk and 4-wk respectively). Evaluation date also affected CP, with lower CP in the second evaluation, followed by evaluation three. Nutritive value of alfalfa-bermudagrass and tropical forages in general declines with plant development, due to a lower leaf/stem ratio (Varonesi et al., 2010; Sule et al., 2021), increase in plant maturity, increase in the fiber fraction in the cell-wall, and accumulation of more stem than leaf (Kim et al., 2021). On the other hand, another factor to affect the nutritive value is the water stress. In the first evaluation (April) there was greater rainfall precipitation than the evaluation two and three (Figure 1). A similar CP result was reported by Xu et al. (2021) evaluating different alfalfa varieties (Hi-Gest 360, Gunner and RR Tonnica) under different harvest frequencies, with values ranging from 170 to 190 g kg^{-1} and 159 to 175 g kg^{-1} cutting every 4-wk and 5wk respectively. Crude protein was likely greater in the first and second evaluation of this study because of the increase in alfalfa contribution (Figure 4) during the growing season.

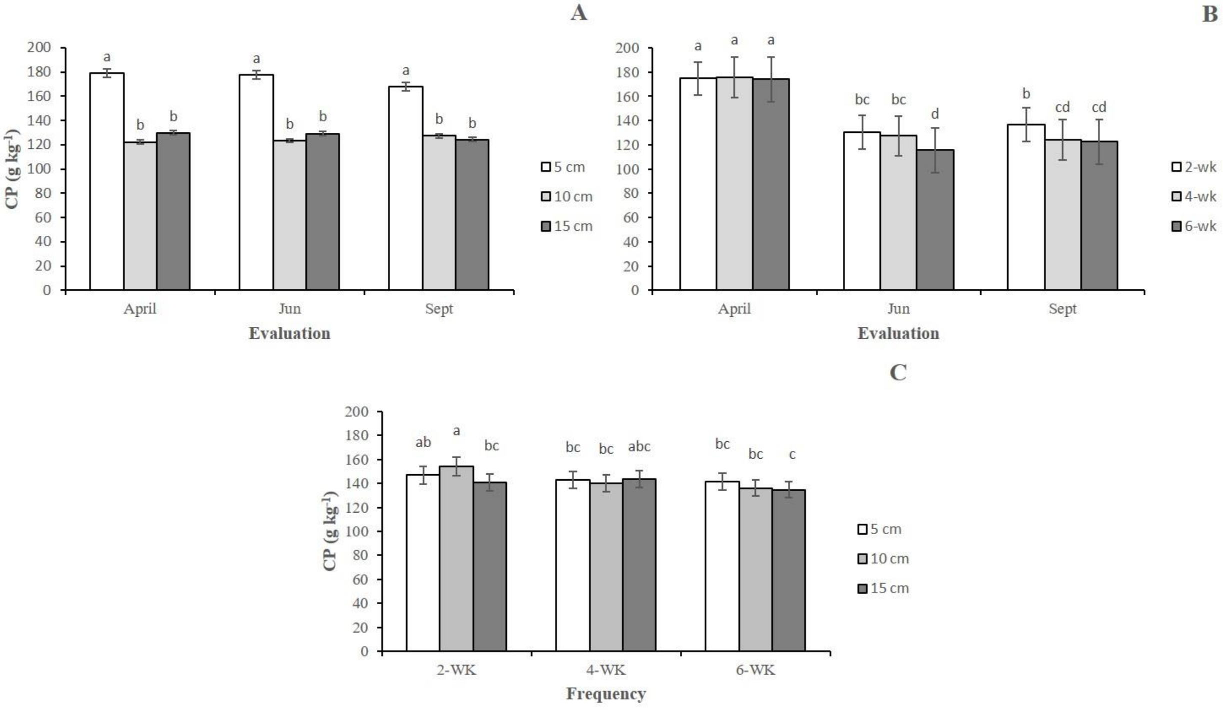

Figure 8. Evaluation × stubble height (A), evaluation × frequency (B), frequency × stubble height (C) interactions on crude protein (CP). Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

There was a harvest frequency evaluation interaction affecting in-vitro digestible organic matter (IVDOM). The frequency of the evaluation interaction had an effect on the IVDOM values, with higher (P 0.05) values in April, cut every 4 weeks. However, in September, these IVDOM values decreased regardless of harvesting frequency. The values ranged from 421 to 539 g kg^{-1} of DM, differing among them (P 0.05). The IVDOM value, cut every 4 wk, showed a linear decrease over the three evaluations (Figure 10). Increasing temperatures result in rapid growth, resulting in the increasing appearance of lignified tissues and reduced digestibility (Mislevy et al., 2001; Ezenwa et al., 2006). Hendricks et al. (2021) reported an IVDOM average value of bermudagrass-alfalfa mixture (Tifton 85 and Bulldog 805) of 80% harvested at 2-cm stubble height every 28–35 days.

In general, regardless of the harvesting interval and stubble height, the nutritive value of grass and legume mixtures declined as the season progressed (Figure 10). However, the nutritive value tended to increase as the legume contribution in the mixture increased in a new regrowth period (Hendricks et al. 2021). Garcia et al. (2021) reported similar results to IVDOM of legume-grass mixture in a study with unfertilized bahiagrass (*Paspalum notatum* Flüggé) overseeded with rye-oat-clovers (*Secale cereale* L.; *Avena sativa* L; *Trifolium* sp.) mixture and rhizoma peanut-bahiagrass mixture overseeded with rye-oat. The author reported greater IVDOM in the grass-legume mixture (465 to 522 g kg^{-1}) than in grass as a monoculture (522 g kg^{-1}). Under the same legume-grass components, Jaramillo et al. (2022) reported IVDOM values of 668 g kg^{-1}, superior to that reported by Garcia et al., (2021) and shown in this study, which ranged from 421 to 539 g kg^{-1}.

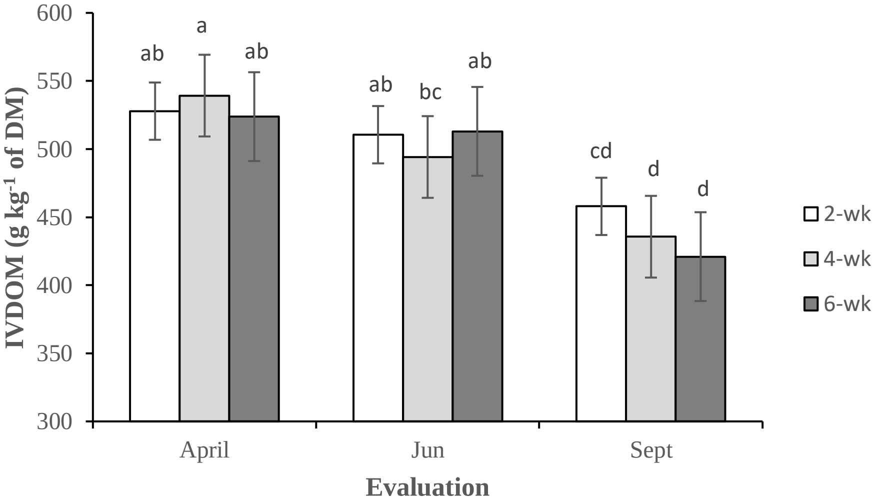

Figure 9. In vitro digestible organic matter (IVDOM) of alfalfa-bermudagrass mixture over evaluation. Column followed by a different small letter are significantly different at 5% probability level by Tukey test. Bars refer to the standard error of mean.

4 CONCLUSIONS

The alfalfa UF2015-AP resulted in greater THA, LC, Ny, and BNF than Bulldog 805 in 1 (April) out of 3 evaluations. For both alfalfa cultivars, harvesting less frequent (6 weeks) resulted in greater overall performance. UF2015-AP is a promising cultivar for release and should be tested in other different environments and management practices. The alfalfa-bermudagrass mixture tended to accumulate more forage mass, cut at 5 cm and 10 cm of stubble height every 4 and 6-wk, but regardless of the harvested frequency and stubble height, all the productive and nutritive value traits were affected by the period of evaluation, being in April the highest values and decreasing from June to September, thus showing the low persistence of both alfalfa varieties when mixed with bermudagrass in tropical conditions.

Nitrogen responses such as Ny, TNy and %Ndfa are strongly influenced by the harvest frequency and clipping height, except for BNF is affect by harvest frequency. The legume-grass mixture accumulated and fixed more N when harvested at a height of 5 and 15 cm, clipped every 6 weeks. This type of management for legume-grass mixtures could be an option for having high nitrogen use efficiency in grassland systems while also avoiding nitrogen losses due to leaching or byproducts of the denitrification process such as N_2O.

The nutritive value (CP and IVDOM) of the alfalfa-bermudagrass mixtures were affected by the evaluation dates, decreasing their values as much as the plant reached maturity or changed the season. Therefore, more studies are needed to evaluate the productive and nutritive values of other alfalfa varieties that are resistant to warm temperatures and in mixtures with grasses in North Florida under different management methods.

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CONCLUDING REMARKS

NITROGEN UTILIZATION AND CYCLING IN FORAGE PRODUCTION SYSTEMS

Through the studies developed in this dissertation, the importance of grassland ecosystems components interactions on nitrogen cycle dynamics was demonstrated, thus providing information about the nitrogen utilization strategies. More specifically, we found that coprophagous insects such as dung beetles have effects on GHGs and nutrient cycling from cattle dung. That means, all dung beetle in this experiment had a capacity to increase the N_2O fluxes from cattle dung, whereas the *Phaneus vindex* increased more N_2O fluxes than the *Digitonthophagus gazella*. Nevertheless, the dung beetle species *D. gazella* presented a tendency to reduce N_2O fluxes from cattle dung over time and showed an efficiency to remove dung on soil surface, which might be linked to the Pear millet improved performance. These results suggest that despite the beetle presence increases N losses, it could represent an alternative to raise forage N uptake from the soil and use it for production. Furthermore, this trial highlights the importance to preserve soil macrofauna and shows the need for more studies to understand the dung beetle effect on nitrate soil concentration and soil microorganisms.

Another important finding regarding nitrogen utilization strategies was that different genotypes of bermudagrass could be used to control the nitrogen losses in grassland systems, in addition to providing more forage mass and greater quality (crude protein and digestibility) to animal feed. Given this, Newell bermudagrass, also named in this study as 322, the genotype 323 and 286, exhibited a greater nitrogen yield in the growing season than other entries of this study, representing an alternative to enhance nitrogen efficiency use and cycling. The PC analysis revealed that the 286 genotype had a strong productive and nutritive value relation and could also be a candidate to be released as a cultivar. Furthermore, it was revealed that the Missouri genotype had low productive performance and nitrogen extraction.

In the last study, the effect of different harvest management methods was tested on productive and nutritive value traits of alfalfa (UF2015-AP and Bulldog 805)-bermudagrass (Tifton 85) mixture. As much as soil macrofauna and forages genotypes have a strong influence on nitrogen dynamics, the harvest frequencies have an important role on nitrogen responses, such as BNF. The UF2015-AP tended to be greater than Bulldog in many productive traits (THA, LC, Ny, and BNF) clipped 5 or 15 cm every 6 weeks. The grass-legume mixture accumulated more forage mass, cutting at 5 or 10 cm of stubble height every 4 or 6-wk. Low alfalfa persistence was observed over the evaluations due to the least alfalfa proportions in the mixture from June to September. The mixture tended to accumulate and fix more nitrogen clipped at 5 and 15 cm of stubble height, every 6 weeks. This management strategy might be an alternative for having high nitrogen use efficiency in grassland systems while also reducing nitrogen losses to the environment.

Finally, the take-home message from this study is that dung beetles, bermudagrass genotypes as monoculture and their mixture with alfalfa and forage management methods have a direct effect on nitrogen dynamics, making it important to pay attention to their interactions before establishing a forage system, in order to avoid nutrient losses, especially nitrogen.

APPENDIX

Figure 1. Dung beetle trial procedures.
A: dung collection and weight, B and C: pitfall trap installing on field, D: placing dung on the trap surface, E: dung beetle collection after 24 hours from installing on field, F and G: dung beetle classification and forming treatments.

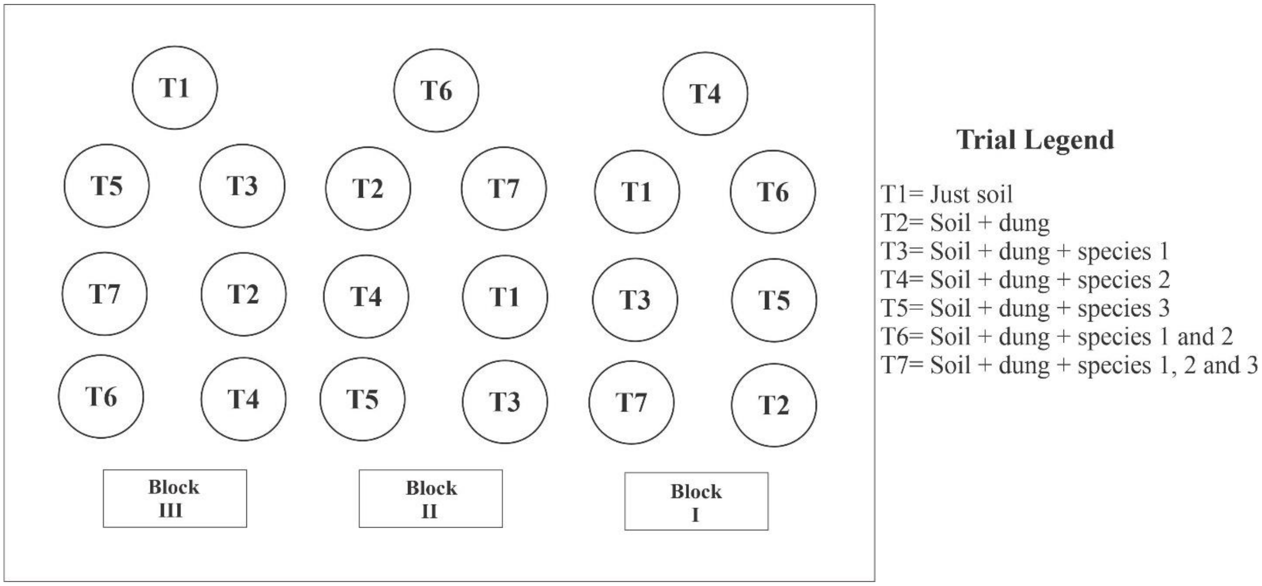


Figure 2. Dung beetle treatments map in Greenhouse.

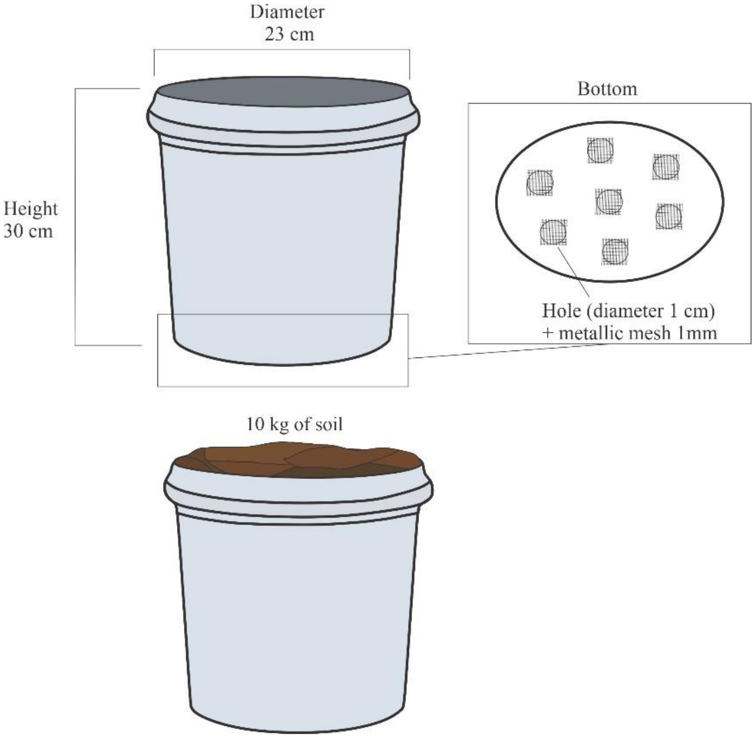


Figure 3. Terraria (plastic bucket) details for Dung beetle trial.

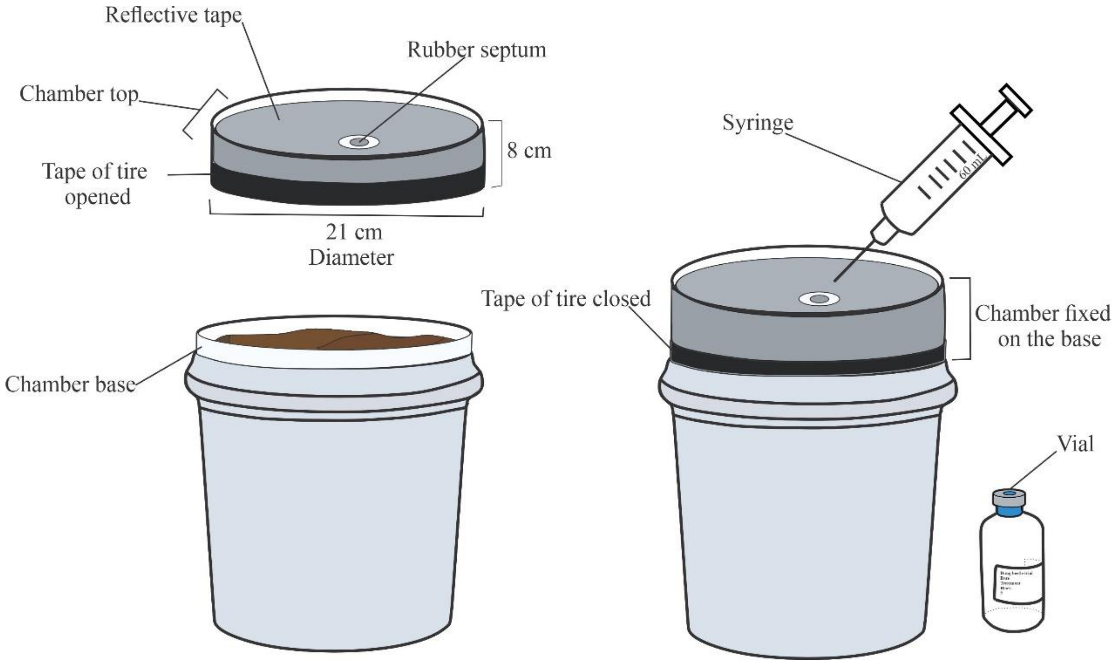


Figure 4. Static chamber details and instruments for GHG collection in Dung beetle trial.

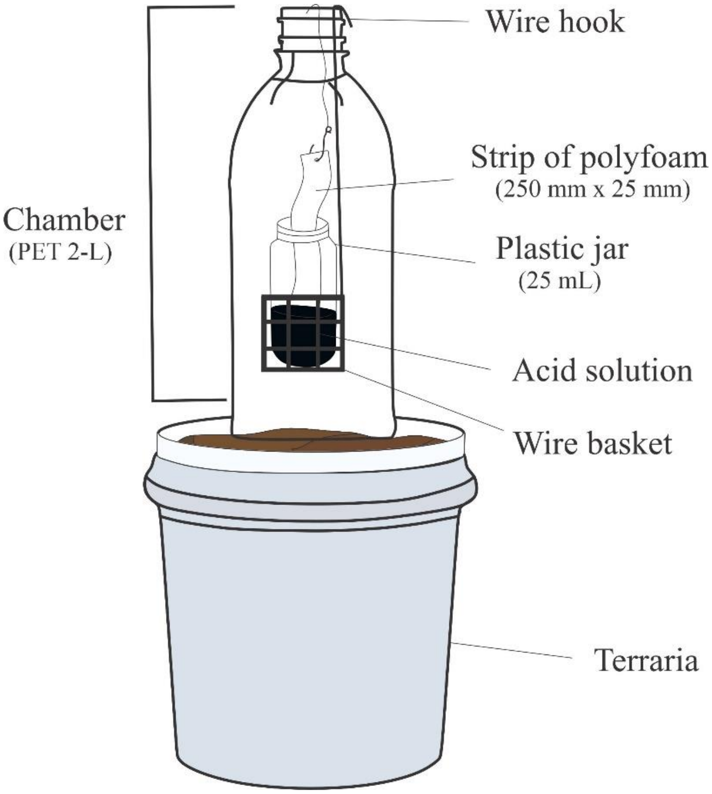


Figure 5. Mobil chamber details for ammonia (NH_3) sequestration in Dung beetle trial.

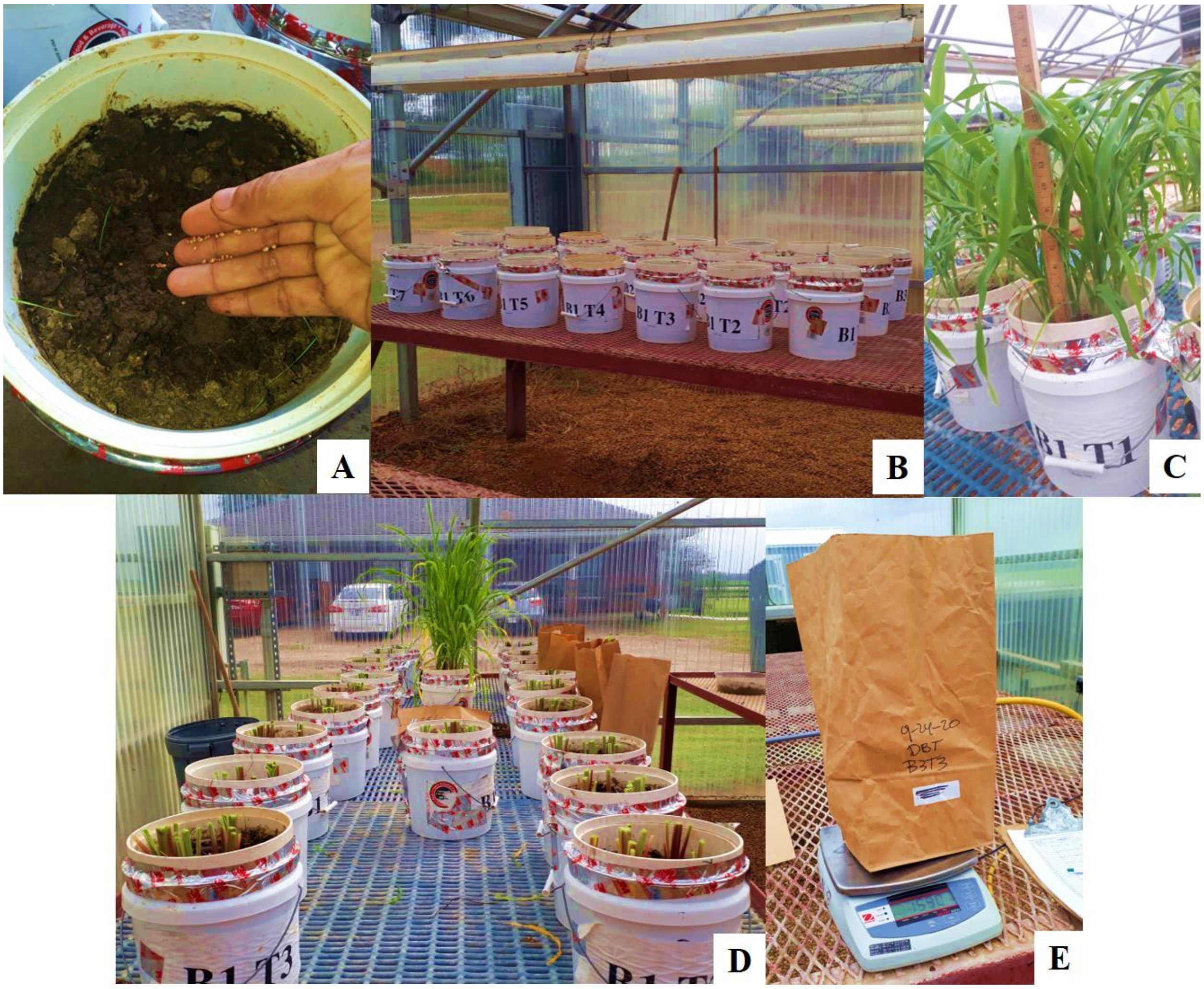


Figure 6. Dung beetle trial: nutrient cycling procedures.

A: pear miller sowing on the buckets after GHG evaluations, B: buckets distribution on the greenhouse, C: height measurements, D: pear millet harvest, E: pear millet fresh mass.

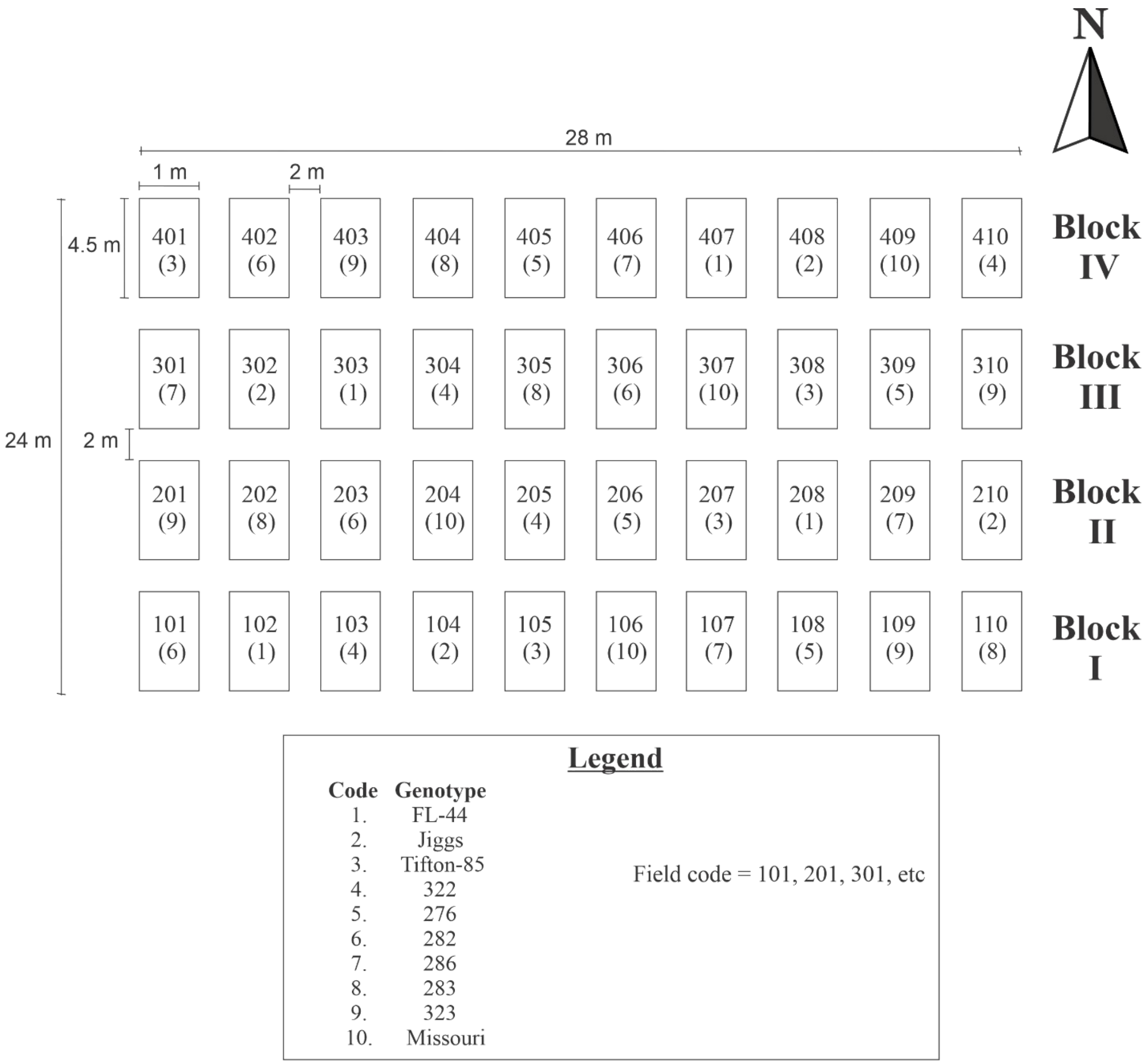


Figure 7. Bermudagrass treatments map.

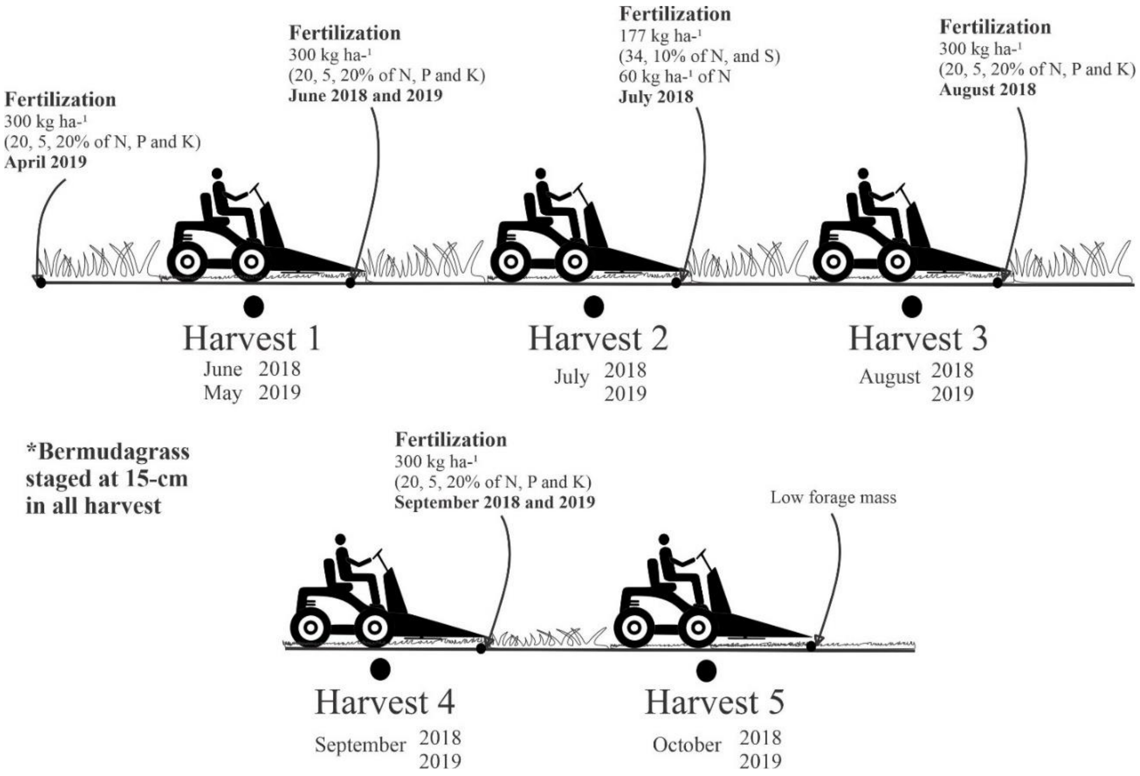


Figure 8. Timeline of bermudagrass trial harvest and fertilization 2018 and 2019.

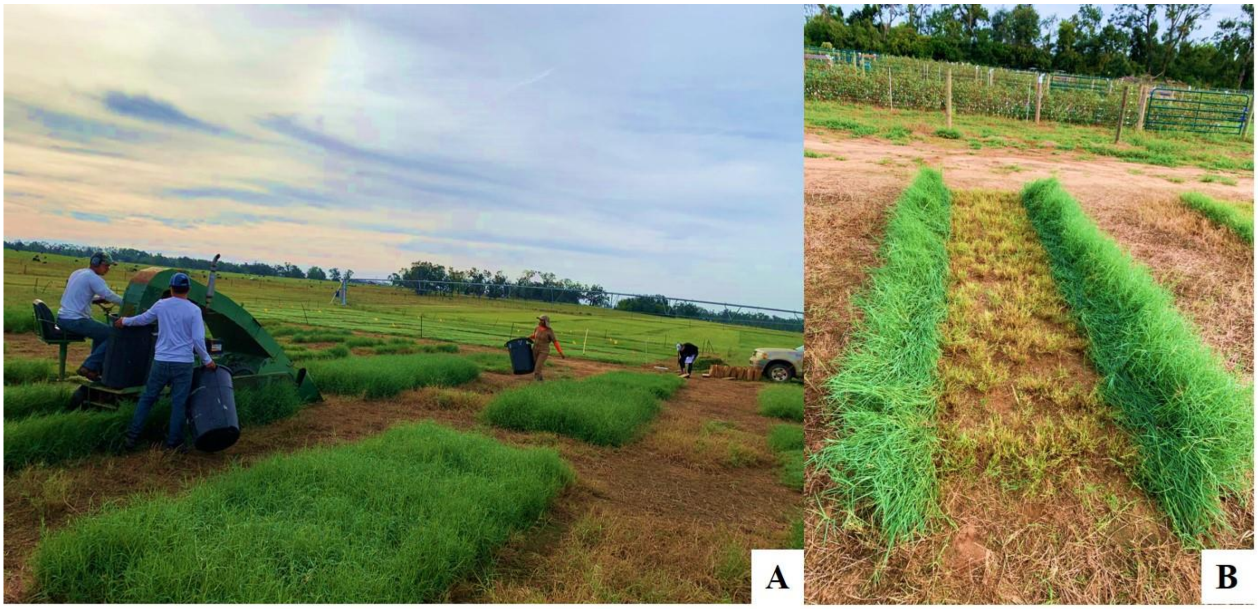
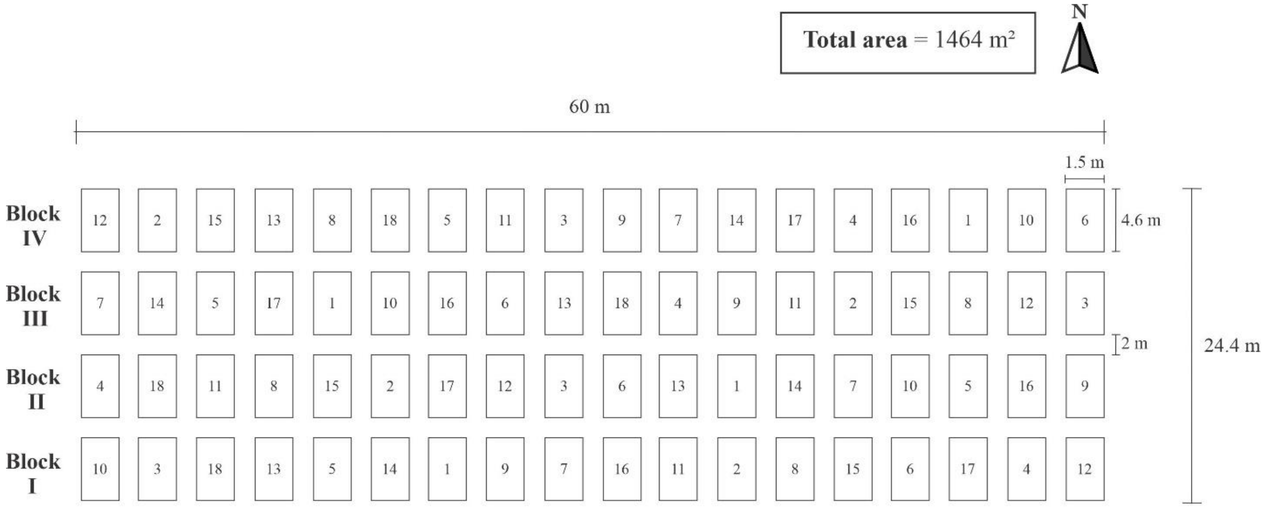


Figure 9. Bermudagrass treatments managements.
A: bermudagrass harvest per plot, B: plot area harvested



Treatment	Alfalfa variety	Frequency	Height
1	Bulldog 805	2 weeks	5 cm
2	Bulldog 805	2 weeks	10 cm
3	Bulldog 805	2 weeks	15 cm
4	Bulldog 805	4 weeks	5 cm
5	Bulldog 805	4 weeks	10 cm
6	Bulldog 805	4 weeks	15 cm
7	Bulldog 805	6 weeks	5 cm
8	Bulldog 805	6 weeks	10 cm
9	Bulldog 805	6 weeks	15 cm
10	UF2015-AP	2 weeks	5 cm
11	UF2015-AP	2 weeks	10 cm
12	UF2015-AP	2 weeks	15 cm
13	UF2015-AP	4 weeks	5 cm
14	UF2015-AP	4 weeks	10 cm
15	UF2015-AP	4 weeks	15 cm
16	UF2015-AP	6 weeks	5 cm
17	UF2015-AP	6 weeks	10 cm
18	UF2015-AP	6 weeks	15 cm

Figure 10. Alfalfa-bermudagrass treatments map.

Figure 11. Alfalfa-bermuda trial procedures.
A and B: legume-grass harvest per plot, Cand D: forage collecting from machine and weight.

Table 1. Soil and temperature average over time of dung beetle experiment during 2019 and 2020

Day	**Parameter**	**Treatments**						
		1	**2**	**3**	**4**	**5**	**6**	**7**
1	Soil temperature (ºC)	26	25	23	22	22	22	23
	Soil moisture (%)	66	-	-	-	-	-	-
2	Soil temperature (ºC)	26	25	25	25	25	25	26
	Soil moisture (%)	58	-	-	-	-	-	-
3	Soil temperature (ºC)	27	27	26	27	26	26	27
	Soil moisture (%)	47	-	-	-	-	-	-
4	Soil temperature (ºC)	28	27	26	27	26	26	26
	Soil moisture (%)	34	-	-	26	-	-	27
6	Soil temperature (ºC)	28	26	26	26	26	27	26
	Soil moisture (%)	38	-	-	27	-	19	21
12	Soil temperature (ºC)	28	26	26	26	26	27	26
	Soil moisture (%)	29	-	-	24	-	27	25
24	Soil temperature (ºC)	25	25	25	24	25	25	25
	Soil moisture (%)	24	47	32	35	83	75	83