

**UNIVERSIDADE DE SÃO PAULO
CENTRO DE ENERGIA NUCLEAR NA AGRICULTURA**

BEATRIZ ELISA BIZZUTI CREMOSTIM

**Greenhouse gas emissions and carbon and nitrogen stock in different forage
production systems**

**Piracicaba
2024**

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production systems**

Versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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Agriculture of the University of Sao Paulo as
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ABSTRACT

CREMOSTIM, B. E. B. **Greenhouse gas emissions and carbon and nitrogen stock in different forage production systems.** 2024. 79 p. Thesis (Ph.D.) – Center for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, 2024.

Plant biodiversity in grazing lands is reported to increase productivity and soil health compared to monocultures, as well as change carbon, nitrogen stocks, and greenhouse gas emissions. This comprehensive study explores the effects of including shrubs (silvopastoral) and leguminous forages (grass-legume) in the grazing system, exploring the impacts of these systems on greenhouse gas (GHG) emissions, soil quality, and soil biological activity. Two experiments were conducted. Experiment 1 was in southeastern Brazil evaluating the effects of exclusive grass systems (EGS) and silvopastoral systems (SPS) incorporating *Tithonia diversifolia* in GHG emissions, emission intensity, soil carbon (C) and nitrogen (N) stock, organic matter fractionation, and isotopic composition. While cumulative GHG emissions were comparable, emission intensity was lower in SPS. No significant differences were found in carbon and nitrogen stocks, but a tendency for higher particulate organic carbon in SPS and a distinct carbon isotopic signature indicate *T. diversifolia*'s influence on soil organic carbon levels despite the short-term establishment. Experiment 2, conducted at the North Florida Research Education Center – NFREC in Marianna, Florida, USA, assessed soil biological activity (STBA), C mineralization, and $\delta^{13}\text{CO}_2$ in contrasting grazing systems 10 years old. The treatments included bahiagrass (*Paspalum notatum* Flugge) with nitrogen fertilizer (BGN), bahiagrass (BG) with a winter legume in the cool season, and bahiagrass with perennial rhizoma peanut (*Arachis glabrata* Benth.) and a winter legume (BGRP). Soil cumulative CO_2 emission, soil-test biological activity, and carbon mineralization were not different among treatments. Even though BGRP reduced carbon and nitrogen stocks, it exhibited biological activity comparable to that of the other treatments, BG and BGN.. Incorporating legume forage into grassland systems emerges as a significant alternative to reduce the need for nitrogen fertilizer without impacting soil biological activity. These experiments highlight the complex interplay between land management practices, and C4/C3 integration in grazing systems, emphasizing the need for more studies to fully understand the potential of sustainable agricultural systems in mitigating climate change and optimizing soil health.

Keywords: Carbon sequestration. C4/C3 integration. Soil biological activity. Plant diversity.

RESUMO

CREMOSTIM, B. E. B. **Emissões de gases de efeito estufa e estoque de carbono e nitrogênio em diferentes sistemas de produção de forragem.** 2024. 79 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2024.

A diversidade de plantas em sistemas de pastagens está associada ao aumento da produtividade e à melhoria da saúde do solo, além de exercer influência sobre os estoques no solo de carbono e de nitrogênio e as emissões de gases de efeito estufa. Este estudo investiga os efeitos da inclusão de arbustivas (silvopastoril) e leguminosas forrageiras (gramínea-leguminosa) em sistemas pastoris, explorando os impactos destes sistemas nas emissões de gases de efeito estufa (GEE), na qualidade e na atividade biológica do solo. Dois experimentos foram conduzidos. O Experimento 1 foi realizado no sudeste do Brasil avaliando os efeitos de um sistema de gramínea exclusivo (EGS) e sistema silvopastoril (SPS) com *Tithonia diversifolia* nas emissões de GEE, intensidade de emissão, estoque de carbono (C) e de nitrogênio (N), fracionamento de matéria orgânica e composição isotópica do C no solo. Embora as emissões cumulativas de GEE fossem comparáveis, a intensidade de emissão foi menor no SPS. Não foram encontradas diferenças significativas no estoque de carbono e nitrogênio, porém SPS demonstrou uma tendência para maior carbono orgânico particulado, e assinatura isotópica do carbono distinta do EGS, indicando a influência da *T. diversifolia* nos níveis de carbono orgânico do solo, apesar do curto período de estabelecimento dos sistemas. O Experimento 2, realizado na North Florida Research Education Center – NFREC, Florida, EUA, avaliou a atividade biológica do solo (STBA), mineralização de C e $\delta^{13}\text{CO}_2$ em sistemas de pastejo contrastantes com 10 anos de idade. Os tratamentos incluíram capim-bahia (*Paspalum notatum* Flugge) com fertilizante nitrogenado (BGN), apenas capim-bahia (BG) com leguminosa no inverno e capim-bahia com amendoim forrageiro (*Arachis glabrata* Benth.) e leguminosa no inverno (BGRP). A emissão cumulativa de CO_2 do solo, a atividade biológica e a mineralização do carbono não foram diferentes entre os tratamentos. Embora o BGRP tenha reduzido os estoques de carbono e nitrogênio, este apresentou atividade biológica comparável à dos outros tratamentos, BG e BGN.. A incorporação de leguminosas forrageiras em sistemas de pastagens surge como uma alternativa significativa para reduzir a necessidade de fertilizantes nitrogenados sem impactar a atividade biológica do solo. Estes experimentos destacam a complexa interação entre o manejo e a integração de plantas de ciclos fotossintéticos C4 e C3 nos sistemas de pastoreio, enfatizando a necessidade de mais estudos para compreender o potencial dos sistemas agrícolas sustentáveis na mitigação das alterações climáticas e na otimização da saúde do solo.

Palavras-chave: Atividade microbial do solo. Diversidade de plantas. Integração C4/C3. Sequestro de carbono.

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1. INTRODUCTION

The increasing global demand for animal products driven by population growth has encouraged both research efforts and public interest in improving livestock efficiency and comprehending its environmental implications (MOTTET et al., 2018). Livestock, particularly ruminants, are pivotal in converting low-quality and inedible fiber into nourishing and consumable products, including milk and meat (MOTTET et al., 2017). Therefore, the adoption of forage-based systems becomes essential in the continual effort to convert inedible biomass into high-quality, consumable food.

Besides the ruminant's ability to convert low-quality fiber into high-quality products, the forage-based systems are an option to active the sustainability of production. Forage-based systems have a multifunctional role, and an interaction between soil, plants, and animals (PETERS et al, 2013). Consequently, the utilization of forage-based systems becomes pivotal in Cattle farming in tropical and subtropical regions (OLIVEIRA et al., 2020). Livestock production with forage-based systems doesn't produce just milk and meat, but also brings some ecosystem services, such as erosion control, biodiversity, restoration of degraded lands, soil improvements, resource use efficiency, and climate change mitigation (PETERS et al, 2013). Increasing intensification and biodiversity of forage systems can provide the ecosystem functioning (GROSS et al., 2017).

There are many types of forage-based systems. Here, will be discussing about two of them, the silvopastoral (SPS) and the grass-legume (GLS). SPS is a type of agroforestry system, that combines grass, trees/shrubs, and livestock in one (RAMACHANDRAN NAIR et al., 2010; MONTAGNINI; IBRAHIM; MURGUEITIO RESTREPO, 2013). The SPS brings many services to the system and has the capacity for increased carbon (C) sequestering, above and belowground, compared with other land uses (NAIR et al., 2010). The presence of trees or shrubs in SPS can create microenvironments that support a wider variety of flora and fauna (RAMACHANDRAN NAIR et al., 2010), and can capture and store carbon (CERDA et al., 2019). The SPS can provide shade, the shade can reduce heat stress in cattle (GALLOSO-HERNÁNDEZ et al. 2020) and interfere with the soil temperature.

The GLS is a combination of perennial warm-season tropical grass and perennial forage legumes. This integration increases forage quality and improves soil health, in addition, the legume has the biological nitrogen fixation ability (BAIJUKYA et al., 2016). For all the benefits that legumes can bring to the system, Dubeux et al. (2017) found that new rhizoma peanut cultivars have the potential to increase forage production of 6020 to 10275 kg DM ha⁻¹, while Jaramillo et al. (2021) concluded that rhizoma peanut and clovers contribute to developing

sustainable grazing practices with reduced off-farm inputs, as nitrogen. The grass systems mixed with rhizome peanut and/or clover applied 190 kg N ha⁻¹ less than in the grass system only.

In both systems, SPS and GLS, were integrated C3 and C4 pathway plants. C3 plants, typified by many trees, crops, and legumes, employ the C3 photosynthetic pathway. On the other hand, C4 plants employ the C4 pathway in warm-season tropical grasses, recognized for their efficiency in high-temperature and arid environments. The coexistence of C3 and C4 plants within ecosystems gives rise to ecological interactions that produce a variety of ecosystem services (HAILE; NAIR; NAIR, 2010).

Grass systems are essential for livestock production and can mitigate climate change by sequestering atmospheric carbon dioxide (CO₂) through accumulating organic matter in soils (FOLLETT; REED, 2010), bringing also other important ecosystem services. Despite this, further research is required with forage-based systems, to understand how the different managements interfere in the soil, plant, and animal interface. In this dissertation, two experiments were conducted to understand the soil-plant interface at the two forage-base systems, SPS and GLS.

1.1 Hypothesis and Objectives

The hypothesis of study 1 (respective to chapters 3 and 4) was that the perennial C4 tropical grass *Urochloa brizantha* (Hochst. ex A. Rich.) R.D. Webster cv. Xaraes (syn. *Brachiaria brizantha* cv. Xaraes) when integrated with a tropical C3 shrub *Tithonia diversifolia* ssp., in a silvopastoral system, can bring benefits to the system such as reduction in the greenhouse gases emission intensity and greater positive effect on soil carbon stock compared to respective monoculture (Figure 1.1). The hypothesis of study 2 (respective to Chapter 5) is that the grass-legume grazing system can improve soil biological activity compared with only the grass grazing system and impact the N mineralization.

The main objective of the studies was to understand the effects of different forage systems, integrating C4 and C3 plants, in the greenhouse gas emission and soil parameters.

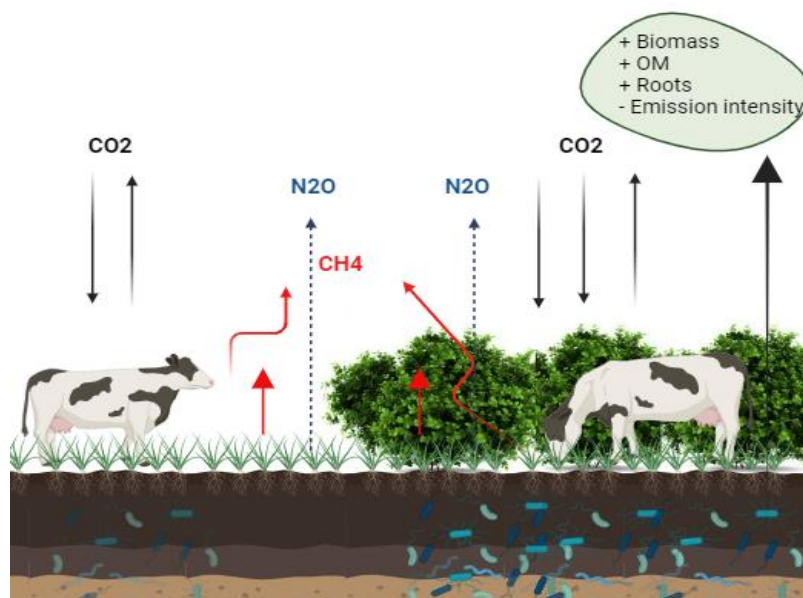


Figure 1.1 Infographic representing the soil-plant dynamic.

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2. LITERATURE REVIEW

2.1 Forage-based systems

2.1.1 Silvopastoral system and integration of C3 and C4 plants.

Pasture areas are a crucial component of Brazil's agriculture sector, playing a central role in livestock production, covering approximately 25% of the world's total land area and around 175 million hectares of Brazil's areas (IBGE, 2017a), contributing to the country's economy.

However, the conventional management of pastures has raised concerns related to environmental sustainability. In Brazil, approximately 12 million hectares are in stage of degradation (IBGE, 2017a) and about 50% of the Brazil's pastures are in some stage of degradation. The degradation of pastures results in low livestock productivity in grazing systems and leads to degraded soil and higher amounts of greenhouse gas emissions (MORAES et al., 2019).

Well-managed pastures can sequester atmospheric carbon by depositing it in the form of biomass above and below the soil (NAIR et al., 2010). The integration of shrubs and trees in pastures is a promising alternative to prevent stages of pasture degradation, reduce production seasonality, and increase the productive efficiency of livestock based on grazing systems (JOSE et al., 2019). In addition to providing various ecosystem benefits such as improved water retention, increased soil aeration, better nutrient utilization, and greater organic matter deposition.

In response to growing environmental concerns associated with agriculture, silvopastoral systems (SPS) are gaining support and advocacy from various stakeholders.

Based on the latest census by the IBGE (2017b), approximately 13.8 million hectares in Brazil are under agroforestry systems. The Empresa Brasileira de Pesquisa Agropecuária (Embrapa) has played a significant role in promoting the adoption of this technology among farmers. Silvopastoral systems involve the deliberate integration of trees or shrubs with pasture for livestock production (MONTAGNINI; IBRAHIM; MURGUEITIO RESTREPO, 2013). These systems offer numerous benefits, such as biodiversity enhancement, carbon sequestration, improved livestock productivity, and soil improvement (MONTAGNINI; IBRAHIM; MURGUEITIO RESTREPO, 2013).

The presence of trees or shrubs in SPS can create microenvironments that support a wider variety of flora and fauna (NAIR et al., 2010), and can capture and store carbon (CERDA et al., 2019).

The mix of C3 and C4 plants plays an important role in SPS. C3 plants, typified by many trees and crops, employ the C3 photosynthetic pathway (HAILE; NAIR; NAIR, 2010). In contrast, C4 plants utilize the C4 pathway, known for its efficiency under high-temperature and arid conditions. The coexistence of C3 and C4 plants with ecosystems creates ecological interactions that result in a range of ecosystem services.

Some of the ecosystem services provided by integrated C3 and C4 plants are carbon cycling, water resource utilization, and soil health (NING; HE; HOU, 2020). C3 plants are notably effective in long-term carbon sequestration, while C4 plants exhibit rapid carbon fixation, contributing to short-term carbon sequestration. C3 plants demonstrate higher water use efficiency in temperate climates, while C4 plants can be more resistant in hot and arid conditions. C3 plants contribute to soil organic matter through litterfall and root decomposition, while C4 plants, especially grasses, improve soil structure and prevent erosion. These plants, with their distinct photosynthetic pathways, provide complementary advantages and play a pivotal role in fortifying ecosystem services and ecological equilibrium.

Tithonia diversifolia is a C3 plant pathway from the Asteraceae family, native to Central America (PÉREZ, et al., 2009). It is adapted to acidic and low-fertility soils and tolerant to water deficit. It is a species with high biomass production and rapid growth, coupled with low input requirements, capable of reaching heights of 1.5 to 4.0 meters. Its morphological characteristics include alternate and large leaves, as well as substantial branches (PÉREZ et al., 2009).

Tithonia is widely used as green manure (OLABODE et al., 2007; SCRASE et al., 2019) due to its low C:N ratio, which facilitates and accelerates the decomposition process. In addition to rapid decomposition, this plant has high concentrations of nitrogen (N), phosphorus (P), and potassium (K) in its biomass. When recycled by soil microorganisms, these nutrients improve soil fertility, and its roots form symbiotic relationships with mycorrhizal fungi, enhancing nutrient absorption through the roots (PHIRI et al., 2003; SHARROCK et al., 2004).

This plant can be propagated by cuttings or seeds. There is evidence that *T. diversifolia* accumulates as much nitrogen in its leaves as leguminous plants, and it also has high phosphorus content (JAMA et al., 2000). Its nutritional quality varies depending on the phenological stage, so this is an important factor in management within the SPS. *T. diversifolia* leaves have a high total nitrogen content, a high proportion of crude protein, a high phosphorus proportion, rapid degradability, and ruminal fermentation, as well as good palatability (REIS et al., 2015).

The aforementioned soil benefits, combined with the interesting nutritional composition of this shrub, led us to study its implementation as a component in the silvopastoral system.

2.1.2 Grass-legume grazing system

Grass-legume grazing system is an agricultural management approach that involves the intentional combination and simultaneous cultivation of grasses and legumes for livestock grazing. Perennial, warm-climate grasses are the most used in tropical regions in grazing systems. Warm-climate C4 grass is generally the primary feed source due to its environmental adaptability (PETERS et al., 2013), but this grass normally provides most of its forage mass during the spring through early autumn, and a shortage of forage mass during winter (GATES et al., 2001). Besides the seasonality production, warm-climate C4 grasses also have a low-quality litter (i.e., high C/N and lignin/N ratio), and aggressive soil nutrients immobilization (WEDIN, 2004).

Incorporating perennial forage legumes into grazing systems is an alternative to increase forage quality, and improve soil health, addition that this species has the capability for biological nitrogen (N₂) fixation (BAIJUKYA et al., 2016). Forage legume adoption has been related to a higher degree of difficulty in implantation and establishment, due to persistence problems. However, there are a lot of studies with different varieties of legume species to improve these unpleasant features.

One of the forage legume species that is well reported in the literature is rhizoma peanut (RP; *Arachis glabrata* Benth.). RP is a warm-season perennial legume, well adapted to the Southeastern United States with a long development and research in Florida (DUBEUX et al 2017). The adoption of RP in forage systems has been limited due to the high cost and slow rate of establishment, but new technologies and varieties have been increasing the adoption rate. The use of forage legumes in warm climates has also been growing due to increasing fertilizer costs, decreasing cost of production, and increasing the grasses production, especially in low fertility input systems.

Biological N₂ fixation is the process that changes atmospheric N₂ into biologically useful NH₃, mediated by N-fixing rhizobia bacteria (Rhizobiaceae, α -Proteobacteria) (SØRENSEN; SESSITSCH, 2007). The legume plant supplies the rhizobia with energy (i.e. carbon from photosynthesis), and in return, the rhizobia fixes nitrogen in a way that is used for the plant. Almost all of the fixed nitrogen goes directly into the plant, but some nitrogen can be “leaked” or “transferred” into the soil for the neighboring non-legume plants (SCHWEMBER

et al., 2019). Besides this “leak”, most of the nitrogen returns to the soil for the non-legume vegetation by legumes roots and leaves decomposition.

For all the benefits that legumes can bring to the system, many authors have been studying this integration, and bringing together all the improvements that the legume inserts to livestock systems. Dubeux et al. (2017) investigating new RP cultivars found a potential to increase forage production. Jaramillo et al. (2021) studying RP and clovers in grazing systems concluded that these legumes contribute to developing sustainable grazing practices with reduced off-farm inputs, as nitrogen application.

Besides all the great forage mass in warm-season grass intercropped with warm-season legumes, the seasonality of production is a gap in the grazing system. This gap in the forage mass during the winter can be alleviated by overseeding cool-season annual forages into perennial warm-season grass pastures. Cool-season annual forages can reduce feeding costs and enhance animal performance (DUBEUX et al., 2016), due to having a greater nutritive value than the warm-season grasses. One of the most typical mixed cool seasons it is oat (*Avena sativa* L.), annual ryegrass (*Lolium multiflorum* Lam.), and a mix of clover crimson (*Trifolium incarnatum* L.), red (*T. pratense* L.), and ball clover (*T. nigrescens* Viv.). Cool-season annual forages decrease the cost of production, decreasing the days without grazing, and decreasing the necessity of hay supplementation (DILLARD et al., 2018). So Cantarutti et al. (2002), studying the effect and presence of legumes on pastures, concluded that the presence of legumes increases nitrogen supply to the soil, which stimulates the accumulation of soil organic matter and brings long-term sustainability to pasture systems.

2.2 Greenhouse gas emissions from agricultural systems

The concentrations of greenhouse gases (GHGs) worldwide have been rapidly increasing due to human activities since pre-industrial times (IPCC, 2020). Agriculture is considered one of the main sources of GHGs, as it can be both a source and a sink of these gases, and land use intensification can increase the exchange of carbon (C) and nitrogen (N) between soil and atmosphere (SAGGAR et al., 2008).

The livestock sector is a significant contributor to global GHG emissions, mainly methane (CH₄) and nitrous oxide (N₂O). These emissions result from enteric fermentation, manure management, and land-use change associated with livestock production. While the environmental impact is a cause of concern, innovative pasture system offers an opportunity to mitigate GHG emissions (PETERS et al, 2013).

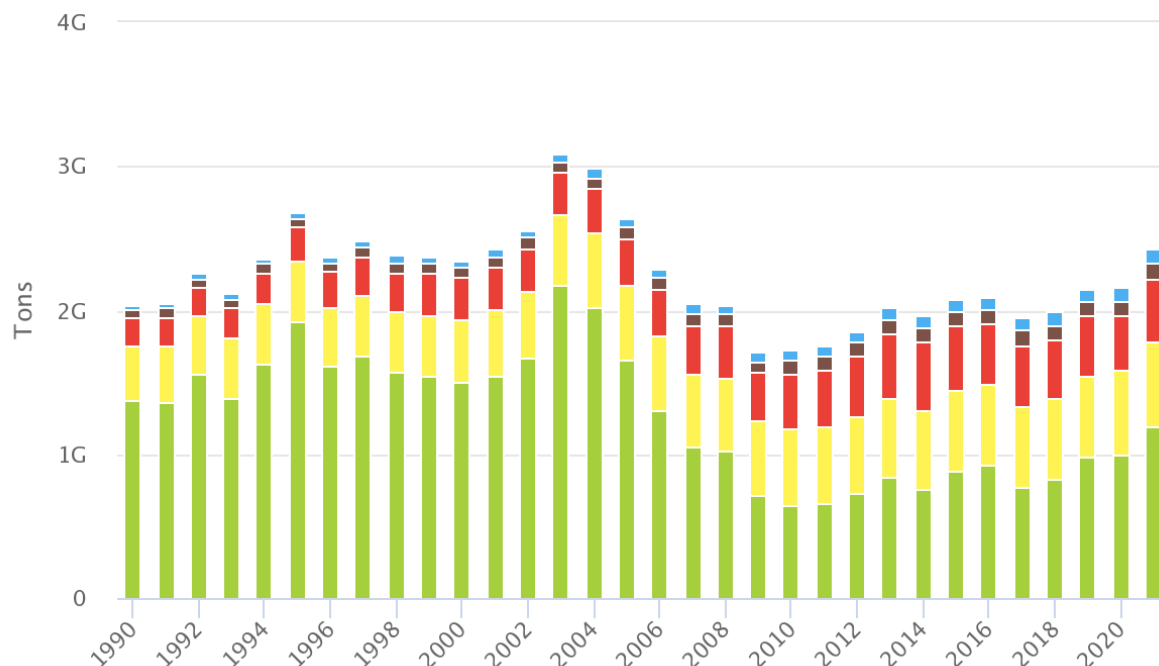


Figure 2.1 Brazil's emissions per sector are expressed in equivalent carbon dioxide. Blue = Residue, Brown = Industry, Yellow = Agriculture and livestock, Red = Energy, Green = Land change use. Adapted from SEEG (2023).

Carbon dioxide (CO₂) is the most abundant greenhouse gas emitted into the atmosphere, primarily through the burning of fossil fuels (34% energy and 15% transport), but also through biological processes within ecosystems and agriculture (22%). In this review, our focus is on emissions and sequestration of greenhouse gases (GHGs) by agriculture. It is estimated that CO₂ is responsible for approximately 70% of global warming, with its concentration in the atmosphere increasing by about 40% since the Industrial Era (IPCC, 2020). Currently, its concentration is approximately 415 ppm (<https://gml.noaa.gov/ccgg/trends/>). In agriculture (including livestock activities), CO₂ is emitted through the respiratory processes of living organisms and the loss of organic carbon from the soil. Carbon (C) derived from plants, such as root respiration and decomposition of plant material, contributes to over half of the total CO₂ emissions from soils (KUZUYAKOV, 2006). Soil disturbance, particularly in agricultural soils, also leads to significant loss of C in the form of CO₂ to the atmosphere (ONTL; SCHULTE, 2012).

Despite the mentioned sources of CO₂, agriculture plays a significant role in carbon (C) sequestration. Plants, as autotrophic organisms, use sunlight as an energy source and atmospheric CO₂ in the process of photosynthesis to synthesize tissues (leaves, stems, roots), storing this C in the produced tissues, both above and below the ground (ONTL; SCHULTE, 2012).

Methane (CH_4), another greenhouse gas, is also increasing its concentration in the atmosphere (IPCC, 2020). Currently, its concentration is approximately 1886 ppb (<https://gml.noaa.gov/ccgg/trends/>), and its global warming potential (GWP) is 29 times higher than that of CO_2 (IPCC, 2020). This gas is considered a global problem particularly because of ruminant animal trough eructation (belching), but in agriculture, CH_4 is also emitted by the soil, especially in waterlogged soils. However, well-drained soils can act as a sink for this gas through CH_4 oxidation.

CH_4 is synthesized in the soil by the microorganisms and the presence or absence of oxygen in this environment defines if methanogenic or methanotrophic microorganisms will be favored (SERRANO-SILVA et al., 2014). Methanogenic microorganisms are responsible for CH_4 production in anaerobic environments. On the other hand, methanotrophic microorganisms consume methane as a source of carbon and energy for their growth. Methanotrophic microorganisms account for approximately 10% of methane consumption.

Nitrous oxide (N_2O) has a global warming potential (GWP) about 298 times higher than CO_2 (IPCC, 2020). The concentration of this gas has been increasing due to anthropogenic activities such as industry and agriculture. In agriculture, N_2O is emitted from the soil through natural processes of nitrogen (N) nitrification and denitrification. However, agricultural practices such as fertilizer use and livestock manure, intensify these processes in the soil, leading to increased emissions of this gas into the atmosphere (OERTEL et al., 2016).

As discussed above, microbial activity, root respiration, organic matter degradation processes, as well as heterotrophic respiration of fauna, produce greenhouse gases (GHGs) in the soil (OERTEL et al., 2016). The rate of gas fluxes in the soil is dependent on various factors, such as soil moisture content, soil temperature, nutrient availability, pH, carbon-to-nitrogen ratio, and vegetation type (LUDWIG et al., 2001; OERTEL et al., 2016). The SPS offers greater opportunities than monoculture grazing systems for the capture and storage of atmospheric CO_2 in biomass and soil (NAIR et al., 2010; CHATTERJEE et al., 2018).

2.3 Automated GHG measurement system

Recent technologies have enabled the development of automated measurement systems, providing a higher resolution of rapid changes in soil greenhouse gas emissions over temporal measurements (PETRAKIS et al., 2017).

The G2508 instrument developed by Picarro Inc. (Santa Clara, California) and used in this study, simultaneously analyzes the five main gases released at the soil-atmosphere interface: CO_2 , CH_4 , N_2O , NH_3 , and H_2O (FLECK et al., 2013). It allows us to obtain gas

measurements with a temporal resolution of 1 second. High-frequency temporal measurements can enhance our understanding of the different biophysical mechanisms that influence greenhouse gas production and fluxes in the soil (PETRAKIS et al., 2017).

This equipment utilizes a cavity ring-down spectroscopy (CRDS) analyzer. The CRDS is an analytical technique used to measure the concentration of trace gases. It operates on the principle of how light interacts with gas samples. This analyzer works by adjusting light rays to the unique molecular fingerprint of the sample species and measuring the time that the light takes to ring down.

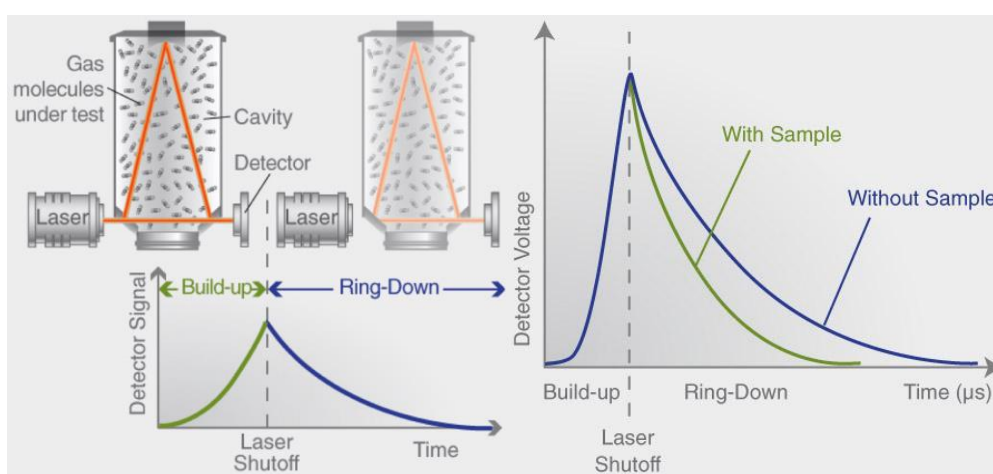


Figure 2.2 Infographic representing the CRDS system. (Source: © 2023 Picarro, Inc.)

The CRDS analyzer (Figure 2.2) starts with a highly stable laser source, where this laser emits light at a specific wavelength that corresponds to an absorption line of the gas of interest. The laser light is directed into an optical cavity, which is a highly reflective chamber with mirrors (extremely reflective, more than 99.9%) at each end. The gas sample of interest is introduced into the optical cavity through the sample inlet. The laser interacts with the gas sample and the gas molecules absorb light. The extent of absorption depends on the concentrations of the gas in the sample. The CRDS will measure the time it takes for the laser light to “ring-down” in intensity within the optical cavity. The “ring-down” rate of the sample is compared to a calibration curve, so the concentration can be determined (VERMA; SHRIVASTAVA; SHARMA, 2023). The CRDS is extremely sensitive because it can detect even very low concentrations of gases due to the precision of measuring the decay rate of light. This renders it highly beneficial in a wide range of applications, encompassing environmental monitoring, atmospheric research, and the analysis of trace gases.

This technique has been used by many authors to measure GHG emissions from soil in field experiments. Eihe et al. (2019) utilized the CRDS to measure ammonia emission. Martin and Moseman-Valtierra (2015) studied the GHG fluxes in different vegetation. Hawthorne et al. (2017) investigated GHG emissions under the influence of biochar and nitrogen application. In a study carried out by Christiansen, Outhwaite and Smukler (2015), they conducted a comparison between CRDS and gas chromatograph (GC) and illustrated the superior precision of CRDS.

However, measurements of greenhouse gas emissions using automated systems with high temporal frequency, along with spatial variability of measurements, are necessary to obtain a better understanding of the soil biogeochemical cycle and the results obtained in greenhouse gas studies (PETRAKIS et al., 2017). For this reason, this study utilizes the Picarro G2508 as a real-time gas analysis.

2.4 Soil-test biological activity methodology

Soil biological activity represents the heterotrophic activity, in other words, the respiration and biological process derived from the soil organisms. In soils, the microorganisms dominate the biological activity, and this can be measured by soil respiration. Soil respiration is one of the most important processes in the carbon cycle and is responsible for converting organic materials (from plants and animals) into carbon dioxide (CO₂) (FRANZLUEBBERS et al., 2000).

The microorganisms are responsible for this carbon dioxide emission, because they being the key mediations of this process, using organic compounds as energy and to proliferate. The measurement of soil respiration can reflect the functional capability of the soil to cycle nutrients, decompose organic matter, and stabilize organic carbon (SPRUNGER; MARTIN, 2023). All this functional capability is dependent on a diverse community of microorganisms. The diversity of soil microorganisms' community is directly related to the diversity of plants, amount of organic matter, quality of organic matter, and C/N ratio (BASTIDA et al., 2021).

Soil-test biological activity (STBA; Figure 4) is a test used as an indicator of soil biological activity. This test gives information about the potential carbon mineralization through the flux of CO₂ released by the soil in a laboratory-controlled incubation (FRANZLUEBBERS et al., 2018). Other variables can be measured using this test, such as nitrogen mineralization, nitrogen available to the plant at that moment, decomposition rate, and microbial biomass. This test is a reliable indicator of available carbon substrates, that have the potential to be processed by the resident soil microbial community.

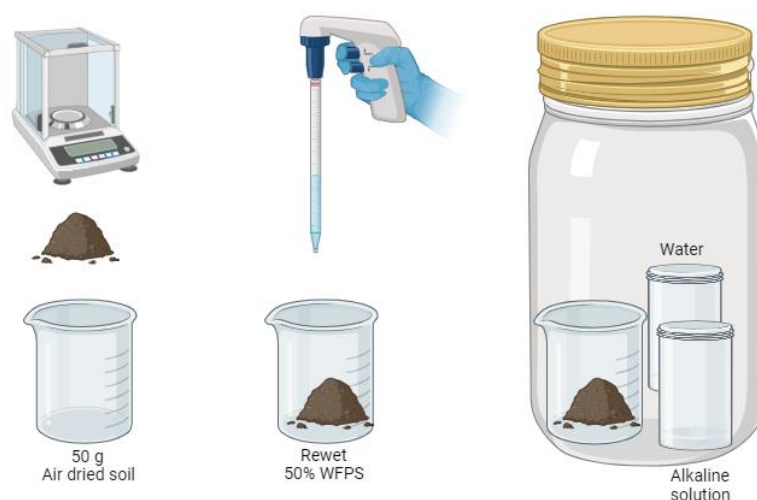


Figure 2.3 Schematic soil-test biological activity methodology used in this work.

According to Franzluebbers et al. (2018), the soil-test biological activity is also directly related to organic nitrogen in the soil. Higher values of SBTA can indicate higher values of organic nitrogen. Using this methodology in grass-legume soil can be a good indicator of the availability of organic nitrogen in the system. Franzluebbers et al. (2018) found that the flush of CO₂ is strongly associated with net nitrogen mineralization, with an average coefficient of correlation (r^2) around 0.83, for a wide range of soil types.

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3. CAN *TITHONIA DIVERSIFOLIA* BE USED IN SILVOPASTORAL SYSTEMS TO MITIGATE GREENHOUSE GAS EMISSIONS?

Abstract: Greenhouse gas (GHG) emissions were quantified in an exclusive grass system (EGS) and a silvopastoral system (SPS) over one year in southeastern Brazil, utilizing *Tithonia diversifolia*. Our objective was to assess in both EGS and SPS the microclimate conditions, GHG emissions, and emission intensity. Four grazing cycles were conducted over one experimental year to measure GHG fluxes from the soil, employing the static chamber technique linked to a cavity ring-down spectroscopy (CRDS) analyzer. Soil samples were collected at the end of the experimental period. Treatments were assigned using a completely randomized design with four replicates. GHGs were expressed in CO₂ equivalent (CO₂eq). The soil temperature in the SPS was 0.6°C lower than in the EGS ($p=0.024$). Cumulative emissions in CO₂eq were comparable among treatments but varied across cycles ($p<0.001$). Emission intensity (mg CO₂eq kg⁻¹ accumulated forage mass) was lower in SPS than in EGS ($p=0.002$). The intensification of the pasture system emerges as a crucial factor in achieving climate change mitigation, and the utilization of *T. diversifolia* in SPS appears promising as an alternative for reducing emission intensity.

Keywords: Climate change; Emission intensity; Forage; Mexican sunflower.

3.1 Introduction

The growing demand for animal products due to population growth has encouraged research and public interest in enhancing livestock efficiency and understanding its environmental consequences (MOTTET et al., 2018). Livestock, particularly ruminants, are crucial in transforming low-quality and inedible fiber into nutritious and edible foods, such as milk and meat (MOTTET et al., 2017). Consequently, the utilization of forage-based systems becomes pivotal in the ongoing effort to convert inedible biomass into high-quality, consumable food.

Cattle farming in tropical and subtropical regions is primarily conducted on pastures, with widespread extensive grazing practices (OLIVEIRA et al., 2020). A significant portion of these established pastures is undergoing degradation, contributing to greater greenhouse gas (GHG) emissions due to lower forage quality and quantity, increasing the sector's emission intensity.

Increasing intensification and biodiversity in grasslands generates GHG mitigations and can provide ecosystem functioning and services (GROSS et al., 2017). The use of

agroecosystems is a way to intensify the system and achieve biodiversity. Silvopastoral (SPS) systems are a type of agroecosystem management and constitute an ecologically integrated land-use strategy that combines livestock grazing with carefully managed tree/shrub cover (MONTAGNINI et al., 2013). This progressive agroforestry approach has gained significant recognition for its dual capacity not only to promote sustainable livestock farming but also to be a potential strategy to mitigate GHG emissions (MOSQUERA et al., 2012; BRETAS et al., 2020). Furthermore, with the increasing global environmental apprehensions, the examination of soil GHG emissions within silvopastoral systems arises as an essential research necessity.

Silvopastoral systems offer a multifaceted perspective on mitigating GHG emissions, as they combine livestock, perennial tree/shrub cover, and soil management practices within a single integrated framework (MONTAGNINI et al., 2013). Understanding the complexity of soil GHG emissions in such systems is essential, as they hold the potential not only to sequester carbon but also to reduce emissions associated with conventional grazing practices. In the face of the critical intersection of agriculture, climate change, and biodiversity conservation, the exploration of soil GHG emissions within silvopastoral systems carries profound implications.

One shrub that has been used in SPS arrangements is *Tithonia diversifolia*. This shrub from the Asteraceae family originates from Central America and exhibits remarkable adaptability to tropical conditions. Moreover, it grows well in acidic soils with minimal water requirements (PÉREZ et al., 2009). Notably, *T. diversifolia* has demonstrated consistent nutritional value even during arid seasons, distinguished by its protein and phosphorus content. Additionally, reports indicate its potential to enhance soil fertility and carbon input (RIBEIRO et al., 2016). The consortium among different species can provide numerous ecosystem services, such as improved soil biodiversity conservation, better use of water, erosion reduction, atmospheric carbon sequestration, and GHG mitigation (PAGIOLA, 2004; ANDRADE et al., 2008).

Rivera et al. (2019) found that intensive SPS reduces GHG emissions from pastures. Rivera et al. (2023), in a specific study with *T. diversifolia* in SPS, observed that SPS generates lower emissions factors from manure deposition compared to pasture monoculture. The authors recommended to better understanding of the GHG dynamic in SPS with *T. diversifolia*, and the necessity to study the GHG emissions in the soil without manure.

Given these attributes, we hypothesize that *Tithonia diversifolia* in an SPS brings improvements to the ecosystem and mitigates GHG emissions. We aim to investigate the system's ability to influence microclimate conditions, greenhouse gas (GHG) emissions and, emission intensity in comparison with a conventional grazing system.

3.2 Material and Methods

3.2.1 Site description and environmental conditions

The study was carried out in the Center for Nuclear Energy in Agriculture, in Piracicaba, São Paulo state, Brazil (22°42'23.31"S, 47°38'41.50"W, 517 m), from February 2020 to January 2023. The climate of the region according to Köppen-Geiger's classification is Cwa (ALVARES et al., 2013), characterized by well-defined dry winter and warm summer with mean annual temperatures of 23 °C and mean annual precipitation of 1310 mm. Weather conditions (Figure 3.1) during the GHG data collections (January 2022 to November 2022) were obtained from the meteorological station located at the Luiz de Queiroz College of Agriculture (ESALQ - USP), at 2.2 km from the study site.

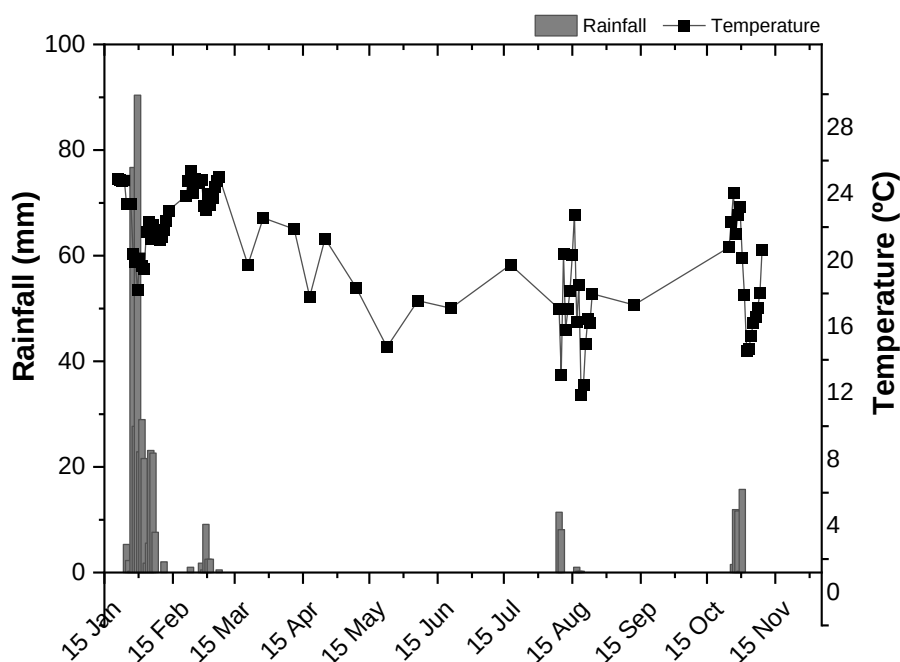


Figure 3.1 Daily temperature means and precipitation (mm) during the evaluation year (2022).

Soil is classified as typical Eutroferic Red Nitosol (FAO, 2015), with a bulk density of 1.36 g cm⁻³ in the 0-20 cm layer, comprising 32% clay, 24% silt, and 44% sand.

Soil chemical properties at the establishment of the treatments (January 2020) were determined by compositing soil samples: pH = 6 (CaCl₂); organic matter = 37 g dm⁻³ (Calorimetry); P = 87 mg dm⁻³ (ion-exchange resin extraction method); S = < 10 mg dm⁻³ (Calcium phosphate 0.01 mol L⁻¹); K = 1.6 mmol dm⁻³ (ion-exchange resin extraction method); Ca = 90 mmol dm⁻³ (ion-exchange resin extraction method); Mg = 41 mmol dm⁻³ (ion-exchange resin extraction method) Al = < 0.02 mmol dm⁻³ (KCl 1 mol L⁻¹);

$H+Al = 22 \text{ mmol dm}^{-3}$ (extraction by SMP), sum of bases = $132.6 \text{ mmol dm}^{-3}$; cation exchange capacity = $154.6 \text{ mmol dm}^{-3}$; base saturation = 86%, and Al saturation = 0%. No fertilization was conducted either before or during the experimental period.

3.2.2 Forage establishment and pasture management

The area was first desiccated in February 2020 using Glyphosate ($1\text{L } 100\text{L}^{-1}$ water, N-(phosphonomethyl) glycine), and the *Urochloa brizantha* (Hochst. ex A. Rich.) R.D. Webster cv. Xaraes (syn. *Brachiaria brizantha* cv. Xaraes) was sown through hand broadcast seeding (5 kg ha^{-1}) as the C_4 grass of the systems. In November 2020 the area was divided into 8 paddocks of 144 m^2 each, and the *Tithonia diversifolia* was planted as the C_3 in four of the paddocks. The *T. diversifolia* was planted in rows, in a 3 m between rows and 1 m between plants, with a stand of 6 plants per line, and a total of 6 lines per paddock. *T. diversifolia* was planted manually using stem cuttings collected from plants previously propagated by seeds. The stem cuttings were obtained from the central part of the *T. diversifolia* shrub during the pre-flowering stage (i.e., $\sim 150 \text{ cm}$ in height). Each stem cutting was 30 cm long, cut at a bevel, with the basal part and apex discarded to avoid using lignified or tender portions. Treatments were then considered as two production systems: Exclusive grass system (EGS) and Silvopastoral system (SPS) which were distributed in a completely randomized design, with four replicates. Paddocks were managed under the rotational stocking method with a variable stocking rate, following the put-and-take stocking approach (WHEELER et al., 1973), adjusted based on grass height (i.e., xaraés palisadegrass; pre-grazing 30 cm and post-grazing 15 cm). The paddocks were grazed by Santa Ines male sheep with a body weight of 27.9 ± 6.36 (mean $\pm$ SD) in four days of grazing, using 3 testers per treatment, passing paddock to paddock. The data collection started in January 2022, when the systems were established.

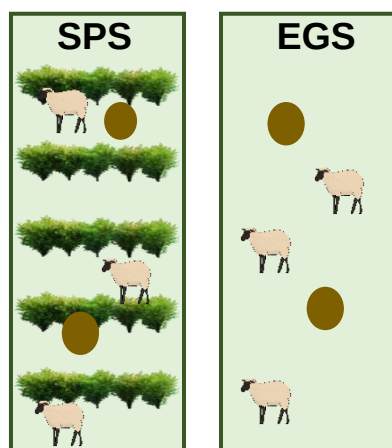


Figure 3.2 Experimental arrangement of treatments (SPS: Silvopastoral System; EGS: Exclusive Grass system; shrubs in the rows represent the *T. diversifolia*; brown circles represent the static chambers, and the animals represent the grazers).

Experimental data from forage mass (FM) and forage accumulated (FA) (EGS: Grass mass; SPS: Grass + shrub mass) were determined by the destructive method. The grass mass was assessed, for each paddock, harvesting two pre-grazed samples within a 0.25m²-quadrat, at 30 cm height, and two post-grazed samples at 15 cm stubble height. For *T. diversifolia*, FM in SPS was sampled by harvesting two pre-grazed plants per SPS paddock cut at 30 cm stubble height. The total FA of SPS was calculated by summing xaraés palisadegrass FA + *T. diversifolia* FA. The FA for both systems was determined by mass in space (0.25 m²) and estimated per hectare after subtracting the post-graze FA from the pre-grazed FA.

3.2.3 Greenhouse gas emission measurements

Greenhouse gases: carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O) were evaluated from January to November 2022, in four grazing cycles (referred as C1, C2, C3 and C4): January to February (01/30/2022 to 02/16/2022; C1), February to March (02/21/2022 to 03/08/2022; C2), August (08/09/2022 to 08/24/2022; C3) and October to November (10/25/2022 to 11/09/2022; C4). Among the cycles, one gas sampling was conducted every 15 days.

The GHG was measured using a static chamber technique (non-steady state chambers; DE KLEIN et al., 2020). Chambers were made of a base and a lid, both built out of polyvinyl chloride (PVC). Bases were buried at 5 cm depth and had 20 cm height and 30 cm diameter. Lids were 7 cm in height and 30 cm in diameter. The lid had a vent to balance the pressure when the chamber was closed (MARTIN; MOSEMAN-VALTIERRA, 2015; BRANNON et al., 2016), and a valve for the polytetrafluoroethylene (PTFE) coated tubes (4 mm diameter).

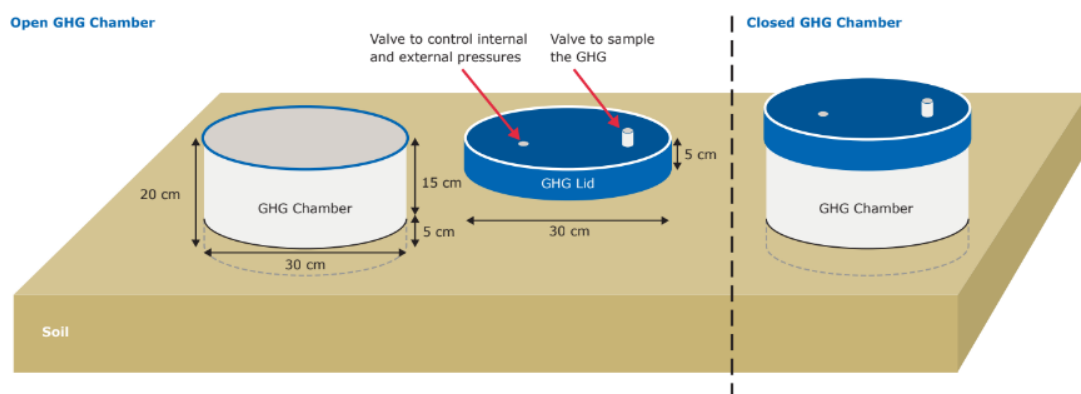


Figure 3.3 Infographic for the static chamber used in the experiment (adapted from GILLER et al., 2023).

Two chambers were installed per paddock, 10 days before the first gas sampling. The chambers were isolated with a fence to protect them from animal interference during experimental periods. The grass inside the fence and the chambers were cut, simulating animal grazing. One chamber from EGS (n=4) was used to measure the soil gas exchange, defined as soil respiration (SR), placed on the soil surface that was cleared from vegetation (JONES et al., 2005). In SPS, to represent the system, one chamber was placed under *T. diversifolia*, and another chamber were placed in the middle of the grass strip (n=8).

Gas samples were analyzed for CO₂, CH₄, and N₂O, using a Picarro (G2508 Greenhouse Gas Analyze; Picarro Inc., Santa Clara, CA, USA). The gas collection started at 09:00 am, which is more representative of the emissions (ALVES et al., 2012; MARTIN; MOSEMAN-VALTIERRA, 2015; PERRY; MOSEMAN-VALTIERRA; THORNBUR, 2020). All GHG measurements occurred for 5 minutes per chamber (with approximately 1-second sampling intervals), according to Martin and Moseman-Valtierra (2015), as the ideal period for linear rates of gas concentrations change, using a cavity ring-down spectroscopy (CRDS) analyzer. The CRDS instrument Picarro G2508 measured gases in an average of five consecutive minutes, with a precision rate as follows: CO₂ <200 ppb +/- 0.05%; CH₄ <5 ppb +/- 0.02%; N₂O <5 ppb +/- 0.008%.

3.2.4 Flux measurements and calculations

Fluxes of CO₂, CH₄, and N₂O (in mg m⁻² h⁻¹) were calculated considering the linear increase (or decrease) of gas concentration, air temperature and pressure, chamber volume, and the area of the bases of the cation period, using the Ideal Gas Law equation (MARTIN; MOSEMAN-VALTIERRA, 2015; ZAMAN et al, 2021; Equation 1):

$$F_{GHG} = [(\delta C / \delta t) \times 0.000001 \times V] \times P \div (R \times T) \div 0.1 \times M \quad (1)$$

where, F_{GHG} is the greenhouse gas flux (CO₂, N₂O or CH₄; g m⁻² h⁻¹); $\delta C / \delta t$ is the change in the gas concentration in the chamber during the deployment time; 0.000001 is a conversion factor; V is the chamber volume (m³); P is the local atmospheric pressure (Pa); R is the gas constant (J mol⁻¹K⁻¹); T is the temperature (in Kelvin); 0.1 is used to transform the flux in m⁻²; M is the molecular weight of the gas.

Cumulative emissions (g m^{-2}) of all gases were calculated by integrating the hourly fluxes over time (area under the curve). The carbon dioxide equivalent (CO_2eq) emission intensity was estimated considering the amount of CO_2eq (mg) emitted per cycle divided by the amount of forage mass produced (kg).

The CO_2eq was calculated considering the global warming potential (GWP) of the gases (Methane, 29.8; Nitrous oxide, 273) about CO_2 GWP (1) (IPCC, 2021), as described in Equations 2, 3 and 4:

$$\text{CO}_2\text{eq}(1) = \text{CH}_4(\text{ppm}) \times 29.8 \quad (2)$$

where CH_4 is the concentration of methane and 29.8 is the CH_4 GWP.

$$\text{CO}_2\text{eq}(2) = \text{N}_2\text{O}(\text{ppm}) \times 273 \quad (3)$$

where N_2O is the concentration of nitrous oxide and 273 is the N_2O GWP.

$$\text{CO}_2\text{eq} = \text{CO}_2(\text{ppm}) + \text{CO}_2\text{eq}(1) + \text{CO}_2\text{eq}(2) \quad (4)$$

where CO_2eq is the sum of equations 1 and 2 plus the CO_2 concentration.

3.2.5 Soil parameters and others variables

Soil samples were collected to determine Water-filled pore space (WFPS) every three days during the grazing cycles, while between cycles, soil collections were conducted every 15 days. The WFPS was calculated from the gravimetric water content and bulk density using a particle density of 2.65 g cm^{-3} (BLAKE; HARTGE, 1986). As the systems had no N inputs during the experimental period, the soil sample to determine inorganic N was collected once per cycle, as previously described. Inorganic N analyses (Ammonium-N and Nitrate-N) were conducted by using 2M KCl on fresh soil samples (BREMNER, 1965) and Kjeldahl methodology. Soil temperature, air temperature, and chamber temperature during the gas sample were measured using a digital thermometer. To represent the SPS system, the temperature was taken under the *T. diversifolia* and in the middle of the grass strip.

3.2.6 Statistical analysis

Fluxes of CO₂, CH₄, and N₂O were reported in CO₂eq as means and standard error of the mean. Normality of distribution and homogeneity of variances were evaluated by applying the Shapiro-Wilk test and Levene's test, using R software version 4.1.2 (R CORE TEAM, 2021) for all data. Integrated data for each grazing cycle (cumulative emissions), soil parameters, temperature, and emission intensity were submitted to ANOVA and t-test. Chamber was considered the experimental unit for GHG variables and the model included treatment (fixed effect), grazing cycle (treated as repeated measure), and their interactions according to the following model:

$$Y_{ij} = \mu + T_i + G_j + T_i * G_j + e_{ij} \quad (5)$$

where Y_{ij} is the response variable for the i treatment and j grazing cycle; μ is the general average; T_i is the effect of treatment; G_j is the effect of the grazing cycle; $T_i * G_j$ is the effect of interaction and e_{ij} represents the random error term associated with the i treatments and j grazing cycle.

Linear correlations between CO₂eq fluxes and soil temperature, WFPS, and air relative humidity (RH) were calculated using Origin software (ORIGINLAB CORPORATION, 2022). Soil respiration was considered the baseline.

3.3 Results

3.3.1 Environmental conditions

In 2022, the total precipitations at the study site amounted to 1244 mm, with distinct amounts recorded during each of the four cycles: 407 mm in C1, 129 mm in C2, 28 mm in C3, and 76 mm in C4. Temperature fluctuations occurred throughout the cycles, with mean temperatures of 25.0 °C, 25.3 °C, 19.5 °C, and 22.7 °C during C1, C2, C3, and C4, respectively (data sourced from the ESALQ meteorological station). The temperatures within the chambers (Table 1) during gas sampling varied from 15 °C to 40 °C, with means of 28.8 °C and 29.8 °C for SPS and EGS, respectively ($p=0.013$). Soil temperature means ($p=0.024$) were 23.2 °C for SPS and 23.8 °C for EGS.

The WFPS varied seasonally in response to rainfall, as shown in Figure 3. The highest WFPS was observed for C1, reaching approximately 62%. This was followed by C2 and C4, which had a WFPS of around 38%. The lowest WFPS was recorded for C3, with a value of around 32%, likely due to lower precipitation during this period.

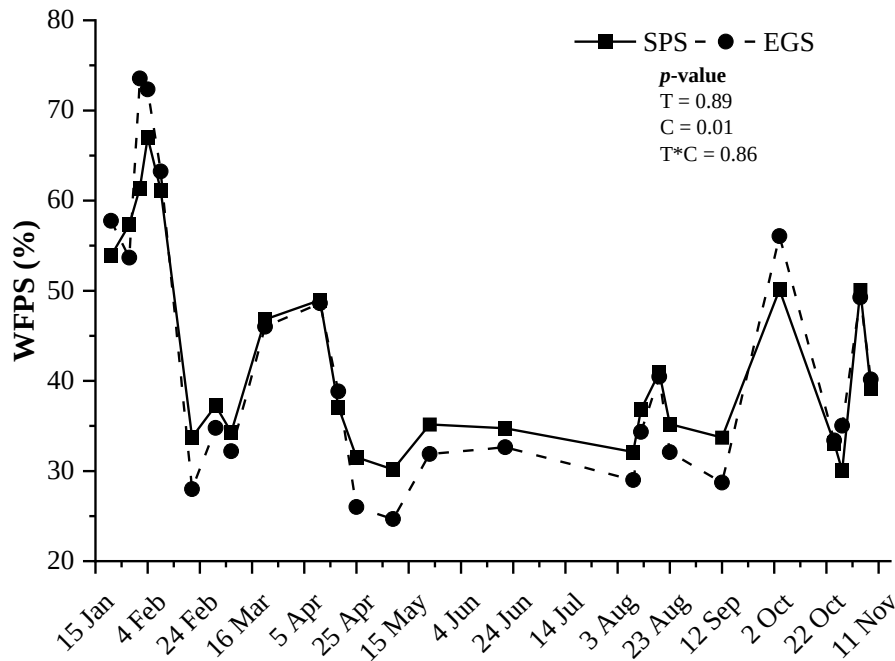


Figure 3.4 Water-filled pore space (WFPS %) during the evaluation year (2022) in the SPS and EGS systems. T = treatment, C = cycle, and T*C = interaction between treatment and cycle. Statistical significance tested by 5% of significance.

3.3.2 Soil parameters

In all treatments, the ammonium soil concentration (NH_4^+ ; approximately 22.4 mg kg^{-1} of soil) was greater than that of nitrate soil concentration (NO_3^-) (approximately 4.9 mg kg^{-1} of soil). However, these values are considered very low for both variables, and there was no significant difference between treatments at the end of the experiment (Table 1). Regarding soil and chamber temperatures, SPS had lower values than those observed in EGS.

Table 3.1 Inorganic nitrogen in the 0-10 cm soil layer and soil, chamber temperature for SPS and EGS.

Variables	SPS	EGS	SE	p-value
NH_4^+ (mg kg^{-1} of soil)	23.3	21.5	4.9	0.110
NO_3^- (mg kg^{-1} of soil)	4.8	5.02	2.4	0.983
Soil Temperature ($^{\circ}\text{C}$)	23.2 b	23.8 a	2.5	0.024*
Chamber Temperature ($^{\circ}\text{C}$)	28.8 b	29.8 a	2.94	0.013*

SPS = silvopastoral system; EGS = Exclusive grass system; NH_4^+ (Ammonium-N); NO_3^- (Nitrate-N); SE = stander error; * indicate the significance a 5%. Letters differ from each other.

3.3.3 Emissions in Carbon Dioxide Equivalent

The CO_2eq emissions (Figure 3.5) were highest in C1, C2, and C4 and lower in C3. The maximum rate of CO_2eq was recorded during the C1 grazing cycle in January 2022, with a mean flux of $680 \text{ mg CO}_2\text{eq m}^{-2} \text{ h}^{-1}$. The annual means CO_2eq fluxes were 471 and 517 to SPS

and EGS, respectively and the mean fluxes for the cycles were 680, 557, 269, and 470 mg CO₂eq m⁻² h⁻¹ (C1, C2 C3, and C4, respectively). In C3 and C4, it was observed that the flux between treatments (SPS and EGS) varied less compared to C1 and C2. Soil respiration (SR) was considered as the baseline.

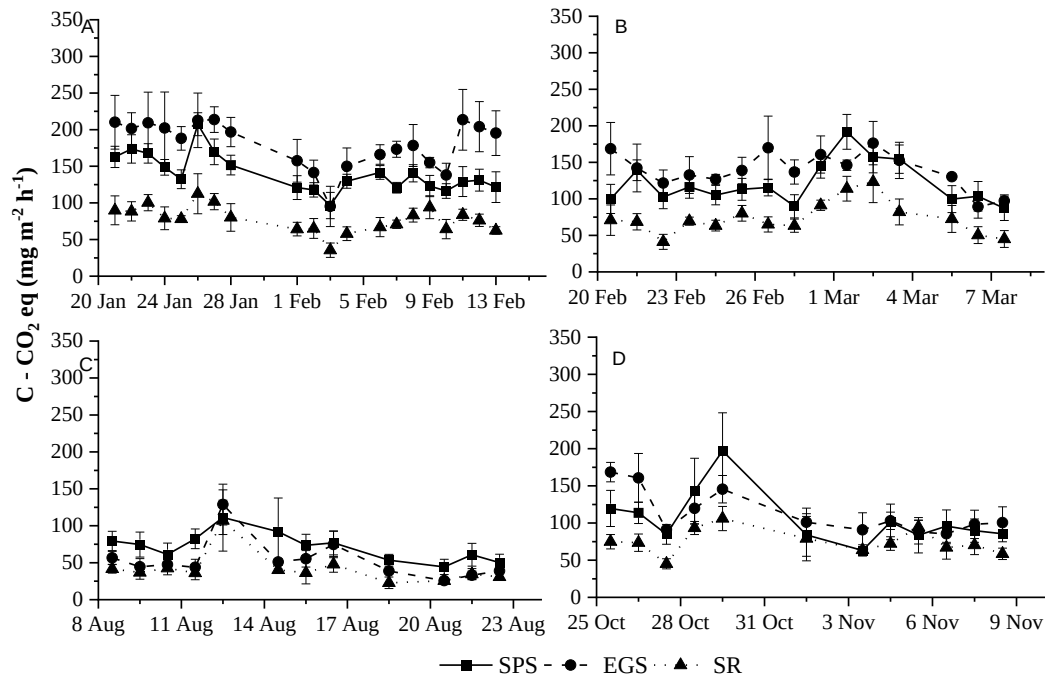


Figure 3.5 Mean daily fluxes in SPS and EGS in the grazing cycles (A = C1; B = C2; C = C3 and D = C4) along the year represented in C - CO₂eq (mg m⁻² h⁻¹). The bars represent the standard error of the means. SR is the soil respiration (baseline).

Soil temperature, WFPS, and RU were found a linear correlation with CO₂eq fluxes (Figure 3.6). Of the three variables, soil temperature had the strongest and most significant correlation with CO₂eq emissions, for both treatments (p -value < 0.01), compared with WFPS and RU. Both soil temperature and WFPS exhibited a higher coefficient of determination (R^2) and Pearson's r to EGS ($R^2 = 0.62$ and 0.14 ; Pearson's $r = 0.79$ and 0.38) than in the SPS ($R^2 = 0.43$ and 0.06 ; Pearson's $r = 0.66$ and 0.25). Concerning WFPS, only the EGS showed a significant correlation with CO₂eq emissions (p -value = 0.03).

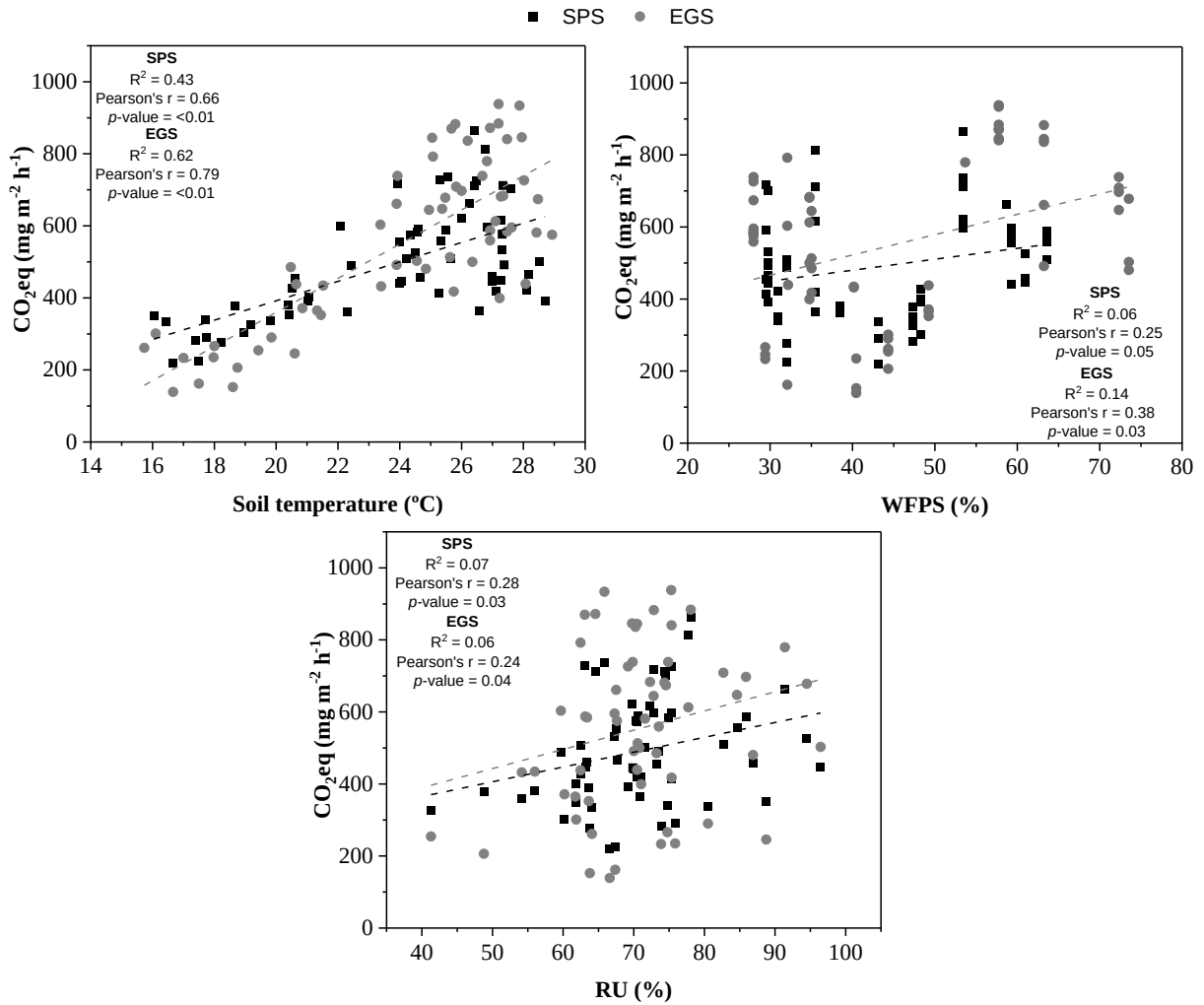


Figure 3.6 Daily CO₂ equivalent regression with soil temperature, water-filled pore space (WFPS), and air relative humidity (RU). Regression fit, coefficient of determination (R²) and Pearson's correlation were shown.

3.3.4 Cumulative emissions

Cumulative emissions for all GHG gases were not different between systems (Table 3.2). Although, significant differences between cycles were found for CO₂ and N₂O (p -value < 0.001 and 0.021, respectively), but not for CH₄. Negative values were observed for CH₄ and N₂O in both systems and all cycles, showing the ability of soils to be a sink of these gases. The smallest negative values were found for N₂O in C2 and C3. CO₂eq cumulative emissions were statistically different from cycles. The highest cumulative emissions values for CO₂eq were found in C1, C2, C4, and C3, respectively. No differences were found for the total year cumulative emission of CO₂eq (p -value = 0.741).

Table 3.2 Cumulative emissions (mg m^{-2}) in SPS and EGS systems during grazing cycles.

Variables	EGS	SPS	SE	p-value
CO ₂	196	186	9.6	0.592
CH ₄	-0.0155	-0.0101	0.008	0.649
N ₂ O	-0.0060	-0.0040	0.002	0.264
CO ₂ eq	194	185	9.7	0.626
CO ₂ eq Total year	2410	2340	650	0.741

	C1	C2	C3	C4	SE	P-value
CO ₂	364 a	187 b	88.7 c	152 b	16.0	<0.001*
CH ₄	-0.0105	-0.0240	-0.0080	-0.0092	0.009	0.549
N ₂ O	-0.0083 b	-0.0020 a	-0.0015 a	-0.0080 b	0.002	0.003*
CO ₂ eq	361 a	186 b	87.9 c	123 bc	16.1	<0.001*

C1 = cycle 1; C2 = cycle 2; C3 = cycle 3; C4 = cycle 4; SE = standard error. * indicate the significance at 5%. Letters differ from each other.

Despite there being no variations in cumulative CO₂eq emissions between treatments, differences in emission intensity were observed. SPS had a lower emission intensity than EGS, and the emission intensity was also affected by the cycle (Figure 3.7).

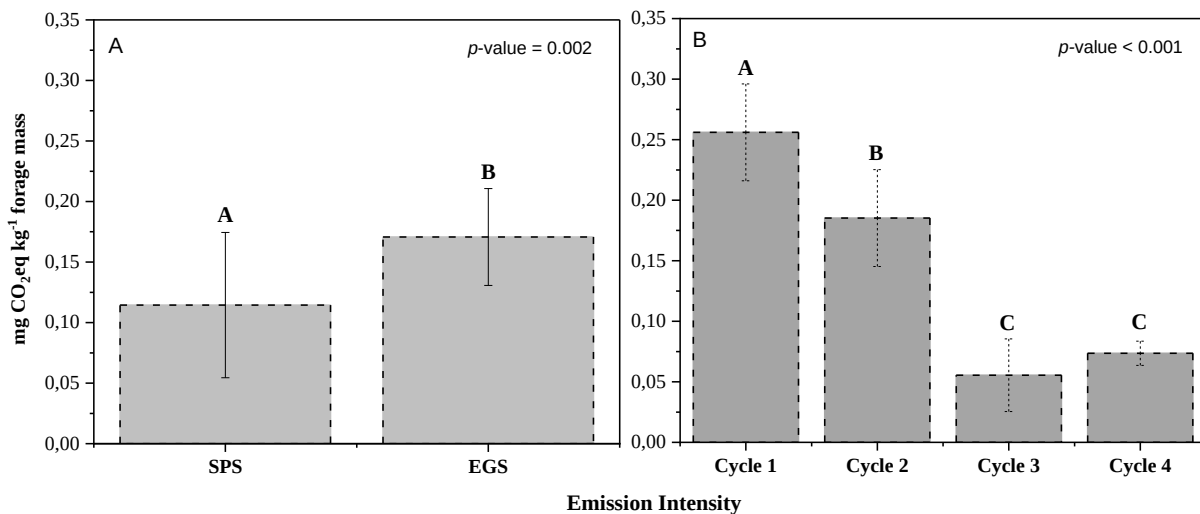


Figure 3.8 Mean emission intensity for treatments (A) and mean emission intensity for cycles (B). The bars represent the standard error of the means and the letters differ from each other by $p < 0.05$.

3.4 Discussion

To investigate GHG emissions in the absence of fertilizer inputs, no fertilization was applied before or during the experimental period. As a result, any inorganic N found in the systems likely originated from prior applications or N mineralization. This explains the lower levels of inorganic N for both systems (Table 3.1).

The climatic conditions prevailing in the region exhibit a tropical climate, characterized by abundant rainfall during the summer months and reduced precipitation during the autumn and winter. The response of WFPS was intricately tied to these seasonal variations (Figure 3.3), with elevated WFPS values observed during the summer season and diminished values during the winter, reflecting distinct rainfall patterns (Figure 3.1). In addition to seasonal influences, the presence of the shrub *Tithonia diversifolia* in SPS can also exert an impact on WFPS levels (RIVERA et al., 2023), this shrub it was managed with 130 to 200 cm height. The canopy of shrubs plays a significant role in modifying rainfall interception, while the shade provided by these shrubs contributes to the preservation of atmospheric and soil moisture (MONTAGNINI; IBRAHIM; MURGUEITIO RESTREPO, 2013). This microclimate, created by the presence of shrubs within SPS directly influences temperature dynamics (CASTILLO; TIEZZI; FRANZLUEBBERS, 2020). Perhaps these factors may explain the lower soil temperature observed in SPS compared to that in EGS (Table 3.1).

For the GHG emission, the fluxes emphasize the significant influence of humidity and temperature on these gases, with no differences between the systems, but higher CO₂eq fluxes observed during the rainy season (C1 and C2) and lower fluxes during the dry season (C3 and C4) (Table 3.2 and Figure 3.5). This observation aligns with previous findings by Oertel et al. (2016), Lal et al. (2018), and Bretas et al. (2020), who highlighted soil temperature and humidity as key variables explaining variations in GHG emissions. Furthermore, the WFPS, humidity, and temperature are recognized as primary drivers of GHG emissions in the literature (LUDWIG et al., 2001).

Cumulative emissions exhibited similar patterns to fluxes, varying between cycles (Table 3.2) and not between treatments. Although vegetation plays a role in shaping GHG emissions, the short-term establishment of the SPS may not have led to significant differences in cumulative emissions compared to the EGS. As noted by Torres et al. (2017), the potential GHG mitigation of SPS varies based on system implementation and plant maturity. Litton et al. (2003) also discussed the impact of aboveground biomass quantity and age on GHG emissions, finding that these variables influence emissions. Both systems in the present study had a short-term establishment.

The release of CO₂ from the soil surface results from the collective contributions of microorganisms and plant roots, serving as an indicator of below-ground metabolic activity (ADAME-CASTRO et al., 2020). Our results are in accordance with Adame-Castro et al. (2020) who found no variations between systems, but variations for seasons with higher CO₂ fluxes in the rainy season when the soils contained a greater amount of moisture.

For the CH₄ cumulative fluxes, the soil acted as a sink of this gas for both systems and all cycles (Table 3.2), No differences were found between systems or cycles. CH₄ in soil is produced by methanogenic microorganisms under anaerobic conditions and it is consumed by methanotrophic microorganisms that use O₂ and CH₄ for their metabolism under aerobic conditions (DUTTAUR; VERCHOT, 2007). CH₄ emission or uptake is positively correlated with soil humidity (GAO et al., 2013) and is mostly influenced by microbial processes, so the availability of substrate to the microorganisms interferes with gas production/consumption (TANG et al., 2019). Many studies have indicated that N status can inhibit, stimulate, or have no effect on soil CH₄ uptake. As observed by Saggar et al., (2008), the lower N-availability can stimulate CH₄ uptake, due to the higher presence of CH₄-assimilating bacteria (MAB) and less presence of NH₃-oxidising bacteria (AAOB), which use NH₃ oxidation as an energy source instead of CH₄. These explanations elucidate the CH₄ sink found in this experiment (Table 3.2) and show that N can drive CH₄ production.

As already mentioned, no N was added to the systems, so as observed for CH₄, N₂O consumption was also found, for both systems and cycles (Table 3.2). Chapuis-Lardy et al. (2007) have discussed N₂O consumption and found that low mineral N levels and high moisture can promote this process due to favor of the denitrification process, which is responsible for reducing N₂O to N₂, decreasing the ratio N₂O/N₂ in the soil. This ratio can be increased by two main factors, first the decrease in the enzyme responsible for the N₂O reduction and second by the higher amount of NO₃⁻ in the soil, since NO₃⁻ is preferred as an electron acceptor over N₂O in the denitrification process (GLATZEL; STAHR, 2001). These explanations clarify the consumption of N₂O observed in this experiment and provide evidence that the most important driver of N₂O consumption is the N-limitations (WU et al., 2013).

The correlations between flux (CO₂eq) and soil temperature (Figure 3.6) underscore the importance of this factor in the soil process responsible for the GHG efflux. The WFPS and relative air humidity also showed a correlation with CO₂eq, but lower than the soil temperature. The EGS had a higher correlation to soil temperature and WFPS than SPS, probably due to lesser variations in the data. Several studies have demonstrated that diurnal patterns of CO₂ emissions are correlated with temperature and/or WFPS and that these are the main drivers of GHG production (OERTEL et al., 2016; BARBA; POYATOS; VARGAS, 2019), confirming the results found in this experiment.

Emission intensity, defined as the volume of GHG emissions per unit of product produced, serves as a crucial metric. Reducing emission intensity implies lower pollution per unit of product, in this case, per kilogram of forage mass produced. An alternative to decrease

emission intensity is improving productive efficiency. According to Rivera et al. (2021a) the inclusion of *T. diversifolia* in ruminant's diet, reduced enteric CH₄ emission and increased milk productivity, especially in moderate rainy season. The results of the present study demonstrate that the SPS, using *T. diversifolia*, decreases emission intensity within the system, emitting the same amount but producing more FM than exclusive grass (Figure 3.7). Rivera et al. (2023) working with the same shrub in SPS showed that *T. diversifolia* can also decrease the emissions of CH₄ and N₂O from manure. Rivera et al. (2021b) observed that *T. diversifolia* could adapt to different edaphoclimatic conditions, having a wide plasticity, and keeping productive in adverse conditions when the grass stops growth. The SPS with *T. diversifolia* was able to produce a great amount of FM without decreasing in the dry season. Mauricio et al. (2017) concluded that *T. diversifolia* when planted along grasses in SPS contributes to an increase the animal production per hectare, due to a greater amount of mass and higher quality.

3.5 Conclusions

The intensification of the pasture system emerges as a crucial factor in achieving climate change mitigation, and the utilization of *Tithonia diversifolia* in SPS appears promising as an alternative to bring sustainability to the system, reducing emission intensity while maintaining forage mass production.

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4. CARBON STOCK AND CARBON ISOTOPIC COMPOSITION IN SILVOPASTORAL WITH *T. DIVERSIFOLIA* AND EXCLUSIVE GRASS SYSTEM

Abstract: Silvopastoral Systems (SPS) integrates grass, trees/shrubs, and livestock to enhance production, mitigate climate change, and optimize land use. This study focuses on including *Tithonia diversifolia* in SPS, exploring its potential to increase carbon sequestration. This study aimed to compare the soil carbon (C) and nitrogen (N) stock, organic matter fractionation, and soil isotopic composition in an SPS and an exclusive grass system (EGS). Soil samples were collected at the beginning and the end of the experimental period (January 2020 to January 2023). Carbon and nitrogen were determined by combustion in an EA-IRMS (mass spectrometer). Soil organic matter fraction was calculated and divided into particulate organic matter (POM) and mineral-associated organic matter (MAOM). The isotopic composition of ^{13}C was determined at the Isotopic Ecology Laboratory at CENA/USP. No differences were found between treatments to stock in carbon, mineral-associated organic carbon, and particulate organic carbon (p -value=0.164, 0.198, and 0.163, respectively). For nitrogen stock, no difference was found, neither to nitrogen in the organic matter fraction MAOM and POM (p -value=0.666, 0.574, and 0.419, respectively). The variation in particulate organic carbon (ΔPOC) was not different between treatments (p -value=0.071) but showed a tendency to stock more carbon in this fraction by the SPS system. The carbon isotopic signature ($\delta^{13}\text{C}$) differed between systems (p -value = 0.035), showing that *T. diversifolia* contributes to the soil carbon in SPS systems, despite the short-term establishment. The SPS with *T. diversifolia* was shown to be an alternative to increase organic carbon deposition in the soil, especially in the POC.

Keywords: Agroforestry; Organic matter; Shrub.

4.1 Introduction

The global challenge of climate change has intensified the need for comprehensive research on carbon dynamics in agricultural systems. As agriculture plays a crucial role in mitigating and contributing to atmospheric carbon dioxide levels understanding the carbon stock is essential for developing sustainable practices.

Silvopastoral (SPS) is a type of agroforestry system, that combines grass, trees/shrubs, and livestock in one system to increase production, mitigate climate change, and improve land use (NAIR et al., 2010; MONTAGNINI; IBRAHIM; MURGUEITIO RESTREPO, 2013). Besides these characteristics, SPS has the capacity for increased carbon (C) sequestration, above and belowground, compared with other land uses (NAIR et al., 2010).

The SPS can consist of a large combination of trees/shrubs with grass and can be used with many tree/shrub species. In this study, it was used, as a shrub component of the system, *Tithonia diversifolia*. This plant belongs to the *Asteracea* family, the C3 plant, which combines resilience and production. This plant is known for its capacity to grow in acidic and low-fertility soils and keep its production and nutritional value even during the dry and cold season (PÉREZ et al., 2009). As a shrub, *T. diversifolia* has a pivotal root, that contrasts with the fasciculate roots from the grasses and increases the nutrient and water use efficiency. Additionally, reports indicate its potential to enhance soil fertility and C input (RIBEIRO et al., 2016).

According to Nair, Kumar and Nair (2009) SPS has the potential to sequester C in a range from 1.1 to 6.5 Mg/ha/yr. Many factors are determinants in the dynamic of C in the soil, such as the type of crops, permanence of the system in the area, edaphoclimatic conditions, weather conditions of the region, and the presence of animals (CARVALHO et al., 2010). The vegetation type is a drive to contribute to the soil organic matter (SOM), due to being the source of new C deposition and changes the soil parameters as microbial activity, interfering in the permanence and stabilization of SOM (GUILLOT et al., 2019). The consortium among different species can provide numerous ecosystem services, such as improved soil biodiversity conservation, better use of water, erosion reduction, and GHG mitigation (PAGIOLA, 2004; ANDRADE; BROOK; IBRAHIM, 2008).

Given this above, we hypothesized that (i) the introduction of the SPS can promote increases in soil C; and (ii) besides the short-term experiment, the SPS can start to express the contribution of the *T. diversifolia* in the soil organic C by changing the soil C isotopic composition. So, this study aimed to compare the soil C stock, organic matter fractionation, and soil isotopic composition in an SPS and an exclusive grass system (EGS).

4.2 Material and Methods

4.2.1 Site description and environmental conditions

The study was carried out in the Center for Nuclear Energy in Agriculture, in Piracicaba, São Paulo State, Brazil (22°42'23.31"S, 47°38'41.50"W, 517 m), from January 2020 to January 2023. The climate of the region according to Köppen-Geiger's classification is Cwa (ALVARES et al., 2013), characterized by well-defined dry winter and warm summer with mean annual temperatures of 23 °C and mean annual precipitation of 1310 mm.

Soil is classified as typical Eutroferric Red Nitosol (FAO, 2015), with a bulk density of 1.36 g cm⁻³ in the 0-20 cm layer, comprising 32% clay, 24% silt, and 44% sand. Soil chemical

properties at the establishment of the treatments (January 2020) were determined by compositing soil samples: pH = 6 (CaCl₂); organic matter = 37 g dm⁻³ (Calorimetry); P = 87 mg dm⁻³ (ion-exchange resin extraction method); S = < 10 mg dm⁻³ (Calcium phosphate 0.01 mol L⁻¹); K = 1.6 mmol dm⁻³ (ion-exchange resin extraction method); Ca = 90 mmol dm⁻³ (ion-exchange resin extraction method); Mg = 41 mmol dm⁻³ (ion-exchange resin extraction method) Al = < 0.02 mmol dm⁻³ (KCl 1 mol L⁻¹); H+Al = 22 mmol dm⁻³ (extraction by SMP), sum of bases = 132.6 mmol dm⁻³; cation exchange capacity = 154.6 mmol dm⁻³; base saturation = 86%, and Al saturation = 0%. No fertilization was conducted either before or during the experimental period.

4.2.2 Forage establishment and pasture management

In February 2020, the vegetation in the area was desiccated using Glyphosate (1L 100L⁻¹ water, N-(phosphonomethyl) glycine), followed by the manual broadcasting of the *Urochloa brizantha* (Hochst. ex A. Rich.) R.D. Webster cv. Xaraes (syn. *Brachiaria brizantha* cv. Xaraes) (xaraés palisadegrass) seeds at a rate of 5 kg ha⁻¹, establishing it as the C4 grass. In November 2020, the area was partitioned into 8 paddocks, each measuring 144 m². Four of these paddocks were designated for planting *Tithonia diversifolia* as the C3 component in a silvopastoral system (SPS), and the other four were established with only grass in an exclusive grass system (EGS). Paddocks were managed using rotational stocking with variable stocking rates based on grass height (xaraés palisadegrass; pre-grazing 30 cm and post-grazing 15 cm), employing the put-and-take stocking approach (WHEELER et al., 1973). Grazing was carried out by Santa Ines male sheep, weighing 27.9 ± 6.36 kg (mean ± SD) over four days. Data collection commenced in January 2022 upon the establishment of the systems.

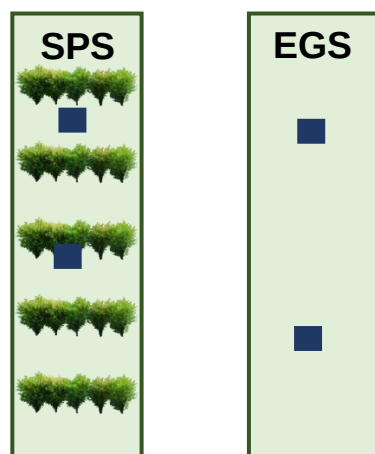


Figure 4.1 Experimental arrangement of treatments (SPS: Silvopastoral System; EGS: Exclusive Grass system; shrubs in the rows represent the *T. diversifolia*; blue squares represent the soil sample (following for all the paddocks).

4.2.3 Carbon and Nitrogen Stock

At the beginning of establishment, in January 2020 undisturbed soil samples were collected, 2 per plot, with a soil core sampler cup (50 cm³) at 0-10 cm layer. At the end of the experiment (January 2023), the soil sample was repeated as the same in 2020. Subsamples were oven-dried at 105°C for 48 hours to determine soil bulk density (SBD) (TEIXEIRA et al., 2017), and the other subsamples were oven-dried at 55°C until constant weight to determine carbon (C) content. For determination of soil C content, after drying, the samples were passed in a sieve (0.25 mm) and then ball-milled to a fine powder in a Mixer Mill (MM 400, Retsch) at 25 Hz for 9 min. The C concentration was determined by dry combustion using an EA-IRMS (elemental analyzer isotope ratio mass spectrometer Carlo Erba Na 1500. Model Isoprime (2006), Manchester, UK). Carbon (Mg ha⁻¹) was calculated by multiplying the C content for the SBD and depth, according to the equation:

$$\text{Stock (C or N)} = \text{SBD} \times D \times \% \text{C or N} \quad (1)$$

Where Stock of C or N in soil (Mg ha⁻¹); SBS is the average soil bulk density; D is the depth; %C or N is the total C or N content in the soil.

4.2.4 Organic matter fraction and isotopic signature

For fractioning organic matter into particulate organic matter (POM) and mineral-associated organic matter (MAOM), the particle-size method (CAMBARDELLA; ELLIOTT, 1992; LAVALLEE; SOONG; COTRUFO, 2020). A sub-sample of 5 g of soil was used and mixed with 15 mL of sodium hexametaphosphate and dispersed in a horizontal shaker for 16 hours (140 rpm). After 16 hours of shaker, the solution was passed through a 53 µm mesh by washing it with a weak stream of distilled water. The retained (>53 µm – POM) and flushed (<53 µm – MAOM) materials were oven-dried at 50° until all the water evaporated, and then ground and sieved in a ball-milled to a fine powder to C and N determination.

Soil stocks of particulate organic carbon (POC) and mineral-associated organic carbon (MAOC) were calculated for 0-10 cm with the equivalent soil mass approach (ELLERT; BETTANY, 1995), following the equation:

$$\text{POC stock} = \% \text{POM} \times \% \text{CPOM} \times \text{SBD} \quad (2)$$

where POC stock is the stock of particulate organic carbon (Mg ha^{-1}); %POM is the percentage of the 5 g subsample $>53 \mu\text{m}$ mesh; %CPOM is the carbon content of the retained material; and SBD is the average soil bulk density. For nitrogen, the equation was the same but substituted the %CPOM by the % of nitrogen in the POM fraction.

$$MAOC \text{ stock} = \%MAOM \times \%CMAOM \times SBD \quad (3)$$

where MAOC stock is the stock of particulate organic carbon (Mg ha^{-1}); % MAOM is the percentage of the 5 g subsample $<53 \mu\text{m}$ mesh; %C MAOM is the carbon content of the retained material; and SBD is the average soil bulk density. For nitrogen, the equation was the same but substituted the % MAOM by % of nitrogen in the MAOM fraction.

To evaluate carbon stock changes for each fraction to each treatment during the experimental period, the difference between final (01/2023) and initial (01/2021) stocks was calculated.

The isotopic signals of C ($\delta^{13}\text{C}$) were used to infer the organic matter contribution in the SPS of C4 grass and C3 shrub. The C4 grass metabolism has a lower discrimination of ^{13}C against the lower atomic weight ^{12}C at the carboxylation step, so the C4 grass has less negative (around -12 to -15) isotopic signals ($\delta^{13}\text{C}$) compared with the C3 plant (around -25 to -30). The $\delta^{13}\text{C}$ isotopic ratio variations were determined using an elemental analyzer coupled to a mass spectrometer (Carlo Erba/Delta Plus) at the Isotopic Ecology Laboratory at CENA/USP. The results were expressed in the form of $\delta^{13}\text{C}$ (‰) about the international PDB standard (Fry, 2008), according to the equation:

$$\delta \text{ sample (‰)} = (\text{R sample} - \text{R standard}) / (\text{R standard}) \times 1000 \quad (4)$$

Where R is the ratio of ^{13}C and ^{12}C of the sample or standard.

Table 4.1 Initial carbon stock, nitrogen stock, C/N ratio, and natural abundance of ^{13}C in the soil (year 2020).

Depth	Year 2021			
	C	N	C/N	$\delta^{13}\text{C}$
	Mg ha ⁻¹			(‰)
0-10 cm	31.7	1.44	22.01	-15.7

C = carbon; C/N = ratio of C and Nitrogen.

4.2.5 Statistical analysis

Normality of distribution and homogeneity of variances were evaluated by applying the Shapiro-Wilk test and Levene's test, using R software version 4.1.2 (R CORE TEAM, 2021) for all data. Data were submitted to ANOVA and t-test. The model included treatment (fixed effect), data collection (the years 2021 and 2023) (treated as a repeated measure), and their interactions according to the following model:

$$Y_{ij} = \mu + T_i + G_j + T_i * G_j + e_{ij} \quad (5)$$

where Y_{ij} is the response variable for the i treatment and j date collection; μ is the general average; T_i is the effect of treatment; G_j is the effect of data collection; $T_i * G_j$ is the effect of interaction and e_{ij} represents the random error term associated with the i treatments and j with the date of collection.

Regarding the isotopic composition (C^{13}) the model used was mentioned above:

$$Y_i = \mu + T_i + e_i \quad (6)$$

where Y_i is the response variable for the i treatment; μ is the general average; T_i is the fixed effect of treatment and e_i represents the random error term associated with the i treatments.

The significance level, alpha of 0.05, was employed in the analysis. Additionally, a significance ranges from alpha 0.05 to 0.1 was considered as a trend.

4.3 Results and Discussion

No differences were found between the treatments in terms of total carbon (C), mineral-associated organic matter (MAOM), and particulate organic matter (POM) at the end of the experiment (Table 4.2).

The time of establishment and evaluation period were possibly short, and this may explain the no differences found in this study between the systems. It is already known that the C dynamic in the soil is a complex variable, and it is time-dependent, but there are many studies (MANAYE et al., 2021) demonstrating the importance of SPS systems in atmospheric CO_2 absorption through photosynthesis and showing the importance of tree/shrub components in storage C in living plants, as well in soil as organic detritus. According to Valenzuela Que et

al. (2022), the silvopastoral system with different trees species in a scattered designer has the potential for increasing carbon storage compared to grass monoculture in the long term.

Table 4.2 Total carbon, organic matter fractions, and carbon stocks in organic matter fractions for the treatments in 0 -10 cm depth.

Treat	C total g kg ⁻¹	MAOC g kg ⁻¹	POC	Stock		
				C	MAOC	POC
				Mg ha ⁻¹		
SPS	33.90	28.80	5.10	36.50	30.50	6.00
EGS	31.30	27.40	3.70	31.90	27.70	4.20
SE	1.5	1.4	0.65	2.10	1.5	0.80
<i>p</i> -value	0.315	0.198	0.162	0.164	0.198	0.163

SPS = silvopastoral system; EGS =Exclusive grass system; C = carbon; MAOC = mineral-associated organic carbon; POC = particulate organic carbon; SE = standard error of means.

Almeida et al. (2021) evaluating an SPS system, using eucalyptus planted in rows, with four years of implantation also found an increase in C stocks, with the C stock values around 40 Mg ha⁻¹. Also, Cá et al. (2022) concluded that SPS can maintain or increase soil C stocks in the top layer and depths in a 22-year-old system, and values of 0-10 cm were also around 40 Mg ha⁻¹. These studies are with other tree/shrub components, due to the lack of information regarding silvopastoral systems involving *Tithonia diversifolia*. This underscores the need for further research on this shrub component in SPS.

For nitrogen stock, no difference was found (Table 4.3). Neither to N in the organic matter fraction (MAON and PON).

Table 4.3 Total nitrogen stock and nitrogen stock in organic matter fractions at 0- 10 cm depth for the treatments.

Treat	Stock		
	N	MAON	PON
	Mg ha ⁻¹		
SPS	1.52	1.43	0.09
EGS	1.45	1.35	0.10
SE	0.11	0.10	0.01
<i>p</i> -value	0.666	0.570	0.419

SPS = silvopastoral system; EGS =Exclusive grass system; N = nitrogen; MAOM = mineral-associated organic matter; POM = particulate organic matter; SE = standard error of means.

These results are similar to the values found by Almeida et al. (2021) in the silvopastoral system. The authors found a range from 1.29 to 3.56 Mg ha⁻¹ from N stock. No fertilizers were applied in either system, so, SPS and EGS responded in the same way in the N stock. Despite

the shrub inclusion in SPS, the management of xaraés grass probably promoted good soil cover, leading to a constant turnover of roots in the short term, so this can explain the absence of difference between the systems.

The variation in particulate organic carbon (ΔPOC) was not different between treatments (Figure 4.1, p -value=0.0715), but showed a tendency to stock more carbon in this fraction by the SPS system. While ΔPOC for EGS was around -3, to the SPS was positive, around 0.5. This finding illustrates the SPS potential to increase POC over the years. For mineral-associated organic carbon (ΔMAOC), the variation was not affected by treatments (p -value=0.5701). These fractionations allow the evaluation in short-term management due to the POM fraction being more easily altered by land use and management (higher rate of decomposition) and the MOAM has higher stability and is less sensitive to changes in management (SILVEIRA et al., 2013).

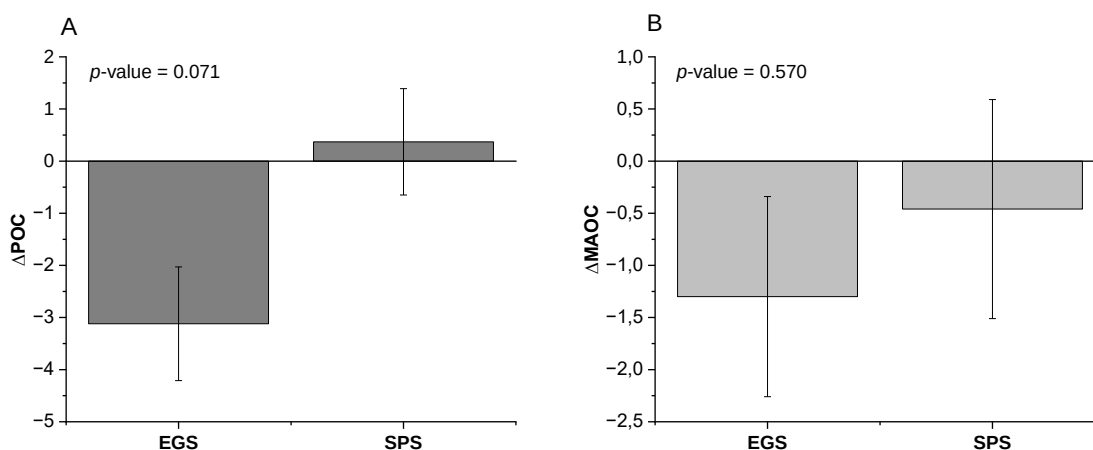


Figure 4.2 Differences of soil particulate organic carbon (ΔPOC – A, Mg ha^{-1}) and mineral-associated organic carbon (ΔMAOC – B, Mg ha^{-1}) of exclusive grass system (EGS) and silvopastoral system (SPS) from the establishment year 2020 and year 2023. Bars indicate standard error of means.

The carbon cycle is complex and depends on many factors as the quantity of carbon fixed via photosynthesis, the organic matter composition, soil chemical, physical parameters, and the abundance of microbial community (LANGE et al., 2015) and all of which affect POC and MAOC in different forms.

The availability of plant carbon for microbial decomposition is a key determinant influencing both POC formation and presentation (LANGE et al., 2015; GUO et al., 2019). Probably, the SPS enhances plant carbon deposition, aboveground and belowground, increasing the POC formation and conservation. The positive correlation between plant carbon input and net soil carbon accumulation is evident, but the organic matter's quality emerges as a crucial

factor in stabilization (LAVALLEE; SOONG; COTRUFO, 2020). The organic matter with lower quality, and a higher C/N ratio, tends to favor POC formation, while greater N content and carbohydrates stimulate microbial activity and carbon stabilization by MAOC formation (COTRUFO et al., 2015).

The C isotopic signature ($\delta^{13}\text{C}$) was different between systems (Table 4.4, p -value=0.0357), showing that *T. diversifolia* starting to contribute to the soil C in SPS systems, besides the short-term establishment. The $\delta^{13}\text{C}$ isotope technique can be used to quantify the relative contribution of plants of each photosynthetic pathway (C3 or C4) to soil organic matter (BALESDENT; MARIOTTI; GUILLET, 1987).

Table 4.4 Soil isotopic signature of SPS and EGS systems.

Treatment	$\delta^{13}\text{C}$ (‰)
SPS	-16.60 a
EGS	-15.40 b
SE	0.34
p -value	0.035*

SE = standard error of means. * indicate the significance at 5%. Letters differ from each other.

In a site where the photosynthetic pathway is C4, when integrated the C3 plant takes time to the C3 start to contribute to the soil organic matter. This time can depend on many factors, such as biomass deposition, turnover rate, C/N rate, microbial activity, and others. Haile, Nair and Nair (2010) using the $\delta^{13}\text{C}$ isotopic technique showed that in the long term, SPS may help sequester more soil organic carbon in the soil. These authors also found that integrating trees in pasture increases C storage in lower soil depths due to the tree roots. In this study, we observed that the C3 (*T. diversifolia*) plant started contributing to the soil organic matter (Table 4.4).

Conclusions

The lack of differences in soil carbon stocks between systems can be explained by the short-term establishment. However, the tendency to change the particulate organic carbon (ΔPOC) and the difference in $\delta^{13}\text{C}$ isotopic indicates that, besides the short-term establishment, *T. diversifolia* began to exert a discernible influence on soil organic carbon levels. Further studies are necessary, spanning subsequent years post-establishment, to assess and confirm the role of *T. diversifolia* in soil carbon dynamics within the silvopastoral system.

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5. SOIL BIOLOGICAL ACTIVITY IN GRASS OR GRASS-LEGUME MIXED GRAZING SYSTEMS

Abstract: Understanding the dynamics of soil biological activity, and carbon and nitrogen mineralization in forage-based systems is vital for sustainable land management. Enhancing plant diversity is acknowledged as a strategy to improve soil health and productivity. The objectives of this investigation were to assess the soil biological activity (STBA), the carbon (C) mineralization, and the $\delta^{13}\text{CO}_2$ of contrasting grazing systems. Treatments were bahiagrass plus nitrogen fertilizer (BGN) that consisted of ‘Argentine’ bahiagrass during the summer N-fertilized ($224 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), overseeded with a mixture of annual ryegrass (*Lolium multiflora* Lam.) and oat (*Avena sativa* L.) during the cool season; only bahiagrass (BG) that consisted of unfertilized bahiagrass during the summer, overseeded with a ryegrass-oat-‘Dixie’ crimson (*Trifolium incarnatum* L.) - ‘Southern Belle’ red (*Trifolium pratense* L.) - ball (*Trifolium nigrescens* Viv.) clover mixture and received 34 Kg N ha^{-1} during fall; and bahiagrass plus rhizoma peanut (*Arachis glabrata* Benth.) (BGRP) mixed during the summer, with a similar oat-annual ryegrass and clover mixture, and fertilization scheme in the fall as the BG system. The treatments were replicated three times in a randomized complete block design, and each pasture measured 0.85 ha . Soil samples (0- to 10-cm layer) were collected for soil incubation for 24 days. Soil samples were analyzed for total C and nitrogen (N). Soil cumulative CO_2 emission in 24 days, soil test biological activity (STBA) in 3 days, and carbon mineralization (CMIN) were not different among treatments (p -value = 0.692; 0.387, respectively). Carbon stock showed a tendency to be greater in BGN and BG than in BGRP (p -value=0.064). Carbon mineralization rate and nitrogen stock were different among treatments (p -value=0.042 and 0.025, respectively), following the same pattern of carbon stock. There was a time effect on the carbon mineralization rate (p -value<0.0001), showing a greater rate 3 days after incubation (after rewet soil) and a decreasing pattern in the later days. The isotopic composition of $\delta^{13}\text{CO}_2$ was significantly different among treatments and days of incubation (p -value=0.0001 and 0.003, respectively). Even though BGRP reduces carbon and nitrogen stocks, it exhibits comparable biological activity to the other treatments (grass plus nitrogen fertilization). Nevertheless, incorporating legume forage into grassland systems represents a significant alternative to reduce the need for nitrogen fertilizer without negatively impacting soil biological activity.

Keywords: Carbon; biological nitrogen fixation; soil respiration.

5.1 Introduction

Soil health and nutrient cycling are fundamental components of sustainable agriculture, affecting food production and environmental quality. Grasslands have a crucial role, serving as the primary source of forage for livestock and acting as carbon (C) sink in terrestrial ecosystems (ELIAS et al., 2023). Grass systems are essential for livestock production and can mitigate climate change by sequestering atmospheric carbon dioxide (CO₂) by soil organic matter accumulation (FOLLETT; REED, 2010).

Perennial warm-season grasses, such as bahiagrass (*Paspalum notatum* Flüggé), are key components of beef cattle production systems. These grasses provide most of the forage from spring to early autumn, but they have reduced herbage accumulation during the cool season (GATES; MISLEVY; MARTIN, 2001). Integrating perennial warm-season grasses with perennial warm-season legumes can improve the system, and one of the perennial warm-season legumes that have been used is Rhizoma peanut (RP) (*Arachis glabrata* Benth.) (DUBEUX et al., 2017). Another important applicability of legumes is used as cool-season annual forages. Overseeding cool-season annual forages onto perennial warm-season grass pastures can reduce seasonality by providing high-quality cool-season forages, including legumes and cool-season grasses (DUBEUX et al., 2016).

Integrating legumes not only provides high-quality forage, but also adds additional benefits to the system, such as nitrogen (N) fixation, improved litter decomposition (lower C: N ratio), and lower water footprint (SOLLENBERGER et al., 2019; JARAMILLO et al., 2021). One of the major benefits, however, is the biological nitrogen fixation (BNF), which potentially enhances N availability, reducing the need for industrial N fertilizers (CANTARUTTI et al., 2002). Legumes can improve soil health by enhancing soil microbial diversity (ERHUNMWUNSE et al., 2023a), adding N from BNF, and improving nutrient cycling efficiency (ERHUNMWUNSE et al., 2023b). Understanding the dynamics of soil biological activity and carbon mineralization in these forage-based systems is vital for sustainable land management.

Soil-test biological activity (STBA) is measured by the flush of CO₂ after rewetting of dried soil in a 3-day soil incubation (FRANZLUEBBERS et al., 2000). This measurement is related to the activities of microorganisms that play a crucial role in utilizing both simple and complex organic compounds for energy through respirations (SOONG et al., 2020) and has been well correlated with N mineralization (FRANZLUEBBERS et al., 2018).

It is known that biodiversity and plant functional diversity increase the productivity of grasslands and improve resistance to disturbance (COTRUFO et al., 2015; TRACY et al.,

2018). However, there are few studies integrating species diversity, soil biological indicators, and carbon accumulation. Santos et al. (2019) found that incorporating perennial rhizoma peanut led to a 22% increase in soil particulate organic matter concentrations (0-15 cm layer). However, no differences were observed in soil carbon or nitrogen stocks among the various forage treatments, including bahiagrass and rhizoma peanut monocultures, and bahiagrass-rhizoma peanut mixtures.

Kohmann et al. (2019) found that the litter decomposition rate was not affected by the inclusion of RP but with more N mineralization in legume treatment. Liu et al. (2017) found, in a semiarid region of China, that forage legume monocultures increased soil carbon accumulation by 0.64 Mg C ha⁻¹ yr⁻¹ more than grass monocultures. Wright; Hons; Rouquette (2004) observed only a 58% increase in soil organic carbon (SOC) concentration under N-fertilized bermudagrass-annual ryegrass (*Lolium multiflorum* Lam.; 350 kg N ha⁻¹ yr⁻¹) pasture compared to a bermudagrass-clover (*Trifolium* spp.) mixture receiving no N fertilizer over 19 years. Studies on legumes are limited, but including legumes in pastures has been shown to increase soil organic carbon (JENSEN et al., 2012).

Assessing soil respirations can serve as an indicator of the soil's functional capacity in nutrient cycling and organic matter decomposition (FRANZLUEBBERS, 2020). This study hypothesized that different managements in forage-based systems could induce changes in soil biological activity, thereby influencing the carbon (C) and nitrogen (N) mineralization. The objectives of this investigation were to (a) assess the soil biological activity, (b) estimate the C and N mineralization, and c) $\delta^{13}\text{CO}_2$ of contrasting grazing systems.

5.2 Material and Methods

5.2.1 Site description and experimental design

Soil samples (0- to 10-cm layer) were collected from an 8-yr grazing system (established in July 2014) at the University of Florida, North Florida Research and Education Center, located in Marianna, FL (30°52'N, 85°11'W, 35 m a.s.l.). Soils at the experimental site were classified as Orangeburg loamy sand (fine-loamy-kaolinitic, thermic Tyouc Kandudults). Treatments were three grazing systems: bahiagrass plus nitrogen fertilizer (BGN) that consisted of N-fertilized (224 kg N ha⁻¹ yr⁻¹) 'Argentine' bahiagrass during the summer, overseeded with a mixture of annual ryegrass (*Lolium multiflora* Lam.; 16.8 kg ha⁻¹) and oat (*Avena sativa* L.; 89.6 kg ha⁻¹) during the cool season; only bahiagrass (BG) that consisted of unfertilized bahiagrass during the summer, overseeded with a ryegrass-oat-'Dixie' crimson (*Trifolium incarnatum* L.;

16.8 kg ha⁻¹) - ‘Southern Belle’ red (*Trifolium pratense* L.; 6.7 kg ha⁻¹) - ball (*Trifolium nigrescens* Viv.; 3.4 kg ha⁻¹) clover mixture and received 34 Kg N ha⁻¹ during fall; and bahiagrass plus ‘Ecoturf’ rhizoma peanut (*Arachis glabrata* Benth.) (BGRP) mixed during the summer, with a similar oat-annual ryegrass and clover mixture, and fertilization scheme in the winter as the BG system. All systems received 20 kg P ha⁻¹, 46.5 kg K ha⁻¹, and 13.4 kg S ha⁻¹ annually. The N application on N-fertilized treatment was split into 4 applications, 2 in the summer and 2 in the cool season. The treatments were replicated three times in a randomized complete block design, and each pasture measured 0.85 ha.

5.2.2 Soil-test biological activity, C and N mineralization

To assess soil-test biological activity (STBA), carbon (C), and nitrogen (N) mineralization, soil samples were collected from all the paddocks in the three blocks at 0- to 10-cm layer with a hand probe (4-cm diameter). Twenty cores from each paddock were composited in a paper bag, transported to the laboratory, and dried by placing them in an oven at 55°C for at least 3 days until constant weight. The soil was then gently crushed with a pestle over a screen with 4.75-mm openings. The residue that was not passing the screen was removed from the sample.



Figure 5.1 Apparatus for soil sampling at the site of the assay (Personal file).

To carry out this study, we utilized 50-g soil samples placed in 50 mL becker and two replicates per pasture. Each soil sample was subsequently wetted at 50% water-filled pore space and positioned inside a 1-liter canning jar, which also held a vial containing 10 mL of 1 mol L⁻¹ NaOH to capture CO₂ and a vial of water to maintain humidity levels. The jars

were placed in a temperature-controlled room at 25°C and incubated for 24 days as described by Franzluebbers et al. (2018). We replaced the alkali traps at both the 3-day and 10-day marks of the incubation period.

The emission of CO₂ after the rehydration of the dried soil was assessed over three days. Additionally, it was quantified the cumulative mineralization of carbon (C) through the flush of CO₂ over a 24-day incubation. The calculation of C mineralization (CMIN) was based on cumulative mineralization.

The amount of water needed to achieve 50% WFPS was determined by the equation:

$$mL\ H_2O = 0.50 \times total\ porosity \times volume \quad (1)$$

Where porosity is determined from weight and bulk density as shown in equation 2, and volume is the volume occupied by 50 g of soil (mL).

$$Total\ porosity = \left(1 - \frac{BD}{PD}\right) \quad (2)$$

Where BD = bulk density (Mg m⁻³) and PD = particle density (assume 2.65 Mg m⁻³).

The CO₂-C was determined through titration using 1 mol L⁻¹ HCl, thoroughly stirring in the presence of BaCl₂ (which precipitated to form BaCO₃) until a phenolphthalein endpoint was reached. Soil organic C and N fractions were determined by dry combusting using EA-IRMS (Elemental analyzer isotope ratio mass spectrometer Carlo Erba Na 1500, model Isoprime, 2006, Manchester, UK), and C and N stock calculated, as equation 3.

$$Stock = SBD \times D \times \%C \quad (3)$$

Where Stock of C in soil (Mg ha⁻¹); SBS is the average soil bulk density; D is the depth; %C is the content of total C in the soil.

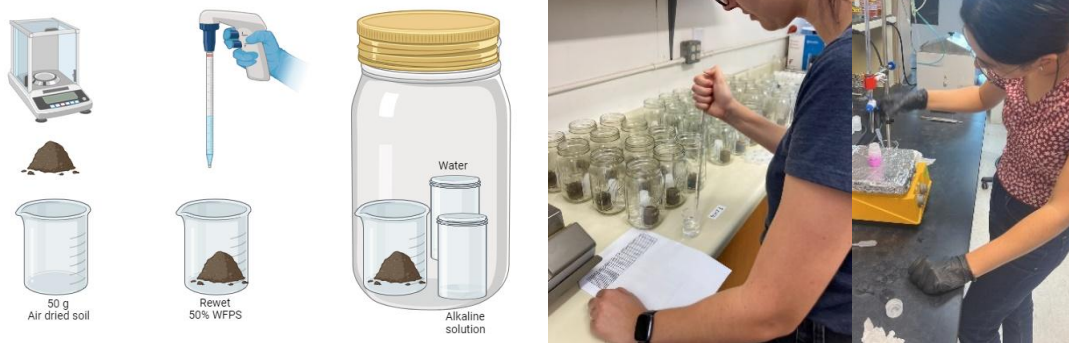


Figure 5.2 Schematic soil biological activity assay and methodology procedures (Personal file).

5.2.3 ^{13}C dynamic from carbon dioxide emission

A parallel assay was run, following the same incubation protocol, to collect gas for ^{13}C determination due to the treatments being compounds of C4 and C3 pathway plants (Figure 5.3). The gas was collected in three periods: 3, 10, and 24 days of incubation. Within each period, gas samples were collected three times, initial time (before closing the jars), middle, and final time. After each period, the jars were opened to ventilate and closed again. In all sampling times, gas was collected to determine the ^{13}C from CO_2 .



Figure 5.3 Gas collection to determine the ^{13}C from CO_2 during assay (Personal file).

5.2.4 Statistical analysis

After testing the normality of distribution and homogeneity of variances by applying the Shapiro-Wilk test and Levene's test, the data were submitted to an ANOVA using the mixed-model method (PROC MIXED) of SAS[®] software, version 9.4 (SAS, 2014). For the soil parameters, the model included treatment (fixed effect) and block (random effect) as follows:

$$Y_{ij} = \mu + T_i + B_j + e_{ij} \quad (4)$$

Where Y_{ij} is the response variable for the i treatment; μ is the general average; T_i is the fixed effect of treatment; B_j is the random effect of block; and e_{ij} represents the random error term associated with the i treatments and j block.

For the cumulative and ^{13}C variables, the model included treatment (fixed effect), data collection (treated as a repeated measure), and their interactions according to the following model:

$$Y_{ij} = \mu + T_i + G_j + T_i * G_j + e_{ij} \quad (5)$$

Where Y_{ij} is the response variable for the i treatment and j date collection; μ is the general average; T_i is the effect of treatment; G_j is the effect of data collection; $T_i * G_j$ is the effect of interaction and e_{ij} represents the random error term associated with the i treatments and j with the date of collection.

The significance level, alpha of 0.05, was employed in the analysis. Additionally, a significance ranges from alpha 0.05 to 0.1 was considered as a trend.

5.3 Results

The STBA, CMIN, and C stock were not different among treatments ($p < 0.05$, Table 5.1), but C stock showed a tendency to be greater in BGN and BG compared to BGRP.

Table 5.1 Carbon mineralization rate, SBTA, carbon mineralized, carbon stock, nitrogen stock after 24 days of soil incubation from 0-10 cm depth.

Treatment	C mineralization rate	STBA	CMIN	C stock	N stock
	CO ₂ -C (mg kg ⁻¹ soil day ⁻¹)	CO ₂ -C (mg kg ⁻¹ soil)	CO ₂ -C (mg kg ⁻¹ soil)	Mg ha ⁻¹	
BGN	22.1a	111	379	26.2	0.21a
BG	22.5a	118	370	22.3	0.17b
BGRP	20.1b	101	349	17.8	0.14c
SE	1.19	8.30	25.4	2.27	0.02
p-value	0.042	0.099	0.387	0.063	0.025

BGN = bahiagrass + nitrogen fertilizer + cool-season grass; BG = bahiagrass without fertilizer + cool-season legume; BGRP = bahiagrass mixed with rhizoma peanut + cool-season legume; STBA – soil-test biological activity (3 days of incubation); CMIN – carbon mineralized (24 days of incubations); SE – standard error of means;

Carbon mineralization rate and N stock were different among treatments. The carbon mineralization rate was higher in BGN and BG compared to BGRP ($p < 0.05$). N stock followed

a similar pattern, with higher values in BGN, followed by BG, and lower values in BGRP (Table 5.1).

There was no difference among treatments for cumulative C mineralization (Figure 5.4a). However, there was a difference among incubation days (Figure 5.4b). Despite the absence of difference among the treatments, there was a tendency for greater cumulative C mineralization in BGN and BG ($P=0.08$) than in BGRP.

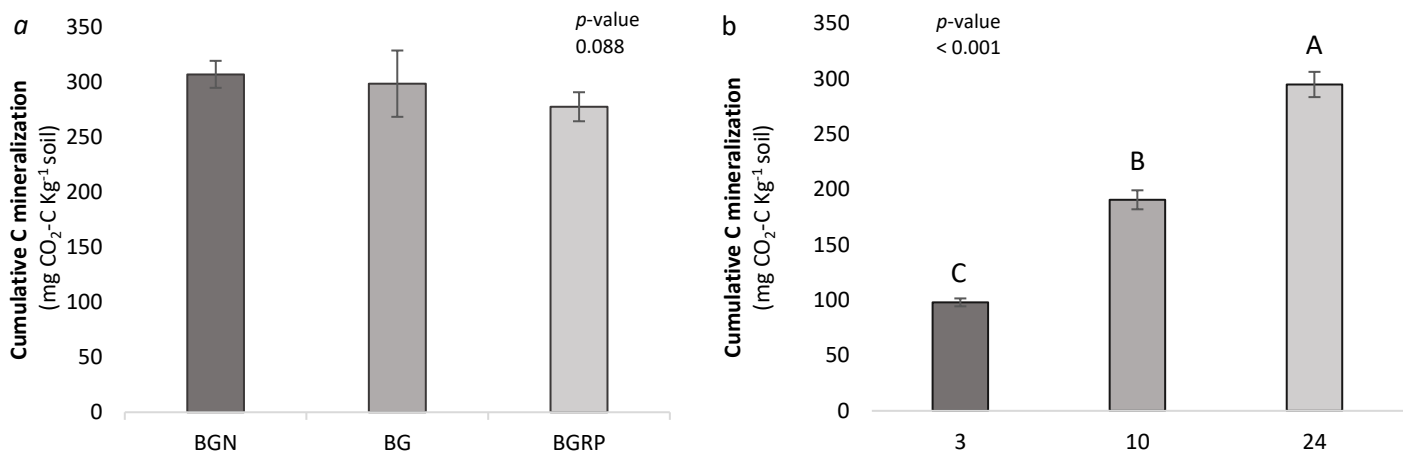


Figure 5.4 (a) Cumulative C mineralization for treatments. **(b)** Cumulative C mineralization during the incubation time. The bars represent the standard error of means. Letters differ from each other by $p<0.05$.

BG had a greater average C mineralization rate (22.58 mg CO₂-C kg⁻¹ soil day⁻¹) compared to BGRP (20.13 mg CO₂-C kg⁻¹ soil day⁻¹), with a similar rate to BGN (22.13 mg CO₂-C kg⁻¹ soil day⁻¹) ($p<0.05$; Figure 5.5a). Regarding the incubation days (Figure 5.5b), day 3 had the higher C mineralization rate (36.35 mg CO₂-C kg⁻¹ soil day⁻¹), following a decreasing pattern in day 10 (17.41 mg CO₂-C kg⁻¹ soil day⁻¹) and day 24 (11.08 mg CO₂-C kg⁻¹ soil day⁻¹) ($p<0.05$).

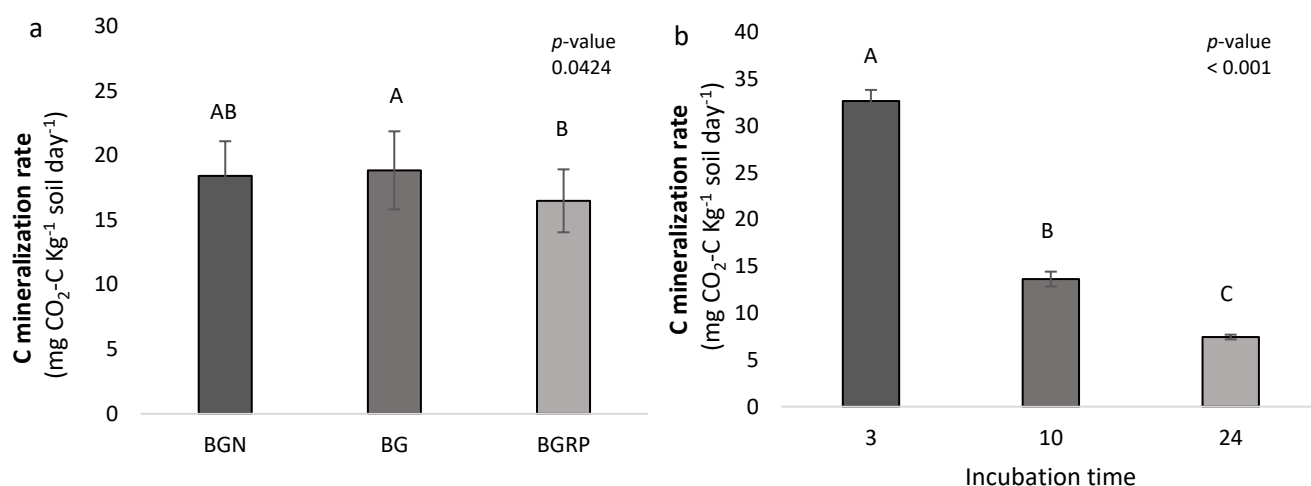


Figure 5.5 (a) Rate of organic C mineralization from different grazing systems. **(b)** Rate of organic C mineralization during the incubation time. The bars represent the standard error of means. Letters differ from each other by $p < 0.05$.

The isotopic composition of CO₂-C was significantly different for treatment and days of incubations (p -value < 0.05 ; Table 5.2). For treatment, BGRP was depleted compared to the other treatments. For days of incubation until day 10, the $\delta^{13}\text{C}$ of CO₂ was the same but different to days 17 and 24, being more depleted in these lasted days of incubation.

Table 5.2 CO₂-C $\delta^{13}\text{C}$ from grazing system and different soil incubation times.

Treatment	BGN			BG		BGRP		SE	p-value
$\delta^{13}\text{C}$	-14.1 b			-13.8 b		-16.2 a		0.27	<0.001*
DI	1.5	3	7.5	10	17	24			
							SE	p-value	
$\delta^{13}\text{C}$	-14.1 b	-14 b	-14.2 b	-14.6 b	-15.6 a	-15.7 a	0.38	0.0025*	

BGN = bahiagrass + nitrogen fertilizer + cool-season grass; BG = bahiagrass without fertilizer + cool-season legume; BGRP = bahiagrass mixed with rhizoma peanut + cool-season legume; DI – days of incubation; SE – standard error; * indicate the significance a 5%.

5.4 Discussion

Soil organic carbon mineralization is an important biochemical process in soil, which is directly related to the release of carbon dioxide (CO₂) and the supply of soil nutrients for soil quality maintenance (ROSS et al., 1999). The cumulative C mineralization represents the potential of CO₂ flush in 24 days and demonstrated a decrease in accumulation over time (Figure 5.4). It can be explained by the theory that in the early mineralization stage, the organic matter decomposed by soil microbes is derived from active organic substances (e.g., sugars and

proteins) which were easily decomposed in the soil, providing a ready carbon source promoting microbial activity (GOUGOULIAS et al., 2014).

After the initial peak in microbial activity occurred in the first 3 days following the rewetting of dried soil, the C mineralization rate gradually declined (Figure 5.5). The C mineralization rate varied among treatments, being lower in the bahiagrass (BG) + rhizome peanut (RP) mixture (BGRP); this variation is associated with the level of CO₂ flush, and it is directly related to the ready carbon availability. The carbon mineralization rate demonstrates a gradual reduction in the release of CO₂ over time. This result is by Guo et al. (2019), who also found a decline in the C mineralization rate over time. According to these authors upon soil rewetting, microorganisms initiate respiration, and this process involves the simplification of complex compounds before absorption and utilization as an energy resource. This was evident in the rapid increase in respiratory rate during the initial stage.

The release of CO₂ during a 3-day incubation period is referred to as soil-test biological activity (STBA). According to Franzluebbers and Pershing (2020), this method is relatively quick and correlates with soil biological properties, as well as soil carbon characteristics of biological origin. STBA is directly linked to carbon, nitrogen mineralization, and microbial activity. In our research, we noted that incorporating legumes into grass had no impact on STBA, as the microorganisms were activated in a manner like the grass N-fertilized treatment. However, studies are absent about soil respiration in grass-legume mixture.

Although Santos et al. (2019) reported that BGRP mixed had a similar effect on soil C stock as N-fertilized bahiagrass, the current study identified lower carbon stock values in the BGRP mixture when compared to N-fertilized bahiagrass. Similar trends were observed in N stock, which was also lower in the BGRP mixture. Silva et al.(2022), studying the grass-legume mixture and grass N-fertilizer, found an increase in C stock to grass N-fertilized compared to a grass-legume mixture of about 53% (17.8 vs. 11.6 Mg C ha⁻¹). Probably the nitrogen-fixing capability via biological nitrogen fixation, and the quantity of nitrogen fixed by RP was likely insufficient to meet the system's requirements for both the growth of BG and RP, as well as the storage of carbon and nitrogen within the system. Another hypothesis that could help comprehend these results is that being a legume, RP may have a lower amount of root biomass deposition than the grass.

Besides the lower C mineralization rate found in this study for the BGRP mixture, legumes are known for their lower C/N ratio and greater and faster decomposition (LYNCH et al., 2016). Kohmann et al. (2019), evaluating the decomposition of litter in BG and RP monoculture and BGRP mixture, found that BGRP had a superior decomposition compared

with the monocultures. Grass-legume mixtures change the litter composition and have different litter half-life. C4 grasses have a litter half-life average of 174 days, while C3 legumes have a half-life of around 67 days (DUBEUX et al., 2007). The shorter half-life of legume litter and the lower C/N ratio can accelerate the decomposition of the mixture, causing the release of more carbon than the system is depositing.

Possibly throughout the incubation period, microorganisms progressively consumed the readily decomposable active organic compounds in the soil. In contrast, the remaining components, such as lignin and cellulose, became more resistant and challenging for microorganisms to break down. This phenomenon is directly tied to the availability of organic compounds serving as an energy source for microorganisms. When more easily degradable organic compounds are present, microorganisms become more activated, explaining the gradual decrease in the mineralization rate over time (GOUGOULIAS et al., 2014). In summary, the reduced soil organic carbon content in the BGRP treatment impacts the mineralization rate of soil organic carbon, leading to a reasonably small cumulative release of CO₂.

When evaluating the sustainability aspects of grass-legume mixtures in comparison to nitrogen-fertilized grass monoculture, it is essential to take additional factors into account. In a review article on legumes for mitigation of climate change, Jensen et al. (2012) reported that the amount of CO₂ respired from root systems of N₂-fixing legumes could be comparable, to, or higher than, the CO₂ generated during N fertilizer production, but the CO₂ respired from the legume roots originated from the atmosphere and it was captured by photosynthesis, being a C neutral, in contrary that one released by the synthesis of N-fertilizer and by the transport and the fertilizer application. They concluded that legumes should be considered an important component in the development of future agroecosystems.

As already known, C3 plants are more depleted in ¹³C than C4 plants (FERNANDEZ; MAHIEU; CADISCH, 2003). BGRP, as an integration between C4 and C3 pathway plants, had a more ¹³C depleted CO₂-C released from soil. This result is in accordance with Fernández et al. (2012) which showed that the isotopic ¹³C composition of solid and CO₂ evaluation for C3 plant is more depleted than C4 plant material. Microorganisms, at the beginning of the incubation, degrade readily carbon, and the easily composted material is less depleted in ¹³C than lignin (FERNANDEZ; MAHIEU; CADISCH, 2003). So, when the easily degradable compounds are exhausted, microorganisms begin to degrade more resistant compounds such as lignin (GUO et al., 2019), and lignin is consistently more depleted in ¹³C compared to cellulose and other compounds (FERNANDEZ; MAHIEU; CADISCH, 2003).

Conclusions

Enhancing plant diversity is acknowledged as a strategy to improve soil health and productivity. Even though BGRP reduces carbon and nitrogen stocks, it exhibits comparable biological activity to the other treatments (grass + nitrogen fertilization). Nevertheless, incorporating legume forage into grassland systems represents a significant alternative to reduce the need for nitrogen fertilizer without negatively impacting soil biological activity.

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6. FINAL CONSIDERATIONS

In conclusion, the findings of this study underscore the potential of integrating C4 perennial forage grasses with C3 photosynthetic pathway plants as a strategy for the sustainable intensification of grasslands, particularly in the context of climate change mitigation. The incorporation of *Tithonia diversifolia* in silvopastoral systems shows promise in reducing emission intensity and maintaining forage mass production across the year. While cumulative greenhouse gas emissions were comparable between exclusive grass systems and silvopastoral systems, emission intensity was lower in the latter. Additionally, although there were no significant differences in carbon and nitrogen stocks between systems, the tendency for higher particulate organic carbon in silvopastoral systems suggests a potential positive influence of *T. diversifolia* on soil organic carbon levels. Further long-term studies are necessary to fully assess its impact on soil carbon dynamics.

The second experiment highlighted the significance of incorporating legume forage into grassland systems as an alternative to reduce the need for nitrogen fertilizer without negatively impacting soil parameters. Despite reductions in carbon and nitrogen stocks observed in the grass-legume mixture compared to the grass plus N-fertilization, overall soil-test biological activity and carbon mineralization were comparable across treatments, emphasizing the resilience of soil biological processes to different managements.

Collectively, these experiments underscore the complexity between soil-plant interface in grazing systems, and show the importance of plant diversity, as it not only improves soil health and productivity but also influences nutrient dynamics and greenhouse gas emissions. These efforts are important for informing future agriculture practices aimed at achieving environmental sustainability and increasing food productivity.