University of São Paulo
"Luiz de Queiroz" College of Agriculture

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Caio Macret Gomes

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Caio Macret Gomes
Agronomist

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Advisor:
Prof. Dr. **SILA CARNEIRO DA SILVA**

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SUMMARY

RESUMO

Associação de gramíneas forrageiras tropicais em pastagens: uma oportunidade para a intensificação sustentável?

Pastagens biodiversas têm a capacidade de aumentar a produtividade, o estoque de carbono do solo e de reduzir as emissões de óxido nitroso (N_2O) em relação a monocultivos. Esses efeitos são atribuídos à complementaridade, por meio da qual diferenças morfofisiológicas entre as espécies permitem o uso mais eficiente dos recursos em escalas variáveis de tempo, espaço e gradientes químicos do solo. Entretanto, não há informações sobre como as características de gramíneas forrageiras tropicais C_4 e sua associação afetam a dinâmica do carbono e do nitrogênio no solo. O objetivo deste estudo foi identificar se uma mistura de gramíneas perenes C_4 com estratégias de crescimento constrastantes pode ser uma alternativa para mitigar as emissões de gases de efeito estufa em relação a seus monocultivos. O experimento foi conduzido em Piracicaba/SP, Brasil, de Janeiro/2020 a Março/2022, segundo um delineamento de blocos completos casualizados, com quatro repetições. Os tratamentos corresponderam a três gramíneas forrageiras tropicais C_4: *Andropogon gayanus* cv. Planaltina (AND), *Megathyrsus maximum* cv. Massai (MAS) and *Urochloa brizantha* cv. BRS Piatã (PIA) cultivadas em monocultura e em associação (ASS – as três espécies semeadas em iguais proporções). As características específicas das espécies em monocultura foram determinantes da emissão de N_2O e do acúmulo/perda de carbono do solo. O capim-andropogon aprensentou menor sistema radicular e a maior concentração de nitrogênio na parte aérea e raiz, o que contribuiu para maiores emissões de N_2O desse tratemento. A maior concentração de nitrogênio do AND também influenciou positivamente o acúmulo de carbono orgânico associado a minerais do solo ($\Delta COAM$), possivelmente por estimular a atividade microbiana. Os cultivares PIA e MAS apresentaram estratégias contrastantes relativas ao desenvolvimento radicular, maior biomassa e maior comprimento, respectivamente. O efeito das estratégias sobre as emissões de N_2O desses dois cultivares variou entre estações do ano, conforme as concentrações de NO_3^- e NH_4^+ do solo. A maior biomassa do PIA influenciou positivamente o acúmulo de carbono orgânico em material particulado (ΔCOP), resultado do maior *input* de matéria orgânica. O maior comprimento e menor concentração de N das raízes de MAS influenciaram negativamente o estoque de C do solo, estimulando a degradação da matéria orgânica existente no solo ('*priming*'). As características contrastantes das espécies utilizadas na associação permitiram o desenvolvimento de complementaridade, o que resultou em maior biomassa e quantidade de N nas raízes em relação ao valor esperado a partir das monoculturas. Ainda, os traços funcionais de MAS e AND foram alterados para um modo mais conservador no uso de recursos na associação, contribuindo para redução da intensidade do ciclo de N no solo (menor abundância do gene relacionado ao processo de nitrificação, menor concentração de NO_3^- e maior NH_4^+ no solo). Os efeitos de complementaridade e alterações no modo de uso de recursos na associação contribuiram para redução das emissões de N_2O. O

incremento na biomassa de raízes na associação também influenciou positivamente o ΔCOP, porém o mesmo efeito não foi observado para o ΔCOAM. As características das espécies e sua associação foram determinantes dos ciclos de C e N do solo, o que pode ser utilizado para a intensificação sustentável de sistemas de produção animal em pastagens.

Palavras-chave: Gases do efeito estufa, Estoque de carbono, Pastagens multiespecíficas, Ciclo do nitrogênio, Microbiologia do solo

ABSTRACT

Association of tropical forage grasses in pastures: an opportunity for sustainable intensification?

Biodiverse grasslands are reported to increase productivity and soil organic carbon stocks (SOC) as well as to reduce nitrous oxide (N_2O) emissions compared to monocultures. The positive effects of biodiversity are generally attributed to complementarity (niche partitioning and facilitation) among different species, through which their morphophysiological dissimilarities allow for greater overall exploitation of light, water and nutrients across space, time and soil chemical gradients. However, there is no information on how traits of tropical C_4 perennial forage grasses commonly used under intensive grazing management and their association affect soil N and C dynamics. The objective of the study was to identify wether a mixture of C_4 perennial grasses with contrasting growth strategies may be an alternative to mitigate GHG emissions relative to their monocultures. The field experiment was carried out in Piracicaba/SP, Brazil, from Jan/2020 until Mar/2022, using a randomized complete block design, with four replications. Treatments corresponded to three tropical perennial forage grass species: *Andropogon gayanus* cv. Planaltina (gamba grass), *Megathyrsus maximum* cv. Massai (guinea grass) and *Urochloa brizantha* cv. BRS Piata (palisade grass) cultivated in monoculture and in association (the three grass species sown in equal proportions). Species traits varied among the monocultures, which influenced both N_2O emissions and SOC stocks. Gamba grass showed poorer root development, but greater plant nitrogen content, which seems to have stimulated N cycling and N_2O efflux during the whole experimental period. The greater nitrogen content of gamba grass also influenced the stabilization of plant derived carbon input into the mineral-associated form (ΔMAOC), presumably by stimulating microbial activity. Guinea and palisade grass presented contrasting rooting strategies of precision (length development efficiency) and scale (greater biomass), respectivelly. The species strategies reduced N_2O emissions during different seasons of the year, according to the respective soil NO_3^- and NH_4^+ content. Palisade grass greater herbage and root biomass positively influenced the soil particulated organic carbon (ΔPOC) accumulation, as it represented greater plant C input. On the other hand, greater length development and lower N content of guinea grass roots were related to lower and negative values of ΔMAOC and ΔPOC, which seems to be the result of 'priming effect'. The dissimilar traits of species used generated complementarity in their association, as shown by increased root biomass and N-stock compared to the expected from the monocultures. Also, guinea and gamba grass shifted their functional traits towards a more conservative mode when grown in association. This shift may have slowed N-cycling in the association, as indicated by the smaller ammonia oxidizing archaea gene abundance, soil NO_3^- content and greater soil NH_4^+ content, compared to the expected values from the monocultures. Both complementarity and intraspecific shift of species grown in association contributed to reduce N_2O

efflux in the multispecies swards. Root overyielding by the association was also related to increased ΔPOC, but no effect was observed in the ΔMAOC. We concluded that the species traits and their association influence soil C and N cycles, allowing for sustainable intensification of animal production systems from pastures.

1. INTRODUCTION

Modern agriculture must increase the level of production of major crops by 50 to 100% in order to keep pace with food demand from the projected more affluent population of 9 to 10 billion people by 2050 (Royal Society 2009; FAO 2011). Such advance in terms of crops and livestock production has been overachieved before, during the last 60 years (FAOSTAT 2022). However, it relied mostly on increased inputs of fertilizers, irrigation water, pesticides and on the expansion of agricultural lands over tropical forests (Matson et al. 1997; Tilman et al. 2002; Gibbs et al. 2010). This model of agriculture development produced important externalities, namely greenhouse gases (GHG – CO_2; N_2O; CH_4) emissions, loss of biodiversity, soil degradation and water pollution, which need to be addressed by researchers of agroecosystems and by food producers (Matson et al. 1997; Tilman et al. 2002; Foley et al. 2011; Paustian et al. 2016). Thus, the challenge is to keep increasing crop and livestock outputs, while being constrained by the finite availability of resources, competing land uses and the imperative necessity of reducing its environmental footprint, which has been worked out under the framework of sustainable intensification (Royal Society 2009; Godfray et al. 2010; Foley et al. 2011).

Managed grasslands play important role in this scenario as it is the major anthropic land use, accounting for 22 to 26% of the Earth's ice-free land (Ramankutty et al. 2008; FAOSTAT 2022), and it can provide essential ecosystems services such as forage, meat and milk production, carbon storage, climate change effects mitigation, water quality and others (Lemaire et al. 2011; Bengtsson et al. 2019; Hanisch et al. 2020). However, there are aspects of animal production in grasslands that can either mitigate or enhance the environmental impacts of the activity, such as fertilization management (Conant et al. 2001; Jones et al. 2005), species and cultivars genetic improvement (Valle et al. 2014), grazing and/or cutting management (Chiavegato et al. 2015; Chen et al. 2015; Congio et al. 2018; Congio et al. 2019) climate and weather conditions (Klein et al. 2014) and others (Congio et al. 2022).

Increasing biodiversity in grasslands can alter ecosystem functioning and services (Naem et al. 1994; Loreau et al. 2001; Hooper et al. 2005; Gross et al. 2017) and it may be an opportunity for creating intensive sustainable agroecosystems, which bring together high yielding and mitigation of environmental impacts (Volaire et al 2014; Tilman et al. 2020; Wright et al. 2021).

1.1. Biodiverse grasslands: general principles and mechanisms

Early experiments with biodiverse grasslands consistently reported aboveground biomass overyielding of multispecific swards compared to monocultures, but rarely transgressive overyielding, i.e. greater yield for mixed species pastures than for the most productive monocultures (Cardinale et al. 2007), which led to a debate on the true ecological meaning of biodiversity effects (Tilman et al. 2014). It was argued that the probability of selecting a very productive species increases with the level of biodiversity in the treatments ('selection effect' – Aarsen 1997; Huston 1997), or that multispecies outcomes were just additive (proportional) results from the individual species comprising the mixtures (Grime 1998). Other researchers suggested that 'complementarity effects' of resource partitioning and/or positive interactions (facilitation) among species resulted in increased total resource use and overyielding of more diverse treatments (Loreau 1998; Hector et al. 1999; Sphen et al. 2000).

Experimental designs with replication of every specific monoculture and mixture allow the calculation of these effects by additive partitioning (Loreau & Hector 2001). It was shown in multi-site biodiversity experiment (pan-European BIODEPTH experiment) that 'selection effect' can vary from negative to positive values, whether the dominating species is the most productive or not, and the average value for this effect was zero in that case (Loreau & Hector 2001). On the other hand, 'complementarity effects' were always positive (Loreau & Hector 2001). Further, a meta-analysis with biodiverse grasslands experiments reported that 'selection effect' was more transient, while 'complementarity effect' increased with the duration of the experiments (Cardinale et al. 2007).

Complementarity is achieved by species with diverging morphophysiological characteristics grown together, which allows for niche partitioning, and consequently more efficient exploration of available resources (light, water, nutrients, etc.) in space, time and soil chemical gradients (Loreau et al. 2001; Hooper et al. 2005; Tilman et al. 2014). The level of complementarity may vary according to the mixed species specific traits and the nature of the resource, i.e. quantity, spatial and temporal distribution, among other factors (Loreau & Hector 2001; Hooper et al. 2005; Hiiesalu et al. 2012; Pärtel et al. 2012; Eskelinen et al. 2022). Further, positive interactions among species (facilitation) such as mineralization of organic matter and nutrient provision (Li et al. 2006) and diseases/pests effects mitigation

(Wright et al. 2017) are also suggested as mechanisms of complementarity that may enhance multispecies grasslands performance (Wright et al. 2017).

In theory, contrasting traits of mixed species should also generate coexistence stability and reduce competition by the same mechanism of niche partitioning (MacArthur & Levins 1967; May 1974; Chesson 2000). However, management of biodiverse pastures may also influence the dynamics of these communities by altering plant traits, local environmental conditions and their interaction, mainly regarding light and nutrients supply/uptake (Holt et al. 2004; Borer et al. 2014; Eskelinen et al. 2022). This condition may offer opportunities and challenges for managing multispecies swards, which still need to be addressed by further researches.

Biodiverse grasslands may also improve stability of the sward biomass and productivity through increased resistance and/or resilience to environmental disturbances and periods of severe stress (Tilman & Downing 1994; Volaire et al. 2014; Tilman et al. 2014; Duchini et al. 2018). Seasonal variation of environmental conditions and other sources of stress for the plants may cause the botanical composition to change throughout the time, allowing for greater stability in multispecific swards biomass (May 1974; Lehman & Tilman 2000; Duchini et al. 2018). This characteristic may also explain part of the increased complementarity effects revealed in long-term compared to short-term experiments (Cardinal et al. 2007; Wright et al. 2021).

1.2. Biodiverse grasslands climate mitigation potential: N_2O emissions and soil carbon storage

By altering ecosystem functioning, mainly productivity of plants, resource utilization and nutrient cycling, biodiverse grasslands may be a tool for sustainable intensification of forage and animal production (Hooper et al. 2005; Tilman et al. 2014; Volaire et al 2014; Tilman et al. 2020; Wright et al. 2021). The emission of greenhouse gases (GHG) is one important externality of agriculture, which accounts for approximately 22% of anthropic emissions, including land conversion to crops, animal, energy and forestry production (IPCC 2021). The soil has an important contribution to the agriculture GHG budget, mainly concerning nitrous oxide (N_2O) emissions from N-fertilizers and soil carbon storage (Amelung et al. 2020; Tian et al. 2020; IPCC 2021).

The soil N_2O emissions are related to nitrogen cycling, a process mediated by plants and microorganisms, which is affected by soil physicochemical characteristics and climatic conditions (Sahrawat & Keeney 1986; Butterbach-Bahl et al. 2013; Abalos et al. 2014; Abalos et al. 2017). Considering biodiverse grasslands, mixtures that are able to capture and store soil N efficiently, due to 'complementarity effects', are reported to reduce N-losses to the environment in the form of N_2O and N leaching (Scherer-Lorenzen et al. 2003; Niklaus et al. 2006; Abalos et al. 2014; Leimer et al. 2016; Niklaus et al. 2016; Piñeiro-Guerra et al. 2019; Cummins et al. 2021). The number of species in pastures is shown to be inversely correlated to soil mineral N concentration (Tilman et al. 1996; Niklaus et al. 2001). Hence, greater biodiversity in grasslands can reduce soil ammonium (NH_4^+) and nitrate (NO_3^-) availability for microbial nitrification and denitrification (Hodge et al. 2000; Scherer-Lorenzen et al. 2003; Fowler et al. 2013). However, species specific traits are shown to be as important as diversity (i.e. number of species) *per se* regarding N-cycling and losses, once complementarity is the result of plant's contrasting characteristics and strategies of niche exploration (Scherer-Lorenzen et al. 2003; Niklaus et al. 2006; Abalos et al. 2014; Leimer et al. 2015; Niklaus et al. 2016).

For example, the presence of legumes in mixtures strongly influence N-cycling, mainly by biological fixation of atmospheric N ($N_{2\ atm} \rightarrow NH_3$), but plant chemical composition and tissue turnover are also tightly related to microbial activities of ammonification (C-$N_{organic} \rightarrow NH_4^+$), nitrification ($NH_4^+ \rightarrow NO_3^-$) and denitrification ($NO_3^- \rightarrow N_2$) (Niklaus et al. 2001; Scherer-Lorenzen et al. 2003; Cummins et al. 2021). Legumes' characteristics generally accelerate N-cycling and related losses in the form of nitrate leaching and nitrous oxide emissions in low diversity mixtures (Scherer-Lorenzen et al. 2003; Leimer et al. 2016; Niklaus et al. 2016). Thus, when legumes are present in associations, increasing the number of highly efficient N-absorbing plants such as grasses may reduce N-losses at the extent to which these plants dominate the sward (Scherer-Lorenzen et al. 2003; Leimer et al. 2016; Niklaus et al. 2016). On the other hand, non-legume associations (grasses and/or other herbs) may reduce N_2O emissions at lower level of diversity (two or three species) and that effect is less pronounced with further increase in number of species, representing a saturation of niche exploration (Niklaus et al. 2006; Abalos et al. 2014).

Although greater yielding of monocultures is related to decreased N_2O emissions (Abalos et al. 2017), complementarity generated by grass mixtures with contrasting rooting

strategies is reported to be effective in reducing soil N_2O efflux, independently of aboveground biomass accumulation (Abalos et al. 2014; Piñeiro-Guerra et al. 2019). In fact, in low-fertility conditions, exploitative species greater yielding and biomass N-content (Wright et al. 2004; Reich 2014) may stimulate N_2O emissions (Abalos et al. 2014; Piñeiro-Guerra et al. 2019), which can be attributed to faster tissue and litter turnover, N mineralization, nitrification and denitrification, i.e. nitrogen cycling (van Der Krift & Berendse 2001; Scherer-Lorenzen et al. 2003). The complexity of the processes involving N-cycling and N_2O emissions in multispecies swards arises from the great number of possible species and plant traits combinations, their intra- and interspecific interactions, which may vary according to local conditions and management. Hence, studies with the objective of understanding how multispecific swards and their management affect nitrogen related ecosystem functions are important to the development of sustainable forage and animal production systems.

While mixed species grasslands may mitigate N_2O emissions, reducing the efflux of GHGs, they are also reported to increase soil carbon storage, which contributes to CO_2 removal from the atmosphere (Steinbeiss et al. 2008; Lange et al. 2015; Yang et al. 2019). The carbon cycling in agroecosystems is influenced mainly by climatic conditions (rain and temperature), organic inputs (quantity and quality, i.e. presence of recalcitrant compounds and C/N ratio) and soil physicochemical characteristics (texture, structure, pH and base saturation) (Oades 1988). In a specific edaphoclimatic condition, the land use (i.e. crop, grassland, forestry, etc.) is determinant of C-cycling, however crop or grassland management may also influence it by increasing or decreasing soil C stock (Paustian et al. 2016; Conant et al. 2017; Amelung et al. 2020).

Generally, it has been argued that carbon input to the soil is enhanced in biodiverse grasslands as the result of both above and belowground overyielding of these communities compared to monocultures or less diverse treatments (Steinbeiss et al. 2008; Dias et al. 2010; Mommer et al. 2010; Yang et al. 2019). Increased C-input to the soil results in greater microbial activity and community size (Kemmitt et al. 2006; Dias et al. 2010; Eisenhauer et al. 2010), which may stimulate the mineralization and loss of soil organic matter (Fontaine et al. 2007). However, it has been reported that this effect is transient in biodiverse grasslands and more related to soil labile carbon, while microbial activity and their by-products

(microbial necromass) positively affects the pool of stabilized soil carbon in the long-term (Steinbeiss et al. 2008; Lange et al. 2015).

In fact, it is shown that stabilization of organic matter into the soil mineral matrix (mineral associated organic carbon – MAOC) is tightly related to microbial activity, while particulated forms of organic carbon (POC) are derived from plant recalcitrant compounds, which have faster soil turnover (shorter-lived) in relation to MAOC (Cotrufo et al. 2015; Lavallee et al. 2019). As plant mixtures are reported to increase microbial activity also independently of enhanced biomass accumulation, this may contribute to increased soil carbon stocks in biodiverse grasslands experiments (Steinbeiss et al. 2008; Lange et al. 2015; Yang et al. 2019). However, species characteristics are determinant of soil C-cycling by specific interactions with the soil microbiota, which depends on root size and architecture, exudates and plant chemical composition (Oades 1988; Eisenhauer et al. 2010; Rossi et al. 2020).

The majority of biodiversity experiments are designed to evaluate the effect of increasing/decreasing the number of species on C-cycling and not all monocultures are replicated, which generates high variability in the outcome of less diverse treatments as result of their species specific traits and their influence on soil carbon (Steinbeiss et al. 2008; Eisenhauer et al. 2010; Lange et al. 2015; Yang et al. 2019). Thus, it is necessary to understand the relations of individual species traits on soil C dynamics as well as their mixture effects (Rossi et al. 2020), which requires fewer species being studied in more detail cultivated as monocultures and as multispecific swards.

Despite the importance of tropical grasslands for sustainable intensification of food production, there is no information on how traits of tropical C_4 perennial grass species commonly used under intensive management and their association in mixtures affect N_2O emission and soil C dynamics.

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2. HYPOTHESIS AND OBJECTIVES

Against that background the general hypothesis of this study is that the perennial C_4 tropical forage grasses Guinea grass - *Megathyrsus maximum* cv. Massai (MAS), Palisade grass – *Urochloa brizantha* cv. BRS Piatã (PIA), and Gamba grass – *Andropogon gayanus* cv. Planaltina (AND) have interspecific variability in functional traits that could generate complementarity in their association when cultivated as a mixed pasture. The expected results from complementarity in the multispecific swards are reduced soil N_2O emissions and greater positive effect on soil C stock compared to the respective monocultures (Figure 1 – Conceptual model).

The objective of the study was to understand the effects of mixing perennial C_4 tropical forage grasses and the contribution of individual species traits on N_2O emissions and soil carbon stocks, exploring possible complementarity effects that generates mitigation of GHG related impacts on intensively managed grasslands.
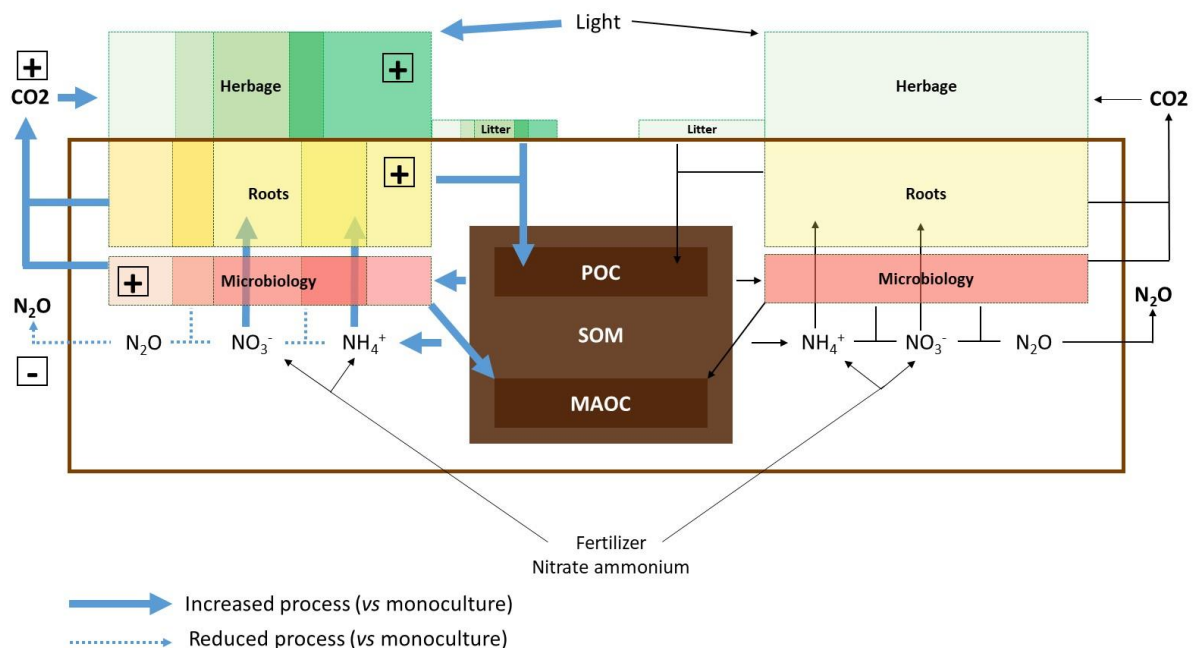
Figure 1. Conceptual model of the study. The hypothesis of the study is that the association of tropical C_4 perennial forage grasses (left) will increase overall resource utilization (e.g. mineral nitrogen, light) compared to monocultures (right) resulting in lower soil N content and N_2O emissions as well as greater organic matter inputs and increased soil particulated (POC) and mineral-associated (MAOC) organic carbon stocks.

3. MULTISPECIES SWARDS OF C₄ GRASSES REDUCE NITROUS OXIDE EMISSION COMPARED TO THEIR RESPECTIVE MONOCULTURES IN INTENSIVE MANAGED PASTURES

Abstract

One major impact of agriculture is the emission of ozone depleting and greenhouse gas nitrous oxide, which is tightly related to the use of N-fertilizers. Studies show that biodiverse grasslands may be more efficient in resource utilization as consequence of complementarity in niche exploration among species. Thus, multispecific swards are reported to increase N uptake, storage and to reduce losses by leaching and N_2O emissions when compared to monocultures. However, species specific traits are shown to be at least as important as biodiversity *per se* regarding soil N-cycling and losses. For tropical C₄ grasses, information on how their traits and their association in pastures may influence N-cycling and eventually N_2O emissions are almost non-existent. Hence, the objective of this study was to evaluate and to relate plant traits and soil nitrogen cycling parameters in order to investigate possible mechanisms of complementarity and mitigation of N_2O emissions. The field experiment was carried out in Piracicaba/SP, Brazil, from Jan/2020 until Mar/2022, using a randomized complete block design, with four replications. Treatments corresponded to three tropical perennial forage grass species: *Andropogon gayanus* cv. Planaltina (gamba grass), *Megathyrsus maximum* cv. Massai (guinea grass) and *Urochloa brizantha* cv. BRS Piata (palisade grass) cultivated in monoculture and in association (the three grass species sown in equal proportions). The treatments were compared among each other and expected values were calculated based on monocultures' actual values and their botanical proportion in the mixture for comparison with the association treatment. Species traits differed greatly and guinea grass had greater root length density and specific root length, palisade grass had greater root and aboveground biomass, while gamba grass was related to greater nitrogen content both above and belowground, but with smaller plant biomass. These dissimilar traits resulted in complementarity and overyield of root biomass and N stocked in the root system for the association compared to the expected values. Concerning aboveground biomass, herbage accumulation and N-stock, the association performed as expected by actual values of monocultures. When associated, guinea grass and gamba grass shifted specific leaf area and leaf N content to a more conservative mode of resource utilization, which influenced weighted values for the association treatment. Also, specific root length showed the same trend and was smaller for the association compared to expected values. This shift may have slowed N-cycling in the association, as indicated by the smaller ammonia oxidizing archaea gene abundance, greater soil NH_4^+ content and smaller NO_3^- compared to expected values. Complementarity in rooting system, overyielding in root biomass and nitrogen stock and the shift to more conservative mode may all have contributed to the reduction in N_2O emissions observed in the association compared to the expected values from the monocultures. We concluded that the C₄ tropical grasses used in this experiment have trait

dissimilarities that can generate complementarity and contribute to increased nitrogen utilization. Moreover, intraspecific changes in mixed swards also seemed to have increased efficiency of nitrogen utilization by plants.

3.1. Introduction

Increasing world's population and *per capita* income within the climate change context represent a challenge for the food supply chain, as production must keep pace with demand while the environmental footprint of the activity must decrease (Royal Society 2009; FAO 2011; Foley et al. 2011). Solutions to this apparent dilemma have been worked out under the framework of sustainable intensification, which proposes increasing productivity in the current agricultural lands with efficient resource utilization and thus mitigating environmental impacts (Tilman et al. 2002; Royal Society 2009 Godfray et al. 2010).

Grasslands play an important role in this scenario as they are the major anthropic land use (Ramankutty et al. 2008; FAOSTAT 2022), providing essential ecosystems services such as forage, meat and milk production, carbon storage, climate mitigation and water quality (Lemaire et al. 2011; Bengtsson et al. 2019; Hanisch et al. 2020). However, the process of intensification and maintenance of high forage and animal yields from grasslands requires regular use of nitrogen fertilizers (Delevatti et al. 2019), which has been reported as the main cause of the increase in anthropic nitrous oxide (N_2O) emissions and atmospheric concentration during the last decades (Thompson et al. 2019. Tian et al. 2020).

Nitrous oxide is a long-lived greenhouse gas (GHG) which accounts for 6% of total effective radiative forcing (Prather et al. 2015; Candell et al. 2021) and is the most important stratospheric ozone-depleting substance (Ravishankara et al. 2009). Production and emissions of N_2O are mainly driven by microbial transformations of nitrogen involving nitrification and denitrification processes (Sahrawat & Keeney 1986; Butterbach-Bahl et al. 2013), occurring directly from the soil and downstream/downwind from leached or volatilized nitrogen that escapes the agroecosystem (Tian et al. 2020). Thus, intensifying forage and animal production by application of N-fertilizers needs to aim efficient utilization of the nutrient in order to improve yield and mitigate direct and indirect N_2O emissions.

Experimentally, it has been shown that biodiverse grasslands frequently make more complete use of available resources which results in greater (at least similar) yield compared

to monocultures or less diverse treatments (Tilman et al. 1996; Hector et al. 1999; Spehn et al. 2000; Niklaus et al. 2006; Cardinale et al. 2007; Gross et al. 2007). The positive effects of biodiversity are generally attributed to complementarity effects of niche partitioning and facilitation among different species, through which their morphophysiological dissimilarities allow greater overall exploitation of light, water and nutrients across space, time and soil chemical gradients (Loreau et al. 2001; Hooper et al. 2005; Tilman et al 2014). Considering nitrogen cycling, it is reported that greater uptake and storage of this nutrient by biodiverse grasslands result in lower soil mineral N concentration (Tilman et al. 1996; Niklaus et al. 2001), which in turn reduces ammonium (NH_4^+) and nitrate (NO_3^-) availability for microbial nitrification and denitrification (Hodge et al. 2000; Fowler et al. 2013). Consequently, leaching and direct N_2O emission are reduced in biodiverse grasslands that are able to capture N efficiently (Scherer-Lorenzen et al. 2003; Niklaus et al. 2006; Abalos et al. 2014; Leimer et al. 2016; Niklaus et al. 2016; Piñeiro-Guerra et al. 2019).

However, these experiments show that species identity or associated functional traits and specific combinations are at least as important as diversity (i.e. number of species) *per se* regarding nitrogen cycling, its utilization by plants and eventually N_2O emission. For example, the presence of N-fixing legumes in mixtures generally increases nitrogen pools and fluxes in the soil, resulting in greater N_2O emissions and N leaching in treatments of low diversity, but these effects are reduced when diversity increases due to the presence and dominance of highly efficient N-absorbing grasses (Scherer-Lorenzen et al. 2003; Niklaus et al. 2006; Leimer et al. 2015; Niklaus et al. 2016; Cummins et al. 2021). Regarding non-legume associations (grasses and/or other herbs), it is reported that lower diversity mixtures (two or three species) can reduce N_2O emissions compared to monocultures and that the effect is less pronounced with further increase in number of species (Niklaus et al. 2006; Abalos et al. 2014). Hence, to predict the effect of increasing biodiversity in grasslands on N_2O emissions it is necessary to understand how plant traits affect N-cycling (Abalos et al. 2017), which might vary according to soil N content (Abalos et al. 2014).

In N fertilized soils, the use and depletion of soil nitrogen is usually related to functional types in the leaf-economic spectrum (Wright et al. 2004; Reich 2014), in which exploitative species (high specific leaf area, N content and specific root length) are more efficient in capturing NH_4^+ and NO_3^- in comparison to conservatives species (Grassein et al. 2015), resulting in greater overall N uptake, soil N depletion, biomass accumulation and

smaller N₂O emission (Abalos et al. 2017). Moreover, specific combinations of high yielding exploitative grass species with dissimilar complementary rooting strategies (large biomass *vs* high specific root length) were reported to decrease up to 50% the N₂O emissions compared to its respective monocultures and mixtures of more conservative species (Abalos et al. 2014). Nonetheless, in low-fertility soils, greater yields can stimulate N₂O emissions (Abalos et al. 2014; Piñeiro-Guerra et al. 2019), which can be attributed to exploitative species faster tissue and litter turnover, N mineralization, nitrification and denitrification, i.e. nitrogen cycling (van Der Krift & Berendse 2001; Scherer-Lorenzen et al. 2003).

The varying outcomes of N₂O efflux in the relatively small set of experiments on biodiverse grasslands regarding this subject is the result of complex interactions among plants and their specific traits, how they affect whole mixture functioning and its influence on nitrogen cycling, all of which can vary within contrasting environmental conditions, specially soil N availability (Abalos et al. 2014; Suter et al. 2021). Despite the importance of tropical grasslands on sustainable intensification of food production, there is no information on how traits of tropical species commonly used under intensive management affects N₂O emission, not to mention their mixtures.

Against that background, we hypothesized that mixing perennial tropical forage grasses in pastures under N fertilization will: i) generate complementarity by dissimilarities in plant traits; ii) increase nitrogen uptake and storage in biomass; iii) reduce soil N content and iv) reduce N₂O emission. The objective of this study was to identify whether a mixture of perennial C₄ grasses with contrasting growth strategies can mitigate N₂O emissions compared to their monocultures.

3.2. Materials and methods

3.2.1. Study site and experimental conditions

The study was carried out in an experimental area of the "Luiz de Queiroz" College of Agriculture, University of São Paulo (ESALQ/USP), located in Piracicaba, São Paulo, Brazil (22°42'35" South Latitude, 47°38'24" West Longitude and 546 m altitude). According to Köppen-Geiger's classification, the climate of the region is Cwa (Alvares et al. 2013), characterized by well-defined dry winter and warm summer (Köppen 1936). The mean air

temperature and annual precipitation (1990-2022) are 22.4°C and 1310 mm, respectively (data of the meteorological station located two kilometers from the experimental site). Soil water balance (WB) was calculated for the experimental period according Thornthwaite & Mather (1955) using ten days resolution and 50 mm of water storage capacity. The WB indicated water surplus during summers, water deficit in autumns and winters and transition from deficit to surplus during springs (Figure 1).
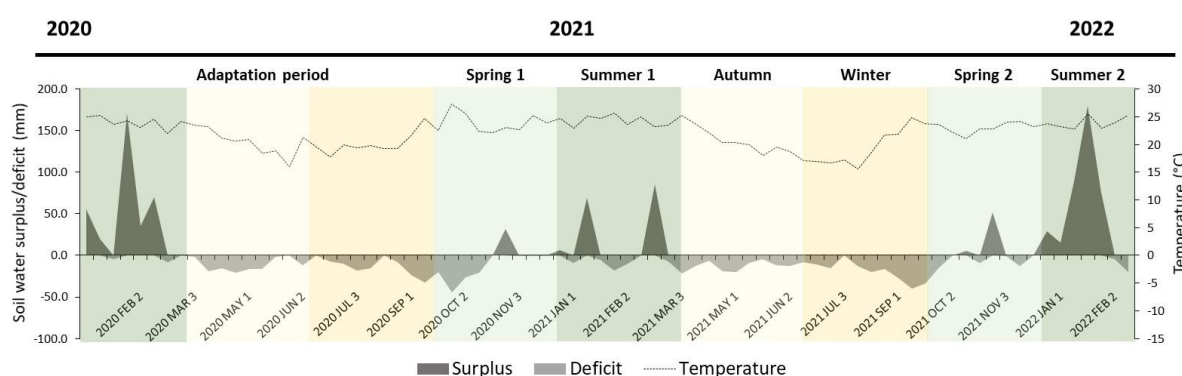

Figure 1. Soil Water Balance from adaptation and experimental periods (from Spring 1 to Summer 2), calculated according Thornthwaite & Mather (1955) and mean temperature (ten days resolution).

The soil of the experimental site is a Distroferric Red Nitisol, which are typically deep, well-drained and have moderate to strong angular blocky structure (FAO 2015). Soil texture is composed by 46% clay, 18% silt and 36% sand, within the 0-20 cm horizon. Mean soil bulk density and porosity (0-20 cm) were 1.43 g cm^{-3} and 0.50 cm^3 cm^{-3}, respectively. Soil sampling was performed on February/2019, one year before the establishment of the experiment, and chemical results were: pH CaCl$_2$ = 4.50; organic matter = 33.8 g dm^{-3} ; P = 49.5 mg dm^{-3} ; K = 3.45 mmol$_c$ dm^{-3} ; Ca = 30.3 mmol$_c$ dm^{-3} ; Mg = 12.5 mmol$_c$ dm^{-3} ; Al = 1.50 mmol$_c$ dm^{-3} ; H + Al = 72.5 mmol$_c$ dm^{-3} ; S = 25.8 mg dm^{-3} ; Cu = 4.51 mg dm^{-3} ; Fe = 176 mg dm^{-3} ; Zn = 5.54 mg dm^{-3} ; Mn = 35.2 mg dm^{-3} ; B = 0.26 mg dm^{-3} ; sum of bases = 46.0 mmol$_c$ dm^{-3} ; cation exchange capacity = 119 mmol$_c$ dm^{-3} ; base saturation = 39.0%; aluminum saturation = 3.50%.

3.2.2. Experimental design, establishment and management

In order to investigate the effects of mixed swards on nitrous oxide emissions three species of C$_4$ perennial grasses were cultivated as monocultures: i) Guinea grass -

Megathyrsus maximum cv. Massai (MAS); ii) Palisade grass – *Urochloa brizantha* cv. BRS Piatã (PIA); iii) Gamba grass – *Andropogon gayanus* cv. Planaltina (AND); and as multispecific pasture: iv) the association of the three species (seeded in equal proportions based on the number of viable seeds) (ASS). These were allocated to 12 x 15 m (180 m^2) paddocks according to a randomized complete block design, with four replications. Paddocks were separated by 3 m-wide raceways to minimize cross influence among treatments.

Considering the great diversity of tropical forage grasses currently used under intensive management (Euclides et al. 2010), species choice was based on defoliation management compatibility, which means that species to make up the mixture had to have similar pre-cutting target heights. Otherwise cutting management *per se* could favor one species while constraining the development of others, or even reduce overall agronomic performance in terms of quantity and quality of herbage production. This assumption was based on extensive literature regarding the ecophysiology of C$_4$ forage grasses, which shows that the ideal moment to interrupt regrowth of monospecific swards is when their canopy reaches the critical leaf area index (95% interception of the incident light), ensuring high rates of herbage accumulation and avoiding stem elongation and reduced nutritive value (Da Silva et al. 2015). This condition may be characterized by the sward's canopy height, which is specific for each grass species. After considering management compatibility for agronomic optimum performance, the next step was to consider species dissimilarities in terms of plant functional traits as an attempt to enhance coexistence stability (Chesson 2000), to increase complementarity (Hooper et al. 2005) and ultimately to increase and diversify resource utilization (Gross et al. 2017). The above mentioned rational was used because experiments regarding C$_4$ grasses mixtures and defoliation management are non-existent and empirical knowledge is scarce. Botanical composition stability, functional characteristics of each species and possible mechanisms of complementarity of their association are further discussed in the manuscript.

Previously to this study, the experimental area was used as extensive managed grassland of *Cynodon* spp. cv. Tifton 85 with scarce nutrient restitution for over 20 years, which caused severe degradation and weed presence. For the establishment of the experiment (Figure S1. Supplementary material), lime was applied (July/2019) at a rate of 4.5 Mg ha^{-1} with the objective of reaching 70% of base saturation, considered adequate for high

levels of herbage production as expected from intensively managed tropical grasslands (Raij et al. 1996). The lime was applied superficially on the soil and incorporated to 20 cm depth by harrowing. In January/2020, the soil was leveled with a rotary hoe and seeding of paddocks carried out according to treatments specifications. Seeding was made by broadcast at the of rate of 300 pure-viable seeds m^{-2}, with 100 viable seeds m^{-2} of each species for the association treatment, followed by compaction with a roller compactor weighing approximately 100 kg.

From establishment (January/2020) until the end of the experiment (February/2022) all paddocks were subjected to a common intermittent cutting regime characterized by a pre-cutting height of 35 cm and a post-cutting height equivalent to 50% of the pre-cutting height (i.e. 17.5 cm – 50% defoliation severity). For maintaining the proposed regime, every regrowth cycle of each paddock was monitored with a sward stick on 40 points along four transect lines. When the target pre-cutting height was reached, pre-cutting sampling procedure were performed and the herbage was cut using a motorized brush cutter. Immediately after cutting, the material was removed by raking.

The canopy height at which MAS, PIA and AND reach 95% of light interception is 55, 35 and 50 cm, respectively (Barbosa et al. 2010; Crestani et al. 2017; Sousa et al. 2010). However, recent study reported that optimum herbage accumulation rates may be maintained relatively stable by the tiller size x population density compensation mechanism in pre-cutting heights up to 40% smaller than those correspondent to the critical LAI (maximum pre-cutting target height), provided that soil fertility is moderate-high and defoliation severity does not exceed 50% (Sbrissia et al. 2018). The 35 cm pre-cutting height encompass the flexibility range of the three forage grass species used in the experiment. This regime ensures frequent and non-severe defoliations, which should reduce light competition among mixed species, favoring diversity maintenance (Eskelinen et al. 2022). As mentioned earlier, these assumptions were used because of the lack of information regarding defoliation management of tropical forage grasses grown in mixtures.

Nitrogen fertilization was applied only during the warm rainy periods (late Spring and Summer). Total amount of N-fertilizer was 200 kg N ha^{-1} for the 2020/21 and 2021/22 growing seasons, while for the establishment/adaptation period (February and March/2020)

the rate was 100 kg N ha^{-1}, since sowing was performed at the middle of the warm rainy period. The total amount of N-fertilizer was divided in instalments, with fertilization applied after every cut proportionally to the regrowth interval using a daily rate of 1.7 kg N ha^{-1} day^{-1} (Table S1. Supplementary material).

3.2.3. Shoot and root analysis

Measurements of herbage accumulation were carried out continuously throughout the experiment. At pre-cutting, two metal frames (0.25 x 1.0 m) were placed on representative areas of the paddocks at the time of sampling (visual assessment of herbage biomass and canopy height) and all the herbage within the frames was cut at the targeted post-cutting height of 17.5 cm. Herbage samples were dried in a forced draft oven at 60 °C until constant weight. Herbage accumulation rate (HAR – kg DM ha^{-1} day^{-1}) was calculated by dividing the dry weight of the samples by the corresponding cutting interval.

Herbage biomass samples were taken once every season of the year (Figure S2. Supplementary material). At pre- and post-cutting, two frames (0.25 x 1.0 m) were allocated on representative areas of the paddocks at the time of sampling (visual assessment of herbage mass and canopy height) and all herbage within the frames were cut to soil level. Samples were hand separated into their morphological components and a subsample of leaves had its area measured on a LI3100 (LI-COR™) leaf area integrator. The material was dried in a forced draft oven at 60 °C until constant weight. Herbage biomass (HBM – kg DM ha^{-1}) was calculated as the average between pre- and post-cutting herbage mass. For the association treatment, samples were further dissected into their botanical components before being put to dry and HBM was calculated for each species in order to determine botanical composition. Specific leaf area (SLA – cm^2 g^{-1}) was calculated as the quotient between area and weight of the leaf subsample, which was performed separately for the three grass species in the case of the association treatment.

Samples from the pre-cutting condition were subsampled, dried and ground in a "Wiley" type mill with a 1 mm sieve for nitrogen content analyses (HNC – %) through dry combustion at 1400 °C using the Leco FP 528 system (Leco Corporation, St. Joseph, MI, USA).

Species leaf nitrogen content (LNC – %) was evaluated once every season of the year from Summer 1 to Summer 2 (Figure S2. Supplementary material). Also at pre-cutting, approximately 20 g of sun-exposed leaves were sampled (top 10 cm of leaves blades – Farruggia et al. 2004; Louarn et al. 2020) for determining nitrogen concentration following the same procedure for HBM samples. Nitrogen stocked in herbage biomass (NHBM – kg N ha^{-1}) was calculated as the product of pre-cutting HBM and HNC.

Roots were sampled once every season of the year (Figure S2. Supplementary material), always at post-cutting. On each paddock, eight samples were taken between tussocks using a steel probe (6.3 cm diameter and 20 cm depth). The eight samples were combined into a composite sample which was washed on 0.5 mm sieves for removing soil. Roots were isolated and taken to the laboratory, where they were digitalized and analyzed by WinRHIZO™ (Regent instrument Inc.) coupled in a scanner (Epson™ Expression 11000XL) (Bouma et al., 2000). The software resolved root diameter (RD – mm), length and volume. Root length density (RLD – m m^{-3}) was calculated by relating length per sample and volume of sampled soil. Root volume density (RVD – cm^3 m^{-3}) was calculated as the quotient between sampled root volume and volume of sampled soil.

Subsequently, samples were dried in a forced draft oven at 60 °C until constant weight and data used to calculate root biomass (RBM – kg DM ha^{-1}). Specific root length (SRL – cm g^{-1}) was calculated as the quotient between root length and root biomass. After weighting, samples were ground in a "Wiley" type mill with a 1 mm sieve and root total nitrogen content (RNC - %) determined by dry combustion at 1400°C using the Leco FP 528 system (Leco Corporation, St. Joseph, MI, USA). The RNC was analyzed from Summer 1 to Summer 2, same period as LNC (Figure S2. Supplementary material). Nitrogen stocked in the root biomass (NRBM kg N ha^{-1}) was calculated as the product between RBM and RNC.

3.2.4. Soil analyses

Soil samples were collected from Spring 1 to Summer 2 (a total of 28 sampling procedures – Figure S2. Supplementary material), always coupled with sampling dates for determining soil nitrous oxide emission. Sampling was made using a Dutch auger at 0 – 10

cm soil depth at three points (within a radius of 1.5 m from the center of the static chambers – section 3.2.5). Subsamples were weighed and dried in a forced draft oven at 60°C until constant weight. Gravimetric soil water content (SWC) was calculated as the quotient between sample water content and dry weight. During the establishment period of the experimental swards and at the end of the experiment, undeformed soil samples were taken with a steel core at 0 – 10 cm soil layer. These samples were analyzed for soil bulk density (SBD) and porosity (SP). The mean value of initial and final SBD and SP for each paddock and SWC were used to calculate water-filled pore space (WFPS), according to equation 1.

$$WFPS\ (\%) = \frac{SWC \times SBD}{SP} \times 100 \tag{1}$$

Soil nitrate and ammonium content (NO_3^- and NH_4^+ – µg N g dry soil^{-1}) were analyzed in 14 sampling dates (Figure S2. Supplementary material). Mineral nitrogen was extracted by solution of KCl 1 mol L^{-1} in subsamples (10 g) and NO_3^- and NH_4^+ were determined by the Kjendahl method of distillation and titrations (Jones Jr. 1987, adapted by Campos et al. 2017). Values were adjusted by SWC for calculating contents on dry soil basis. Soil pH was determined on $CaCl_2$ solution (10 sampling dates).

Soil was sampled for microbiological analyses by quantitative PCR (qPCR) on Summer 1, Winter and Summer 2 (Figure S2. Supplementary material), at 0 – 10 cm soil depth. Soil DNA was extracted from 0.30 g of soil in triplicate using PowerSoil PowerLyzer DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was checked on a 1% agarose gel and DNA concentration was determined using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Tenfold diluted DNA was used in quantitative PCR (qPCR) analysis.

Bacterial populations capable of performing nitrification and denitrification were monitored by targeting the amoA (AOA and AOB) as well as nirK and nosZ genes. The target genes were quantified by qPCR using ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, USA). Each 10-µL reactions contained 1 µL of bovine serum albumin (BSA, 0.10 mg ml^{-1}), 1.25 µL of each primer (0.8 µM for AOA and nosZ, and 0.5 µM for nirK and AOB), 5 µL of qPCR SYBR Master Mix 2x (Applied Biosystems, Frankfurt, Germany) and 10 to 20 ng of DNA. The standard curve for each gene was generated using serial dilutions of 10^2 to 10^9

copies µL^{-1} of a known amount of the gene, previously amplified by PCR. The amplifications were performed as described in Table S2. Three independent qPCR assays were carried out for each gene, and three no-template controls were run for each assay, which resulted in null or negligible values. Melting curve analysis confirmed the specificity of the qPCR amplicons, with a single melting peak (60 °C to 95 °C). Results were only accepted if the R^2 of the standard curve was above 0.995. The amplification efficiencies were 95% ± 9.

3.2.5. Nitrous oxide flux measurements

Nitrous oxide flux (µg N-N$_2$O m^{-2} d^{-1}) was measured using the closed static chamber methodology (Hutchinson & Moiser, 1981; Clough et al. 2020) from Spring 1 to Summer 2 (total of 41 evaluations – Figure S2. Supplementary material), always between 8:00 h – 11:00 h. On each paddock, one aluminum chamber base (20 cm diameter, 22 cm height) was installed between tussocks (i.e. excluding plants from the inside) and buried 0.05 m into the soil, with reallocations every 4 or 5 evaluations. For sampling, PVC caps (7.5 cm height) were placed on the water-filled trough of the chamber base for sealing. The caps were designed with vent opens to prevent pressure gradient between chamber interior and exterior. Gas samples were taken with 20 mL plastic syringes from the cap sampling port, at 1, 15 and 30 min after chamber closure. Samples were transferred to pre-evacuated vials (Labco Limited, Ceredigion, United Kingdom) and analyzed in a Shimadzu gas chromatograph (GC-2014). The outputs were tested for linearity and strong linear relationship was found (average R^2 = 0.91). Atmospheric pressure, chamber and air temperature were recorded during sampling and used to calculate daily N$_2$O flux according to equation 2:

$$N_2O\ flux = \frac{\Delta N_2O}{\Delta T} \times \frac{V}{A} \times \frac{M}{Vm} \qquad (2)$$

where $\frac{\Delta N_2O}{\Delta T}$ is the rate of change of the gas concentration inside the chamber during closure time (µmol mol^{-1} h^{-1}); V is the chamber volume (L); A is the soil area covered by the chamber (m^2); M is the molecular mass of N in N$_2$O (28.0134 g mol^{-1}); Vm is the molar volume of gas at sampling temperature. Hourly fluxes were extrapolated to daily emissions (µg N-N$_2$O m^{-2}d^{-1}).

3.2.6. Calculations and statistical analysis

Expected values for the association of the three grass species were calculated based on the monocultures' actual values and their botanical proportion in the association herbage biomass (Loreau and Hector 2001) according to equation 3:

$$Exp\ value = \sum_i p_i \times V_i \tag{3}$$

where *Exp value* is the expected value for the association's community of plants, considering its actual species proportion; p_i is the proportion of species *i* in the mixture and *Vi* is the actual value obtained in monoculture *i*.

Plants functional traits (HNC, LNC, RNC, SLA and SRL) were averaged among seasons of the year and analysis of variance (Anova) was performed using the MIX procedure of the SAS OnDemand for Academics (SAS Institute Inc.). The model included treatment (ASS, MAS, PIA and AND or association *vs* expected value) as fixed and block as random factors.

Agronomic characteristics, soil parameters and N_2O emissions were analyzed (Anova) throughout seasons of the year and the model included treatment, seasons of the year (treated as repeated measure) and their interaction as fixed and block as random factors. The data were tested for normality (Shapiro-Wilk test) and AOB data were log-transformed to meet this assumption. Structures of the variance-covariance matrices were tested according to the Bayesian Information Criterion for the selection of best fit matrix for each variable. Means were obtained by LSMEANS statement and compared by Least Significant Difference test (LSD) when effects were significant in the Anova ($p < 0.05$).

Using R software (version 4.2.2 – R core team, 2022), a Principal Components Analysis (PCA) was performed with monocultures' plant functional traits (HNC, LNC, RNC and LNC), agronomic characteristics (HBM, RBM, RVD, RLD and NRBM) and N_2O efflux for assessing similarities and differences in functionality of individual species and their relationships. A second PCA was performed with actual and expected values of the association for functional traits (SLA, LNC and SRL), soil parameters (NH4+/NO3- ratio, AOA), agronomic characteristic (NRBM) and N_2O efflux with the objective of understanding the

effects of association that are not simple additive effects of the grass species growing in the mix.

3.3. Results

3.3.1. Plant functional traits

Herbage nitrogen content (HNC), leaf nitrogen content (LNC), root nitrogen content (RNC), all varied with treatments ($p < 0.0001$). Gamba grass (AND) had greatest HNC and LNC, palisade grass (PIA) and association (ASS) smallest, and guinea grass (MAS) intermediate for both variables (Figure 2A-B). Gamba grass also had the greatest RNC, followed by PIA and ASS, and MAS had the smallest (Figure 2C). Specific leaf area (SLA) did not differed among monocultures ($p > 0.05$ – Figure 2D). Greatest value of specific root length (SRL) was recorded for MAS and smallest for PIA, while AND and ASS had intermediate values ($p < 0.0001$ – Figure 2E).
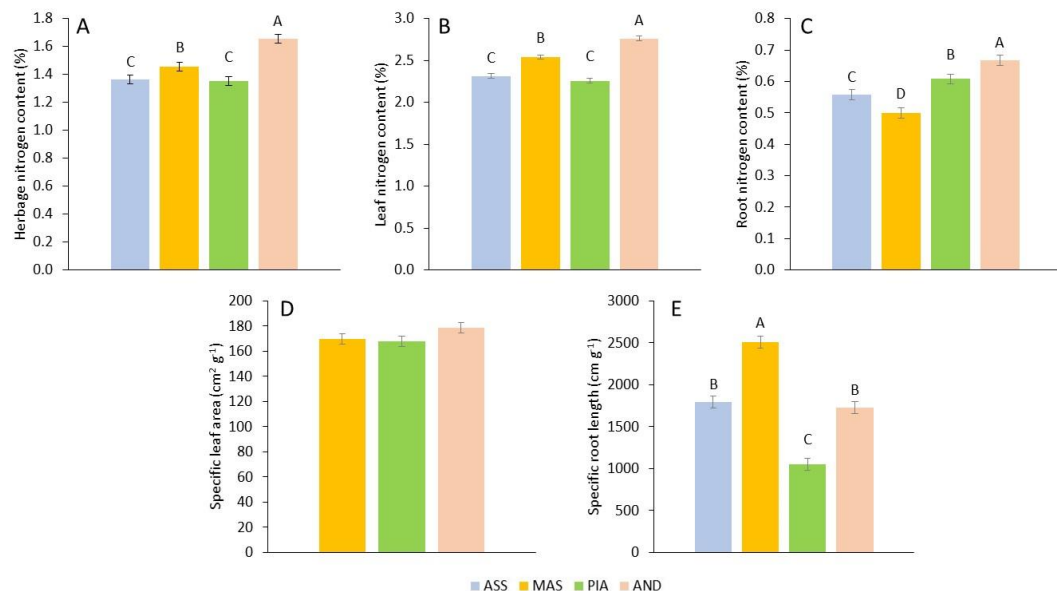

Figure 2. Herbage nitrogen content (A), leaf nitrogen content (B), root nitrogen content (C), specific leaf area (D) and specific root length (E) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences (ANOVA – $p < 0.05$) of mean values by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

3.3.1.1. Plant functional traits for the Association: Actual vs Expected values

Guinea grass showed reduced SLA and LNC when cultivated in association (p = 0.0044 and p < 0.0001, respectively – Table 1). Gamba grass followed the same trend (SLA – p = 0.0009 and LNC – p < 0.0001). For palisade grass, SLA remained stable regardless of being cultivated as monoculture or in association (p > 0.05), but LNC increased (p = 0.0172 – Table 1). The traits SLA (p = 0.0024), LNC (p = 0.0020), HNC (p = 0.0326) and SRL (p = 0.0018) were all reduced for the association relative to the expected values from the monocultures (EXP – Table 2). Overall, there was no difference in root nitrogen content between actual and expected values for the association (p > 0.05).

Table 1. Guinea grass (MAS), palisade grass (PIA) and gamba grass (AND) functional traits in monocultures (EXP) and in association (ASS).

Functional trait	Treatment	Mean	SE	p>F
Specific leaf area MAS $(cm^2\ g^{-1})$	ASS	145 B	3.9	0.0044**
	EXP	169 A	3.9	
Leaf N content MAS (%)	ASS	2.18 B	0.013	<0.0001***
	EXP	2.54 A	0.013	
Specific leaf area PIA $(cm^2\ g^{-1})$	ASS	162	4.8	0.3997
	EXP	168	4.8	
Leaf N content PIA (%)	ASS	2.38 A	0.025	0.0172*
	EXP	2.26 B	0.025	
Specific leaf area AND $(cm^2\ g^{-1})$	ASS	126 B	6.1	0.0009**
	EXP	179 A	6.1	
Leaf N content AND (%)	ASS	2.34 B	0.014	<0.0001***
	EXP	2.76 A	0.014	

Different letters in columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). SE means standard error of the mean.

Table 2. Functional traits for the association (ASS) and the expected values from the respective monocultures (EXP).

Functional trait	Treatment	Mean	SE	p>F
Specific leaf area ($cm^2\ g^{-1}$)	ASS	152 B	2.3	0.0024**
	EXP	168 A	2.3	
Leaf N content (%)	ASS	2.31 B	0.018	0.0020**
	EXP	2.45 A	0.018	
Herbage N content (%)	ASS	1.36 B	0.035	0.0326*
	EXP	1.44 A	0.035	
Specific root length ($cm\ g^{-1}$)	ASS	1795 B	63.8	0.0018**
	EXP	2102 A	63.8	

Different letters in columns indicate differences (ANOVA – $p < 0.05$) of mean values by the LSD test ($p < 0.05$). SE means standard error of the mean.

3.3.2. Agronomic characteristics

Herbage accumulation rate (HAR) did not vary with treatments ($p > 0.05$), but varied with seasons of the year ($p < 0.0001$) and with the interaction treatment x season of the year ($p = 0.0445$). In Summer 1, MAS had the greatest HAR and AND the smallest, with intermediate values recorded for the ASS and PIA. In Winter, PIA had greatest HAR, MAS the smallest and AND intermediate, while the ASS was similar to MAS and AND. In Spring 2, PIA and AND had greater HAR than ASS and MAS (Figure 2). During other seasons of the year, no difference among treatments was observed. Regarding seasonal variation in herbage accumulation, HAR was greater during both Summers, followed by Spring 1, then Autumn and Spring 2, with smallest values recorded during Winter.
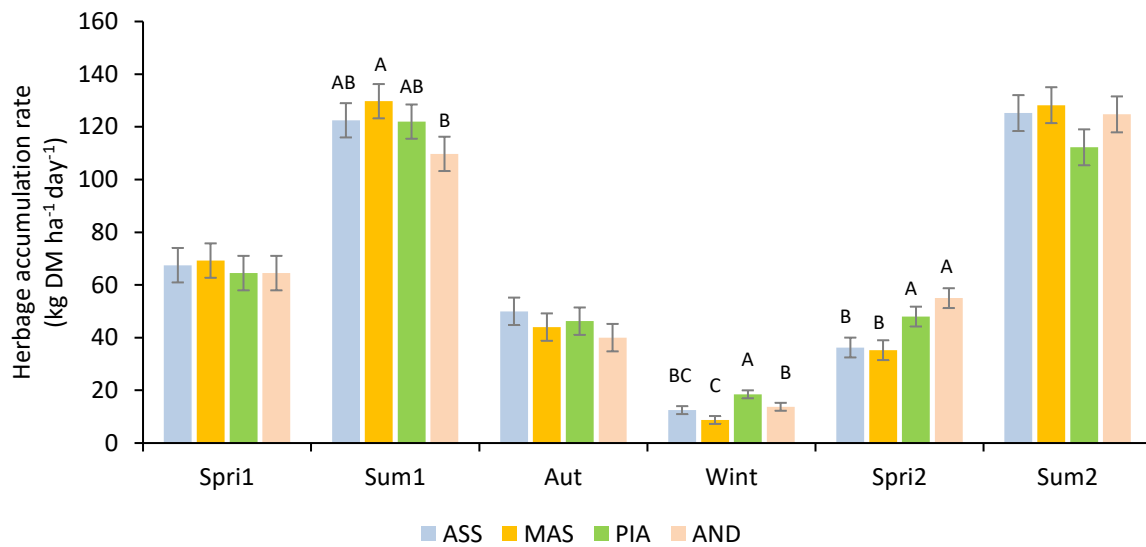
Figure 3. Herbage accumulation rate of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments within seasons of the year (ANOVA – $p_{treatment*season} < 0.05$) by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

Herbage biomass (HBM) varied with treatments ($p < 0.0001$), seasons of the year ($p < 0.0001$) and their interaction ($p < 0.0001$). Palisade grass was consistently among the treatments with greater HBM, except during Spring 2 (Figure 4). Conversely, AND showed smaller values of HBM throughout the experiment, except during Spring 2, when it showed the greatest values (Figure 4). The ASS and MAS had intermediate HBM, which was similar to PIA or AND depending on season of the year (Figure 4). The average HBM was greater during the flowering period, in Autumn, and similar during the remaining seasons of the year.
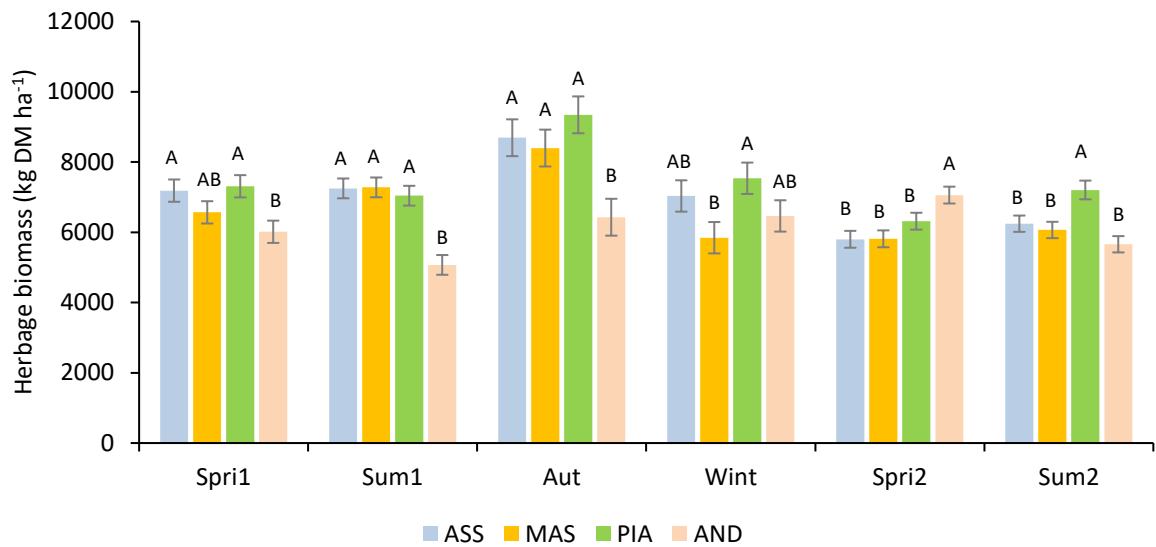
Figure 4. Herbage biomass of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments within seasons of the year (ANOVA – $p_{treatment*season}$ < 0.05) by the LSD test (p < 0.05). Bars indicate standard error of the mean.

Root biomass (RBM) varied with treatments (p < 0.0001), seasons of the year (p < 0.0001) and their interaction (p = 0.0012). Similarly to HBM, Palisade grass had the greatest RBM throughout the experiment and AND the smallest (Figure 5). Recorded values for the association were similar to PIA during all seasons of the year, except during both Summers (Figure 5). Guinea grass presented intermediate RBM between ASS and AND (Figure 5). Considering the warm and rainy seasons of the year (Springs and Summers), ASS and MAS peaked RBM in both Springs, while PIA peaked in both Summers (Figure 6). On the other hand, Gamba grass kept stable smaller values of RBM during the entire experimental period (Figure 6). Generally, RBM increased from the first to the second rainy season (Figure 6), mostly because of ASS, MAS and PIA treatments.
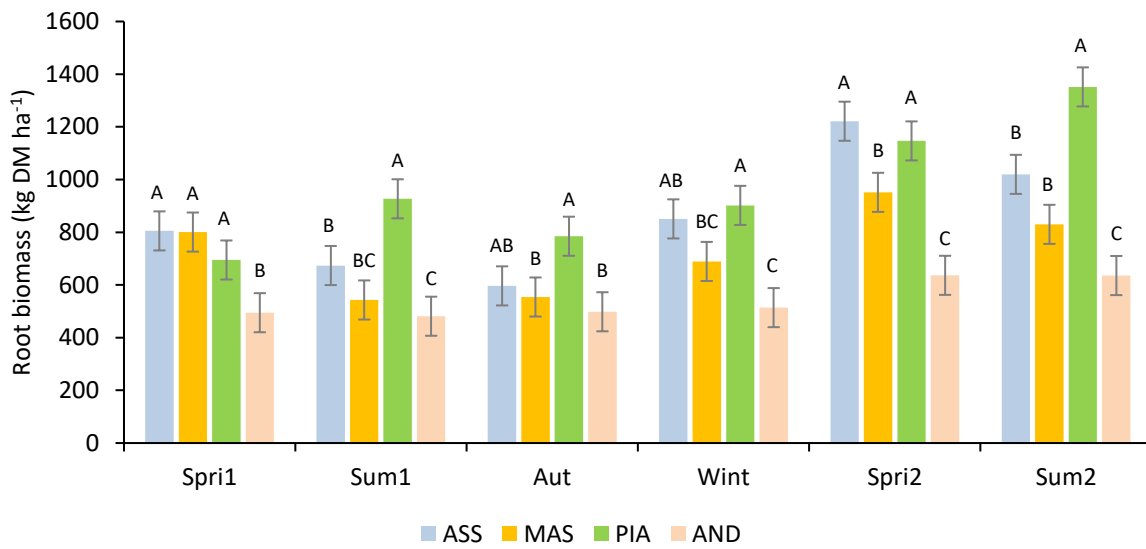
Figure 5. Root biomass of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments within seasons of the year (ANOVA − p$_{treatment*season}$ < 0.05) by the LSD test (p < 0.05). Bars indicate standard error of the mean.
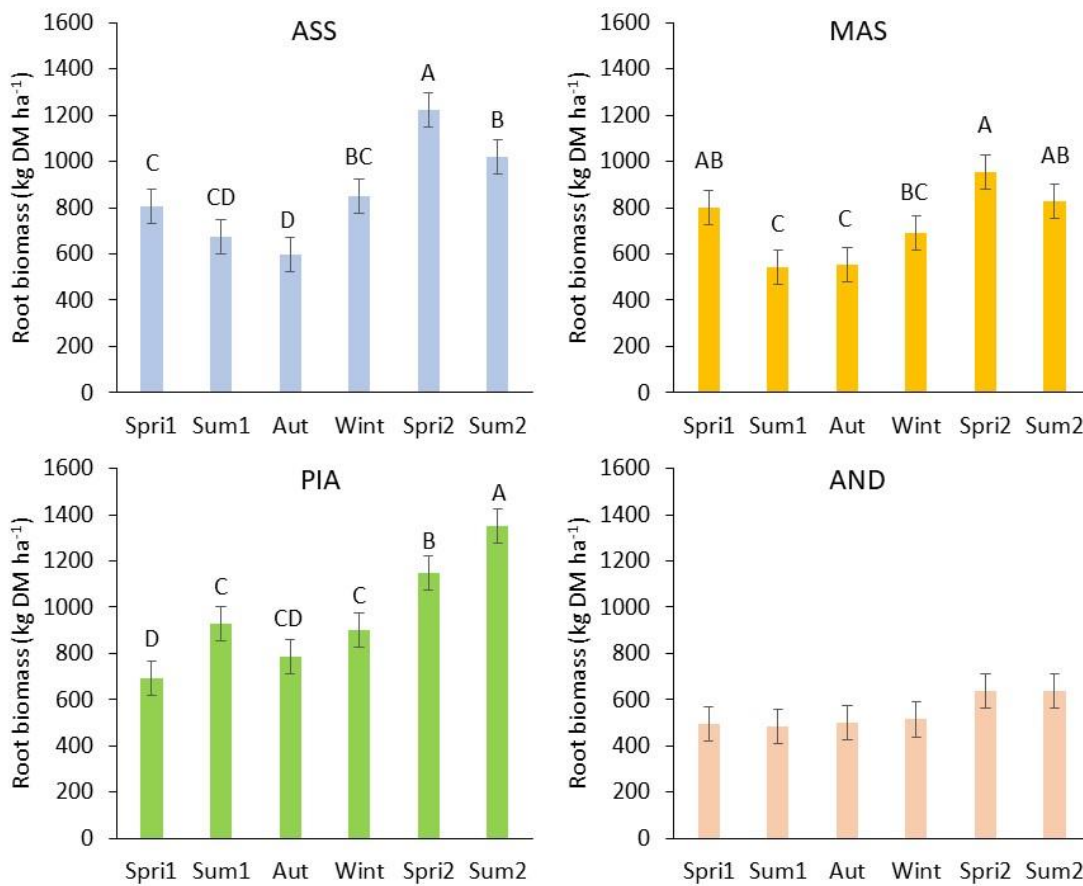

Figure 6. Root biomass of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among seasons of the year within treatments (ANOVA − p$_{treatment*season}$ < 0.05) by the LSD test (p < 0.05). Bars indicate standard error of the mean.

Root length density (RLD) varied with treatments, seasons of the year and their interaction (p < 0.0001, for all sources of variation). Guinea grass showed the greatest values during all seasons of the year, while PIA and AND showed the smallest (Figure 7). The association showed RLD similar to MAS throughout the experiment, except during both Springs (Figure 7). Root length density was greatest in the onset of the experiment (Spring 1 – 9576 ±319.0), decreased until Autumn (3927 ±286.4) and reached similar intermediate values from Winter to Summer 2 (mean values between 5409 ±336.5 and 6046 ±374.3, Table 4).
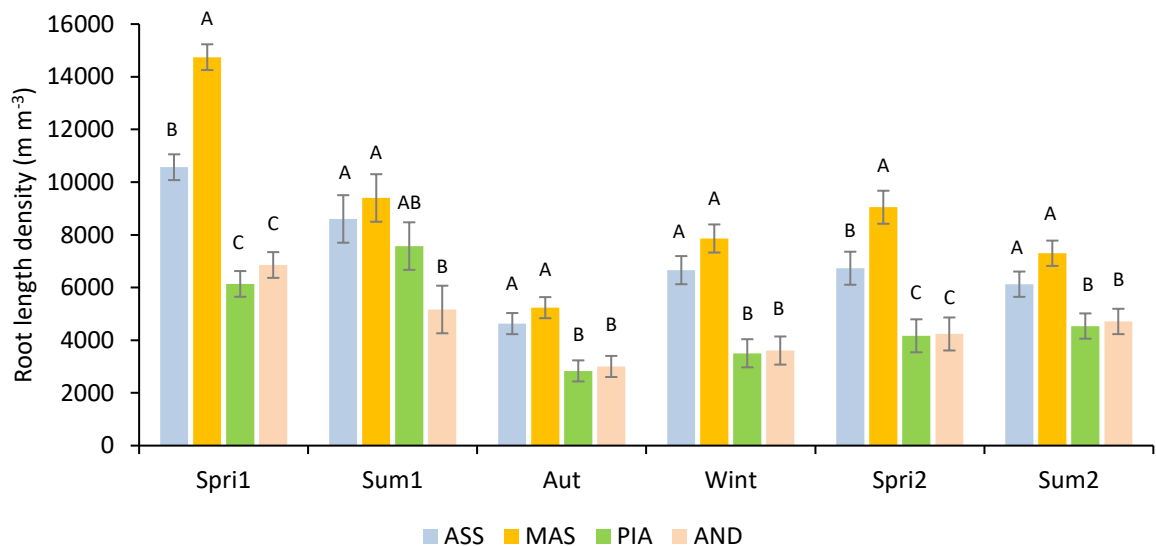

Figure 7. Root length density of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments within seasons of the year (ANOVA – $p_{treatment*season}$ < 0.05) by the LSD test (p < 0.05). Bars indicate standard error of the mean.

Nitrogen stocked in the herbage biomass (NHBM) varied only with seasons of the year (p < 0.0001), with greatest values recorded during Autumn and smallest during Spring 2. Nitrogen stocked in the root biomass (NRBM) varied with treatments (p < 0.0001) and seasons of the year (p = 0.0009). Palisade grass showed greatest NRBM, followed by the association (Figure 8A) and guinea grass, with gamba grass showing the smallest values (Figure 8A). Greatest NRBM was recorded during Summer 1 and Spring 2 and smallest during Autumn (Figure 8B).
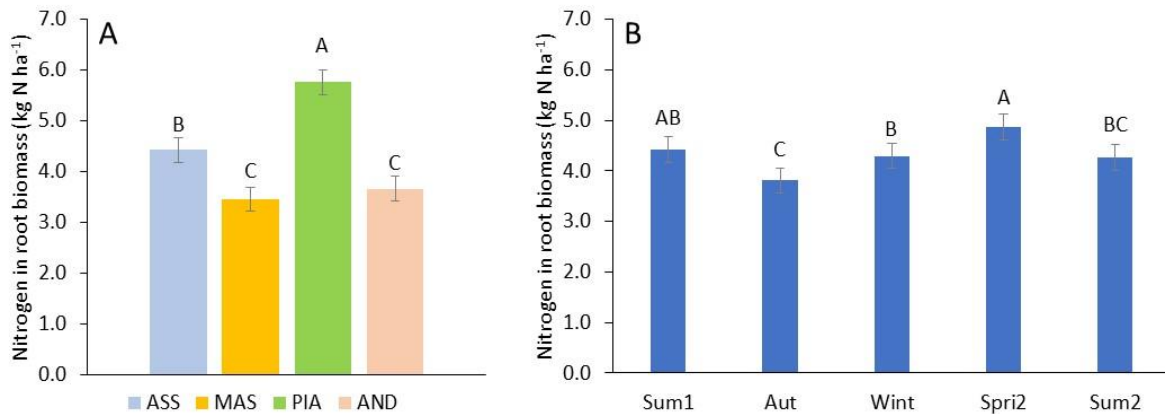
Figure 8. Nitrogen stocked in the root biomass of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments (A - ANOVA – p < 0.05) and seasons (B - ANOVA – p < 0.05) by the LSD test (p < 0.05). Bars indicate standard error of the mean.

3.3.2.1. Agronomic characteristics for the Association: Actual vs Expected Values

Botanical composition of the association was relatively stable from Spring 1 until Autumn. During Winter and Spring 2, the percentage of guinea grass was reduced in the association while that of palisade grass and gamba grass increased. During Summer 2, the percentage of guinea grass increased but did not reach similar values to those recorded during the previous Summer (Figure S3).

Root biomass increased in the ASS, but not significantly (p = 0.0606 – Table 3). Nitrogen stocked in root biomass was 14% greater in ASS relative to EXP (p = 0.0201 – Table 3). Root length density did not vary between ASS and EXP (p > 0.05 – Table 3). Aboveground characteristics such as HBM, NHBM and HAR were not affected by treatment (actual *vs* expected values) (p > 0.05 – Table 3). Agronomic characteristics did not vary with the treatment x seasons of the year interaction.

Table 3. Agronomic characteristics for the association (ASS) and the expected values from the respective monocultures (EXP).

Variable	Treatment	Mean	SE	Treatment p > F	Treatment*Season p > F
Root biomass (kg DM ha^{-1})	ASS	861	52.7	0.0606	0.5926
	EXP	787	52.7		
N stocked in root biomass (kg N ha^{-1})	ASS	4.66 A	0.300	0.0201*	0.6149
	EXP	4.11 B	0.300		
Root length density (m^3 m^{-3})	ASS	7220	289.9	0.2723	0.0573
	EXP	7619	289.9		
Herbage biomass (kg DM ha^{-1})	ASS	7034	143.5	0.4773	0.6303
	EXP	6915	146.3		
N stocked in herbage biomass (kg N ha^{-1})	ASS	111	3.0	0.1699	0.3530
	EXP	117	2.8		
Herbage accumulation rate (kg DM ha^{-1} day^{-1})	ASS	69	1.9	0.7259	0.7444
	EXP	70	1.9		

Different letters in columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). SE means standard error of the mean.

3.3.3. Soil parameters

Soil ammonium content (NH_4^+) varied with treatments (p = 0.0357) and seasons of the year (p < 0.0001). Smallest values were recorded for PIA and greatest for ASS and AND, with intermediate values for MAS (Figure 9A). Soil ammonium content increased from Spring 1 to Summer 1, but decreased until Spring 2 and remained at smallest values during Summer 2 (Figure 9B). Soil nitrate content (NO_3^-) varied only with seasons of the year (p < 0.0001 – Figure 9C-D) and similarly to NH_4^+, it increased from Spring 1 to Summer 1 and decreased henceforward, reaching smallest values during Summer 2 (Figure 9D). The ammonium:nitrate ratio (NH_4^+/NO_3^-) varied with treatments (p = 0.0294) and seasons of the year (p = 0.0036). The association had the greatest NH_4^+/NO_3^- ratio (18% greater than the average for monocultures), PIA and AND had the smallest, and MAS did not differ from other treatments (Figure 9E). From Spring 1 until Spring 2, the NH_4^+/NO_3^- ratio was stable with

values varying between 1.1 and 1.3 ±0.08, but during Summer 2 it increased to 1.5 ±0.08 (Figure 9F).

Soil pH varied slightly with seasons of the year (p = 0.0355), ranging from 4.8 ±0.14 in Spring 1 and Summer 1 to 5.1 ±0.14 in Winter, but it was not affected by treatments or the interaction treatment x seasons of the year (p > 0.05). Soil water-filled pore space (WFPS) was greater for AND, followed by ASS and PIA, while MAS showed the smallest values (p < 0.0001 – Figure 10A). Season of the year also influenced WFPS (p < 0.0001), with greatest values during Spring 1 and Summer 1, then declining until Winter, when the smallest values were recorded, and increasing until Summer 2, but to smaller values than those recorded during the previous Summer (Figure 10B).
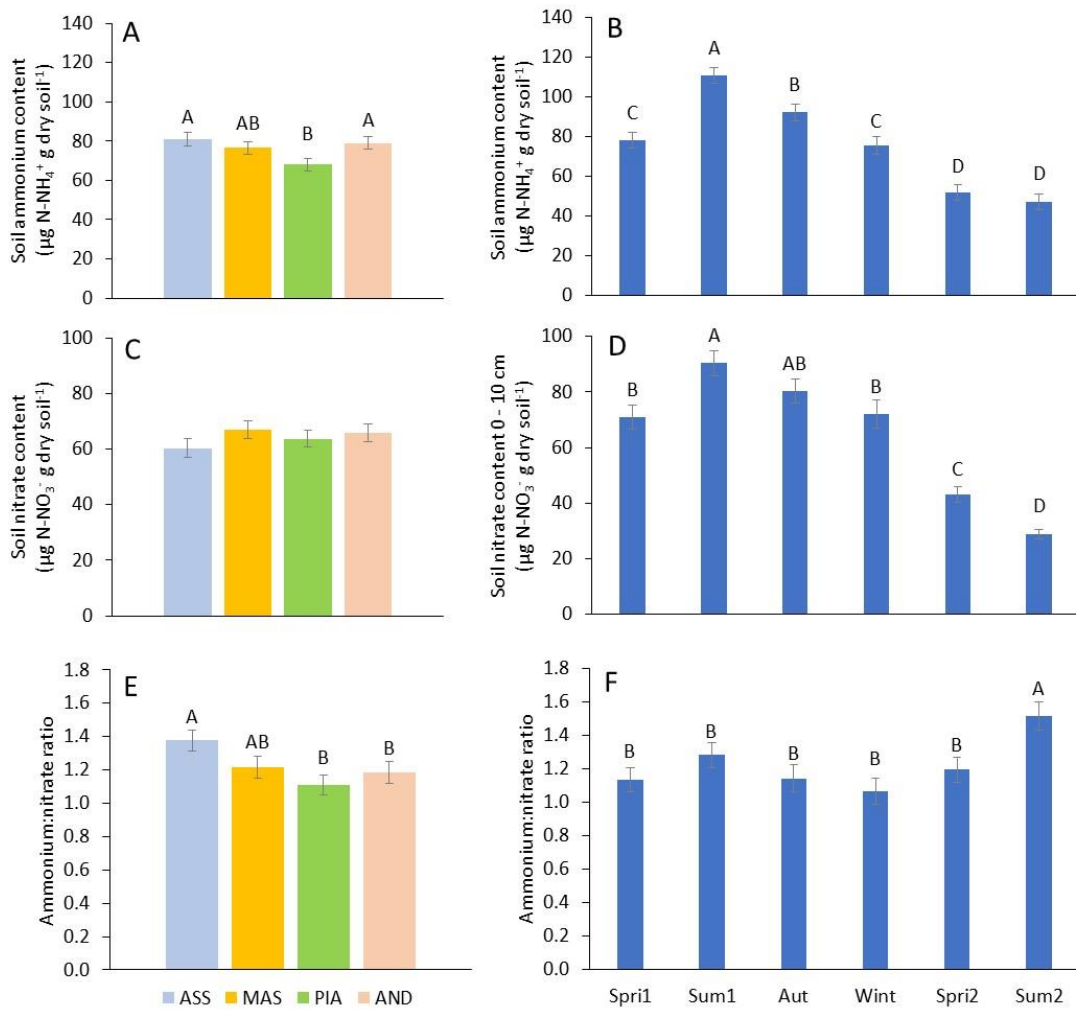
Figure 9. Soil ammonium (A and C) and nitrate (B and D) contents (0 – 10 cm) and their ratio (E and F) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments (A and E - ANOVA – $p < 0.05$) and seasons of the year (B, D and F - ANOVA – $p < 0.05$) by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

Figure 10. Soil water-filled pore space (0 – 10 cm) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments (A - ANOVA – $p < 0.05$) and seasons of the year (B - ANOVA – $p < 0.05$) by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

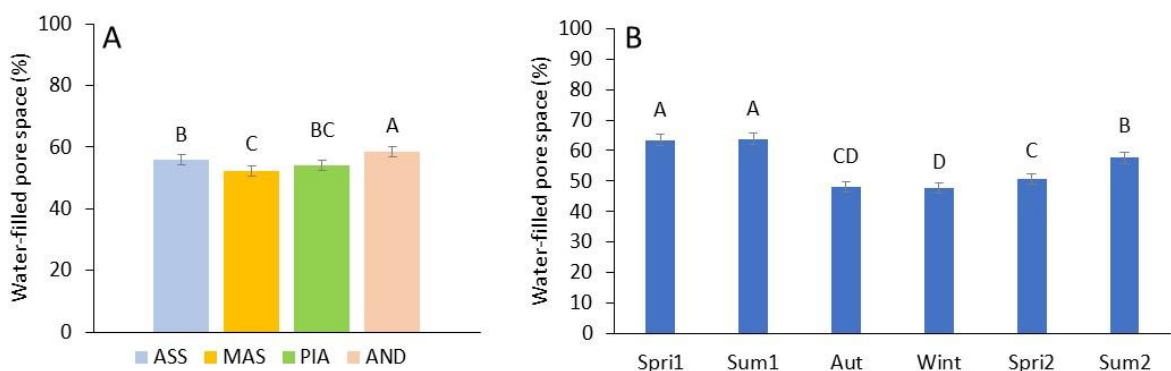Genes abundance did not vary with treatments (p > 0.05), except for ammonia-oxidizing bacteria (AOB – p = 0.0216), which was greater for guinea grass (142 ±20.0) and gamba grass (124 ±20.3) relative to palisade grass (83 ±23.1 thousand gene copies soil g^{-1} – Figure 11). The association presented intermediate value (91 ±20.0 thousand gene copies soil g^{-1}), similar to PIA and AND (Figure 11). There was no treatment x season of the year interaction (p > 0.05), but all genes abundances varied with season of the year (AOA – p = 0.0007; AOB – p = 0.0217; nirK – p < 0.0001; and nosZ – p < 0.0001). AOA, AOB and nosZ increased abundance from Summer 1 to Summer 2, while nirK decreased (Figure 12).

Figure 11. Soil gene abundances of ammonia-oxidizing archaea (A – AOA), bacteria (B – AOB), nitrite reductase coding gene (C – nirK) and nitrous oxide reductase coding gene (D – nosZ) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). Bars indicate standard error of the mean.
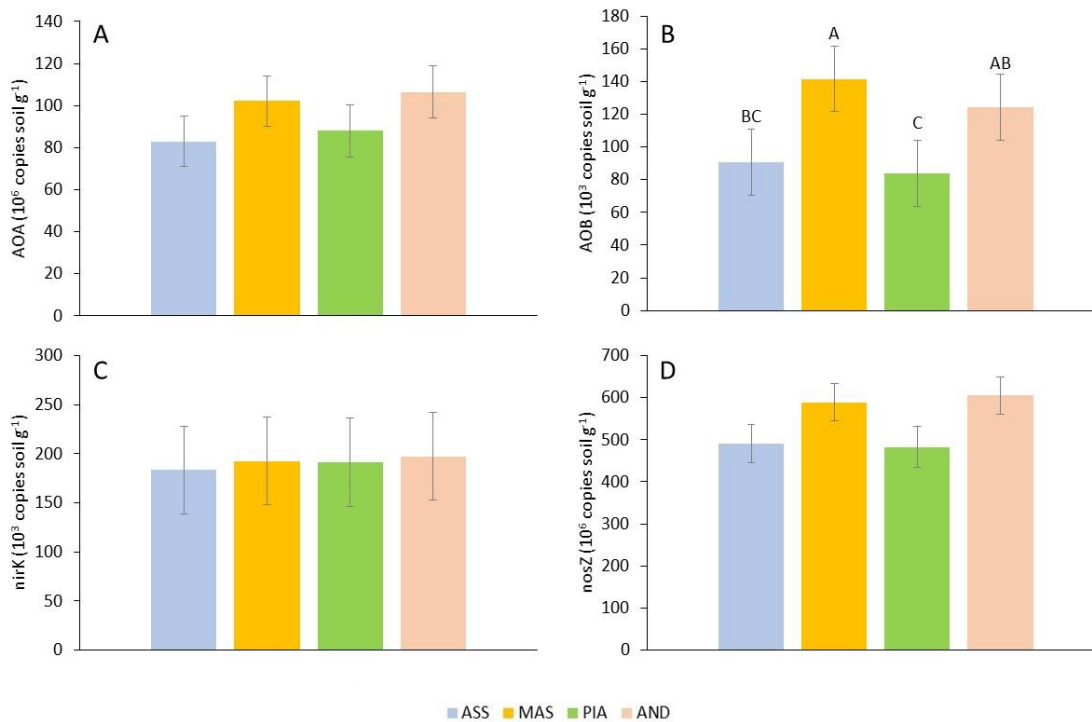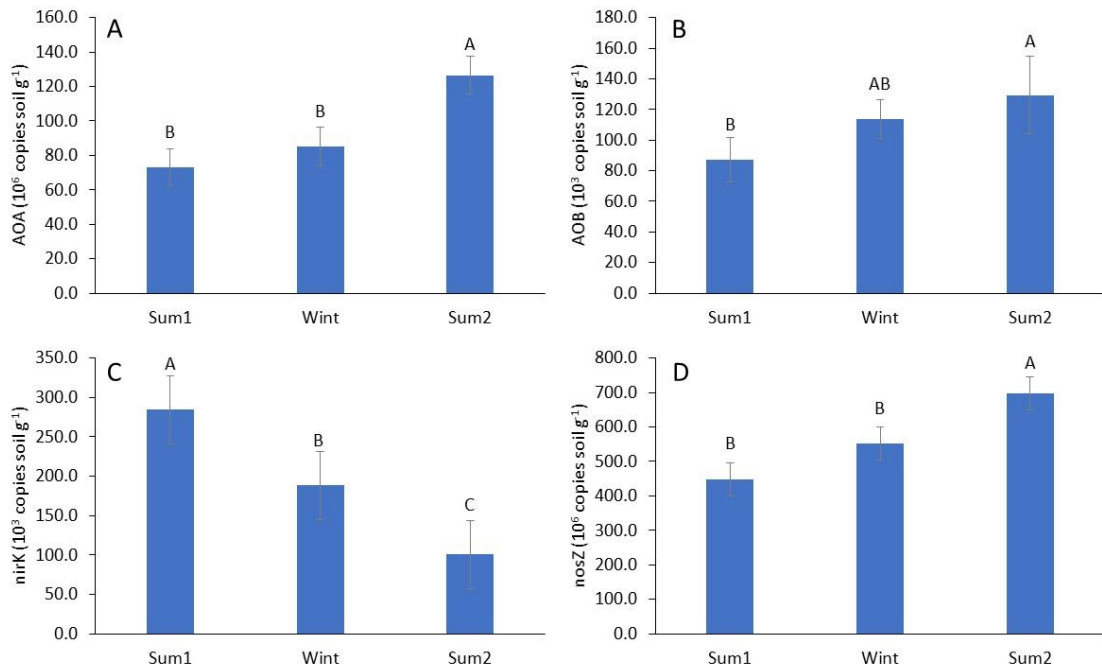
Figure 12. Seasonal variation of gene abundances of ammonia-oxidizing archaea (A – AOA), bacteria (B – AOB), nitrite reductase coding gene (C – nirK), nitrous oxide reductase coding gene (D – nosZ) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). Bars indicate standard error of the mean.

3.3.3.1. Soil parameters for the Association: Actual vs Expected Values

Soil NH_4^+ content was 9% greater for the association relative to the expected values from the monocultures (p = 0.0479). On the other hand, NO_3^- content was 9% smaller, but this difference was not significant (p = 0.0976 – Table 4). As consequence, the soil NH_4^+/NO_3^- ratio was 19% greater for the ASS relative to EXP (p = 0.0077 – Table 4). Values of soil WFPS were 2.7 percentage units greater for the association relative to the expected values from the monocultures (p = 0.0094), with no variation in pH (p > 0.05 – Table 4).

Table 4. Soil parameters for the association (ASS) and the expected values from the respective monocultures (EXP).

Soil parameter	Treatment	Mean	SE	Treatment p > F	Treatment*Season p > F
Soil ammonium content (μg $N\text{-}NH_4^+$ g dry soil^{-1})	ASS	80.8 A	2.41	0.0479*	0.4423
	EXP	73.8 B	2.52		
Soil nitrate content (μg $N\text{-}NO_3^-$ g dry soil^{-1})	ASS	60.3	2.50	0.0976	0.1400
	EXP	66.2	2.43		
Soil ammonium nitrate ratio (-)	ASS	1.38 A	0.056	0.0077**	0.2143
	EXP	1.16 B	0.056		
Soil water-filled pore space (%)	ASS	55.8 A	1.64	0.0094**	0.9350
	EXP	53.1 B	1.64		
Soil pH (-)	ASS	5.0	0.12	0.2411	0.4127
	EXP	4.9	0.12		

Different letters in columns indicate differences (ANOVA – $p < 0.05$) of mean values by the LSD test ($p < 0.05$). SE means standard error of the mean.

AOA gene abundance varied with treatments ($p = 0.0209$), with recorded values for the association being 18% smaller relative to the expected values from the monocultures (83 ±10.25 *vs* 101 ±10.59 million gene copies soil g^{-1}). The abundance of other genes (AOB, nirK and nosZ) did not vary with treatments ($p>0.05$).

3.3.4. Nitrous oxide emission

Nitrous oxide emissions varied with treatments ($p < 0.0001$), season of the year ($p < 0.0001$) and the with treatment x season of the year interaction ($p < 0.0001$). The association showed the smallest emission throughout the experiment and gamba grass the greatest, except in Autumn, when there was no difference among treatments (Figure 13). Palisade grass alternated among seasons of the year from equally smaller emissions as ASS (Summer 1, Winter and Spring 2) or greater as AND (Spring 1, Winter and Summer 2 – Figure 13). Guinea grass showed greater emission in Spring 1 and Summer 1 along with AND, but from Autumn until the end of experiment recorded values were as low as those for ASS (Figure 13). Season of the year had strong influence on emissions ($p < 0.0001$), with values declining throughout the experiment and smallest values recorded in Autumn.
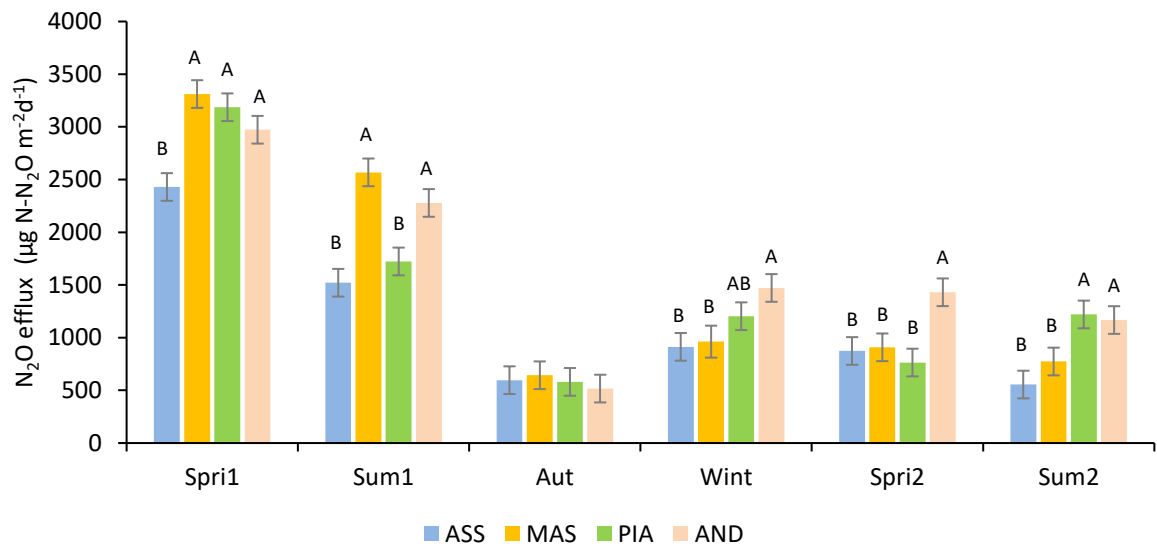
Figure 13. Nitrous oxide emissions of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences among treatments within seasons of the year (ANOVA – $p_{treatment*season} < 0.05$) by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

3.3.4.1. Nitrous oxide emission for the Association: Actual vs Expected Values

On average, the association showed emission 25% smaller than the expected values for the monocultures ($p < 0.0001$). There was a treatment x season of the year interaction ($p = 0.0019$) characterized by the reduction in N_2O efflux from the association during Spring 1 and Summers 1 and 2 (Figure 14). Linear regression between the proportional difference in emissions from ASS and EXP and soil WFPS was significant and negative ($R^2 = -0.83$, $p = 0.04$), i.e. greater reduction in N_2O efflux occurred in wetter soil conditions.

Figure 14. Nitrous oxide emissions of association (ASS) *vs* expected values from its respective monocultures (EXP). Letters over columns indicate differences (ANOVA – $p < 0.05$) within each season of mean values by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

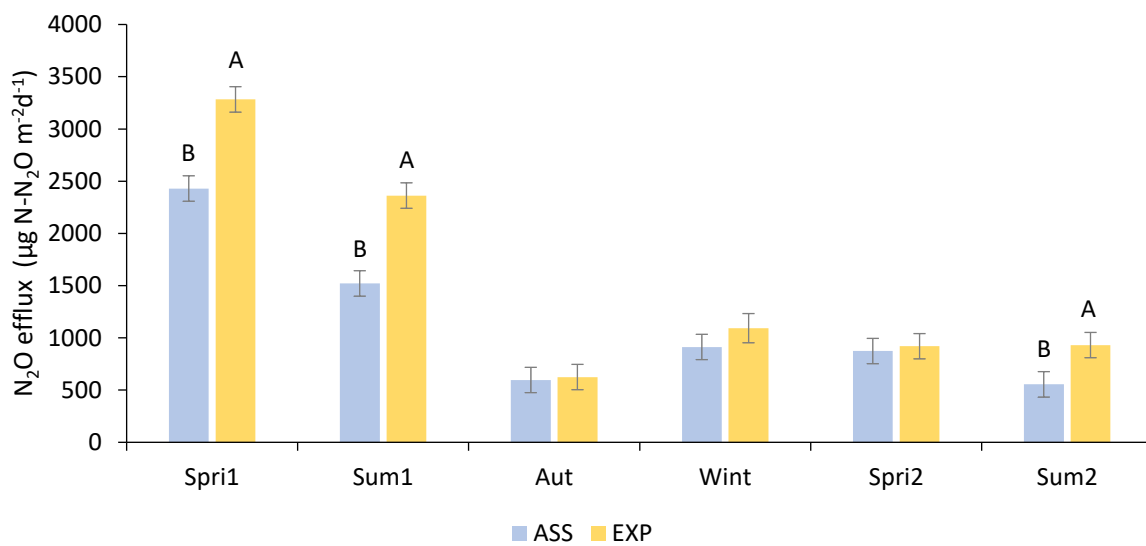### 3.3.5. Multivariate analysis of nitrous oxide emission determinants

Principal component analyses (PCA) of plant functional traits, agronomic characteristics and N_2O emission using the monocultures data resulted in two main principal components (Figure 15A, Table S3. Supplementary material), which combined accounted for 88.4% of the total variation in the dataset. The first principal component (PC1 – explaining 61.0% of the total variation) shows that palisade grass was characterized by large aboveground and root biomass, root volume and amount of nitrogen stocked in roots. These characteristics were contrasted to leaf and herbage nitrogen contents and N_2O emissions, which were features more associated with gamba grass, with guinea grass positioning as intermediate between them. The second principal component (PC2 - explaining 27.4% of the total variation) contrasted root nitrogen content, associated with gamba grass and at a lesser extent to palisade grass, to root specific length and length density, characteristics of guinea grass.

The second PCA performed on dataset regarding actual and expected values for the association reveals differences regarding functional traits, soil parameters and N_2O emission (Figure 15B, Table S4. Supplementary material). PC1 accounted for 73.9% of the total variation in the dataset and related nitrous oxide emissions to greater specific leaf area, leaf

nitrogen content, specific root length and to a lesser extent AOA gene abundance. These parameters were contrasted to the ammonium:nitrate ratio and nitrogen stocked in root biomass, characteristics enhanced by the association relative to the expected values based on the monocultures. PC2 accounted for 15.2% of the total variation and showed negative correlation between AOA gene abundance and nitrogen in root system.
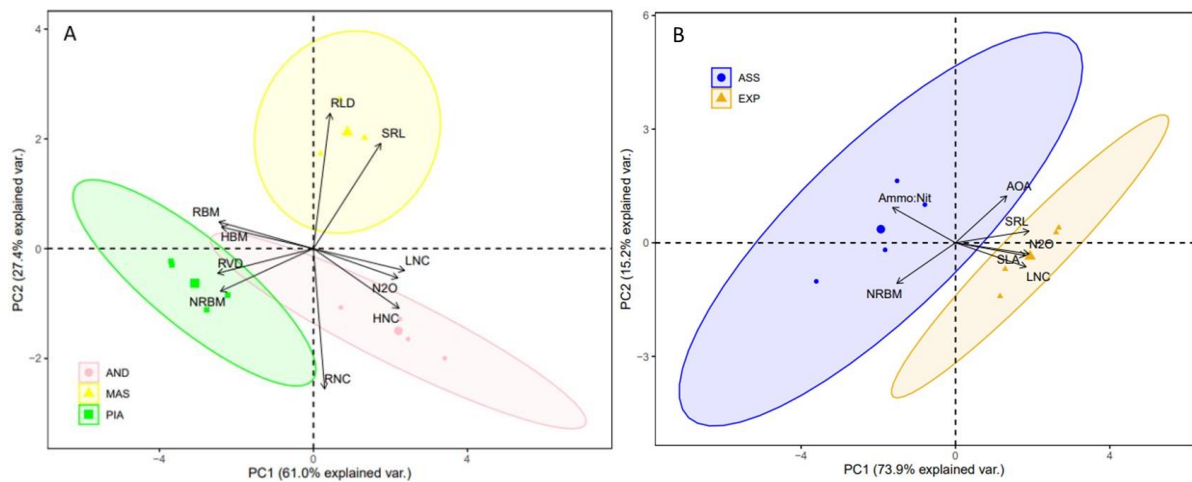
Figure 15. Principal component analysis (PCA), biplot PC1 x PC2 for monocultures (A) and association vs expected (B). RBM, root biomass; RLD, root length density; SRL, specific root length; RVD, root volume density; NRBM, nitrogen in root biomass; RNC, root nitrogen content; HBM, herbage biomass; LNC, leaf nitrogen content; HNC, herbage nitrogen content; N2O, nitrous oxide efflux; Ammo:nit, soil ammonium:nitrate content; AOA, soil ammonia-oxidizing archaea gene abundance; SLA, specific leaf area.

3.4. Discussion

This study showed that multispecific pastures of perennial tropical C_4 grasses under intensive management kept N_2O emissions at smaller values throughout the experiment, while monocultures alternated among seasons of the year with similar or greater emissions (Figure 13), depending on their functional traits and prevailing environmental conditions. Further, the association reduced N_2O efflux compared to the expected values from the monocultures during the "hot moments", characterized by high values of precipitation, temperature, soil water content and N fertilization (Figure 14). The method of comparison between actual and expected emissions from the monocultures (Loreau & Hector, 2001) implies that this reduction was an effect of mixing *per se* rather than caused by the presence of one specific dominating species (Selection effect - Aarsen 1997; Huston 1997). Complementarity in root traits, intraspecific traits variation caused by interspecific

interaction in the association and consequent changes in N-cycling seems to have contributed to the reduction of N_2O emissions.

3.4.1. Complementarity effects on N_2O emissions

Complementarity is the result of the coexistence of plants with contrasting traits, which allows for niche partitioning and better resource exploration (Hooper et al. 2005; Tilman et al. 2014). The PCA of monocultures characteristics revealed great dissimilarity among the grass species used (Figure 15A), with palisade grass being more related to plant greater size (herbage and root biomass), but smaller nitrogen content; guinea grass to rooting precision strategy (i.e. length development efficiency – Maire et al. 2009); and gamba grass to leaf, herbage and root nitrogen content, but smaller root biomass and length.

The most common studied outcome of complementarity among temperate species is aboveground biomass overyielding (Hector et al. 1999; Tilman et al. 1996; Cardinale et al. 2007; Tilman et al 2014), which could result in greater N uptake and smaller N_2O emissions (Abalos et al. 2017), but the association did not increase herbage accumulation, biomass or N stocked in herbage biomass (Table 3, Figures 3 and 4). As light interception by the canopy leaf area is a key feature controlling plant development (Da Silva et al. 2015), aboveground overyielding implies that light would be captured more efficiently in diverse treatments (Naem et al. 1994; Tilman et al. 1996; Sphen et al. 2000), which was not the case in this study (Silva 2023). The unidirectional non-accumulating nature of light as a resource may offer limited opportunity for spatial niche differentiation (Hiiesalu et al., 2012; Pärtel et al., 2012), especially among highly efficient C_4 grasses, which were genetically improved with the objective of rapid leaf area development (Euclides et al. 2010). Despite being related to nitrogen uptake and N_2O emissions in monocultures (Abalos et al. 2017), herbage biomass accumulation is often decoupled from these parameters on mixed swards, which reveals that other forms of complementarity are also important (Abalos et al. 2014; Piñeiro-Guerra et al. 2019).

Indeed, root system was more robust and diversified in the association, which had marginally greater root biomass (p = 0.0606), more nitrogen stocked in belowground tissues and at the same time was able to maintain its length development compared to the

expected values from the monocultures. Root overyielding can be attributed to spatial complementarity generated by contrasting root architectures or facilitation by increased mineralization and nutrient availability (Loreau 1998; Li et al. 2006; Schenk 2006; Mommer et al. 2010; Abalos et al. 2014). Other mechanisms involve reduced species self-inhibition by lower densities on multispecific swards, which occurs with non-dominating species (Mommer et al. 2010) and also root proliferation into soil organic patches, which is stimulated by the presence of other species as a manner of assessing and competing for pools of slowly mineralized nitrogen (Hodge et al. 1999). Independently of the specific mechanism, this enhanced root development presumably influenced the reduction in N_2O emissions for the association as it stocked more N in root biomass and also because its greater root system may have increased N uptake capacity (Aerts & Chapin 2000; Abalos et al. 2014). Though, root traits affected this process differently, as observed on the monoculture treatments.

Among the monocultures, palisade grass (PIA) presented the greatest RBM and guinea grass (MAS) the greatest RLD, while gamba grass (AND) had the smallest RBM and RLD. This poorer root development may have contributed for AND being the grass species with greatest values of N_2O efflux throughout the experiment, while other grass species contrasting strategies reduced emissions to values similar to those of the ASS at varying seasons of the year. In the first Summer, soil mineral nitrogen content (NH_4^+ and NO_3^-) was greater and, in this situation, PIA greater root biomass (scale strategy – Marie et al. 2009) seems to have allowed for greater N uptake and storage capacity (Aerts & Chapin 2000) and thus smaller N_2O emission (Figure 13). At the end of the experiment, PIA and MAS increased root biomass compared to the first seasons of the year (Figure 6) and soil ammonium and nitrate content were smaller (Figure 9); at this condition the precision strategy (Marie et al. 2009) of MAS may have had greater impact providing access to the N hotspots which were not emptied by other grass species (Abalos et al. 2014).

Concerning the association, it matched RBM of palisade grass (greatest values) during all seasons of the year, except when PIA peaked during Summers, and also matched RLD of guinea grass (greatest values) with similar temporal pattern, i.e. except when MAS peaked during Springs (Figures 5, 6 and 7). Mixing plants with contrasting rooting strategies (PIA – scale and MAS – precision) resulted in high complementarity in spatial niche exploration, condition that may have influenced the reduction of nitrous oxide emissions

independently of root overyielding, as reported also for temperate grasses with highly dissimilar root traits (Abalos et al. 2014).

In addition to spatial complementarity, in this study PIA presented smaller soil ammonium content (Figure 9), despite having smaller ammonia-oxidizing bacteria (AOB) gene abundance (Figure 11), which may be due to greater affinity for this N form over NO_3^- compared to the other grass species. Differences in terms of NO_3^- and NH_4^+ absorption by individual plants should also cause chemical complementarity and increase N uptake capacity of multispecies swards (Maire et al. 2009).

However, these complementarity effects that reduce N_2O emissions are usually coupled with soil mineral N depletion (Abalos et al. 2014; Niklaus et al. 2016; Abalos et al. 2017; Piñeiro-Guerra et al 2019), condition that was marginally observed for NO_3^- in this experiment (p = 0.0976), while NH_4^+ increased as effect of plant association (Table 4, Figure 9). The fast cycling of nitrogen in the soil may require greater sampling effort for the accurate detection of treatment differences in N mineral pools (Niklaus et al. 2016), specially during periods of nitrogen fertilizer use. However, the results of this study bring evidences that the outcome in terms of N_2O emissions may have been influenced by other effects of biodiversity on nitrogen cycling, independent of the size of N-pools, which are further discussed.

3.4.2. Association effects on species traits and N-cycling

The effects of species traits and diversity on ecosystem functioning are well documented in the literature and complementarity of niche exploration and facilitation are reported to be important mechanisms generating greater resource exploitation and biomass overyielding (Hooper et al. 2005; Cardinale et al. 2007; Tilman et al. 2014; Gross et al. 2017; Buzhdygan et al. 2020). However, intraspecific changes in functional traits caused by interspecific interactions, as observed in this study, were as important as complementarity regarding N-cycling and are less studied in biodiversity experiments.

In our experiment, gamba grass and guinea grass had SLA and LNC reduced when cultivated in association compared to monoculture (Table 1), indicating a shift to a more conservative mode of the leaf-economics spectrum (Wright et al. 2004). On the other hand, palisade grass was relatively stable regarding these same responses (Table 1), indicating that

intraspecific traits shifting in mixtures is not unidirectional (Liu et al. 2017). Considering that guinea grass + gamba grass represented large percentage of the association botanical composition (Figure S3. Supplementary material), the association actual values for SLA and LNC followed the same trend and shifted to a more conservative mode relative to the expected values from monocultures (Table 2 and Figure 15B). Competition for light and soil nitrogen may have contributed to these effects (Lemaire et al. 1991; Américo et al. 2021; Liu et al. 2017), although the mechanisms involved are not well understood.

In fertilized conditions, conservative species in monocultures have been reported to have smaller N uptake capacity compared to exploitative species (Grassein et al. 2015), smaller biomass production, increased soil mineral N content and greater N_2O emission (Abalos et al. 2017). This was not the case for the more conservative community of plants in the association in this study, presumably because this effect was counterbalanced by complementarity among species, maintaining N stocked in herbage biomass and increasing in the roots (Table 3).

On the other hand, exploitative species with high N content and litter quality (Wright et al. 2004) may stimulate faster N-cycling (Van der Kkrift & Berendsen 2001; Orwin et al. 2010; Mueller et al. 2013), which may explain the greater ammonia-oxidizing (AOA) gene abundance expected from monocultures compared to the actual abundance measured for the association. Among all treatments, this is shown by the smaller AOB gene abundance of ASS and PIA, which presented smaller aboveground N content (Figures 2 and 11).

The above-mentioned differences in N-cycling microbial community influenced the NH_4^+/NO_3^- ratio, which was greater for the association (Figure 9E and Table 4) and is related to a slowed nitrification process ($NH_4^+ \rightarrow NO_3^-$), previously reported as an effect of biodiversity in other mixed grasslands experiments (Niklaus et al. 2001; Scherer-Lorenzen et al. 2003; Mueller et al. 2013). On the contrary, gamba grass, which was associated with large N content on the roots, herbage biomass and leaves, seems to have stimulated N-cycling throughout the seasons of the year (Scherer-Lorenzen et al. 2003; Orwin et al. 2010; Mueller et al. 2013). Along with poor root development, these traits presumably contributed to increase N_2O emissions by AND (Figure 15A), similarly to the effect of the presence of legumes in the mixtures (Niklaus et al. 2006; Niklaus et al. 2016).

Besides shifting to a more conservative mode, slowed nitrification may have been caused by other effects related to grass species association. It has been reported that

Urochloa grasses produces biological nitrification inhibitors when their roots are in contact with patches of high NH_4^+ concentration, as an evolutive adaptation to low N content soils, reducing losses associated with NO_3^- (Subbarao et al. 2013). Considering the greater NH_4^+ content of the ASS compared to PIA and the expected values from the monocultures, this could have had more than an additive effect of palisade grass presence in the mix, slowing down localized nitrification process in the association. Also, as the oxidation of NH_4^+ (nitrification) is an aerobic process, dependent of O_2, greater WFPS in the association could have also contributed to reduce the process, although it did not reach values that would stimulate denitrification (> 60% - Saggar et al. 2013).

As only marginal difference in soil NO_3^- content was observed between the association and the expected values from the monocultures and values were relatively stable among monocultures, the flow between nitrogen pools seems to have had greater effect on N_2O emissions than the actual size of the pools (Jones et al. 2005; Abalos et al. 2014). Moreover, ammonia-oxidizing gene has been shown to be the main gene controlling N_2O emissions in well-drained tropical soils, where nitrification is the main source of this trace gas (Soares et al. 2016). The stability in denitrification gene abundances (nirK, nosZ) among treatments and in the association *vs* expected values from the monocultures also shows that the difference in terms of N_2O efflux is most likely consequence of the nitrification process.

3.4.3. Temporal pattern of N_2O emissions

Nitrification seems to be the key process reducing N_2O efflux in the association relative to the monocultures. Differences were mainly observed during "hot moments", presumably due to higher temperatures, greater soil moisture, but mainly due to the application of ammonium nitrate fertilizer, which stimulates N_2O emissions by increased N-cycling in monocultures compared to the association (Cummins et al. 2021).

On the other hand, the seasonal variation of emissions, soil nitrogen, WFPS and soil microbial community implies that incomplete denitrification rather than nitrification was related to the reduction in N_2O production from the beginning until the end of the experiment. As an overall increase in root biomass was observed throughout the seasons of

the year (Figure 6), NH_4^+ and NO_3^- soil content were both reduced (Figure 9), most likely due to enhanced N uptake capacity (Aerts & Chapin 2000), an effect that was also decoupled from aboveground herbage biomass and herbage accumulation (Figures 3 and 4). The lower availability of NO_3^- during Spring 2 and Summer 2 resulted in smaller nirK gene abundance, as this form of nitrogen is the primary substrate for denitrification (Saggar et al. 2013). Conversely, nosZ abundance increased throughout the experiment, which resulted in higher N_2O potential consumption by the microorganisms (Saggar et al. 2013; Schulz et al 2017) and may also explain the decrease in emissions from the beginning to the end of the experiment. Finally, WFPS was also reduced from Spring 1 and Summer 1 to Spring 2 and Summer 2, which may have resulted in increased O_2 diffusivity in the soil, favoring nitrification and nitrifier microbial populations over denitrification processes (Saggar et al. 2013).

3.4.4. Final remarks

Multispecies swards allowed for reduction in N_2O emissions as hypothesized and reported in other biodiversity experiments (Niklaus et al. 2006; Abalos et al. 2014; Niklaus et al. 2016; Piñeiro-Guerra et al. 2019; Cummins et al. 2021). High dissimilarity in root traits generated complementarity in niche exploration and increased root biomass and N stocked in the roots for the association, which is related to the capacity of absorbing and storing nitrogen from the soil. However, the association resulted in opposite effects on soil NH_4^+ and NO_3^- and no clear soil N depletion was observed, which is not in line with the third hypothesis. Intraspecific shifts to a more conservative mode in response to cultivating plants in association affected N-cycling and reduced nitrification process, which is confirmed by smaller populations of nitrifiers and N_2O efflux for the association. Further, greater N content in gamba grass, both bellow and aboveground, as well as poorer root development resulted in greater N_2O emissions throughout the experiment, while scale and precision rooting strategies from palisade grass and guinea grass, respectively, affected differently the N_2O efflux depending on soil mineral N content.

These results strongly confirm that plant traits and their variation caused by cultivating plants in association can influence ecosystem functions related to N-cycling and N_2O emissions. As these traits and their variation appear to be species specific,

investigations on how tropical C_4 grasses and their mixtures affect ecosystem functions are a powerful tool for the development of intensive and sustainable food production systems based on grasslands. Further, temporal variation in the N_2O emission and soil N content suggest that long-term experiments are required to fully understand the effect of species, their traits and their association on N-cycling processes.

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Supplementary material
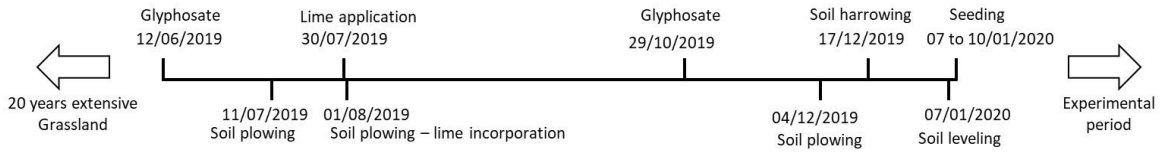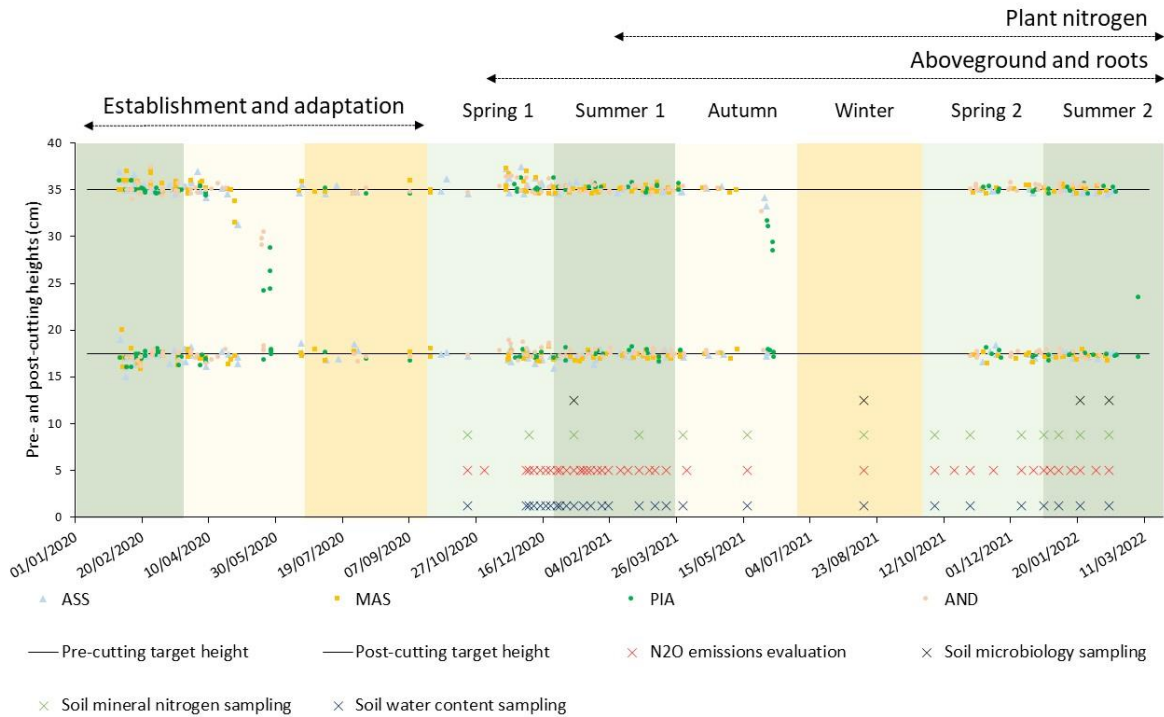
Figure S1. Workflow of pastures establishment.

Figure S2. Sampling chronology along seasons of the year. Dots (ASS, MAS, PIA and AND) in the graph show actual pre- and post-cutting heights for each treatment. ASS means Association treatment; MAS, guinea grass cv. Massai; PIA, palisade grass cv. Piatã; AND, gamba grass cv. Andropogon.
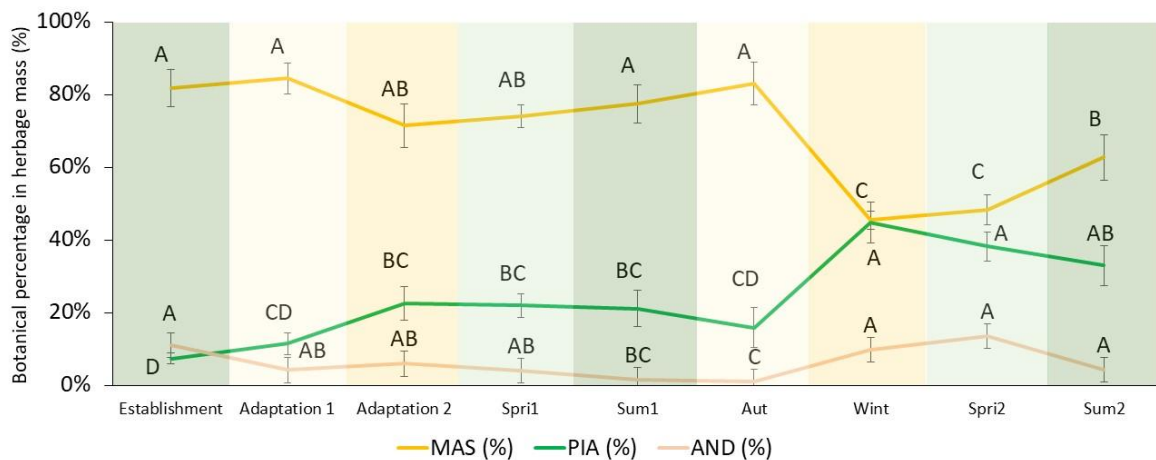
Figure S3. Botanical percentage in herbage biomass seasonal fluctuation for each species in the association (p < 0.0001). MAS means, guinea grass cv. Massai; PIA, palisade grass cv. Piatã; AND, gamba grass cv. Andropogon.

Table S1. Average rate of nitrogen application according to treatment and seasons. ASS means Association treatment; MAS, guinea grass cv. Massai; PIA, palisade grass cv. Piatã; AND, gamba grass cv. Andropogon. Values after ± are the standard error of the mean value.

| Treatment/ Season	Average N rate application^{-1}			
	ASS	MAS	PIA	AND
Establishment	20 ± 0.4	18 ± 0.4	20 ± 0.4	20 ± 0.4
Spring 1	34 ± 2.3	31 ± 2.3	40 ± 2.3	29 ± 2.3
Summer 1	27 ± 0.7	25 ± 0.7	40 ± 0.7	29 ± 0.7
Spring 2	40 ± 0.5	40 ± 0.5	40 ± 0.5	39 ± 0.5
Summer 2	31 ± 1.5	31 ± 1.5	48 ± 1.5	31 ± 1.5

Table S2. Summary of quantitative PCR analysis: Target genes, primers, sequences, amplification conditions, size of fragments (Pb, pair of bases).

Target Gene	Primers	Sequences	Conditions	Fragment size (Pb)	References
AOA	Arch-amoAF	5'-STAATGGTCTGGCTTAGACG-3'	95°C - 5 min; 40 cicles: 95°C - 40 s; 56°C - 30 s; 72°C - 1 min.*	635	Francis et al. 2005
	Arch-amoAR	5'-GCGGCCATCCATCTGTATGT-3'			
AOB	amoA1F	5'-GGGGTTTCTACTGGTGGT-3'	95°C - 10 min; 40 cicles: 95°C - 40 s; 56°C - 30 s; 72°C - 1 min.*	491	Rotthauwe et al. 1997
	amoA2R	5'-CCCCTCKGSAAAGCCTTCTTC-3'			
nirK	NirK876	5'-ATYGGCGGVAYGGCGA-3'	95°C - 10 min; 40 cicles: 95°C - 15 s; 63°C - 30 s; 72°C - 30 s.*	165	Henry et al. 2004
	NirK1040	5'-GCCTCGATCAGRTTRTGGTT-3'			
nosZ	nosZ2F	5'-CGCRACGGCAASAAGGTSMSSGT-3'	95°C - 10 min; 40 cicles: 95°C - 40 s; 63°C - 30 s; 72°C - 40 s.*	267	Henry et al. 2006
	nosZ2R	5'-CAKRTGCAKSGCRTGGCAGAA-3'			

*Fluorescence reading

Table S3. Principal Component Analysis (PCA) performed with subset of plant functional traits, agronomic characteristics and N_2O efflux data for monocultures only (Figure 15A).

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Root biomass	-0.38	0.11	0.40	-0.12	0.13	-0.12	0.10	0.17	-0.71	0.32
Root length density	0.07	0.56	0.28	-0.37	-0.24	-0.15	-0.19	-0.16	-0.07	-0.57
Specific root length	0.27	0.44	-0.08	-0.11	-0.35	0.31	-0.28	0.30	0.07	0.57
Root volume density	-0.38	-0.10	0.30	-0.24	-0.03	0.34	-0.16	-0.63	0.29	0.27
Root nitrogen content	0.04	-0.58	-0.10	-0.16	-0.50	0.14	-0.41	0.03	-0.39	-0.17
Nitrogen in root biomass	-0.37	-0.18	0.29	-0.26	0.03	0.06	-0.07	0.67	0.44	-0.16
Herbage biomass	-0.37	0.09	-0.46	-0.10	0.12	-0.58	-0.51	-0.05	0.08	0.17
Leaf nitrogen content	0.36	-0.09	-0.12	-0.55	0.66	0.22	-0.19	0.02	-0.13	-0.02
Herbage nitrogen content	0.34	-0.25	0.18	-0.44	-0.23	-0.55	0.33	-0.07	0.17	0.30
Nitrous oxide efflux	0.34	-0.12	0.57	0.42	0.20	-0.22	-0.52	-0.01	0.08	0.07
Eigen value	6.10	2.74	0.58	0.35	0.12	0.07	0.03	0.00	0.00	0.00
Proportion of variance	61.0	27.4	5.8	3.5	1.2	0.7	0.3	0.0	0.0	0.0
Cumulative proportion	61.0	88.4	94.2	97.7	98.9	99.6	99.9	100.0	100.0	100.0
Standard deviation	2.47	1.66	0.76	0.59	0.34	0.27	0.18	0.07	0.04	0.02

Table S4. Principal Component Analysis (PCA) performed with subset of plant functional traits, soil characteristics and N_2O efflux data for association and expected values (Figure 15B).

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Nitrous oxide efflux	0.42	-0.14	0.12	-0.40	0.23	-0.72	-0.25
Ammonium:nitrate ratio	-0.36	0.45	0.34	-0.60	-0.09	0.20	-0.38
AOA gene abundance	0.29	0.60	-0.66	-0.08	-0.32	-0.11	-0.02
Specific leaf area	0.40	-0.16	0.40	0.04	-0.80	0.07	-0.05
Leaf nitrogen content	0.40	-0.31	-0.27	-0.14	0.21	0.58	-0.53
Specific root length	0.42	0.15	0.18	-0.40	0.24	0.31	0.68
Nitrogen in root biomass	-0.33	-0.52	-0.41	-0.54	-0.31	-0.04	0.24
Eigen value	5.18	1.07	0.37	0.19	0.14	0.04	0.02
Proportion of variance	73.9	15.2	5.2	2.7	1.9	0.6	0.3
Cumulative proportion	73.9	89.2	94.4	97.1	99.1	99.7	100.0
Standard deviation	2.27	1.03	0.61	0.44	0.37	0.21	0.15

4. ROOT TRAITS OF C$_4$ FORAGE GRASSES AFFECT SOIL CARBON GAIN/LOSS IN SHORT-TERM EXPERIMENT: HOW MIXING SPECIES MITIGATES NEGATIVE EFFECTS?

Abstract

Anthropogenic emissions of greenhouse gases (GHG) contribute substantially to the increasing global temperature and consequent environmental disturbances generally refered to as 'climate change'. In this scenario, soil carbon cycling has become of interest within the scientific community, both by its C accumulation potential as well as for mitigating emissions from agricultural practices. Biodiverse grasslands have been shown to increase soil organic carbon (SOC) through increased plant derived C inputs, which are the result of complementarity among associated species in mixed swards. This process is mediated by the soil microbial community, which is stimulated by biodiversity directly or indirectly via increased C availability. Species traits influence both complementarity effects and microbial activity in plant mixtures. Hence, it is necessary to investigate the combined effect of species specific traits and their association in order to predict the fate of plant C inputs into the soil. Further, SOC turnover and its responses to increased plant diversity may be different in the particulated (POC) and mineral-associated organic carbon (MAOC), which are also affected by edaphoclimatic conditions. For tropical C$_4$ grasses, information on how their traits and their association in pastures may influence soil C-cycling and eventually soil C accumulation/loss in different pools are almost non-existent. Hence, the objective of this study was to evaluate and relate plant traits and soil carbon dynamics in order to investigate possible mechanisms of complementarity and increased stock of soil C. The field experiment was carried out in Piracicaba/SP, Brazil, from Jan/2020 until Mar/2022, using a randomized complete block design, with four replications. Treatments corresponded to three tropical perennial forage grass species (*Andropogon gayanus* cv. Planaltina (gamba grass), *Megathyrsus maximum* cv. Massai (guinea grass) and *Urochloa brizantha* cv. BRS Piata (palisade grass)) cultivated in monoculture and in association (the three grass species sown in equal proportions). Treatments were compared among each other and expected values for the mixture were calculated based on the monocultures' actual values and their botanical proportion in the mixture for comparison with the association treatment. Palisade grass and, to a lesser extent, the association were related with greater plant size and biomass, which resulted in greater POC accumulation. Gamba grass showed high nitrogen content both in herbage and root biomass, which presumably accelerated microbial decomposition and stabilization of by-products of this activity in the MAOC form. On the other hand, guinea grass was related to greater root length development efficiency, characteristic that seem to have contributed to MAOC and POC depletion. The association increased root biomass compared to the expected values from the monocultures, most likely due to complementarity among the species in the mixture. Greater root biomass positively influenced ΔPOC for the association, despite the great proportion of guinea grass in its botanical composition. We concluded that species specific traits may

independently influence the POC and MAOC fractions of SOC and that the association of C_4 perennial forage grasses in pastures has positive effect on the particulated carbon fraction along the 2-year period following establishment, mainly through increased plant derived C input.

4.1. Introduction

Carbon cycle has been disrupted by anthropogenic factors, mainly since the Industrial Revolution (mid-18th century), causing global surface temperature to increase, which is linked to a series of environmental disturbances generally termed as 'Climate Change' (Mitchell et al. 1995; IPCC 2021). The impacts may vary throughout the globe, but severe drought and other extreme events frequency is very likely to increase, demanding costly adaptation strategies and other harms for human society (IPCC 2022). At the current GHG atmospheric concentration, mitigation of emissions has limited capacity of offsetting the consequences of climate change, hence negative emissions technologies (i.e. carbon sinking) are necessary to maintain global warming below 2°C as set by the Paris Agreement (Fuss et al. 2018; Anderson et al. 2019).

Agriculture and land use changes (LUC) play a key role on the GHG emissions/sink budget (Anderson et al. 2019; Tian et al. 2020; IPCC 2021; Friedlingstein et al. 2022). At the same time, these activities provide food and other primary products that are subjected to an increasing demand because of the growing and more affluent population expected by mid-21th century (Royal Society 2009; Godfray et al. 2010; Foley et al. 2011). Thus, climate-oriented land management should conciliate the goals of increasing primary productivity, sparing natural ecosystems from conversion, and reducing environmental impacts, which has been worked out under the framework of sustainable intensification (Tilman et al. 2002; Royal Society 2009; Foley et al. 2011).

Soils are of major interest in this scenario once they store the greatest amount of terrestrial carbon, with larger C stocks than that in the living biomass and atmosphere together (Jobbágy & Jackson 2000). Further, it is estimated that agricultural soils have released approximately 80 Gt of carbon only in the 20th century (Lal 2001), leaving an opportunity for part of this carbon to be restored, offsetting emissions from agriculture as well as from other sectors (Lal 2004; Chabbi et al. 2017; Amelung et al. 2020).

Grasslands are an important ecosystem to global C cycle as they correspond to the largest extension of land in the world (Ramankutty et al. 2008; FAOSTAT 2022) and management options allows for either positive or negative net carbon balance (Conant et al. 2001; Lal 2004; Chang et al. 2021). Intrinsic characteristics of perennial grasslands such as non-tillage, permanent soil coverage and rhizodeposition, plant turnover and robust root systems result in increased capacity of soil C input, stabilization and storage (Conant et al. 2001; Lemaire et al. 2011; Viglizzo et al. 2019).

Increasing biodiversity in grasslands has been reported to influence biogeochemical cycles and ecosystem functioning (Naeem et al. 1994; Loreau et al. 2001; Hooper et al. 2005; Tilman et al. 2014), with potential for improving soil organic carbon (SOC) storage (Yang et al. 2019). Positive effects of biodiversity are attributed to complementarity, i.e. niche partitioning and facilitation among different species, through which their morphophysiological dissimilarities allow greater overall exploitation of light, water and nutrients across space, time and soil chemical gradients (Loreau et al. 2001; Hooper et al. 2005; Tilman et al 2014). Thus, positive correlation between the level of biodiversity and SOC storage or C sequestering rate are reported as the result of above and belowground overyielding, i.e. plant C input to the soil (Cardinale et al. 2007; Fornara & Tilman 2008; Steinbeiss et al. 2008; Mommer et al. 2010; Lange et al. 2015; Yang et al. 2019).

Although the carbon fixed through plant photosynthesis represents potential source of soil organic C, its turnover and stabilization mechanisms should be considered to predict its eventual fate in the ecosystem (Janzen 2006; Cotrufo et al. 2015; Lavallee et al. 2019). The soil microbial community plays an essential role to plant material decomposition, carbon losses (heterotrophic and autotrophic respiration) and stabilization of organic matter (Chapin et al. 2006; Cotrufo et al. 2015). Generally, biodiverse grasslands are reported to increase microbial activity indirectly through greater plant derived C input and also directly, through mechanisms related to species identity (Kemmitt et al. 2006; Dias et al. 2010; Eisenhauer et al. 2010; Lange et al. 2015). The microbial activity stimulated by biodiversity in grasslands is shown to increase labile organic matter decomposition at the same extent that it increases its input, resulting in similar balance between gain and losses on the fast-cycling C pool within levels of biodiversity (Lange et al. 2015). On the other hand, by-products of increased microbial activity (microbial necromass) in more diverse grasslands positively affect the pool of stabilized soil organic carbon in the long-term, both through increased

input and reduced decomposition (Steinbeiss et al. 2008; Cotrufo et al. 2015; Lange et al. 2015). In fact, it is shown that stabilization of organic matter into the soil mineral matrix (mineral-associated organic carbon – MAOC) is tightly related to microbial activity, while particulated forms of organic carbon (POC) are derived from plant recalcitrant compounds, which have faster soil turnover (shorter-lived) in relation to MAOC (Cotrufo et al. 2015; Lavallee et al. 2019).

Further, plant traits and functional group are reported to affect soil C input into these different pools in monocultures and in associations (Lange et al. 2015; Yang et al. 2019; Rossi et al. 2020; Xu et al. 2021; Happonen et al. 2022). For example, grasses characteristics such as greater C/N ratio, lignin and cellulose content, are associated with higher accumulation rate of POC, while legumes greater N and soluble compounds content are related to greater soil respiration and stabilization of SOC in the soil mineral matrix (Rossi et al. 2020). The combination of C_4 grasses and legumes in pastures results in facilitation interactions of N-fixation and transfer among plants, which enables greater SOC input and reduced decomposition (Yang et al. 2019).

Functional trait diversity *per se*, measured by within-community variability of specific leaf area (SLA) and dry matter content (DMC), is shown to increase carbon stocks by the mechanisms of complementarity (Happonen et al. 2022). Also, species specific characteristics such as root biomass, exudates, overall structure (length, diameter, depth, distribution), aboveground biomass, SLA, plant height and chemical composition affect C cycling through changes in plant C input and microbial activity (Lange et al. 2015; Yang et al. 2019; Rossi et al. 2020; Xu et al. 2021; Happonen et al. 2022).

Although general mechanisms on how plant diversity and functional groups affects soil C dynamics are demonstrated in the literature, the integrated effect of multispecies swards and species specific traits are less studied. Further, the tropical environment and C_4 perennial grasses are rarely subjected to investigations on the matter, despite their importance in terms of forage and animal production in grasslands and SOC storage. Hence, the objective of this study was to relate species traits of three C_4 perennial grasses grown as monocultures, the effect of their association on SOC accumulation/loss in the POC and MAOC pools during a two-year experiment following pasture establishment. It was hypothesized that the species used have traits dissimilarities that would allow for niche partitioning and increased biomass production through complementarity effects which

would stimulates POC and MAOC storage in the association compared to the monocultures. Also, that species diverging traits would have different effects on SOC and its pools dynamics.

4.2. Materials and Methods

4.2.1. Study site and experimental conditions

The study was carried out in an experimental area of the "Luiz de Queiroz" College of Agriculture, University of São Paulo (ESALQ/USP), located in Piracicaba, São Paulo, Brazil (22°42'35" South Latitude, 47°38'24" West Longitude and 546 m altitude). According to Köppen-Geiger's classification, the climate of the region is Cwa (Alvares et al. 2013), characterized by well-defined dry winter and warm summer (Köppen 1936 – Figure 1). The mean air temperature and annual precipitation (1990-2022) are 22.4°C and 1310 mm, respectively (data from the meteorological station located two kilometers from the experimental site – Figure 1).
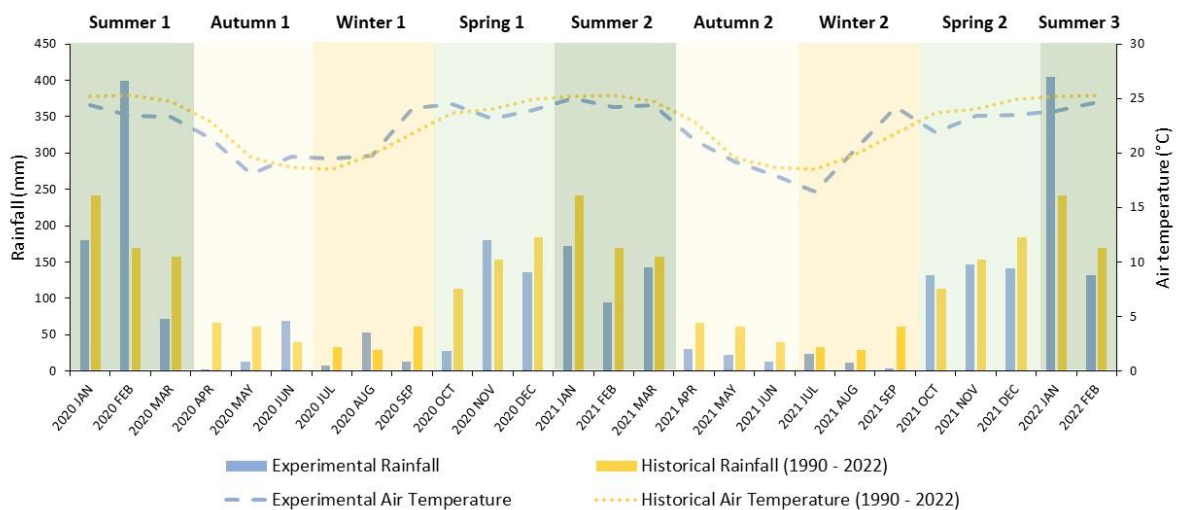

Figure 1. Monthly actual and historical rainfall and air temperature of the experimental site located in Piracicaba/SP, Brazil.

The soil of the experimental site is a Distroferric Red Nitisol, which are typically deep, well-drained soils and have moderate to strong angular blocky structure (FAO 2015). Soil texture is composed by 46% clay, 18% silt and 36% sand, within the 0-20 cm horizon. Mean soil bulk density and porosity (0 - 20 cm) were 1.43 g cm^{-3} and 0.50 cm^3 cm^{-3},

respectively. Soil sampling was performed on February/2019, one year before the establishment of the experiment, and chemical results were: pH $CaCl_2$ = 4.50; organic matter = 33.8 g dm^{-3} ; P = 49.5 mg dm^{-3} ; K = 3.45 $mmol_c$ dm^{-3} ; Ca = 30.3 $mmol_c$ dm^{-3} ; Mg = 12.5 $mmol_c$ dm^{-3} ; Al = 1.50 $mmol_c$ dm^{-3} ; H + Al = 72.5 $mmol_c$ dm^{-3} ; S = 25.8 mg dm^{-3} ; Cu = 4.51 mg dm^{-3} ; Fe = 176 mg dm^{-3} ; Zn = 5.54 mg dm^{-3} ; Mn = 35.2 mg dm^{-3} ; B = 0.26 mg dm^{-3} ; sum of bases = 46.0 $mmol_c$ dm^{-3} ; cation exchange capacity = 119 $mmol_c$ dm^{-3} ; base saturation = 39.0%; aluminum saturation = 3.50%.

4.2.2. Experimental design, establishment and management

In order to investigate the effects of mixed swards on soil carbon dynamics three species of C_4 perennial tropical forage grasses were cultivated as monocultures: i) Guinea grass - Megathyrsus maximum cv. Massai (MAS); ii) Palisade grass – Urochloa brizantha cv. BRS Piatã (PIA); iii) Gamba grass – Andropogon gayanus cv. Planaltina (AND); and as multispecific pasture: iv) the association of the three species (seeded in equal proportions based on the number of viable seeds) (ASS). These were allocated to 12 x 15 m (180 m2) paddocks according to a randomized complete block design, with four replications. Paddocks were separated by 3 m-wide raceways to minimize cross influence among treatments.

Species choice considered dissimilarities in plant traits in order to achieve complementarity and maximize niche exploration (Loreau et al. 2001; Hooper et al. 2005; Gross et al. 2017), as well as to seek coexistence stability (Chesson 2000). Further, the species had to be compatible regarding defoliation management target heights according to the 95% canopy light interception criterion and its flexibility range (Sbrissia et al. 2018), which is important to avoid cutting regime to be a confounding effect on plant performance (Da Silva et al. 2015). All treatments were subjected to a common intermittent defoliation management characterized by a pre-cutting height of 35 cm, which encompass the flexibility range for the three grass species used in the experiment, and a post-cutting height of 17.5 cm (i.e. 50% defoliation severity). This regime allows for frequent and non-severe defoliation, which may reduce light competition among mixed species, favoring their coexistence (Eskelinen et al. 2022). For maintenance of the experimental control, swards were monitored during every regrowth cycle using a sward stick on 40 points along four transect lines. When the target pre-cutting height of 35 cm was reached, pre-cutting

sampling procedures were performed and subsequently herbage were cut using a motorized brush cutter at the post-cutting target of 17.5 cm, and the material removed by raking.

Previously to this study, the experimental area was used as extensive managed grassland of *Cynodon* spp. cv. Tifton 85 with scarce nutrient restitution for over 20 years, which caused severe degradation and weed presence. For the establishment of the experiment (Figure S1. Supplementary material), lime was applied (July/2019) at a rate of 4.5 Mg ha^{-1} with the objective of reaching 70% of base saturation, considered adequate for high levels of herbage production as expected from intensively managed tropical grasslands (Raij et al. 1996). The lime was applied superficially on the soil and incorporated to 20 cm depth by harrowing. In January/2020, the soil was leveled with a rotary hoe and seeding of the paddocks carried out following treatments specifications. Seeding occurred by broadcast at the of rate of 300 pure-viable seeds m^{-2}, with 100 viable seeds m^{-2} of each species in the association treatment, followed by compaction with a roller compactor weighing approximately 100 kg.

Nitrogen fertilization was applied only during the warm rainy periods of the year (late Spring and Summer). Total amount of N-fertilizer was 200 kg N ha^{-1} for the 2020/21 and 2021/22 growing seasons, while for the establishment/adaptation period (February and March/2020) the rate was 100 kg N ha^{-1}, since sowing was performed at the middle of the warm rainy period. The total amount of N-fertilizer was divided in instalments, with fertilization applied after every cut proportionally to the regrowth interval using a daily rate of 1.7 kg N ha^{-1} day^{-1}.

4.2.3. Shoot and root analysis

Herbage accumulation was measured continuously throughout the experiment. At pre-cutting, two metal frames (0.25 x 1.0 m) were placed on representative areas of the paddocks at the time of sampling (visual assessment of herbage biomass and canopy height) and the herbage within the frames was cut at the targeted post-cutting height of 17.5 cm. Herbage samples were dried in a forced draft oven at 60 °C until constant weight. Total herbage accumulation (HA – kg DM ha^{-1}) was calculated for the whole experimental period by the sum of the herbage accumulated during every regrowth cycle, for each paddock.

Herbage biomass samples were taken once every season of the year (Figure S2. Supplementary material). At pre- and post-cutting conditions, two frames (0.25 x 1.0 m) were allocated on representative areas of the paddocks at the time of sampling (visual assessment of herbage mass and canopy height) and all herbage within the frames were cut at soil level. The material was dried in a forced draft oven at 60 °C until constant weight. Herbage biomass (HBM – kg DM ha^{-1}) was calculated as the average between pre- and post-cutting HBM. For the association treatment, samples were further dissected into their botanical components before drying and HBM was calculated for each species in order to determine the botanical composition of the mixture.

Samples from the pre-cutting condition were subsampled, dried and ground in a "Wiley" type mill with a 1 mm sieve for total nitrogen content analyses (HNC – %) through dry combustion at 1400 °C using the Leco FP 528 system (Leco Corporation, St. Joseph, MI, USA). Species leaf nitrogen content (LNC – %) was evaluated once every season of the year from Summer 2 to Summer 3 (Figure S2. Supplementary material). Also at pre-cutting, approximately 20 g of sun-exposed leaves were sampled (top 10 cm of leaves blades – Farruggia et al. 2004; Louarn et al. 2020) for determining nitrogen concentration following the same procedure described for HBM samples.

Roots were sampled once every season of the year from Autumn 1 to Summer 3 (Figure S2. Supplementary material), always at post-cutting. On each paddock, eight samples were taken between tussocks using a steel probe (6.3 cm diameter and 20 cm depth). The eight samples were combined into a composite sample which was washed on 0.5 mm sieves for removing soil. Roots were isolated and taken to the laboratory, where they were digitalized and analyzed by WinRHIZO™ (Regent instrument Inc.) coupled in a scanner (Epson™ Expression 11000XL) (Bouma et al., 2000). The software resolved root diameter (RD – mm), length and volume. Root length density (RLD – m m^{-3}) was calculated by relating length per sample and volume of soil sampled. Root volume density (RVD – cm^3 m^{-3}) was calculated as the quotient between sampled root volume and volume of soil sampled.

Subsequently, samples were dried in a forced draft oven at 60 °C until constant weight and data used to calculate root biomass (RBM – kg DM ha^{-1}). Specific root length (SRL – cm g^{-1}) was calculated as the quotient between root length and root biomass. Root tissue

density (RTD g cm^{-3}) was calculated as the ratio between sample weight and volume. After weighting, samples were ground in a "Wiley" type mill with a 1 mm sieve and root total nitrogen content (RNC - %) determined by dry combustion at 1400°C using the Leco FP 528 system (Leco Corporation, St. Joseph, MI, USA). The RNC was analyzed from Summer 2 to Summer 3, same period as LNC (Figure S2. Supplementary material). Nitrogen stocked in root biomass (NRBM kg N ha^{-1}) was calculated as the product between RBM and RNC.

4.2.4. Soil analysis

At the time of establishment of the experiment (March 2020) a trench was opened in an adjacent area to the experimental site, which was subjected to the same procedures of soil preparation of the experimental area, and undisturbed soil samples were collected with Kopeck rings (5 cm diameter x 5 cm height), at 0 – 5, 5 – 10 and 10 – 20 cm layers, with four replicates. At the end of the experiment (March 2022), trenches were open in all paddocks and undisturbed soil samples were taken, one from each soil depth (0 – 5; 5 – 10; 10 – 20 cm). The samples were oven-dried at 105°C for 48 hours and soil bulk density (SBD) was calculated based on their dry weight and total volume of the sampling rings (Teixeira et al. 2017).

At establishment and at the end of the experiment (two consecutive years) eight soil samples per paddock were collected at 0 – 5, 5 – 10 and 10 – 20 cm depths using a Dutch auger for determination of soil carbon. The samples were air-dried, sieved (< 2 mm) and plant debris removed. For fractioning organic matter into particulated (POM) and mineral associated (MAOM) forms, the particle-size method (Cambardella & Elliott 1992; Lavallee et al. 2019) was used. A sub-sample of 5 g of soil was mixed with 15 mL of sodium hexametaphosphate (5 g L^{-1}) and dispersed in a horizontal shaker for 16 hours (140 rpm). Afterwards, 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°C for 14 days, ground and sieved in a 0.15 mm mesh for C determination.

The percentage of carbon in the POM and MAOM fractions was determined by dry combustion method (Nelson & Sommers 1996) using an elemental analyzer (LECO CN-2000, St. Joseph, Mi, USA). Soil stocks of particulated organic carbon (POC) and mineral associated organic carbon (MAOC – Mg C cm^{-1} ha^{-1}) were calculated for each soil depth with the equivalent soil mass approach (Ellert & Bettany 1995), according to equations 1 and 2:

$$POC\ stock\ = \%POM\ \times\ \%C_{POM}\ \times SBD \tag{1}$$
$$MAOC\ stock\ = \%MAOM\ \times\ \%C_{MAOM}\ \times SBD \tag{2}$$

where *POC stock* is the stock of particulated organic carbon (Mg cm^{-1} ha^{-1}); *%POM* is the percentage of the 5 g subsample retained in the 53 μm mesh; *%CPOM* is the carbon content of the retained material; and *SBD* is the average soil bulk density of all treatments at the calculated depth; *MAOC stock* is the stock of mineral associated organic carbon (Mg cm^{-1} ha^{-1}); *%MAOM* is the percentage of the 5 g subsample which passed through the 53 μm mesh; and *%CMAOM* is the carbon content of that fraction.

To evaluate carbon stock changes for each fraction (ΔPOC and ΔMAOC – Mg cm^{-1} ha^{-1}) during the experimental period, the difference between final and initial stocks at each soil depth were calculated.

4.2.5. *Calculation and Statistical analysis*

Expected values for the association of the three grass species were calculated based on the monocultures' actual values and their botanical proportion in the association herbage biomass (Loreau & Hector 2001) according to equation 3:

$$\boldsymbol{Exp\ value = \sum_i p_i \times V_i} \tag{3}$$

where *Exp value* is the expected value for association community of plants; *p_i* is the proportion of species *i* in the mixture and *Vi* is the actual value obtained in monoculture *i*.

For comparison among treatments (ASS, MAS, PIA and AND), shoot and root characteristics were average amongst seasons of the year and Analysis of Variance (Anova) was performed using the MIX procedure of the SAS OnDemand for Academics (SAS Institute

Inc.). The model included treatment as fixed and block as random factors. Comparisons between association and the expected values from the monocultures (EXP) considered the seasonal variation in order to investigate if the effects of the association varied with time. In this case, the Anova model included treatment (ASS *vs* EXP), season of the year (repeated measure) and their interaction as fixed and block as random factors. Data from soil carbon stock changes (ΔPOC and ΔMAOC) were evaluated according to soil depth (0 – 5 cm; 5 – 10 cm; and 10 – 20 cm). The model included treatment, soil depth and their interaction as fixed and block as random factors.

Before proceeding with Anova, data were tested for normality (Shapiro-Wilk test) and all variables met this assumption. Structures of the variance-covariance matrices were tested according to the Bayesian Information Criterion for the selection of best fit matrix for each variable. Means were obtained by LSMEANS statement and compared by Least Significant Difference test (LSD) when effects were significant in the Anova ($p < 0.05$).

Using R software (version 4.2.2 – R core team, 2022), a Principal Components Analysis (PCA) was performed using a subset containing shoot (HBM, HMNC and LNC) and root (RBM, NRBM, RLD, RTD, RVD, SRL and RNC) characteristics and differences in POC and MAOC stocks for investigating the relationship among those variables and how treatments may have influenced them.

4.3. Results

4.3.1. Shoot and root characteristics

Herbage accumulation (HA) did not vary among treatments ($p > 0.05$ – Figure 2A) and average value for entire experimental period was 57300 kg DM ha^{-1} (±5790). Herbage biomass (HBM) was greater for palisade grass (PIA), which did not differ from the association (ASS), smallest values were recorded for gamba grass (AND) and intermediate for guinea grass (MAS) ($p = 0.0007$ – Figure 2B). Herbage (HNC – Figure 2C) and leaf nitrogen content (LNC – Figure 2D) were greater for AND, followed by MAS, while recorded values for ASS and PIA were smaller ($p < 0.0001$).
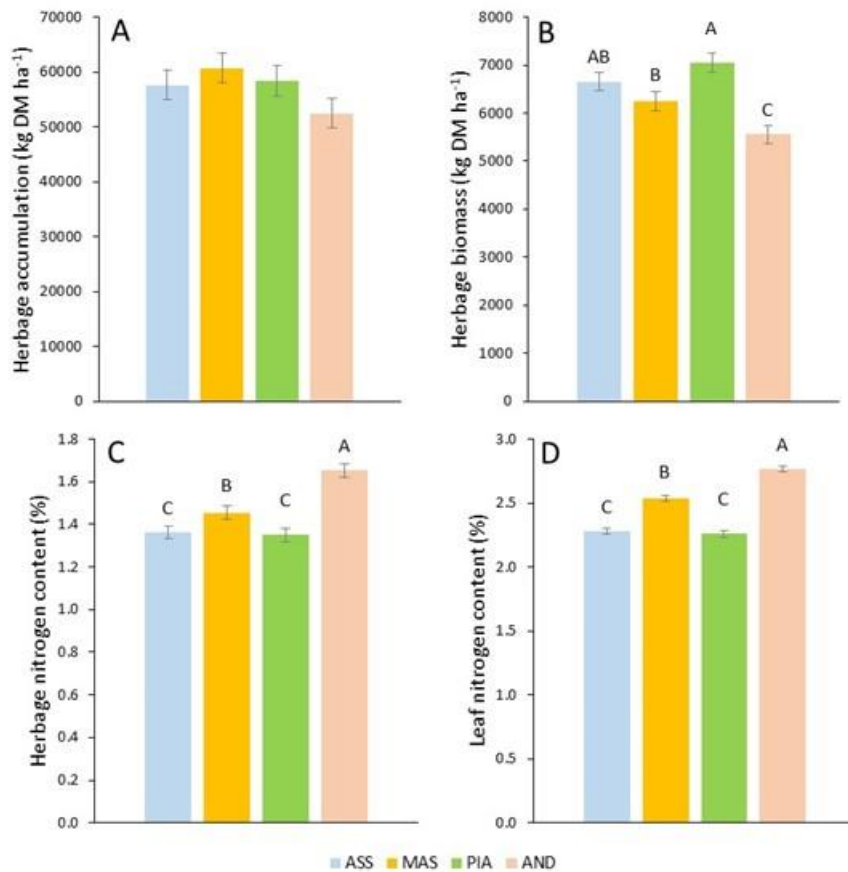
Figure 2. Herbage accumulation (A), herbage biomass (B), herbage nitrogen content (C) and leaf nitrogen content (D) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). Bars indicate standard error of the mean.

Root biomass (RBM) varied among treatments (p = 0.0013 – Figure 3A), with greatest and similar values for PIA and ASS. Gamba grass presented the smallest value and MAS intermediate value between ASS and AND. Root volume density (RVD) was greater for PIA, while other treatments had similar smaller values (p = 0.0009 – Figure 3B). All treatments differed in specific root length (SRL – p < 0.0001), with greatest value recorded for MAS, followed by ASS, AND and PIA (Figure 3C). Root length density (RLD) was greater for MAS, smaller for PIA and AND, and intermediate for ASS (p = 0.0002 – Figure 3D). As for root tissue density (RTD), both MAS and ASS had greater values than AND and PIA (p = 0.0001 – Figure 3E). Root nitrogen content (RNC) varied among treatments (p < 0.0001), with greater value recorded for AND, followed by PIA, ASS and MAS (Figure 3F). On the other hand,

nitrogen stocked in root biomass (NRBM) was greater for PIA and smaller for MAS and AND, with intermediate values recorded for ASS (p = 0.0002 – Figure 3G).
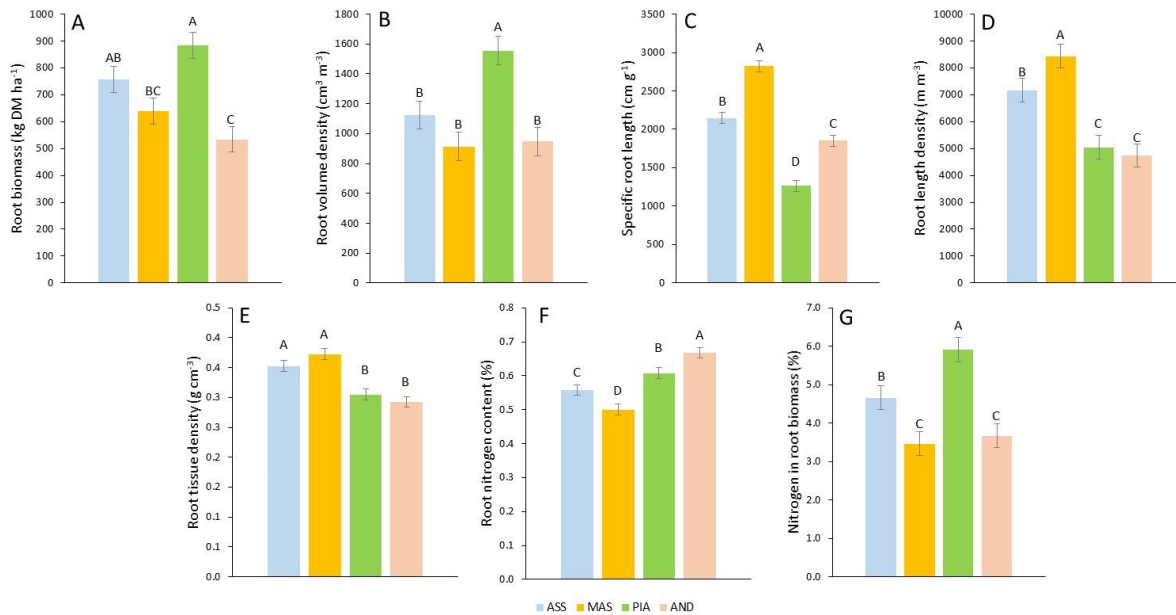

Figure 3. Root biomass (A), root volume density (B), specific root length (C), root length density (D), root tissue density (E), root nitrogen content (F) and nitrogen stocked in root biomass (G) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). Bars indicate standard error of the mean.

4.3.1.1. Shoot and root characteristics for the Association: Actual vs Expected Values

Root biomass increased 9% in the association (ASS) compared to the expected values from the monocultures (EXP) (p = 0.0463 – Figure 4A), while RLD did not differ among treatments (p > 0.05 – Figure 4B). Specific root length for the ASS was 12% smaller relative to EXP (p = 0.0011 – Figure 4C). No differences were observed in RNC (p > 0.05), but the association had 11% greater NRBM relative to EXP (p = 0.0413 – Figure 4D). Root volume density, root tissue density, herbage biomass and herbage accumulation did not vary with treatments (p > 0.05 – ASS *vs* EXP). Leaf nitrogen content varied with treatments (p = 0.0117), with smaller values recorded for ASS (2.32 ±0.035 %) relative to EXP (2.46 ±0.035 %). Herbage nitrogen content (HNC) varied with the treatment x season of the year interaction (p = 0.0133). The association had greater HNC during Summer 2, while the

expected values from the monocultures were greater during Autumn 2 and Winter 2. In Spring 2 and Summer 3, there was no difference between treatments. Actual and expected values from the monocultures for both shoot and root characteristics varied with season of the year ($p < 0.0001$ – Tables S1 and S2. Supplementary material), but no interaction was observed ($p > 0.05$).
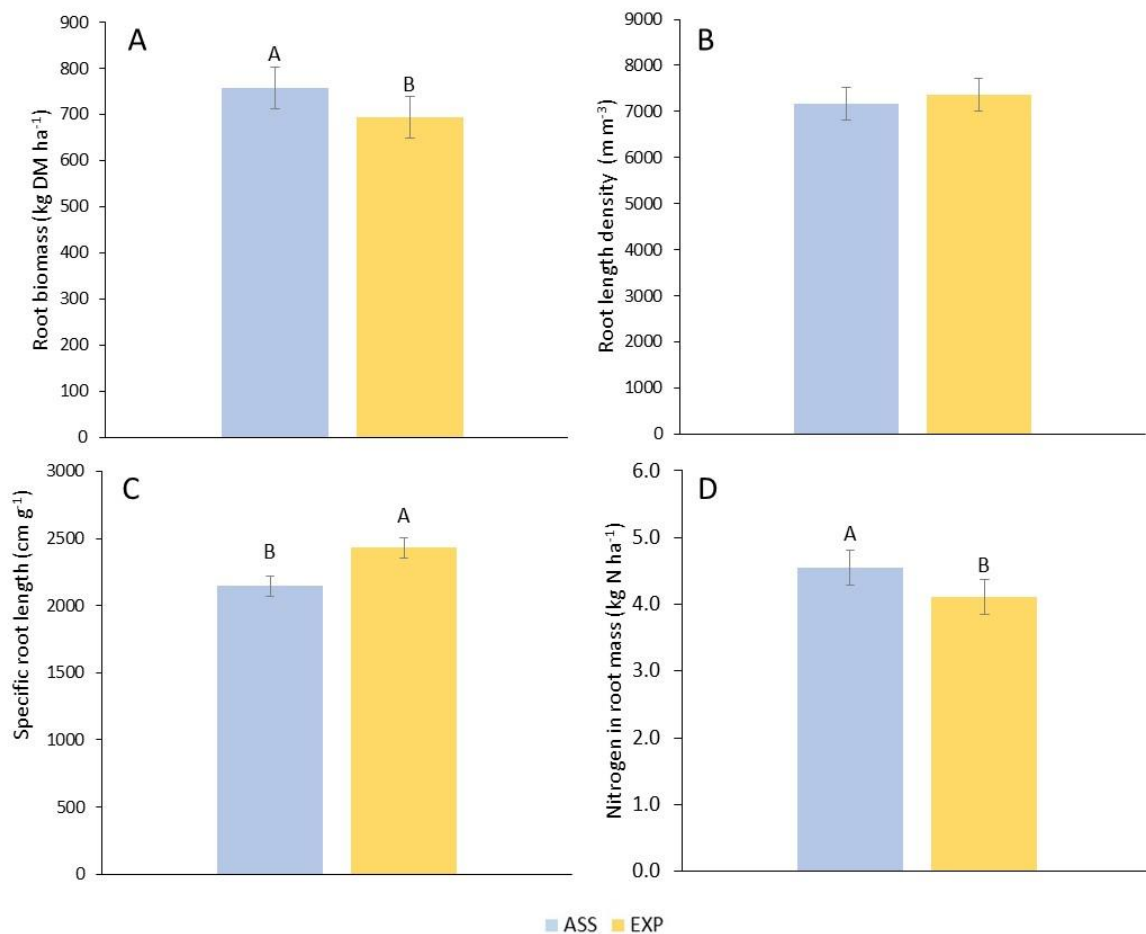

Figure 4. Root biomass (A), root length density (B), specific root length (C) and nitrogen stocked in root biomass (D) for the association (ASS) and the expected values from the monocultures (EXP). Letters over columns indicate differences (ANOVA – $p < 0.05$) of mean values by the LSD test ($p < 0.05$). Bars indicate standard error of the mean.

4.3.2. Soil carbon dynamics

The variation in mineral associated organic carbon stock (ΔMAOC) from the beginning until the end of the experiment was influenced only by treatments ($p = 0.0008$). The ΔMAOC was greater for AND, followed by PIA and then ASS, while MAS showed smallest

and negative values (Figure 5B). For particulated organic carbon stock (ΔPOC), the variation was affected by treatments (p = 0.0004), soil depth (p < 0.0001) and the treatment x soil depth interaction (0.0194). Guinea grass showed negative and smallest values for the 0 – 5 and 5 – 10 cm depths (Figure 5A). For the same soil depths, the ASS and PIA showed greatest values of ΔPOC, with AND being intermediate between MAS and ASS (Figure 5A). For the 10 – 20 cm depth, no differences were observed and the average recorded value was approximately zero (0.01 ±0.011 Mg cm^{-1} ha^{-1} – Figure 5A). The variation in total soil organic carbon (ΔSOC) was influenced by treatments (p = 0.0015) and soil depth (p = 0.0052). Recorded values for MAS were smaller and negative, relative to the remaining treatments which did not differ amongst them (Figure 5C). Considering soil depths, ΔSOC was smaller for the 5 – 10 cm layer (-0.05 ±0.05) and greater for 0 – 5 cm (0.18 ±0.05) and 10 – 20 cm (0.14 ±0.05).

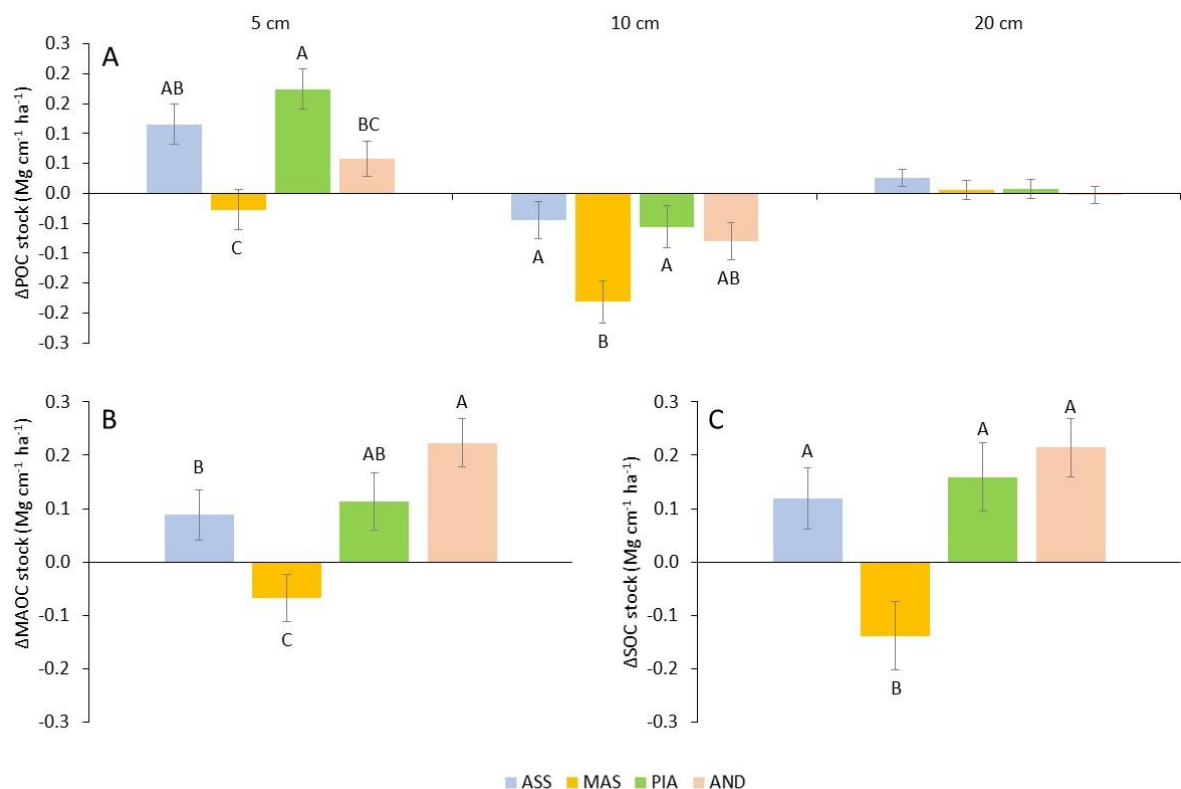**Figure 5.** Differences of soil particulated organic carbon (ΔPOC – A), mineral associated organic carbon (ΔMAOC – B) and total soil organic carbon (ΔSOC – C) of guinea grass (MAS), palisade grass (PIA), gamba grass (AND) and their association in multispecific pastures (ASS). Letters over columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). Bars indicate standard error of the mean.

4.3.2.1. Soil carbon dynamics for the Association: Actual vs Expected Values

Actual values of ΔSOC and ΔPOC were greater than the expected from the monocultures (p = 0.0484; p = 0.0464, respectively), while ΔMAOC did not vary with treatments (p > 0.05) – Figure 6). Total, mineral associated and particulated soil organic carbon did not vary with the treatment x soil depth interaction.
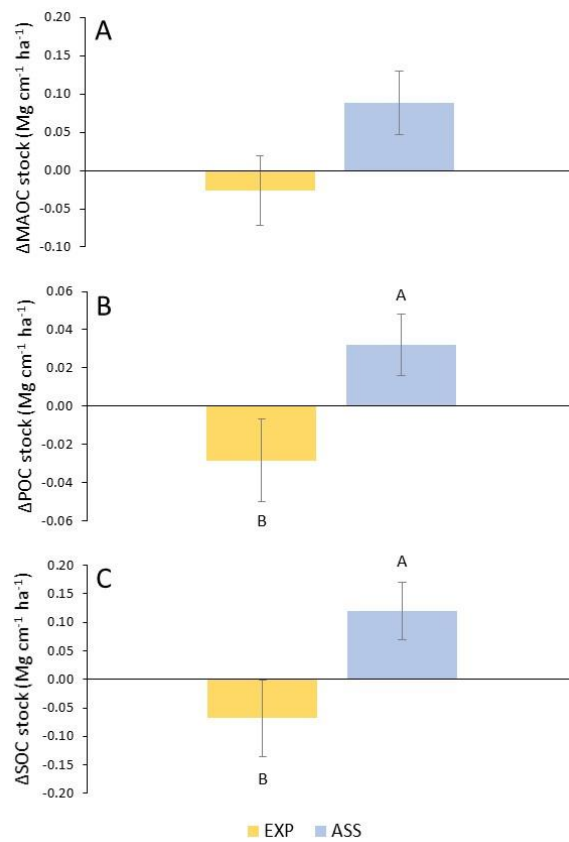

Figure 6. Differences of soil mineral associated organic carbon (ΔMAOC – A), particulated organic carbon (ΔPOC – B) and total organic carbon (ΔSOC – C) for the association (ASS) and the expected values from the respective monocultures (EXP). Letters over columns indicate differences (ANOVA – p < 0.05) of mean values by the LSD test (p < 0.05). Bars indicate standard error of the mean.

4.3.3. Multivariate analysis of soil organic carbon determinants

Principal component analyses (PCA) of shoot and root characteristics and variations in soil carbon stock (ΔPOC, ΔMAOC and ΔSOC) resulted in two main principal components (Figure 7, Table S3. Supplementary material), which combined accounted for 80.2% of the total variation in the dataset. Data of ΔPOC, ΔMAOC and ΔSOC from the 10 – 20 cm soil

depth were excluded from the PCA because previous analysis showed that they were not related to the shoot nor to the root characteristics. The first principal component (PC1 – explaining 45.5% of the total variation) shows that palisade grass was characterized by greater ΔPOC at the 0 – 5 and 5 – 10 cm depths, which were related to greater RVD, RBM and NRBM and smaller SRL (Figure 7, Table S3. Supplementary material). Guinea grass showed opposite characteristics in the first axis, while the association and gamba grass were in an intermediate position (Figure 7, Table S3. Supplementary material). The second principal component (PC2 - explaining 34.7% of the total variation) contrasted nitrogen content in plant parts (HNC, RNC and LNC) and ΔMAOC, mainly related to AND, with root length and tissue densities, characteristics mainly related to MAS (Figure 7, Table S3. Supplementary material). The ASS and PIA were positioned intermediately between AND and MAS in the second axis of the PCA (Figure 7, Table S3. Supplementary material).
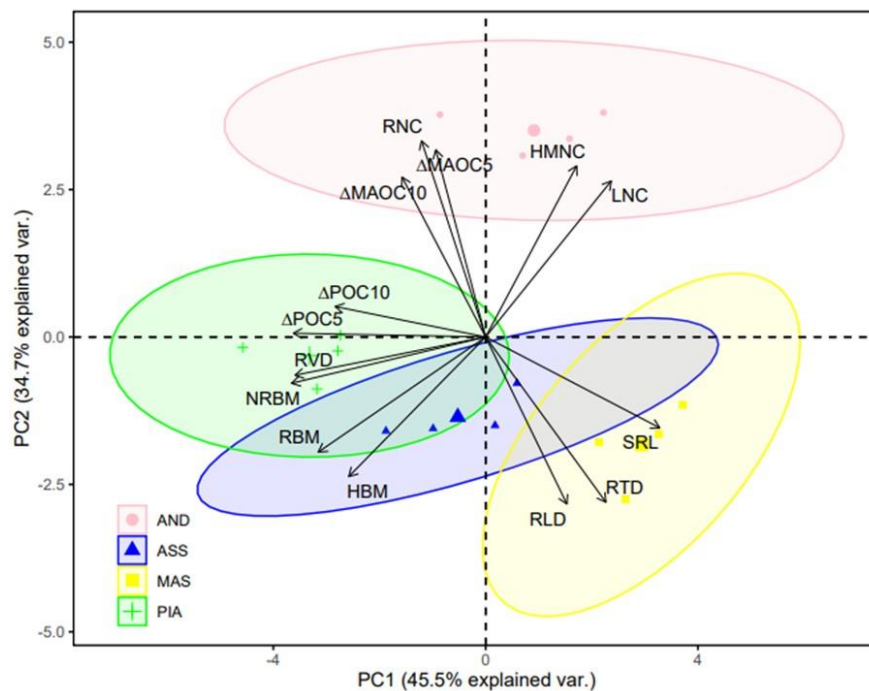
Figure 7. Principal component analysis (PCA), biplot PC1 x PC2 with subset of data from treatments: association (ASS); guinea grass (MAS); palisade grass (PIA) and gamba grass (AND). RBM, root biomass; NRBM, nitrogen stocked in root biomass; RVD, root volume density; RNC, root nitrogen content; SRL, specific root length; RTD, root tissue density; RLD, root length density; HBM, herbage biomass; LNC, leaf nitrogen content; HNC, herbage nitrogen content; ΔPOC5 and ΔPOC10, differences between final and initial soil particulated organic carbon stocks in the 0 – 5 cm and 5 – 10 cm soil depths, respectively; and ΔMAOC5 and ΔMAOC10, differences between final and initial soil mineral associated organic carbon stocks in the 0 – 5 cm and 5 – 10 cm soil depths, respectively.

4.4. Discussion

This study showed that multispecific pastures of perennial tropical C_4 grasses under controlled defoliation management positively affect ΔSOC in the 2-year period following pasture establishment (Figure 6), mainly by altering root traits in relation to the expected values from the monocultures (Figure 4). The monocultures' characteristics (Figures 3 and 7) bring evidences that indicate high belowground complementarity in spatial niches exploration among the community of plants in the association, which may have caused ASS root overyielding (Figure 4). Different set of species specific traits (biomass and nitrogen content) were associated with either ΔPOC or ΔMAOC (Figure 7), through different and independent mechanisms. On the other hand, traits related to soil length development efficiency (root length and specific length) caused losses of carbon in both SOC fractions.

4.4.1. Plant traits effects on ΔPOC, ΔMAOC and ΔSOC

In general, plant size and biomass related traits (RVD, RBM, NRBM and HBM) were associated to treatments with increased ΔPOC (Figure 7). Larger plants, such as palisade grass, have greater aboveground C stock and total CO_2 flux, but inconsistent results in terms of SOC stock and its relation to plant size have been reported (Fornara & Tilman 2008; Rossi et al. 2020; Happonen et al. 2021). That may be due to the complexity of soil carbon cycling, which depends on quantity of carbon fixed via photosynthesis, the organic matter molecular and elemental composition, soil chemical and physical characteristics and microbial community, all of which affect MAOC and POC in different forms (Kallenbach et al. 2015; Lavallee et al. 2019; Chen et al. 2020).

In this study, increased root size and biomass (RBM, RVD, NRBM) presumably represented larger amounts of plant carbon available for microbial decomposition, which may have influenced positively the rate of new POC formation and/or the conservation of the existing POC for larger plants (Lange et al. 2015; Yang et al. 2019; Happonen et al. 2021). Increased plant carbon input, mainly in the root system, has been shown to be positively correlated to net soil C accumulation (Fornara & Tilman 2008; Yang et al 2019), but quality of the organic matter determines its main stabilization process (Cotrufo et al. 2015; Lavallee et al. 2019). Lower quality (i.e. high C/N ratio and lignin content) tends to favor POC formation,

while greater N content and soluble carbohydrates stimulates microbial activity and stabilization of organic C in the form of MAOC (Cotrufo et al. 2013). Hence, the ASS and PIA greater herbage and root biomass and lower herbage nitrogen content may have influenced the larger ΔPOC in the 0 – 5 and 5 – 10 cm depths for these treatments. As for 10 – 20 cm, the ΔPOC was stable among treatments, presumably by lower root and aboveground biomass influence at this depth.

Plant nitrogen content (RNC, HNC, LNC) was tightly related to MAOC formation (Figure 7). Gamba grass' greater RNC, HNC and LNC seems to have increased the stabilization of microbial derived C into the soil mineral matrix, as lower C/N ratio usually allows faster decomposition of organic matter and microbiota turnover resulting in low molecular weight compounds, which are protected of further decomposition by soil matrix interactions (Tjoelker et al. 2005; Cotrufo et al. 2015; Lavalle et al 2019). This result strongly agrees with the theory of microbial efficiency-matrix stabilization, in which labile organic matter input is readily and efficiently used by heterotrophic microorganisms in the soil as the first step for stabilization of SOC via *in vivo* transformation (Cotrufo et al. 2013; Cotrufo et al. 2015). The smaller HBM and RBM of AND may also indicate that microbial activity was the main pathway for SOC stabilization for this treatment, as less organic matter input resulted in greater MAOC accumulation.

On the other hand, efficiency in root length development (RLD, SRL and RTD) showed by guinea grass was related with depletion of both SOC fractions (Figures 5 and 7). These plant traits presumably enable greater soil spatial exploration, giving MAS and its associated rhizosphere's soil microbial community access to greater extent of SOC. Thus, negative values for ΔPOC and ΔMAOC for guinea grass may represent a plant induced 'priming' of organic matter (Kuzyakov et al. 2000; Chen et al. 2020). This effect primarily occurs with unprotected organic matter in the particulated form (Keiluweit et al. 2015; Lavallee et al. 2019), which agrees with POC depletion of MAS, however root exudates are shown to disrupt mineral-organic association, exposing this carbon pool to microbial utilization and also reducing MAOC (Keiluweit et al. 2015). Further, lower N content in the root system of MAS is related to its recalcitrance, which may reduce C use efficiency and stimulate soil organic matter mining ('priming effect') by the microorganisms for nitrogen obtention (Chen et al. 2014; Chen et al. 2018). All of these mechanisms may be enhanced by

the disruption of soil aggregates in the tillage process during pasture establishment, generating a transient 'priming effect' (Lavallee et al. 2019; Chen et al. 2020). Also, higher SRL has been previously shown to increase MAOC stocks in forest ecosystems (Xu et al. 2021), differently from the observed for MAS in this study. Hence, it is important to evaluate the effects of plant traits in longer term experiments, as the soil environment and microbial community interaction with the organic matter may vary within different time frames (Yang et al. 2019).

Partitioning SOC stock into POC and MAOC fractions was effective for understanding the effect of specific traits on each specific carbon pool and the mechanisms which may have been involved (Lavallee et al. 2019; Rossi et al. 2020). For example, PIA, ASS and AND were able to increase ΔSOC to similar values using different pathways, while MAS traits reduced both SOC fractions over the experimental period (Figure 5).

4.4.2. Association effects on ΔPOC, ΔMAOC and ΔSOC

The MAS monoculture showed overall SOC reduction, while values for both ΔPOC and ΔMAOC were higher and positive for the ASS, despite being composed mainly by guinea grass (45 – 84% of herbage biomass – Figure S3. Supplementary material). These results highlight the positive association effect on carbon stock accumulation, which is confirmed by the differences between actual and expected values of ΔPOC and ΔSOC (Figure 6). Generally, mixtures of plants with diverging traits are reported to increase SOC stocks through increased plant derived carbon input (Fornara & Tilman 2008; Steinbeiss et al. 2008; Lange et al. 2015; Yang et al. 2019). The same pattern was observed in this study, as ASS resulted in root overyielding, i.e. greater root biomass than expected from the monocultures and their botanical composition (Figure 4). The method of calculation used (Loreau & Hector 2001) implies that mixing *per se* rather than the presence of one specific dominating species affected both carbon dynamics and plant traits that varied among actual and expected values.

Highly dissimilar root traits among individual species used in this experiment (PIA – larger roots; MAS – greater length development; AND – higher N content - Figure 7) may

have allowed for spatial complementarity, which frequently results in root overyielding of biodiverse communities of plants (Loreau 1998; Mommer et al. 2010; Abalos et al. 2014). Other mechanisms of root overyielding involves increased mineralization of organic matter and nutrient availability (Li et al. 2006; Schenk et al. 2006), reduced species self-inhibition by lower densities on multispecific swards (Mommer et al. 2010) and also root proliferation into soil organic patches, which is stimulated by the presence of other species as a manner of assessing and competing for pools of slowly mineralized nitrogen (Hodge et al. 1999).

Besides increasing C inputs through root biomass by complementarity among species, multispecific swards may also influence SOC stocks by direct influence on microbial activity, which stabilizes the organic matter into the soil mineral matrix (Steinbeiss et al. 2008; Lange et al. 2015). However, the SOC fraction affected by the association in this study was the POC, which indicates that increased C input followed by partial decomposition was the main drive for increased ΔSOC in the association compared to the expected values from the monocultures.

In longer term experiments (> 10 years), increased C input by itself stimulates microbial activity and MAOC stabilization (Lange et al. 2015; Yang et al. 2019), which was not observed in this study. The short duration of this study may have influenced the results once microbial community may take longer than 2 years after pasture establishment to adapt to the new plant cover, especially after soil aggregates disruption caused by tillage (Habekost et al. 2008; Yang et al 2019). Probably the effect of increased plant derived C input can positively affect MAOC in the long-term as demonstrated elsewhere (Lange et al. 2015).

The association also showed reduced SRL in comparison with the expected values from the monocultures, the trait being related to SOC depletion in the PCA (Figure 7). That may have minimized the 'priming effect' showed by MAS, but the mechanism may be coupled with greater root biomass and species specific interactions, once there was no difference in RLD between actual and expected values for the association.

4.4.3. Final remarks

The results of this study showed that species specific traits may influence soil carbon dynamics in the short-term. Moreover, traits may be grouped according to their effect on the different pools of soil organic matter. Greater nitrogen content in AND leaves, herbage and root biomass stimulated MAOC accumulation, presumably due to increased microbial transformations of plant derived organic matter. On the other hand, plant greater biomass and size traits (RBM, NRBM, RVD and HBM) positively influenced the ΔPOC for the ASS and PIA treatments, as they represent greater amounts of plant C input to the soil. Guinea grass great length development and low root N content negatively influenced ΔSOC in both fractions studied. These traits may have allowed for increased 'priming effect', especially as the soil had been harrowed three years prior to the final SOC sampling, exposing particulated and mineral-associated organic matter to microbial community associated to the rhizosphere of the longer and thinner MAS root system. The different relations among plant traits and SOC pools dynamics confirms the hypothesis that species would have different and independent ways of influencing ΔMAOC and ΔPOC.

The association influenced ΔSOC, minimizing the negative impacts showed by the guinea grass monoculture and reaching values similar to those of palisade grass and gamba grass. Complementarity among highly dissimilar root characteristics (biomass and length) probably resulted in increased actual RBM and NRBM for the association compared to the expected values from the monocultures, which represented greater C available for decomposition and stabilization into the POC pool. However, no significant effect of the association was detected on ΔMAOC, probably due to the short duration of the experiment, which did not allow for microbial community adaptation and stabilization of low molecular weight compounds into the soil mineral matrix. Hence the hypothesis regarding positive effects of the association on SOC was partially confirmed as the mixture affected mainly ΔPOC. Longer term experiments may answer if the association could have positive effects also on MAOC through increased root biomass as it did for POC.

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Supplementary material
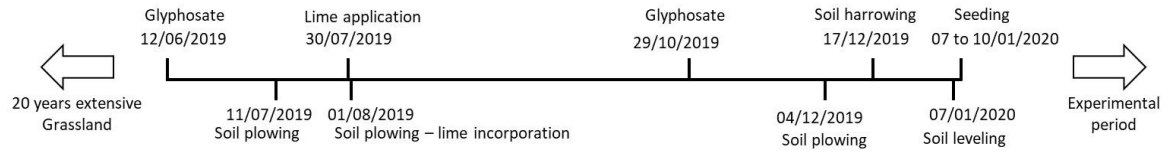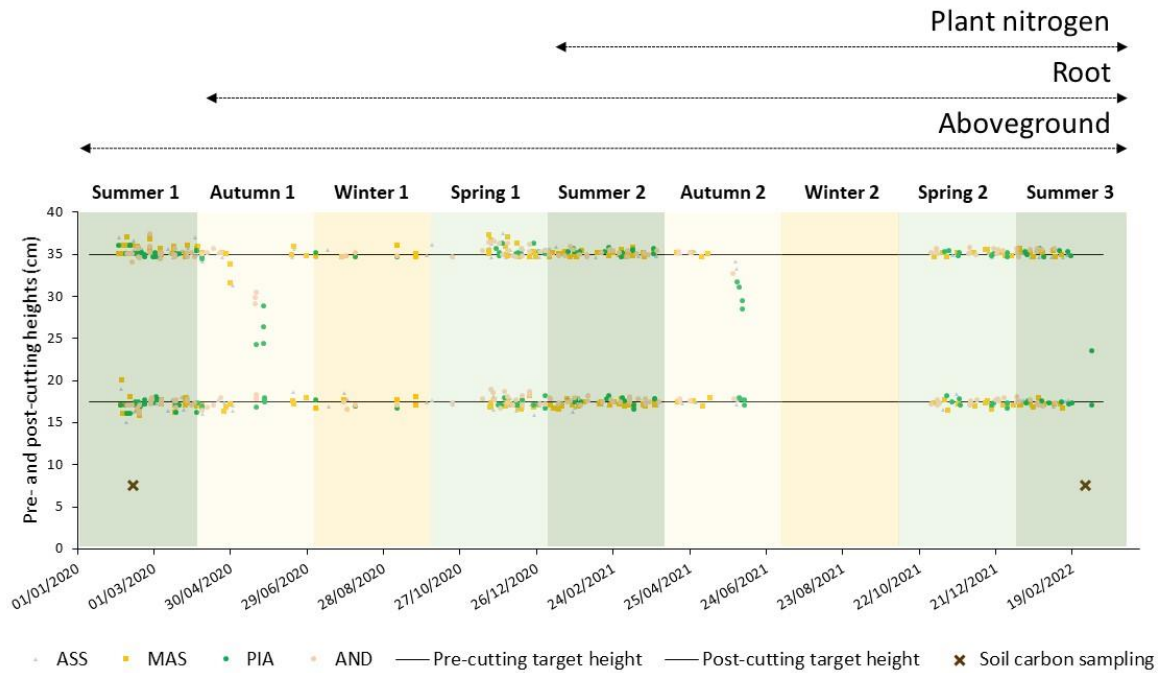
Figure S1. Workflow of pastures establishment.

Figure S2. Sampling chronology along seasons of the year. Dots (ASS, MAS, PIA and AND) in the graph show actual pre- and post-cutting heights for each treatment. ASS means Association treatment; MAS, guinea grass cv. Massai; PIA, palisade grass cv. Piatã; AND, gamba grass cv. Andropogon.

Figure S3. Botanical percentage in herbage biomass seasonal fluctuation for each species in the association (p < 0.0001). MAS means, guinea grass cv. Massai; PIA, palisade grass cv. Piatã; AND, gamba grass cv. Andropogon.

Table S1. Seasonal variation of root characteristics for the association (ASS) and the expected values from the respective monocultures (EXP).

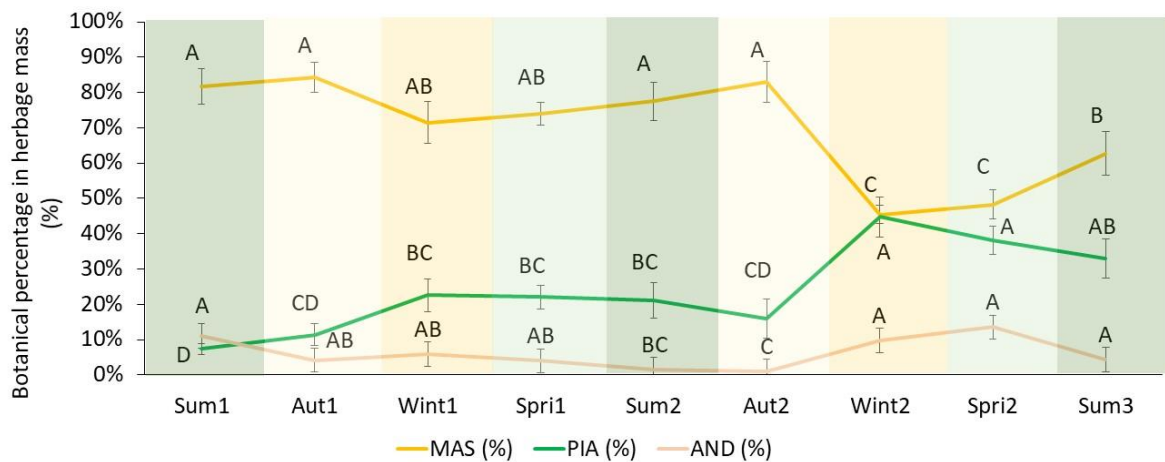| Season	Root biomass (kg DM ha^{-1})	Specific root length (cm g^{-1})	Root length density (m m^{-3})	Nitrogen in root biomass (kg N ha^{-1})
Sum1	-	-	-	-
Aut1	356 E (59.0)	3581 A (196.3)	6246 C (511.8)	-
Wint1	506 D (59.0)	3033 B (80.1)	7397 C (511.8)	-
Spri1	788 B (59.0)	2968 B (137.3)	11616 A (511.8)	-
Sum2	646 C (59.0)	2869 B (143.4)	8906 B (511.8)	4.3 B (0.31)
Aut2	593 CD (59.0)	1662 C (78.4)	4824 D (511.8)	3.4 C (0.31)
Wint2	796 B (59.0)	1609 C (88.3)	6179 C (511.8)	4.4 B (0.31)
Spri2	1110 A (59.0)	1251 D (59.4)	6714 C (511.8)	5.1 A (0.32)
Sum3	1012 A (59.0)	1333 D (86.9)	6281 C (511.8)	4.4 AB (0.31)
p > F	<.0001***	<.0001***	<.0001***	0.0011**

Different letters in columns indicate differences (ANOVA – $p < 0.05$) of mean values by the LSD test ($p < 0.05$). Values in parenthesis are the standard error of the mean

Table S2. Seasonal variation of shoot and root characteristics for the association (ASS) and the expected values from the respective monocultures (EXP).

Season	Root volume density (cm^3 m^{-3})	Root tissue density (g cm^{-3})	Root nitrogen content (%)	Herbage biomass (kg DM ha^{-1})	Leaf nitrogen content (%)
Sum1	-	-	-	4103 F (228.0)	-
Aut1	560 C (112.1)	0.34 BC (0.014)	-	6413 CDE (228.0)	-
Wint1	929 B (112.1)	0.52 A (0.014)	-	6671 BCD (241.3)	-
Spri1	1626 A (112.1)	0.25 D (0.014)	-	6921 BC (228.0)	-
Sum2	1039 B (112.1)	0.36 BC (0.014)	0.68 A (0.022)	7225 B (228.0)	2.4 B (0.02)
Aut2	589 C (112.1)	0.32 C (0.014)	0.58 B (0.022)	8616 A (228.0)	2.9 A (0.07)
Wint2	1077 B (112.1)	0.38 B (0.014)	0.55 B (0.022)	6803 BCD (228.0)	2.0 D (0.03)
Spri2	1616 A (112.1)	0.28 D (0.014)	0.49 C (0.022)	5966 E (228.0)	2.2 C (0.03)
Sum3	1410 A (112.1)	0.37 B (0.014)	0.44 C (0.022)	6318 DE (241.3)	2.4 B (0.08)
p > F	<.0001***	<.0001***	<.0001***	<.0001***	<.0001***

Different letters in columns indicate differences (ANOVA – $p < 0.05$) of mean values by the LSD test ($p < 0.05$). Values in parenthesis are the standard error of the mean.

Table S3. Principal Component Analysis (PCA) performed with subset of shoot and root characteristics and differences between final and initial soil mineral associated carbon in the 0 − 5 cm and 5 − 10 cm soil depths (ΔMAOC5 and ΔMAOC10, respectively) and particulated organic carbon stocks in the 0 − 5 cm and 5 − 10 cm soil depths (ΔPOC5 and ΔPOC10, respectively) (Figure 7).

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14
ΔMAOC5	-0.10	0.37	0.20	0.42	0.30	-0.42	0.13	-0.34	-0.02	0.21	-0.36	-0.08	0.22	0.00
ΔMAOC10	-0.16	0.32	-0.09	0.63	0.03	0.45	-0.18	-0.08	0.07	-0.33	0.26	0.15	-0.13	0.09
ΔPOC5	-0.37	0.01	0.01	-0.02	0.10	-0.61	-0.17	0.40	0.14	-0.36	0.23	0.25	0.10	0.15
ΔPOC10	-0.29	0.06	-0.35	0.26	-0.74	-0.17	0.14	0.12	0.07	0.28	-0.03	-0.15	0.02	-0.12
Root nitrogen content	-0.12	0.39	0.13	-0.31	-0.05	-0.04	-0.63	-0.19	0.21	0.37	0.20	-0.11	-0.22	-0.01
Herbage biomass	-0.26	-0.28	0.26	0.20	0.23	0.30	-0.13	0.49	0.38	0.29	-0.22	-0.22	0.13	-0.06
Herbage nitrogen content	0.18	0.34	-0.43	-0.26	0.03	0.16	0.02	0.02	0.50	-0.14	-0.25	0.09	0.47	0.11
Root length density	0.16	-0.33	-0.51	0.20	0.22	-0.18	-0.32	-0.09	0.12	0.11	-0.28	0.29	-0.35	-0.25
Specific root length	0.34	-0.18	-0.23	0.22	0.05	-0.13	-0.40	0.05	-0.24	0.01	0.24	-0.50	0.40	0.21
Root volume density	-0.37	-0.08	-0.24	-0.10	0.25	0.17	0.06	-0.12	-0.28	0.38	0.34	0.34	0.43	-0.21
Nitrogen in root biomass	-0.37	-0.09	-0.15	-0.15	0.16	0.00	0.04	-0.29	0.12	-0.41	0.03	-0.54	-0.03	-0.47
Root biomass	-0.32	-0.23	-0.24	-0.07	0.15	0.02	0.19	-0.27	0.13	0.13	0.01	-0.15	-0.22	0.74
Root tissue density	0.23	-0.33	0.24	0.16	-0.13	-0.13	0.14	-0.36	0.58	0.06	0.43	0.13	0.16	-0.09
Leaf nitrogen content	0.24	0.31	-0.23	0.04	0.35	-0.11	0.40	0.34	0.13	0.23	0.40	-0.21	-0.31	-0.12
Eigen value	6.38	4.86	0.88	0.70	0.51	0.24	0.18	0.12	0.09	0.02	0.01	0.01	0.00	0.00
Proportion of variance	45.5	34.7	6.3	5.0	3.7	1.7	1.3	0.8	0.7	0.1	0.1	0.0	0.0	0.0
Cumulative proportion	45.5	80.2	86.5	91.5	95.2	96.9	98.2	99.0	99.7	99.8	99.9	100.0	100.0	100.0
Standart deviation	2.52	2.20	0.94	0.84	0.72	0.49	0.42	0.34	0.31	0.14	0.12	0.08	0.05	0.03

5. FINAL CONSIDERATIONS

The results of this study indicate that the association of C_4 perennial forage grasses in pastures may be an alternative for sustainable intensification of grasslands, especially considering the low cost of adoption. The vast area occupied by monospecific grasslands in the tropics represent a great opportunity for enhancing ecosystem services through increased biodiversity. The association of perennial C_4 tropical grasses showed complementarity among species, mainly through dissimilarities in root system, which eventually contributed to reduce N_2O emissions and increase soil organic carbon stocks. Also, intraspecific changes caused by interspecific interactions resulted in slowed N-cycling, which also may have influenced the reduced actual N_2O emissions of ASS in comparison to the expected values from the monocultures. Apparently, the positive results from the association were more related to species specific traits and their interaction in the mixture than to the number of species comprising the association *per se*.

That is corroborated by the different responses of monocultures with diverging traits regarding N and C cycling. Greater herbage and root biomass for the palisade grass were positively correlated to particulated organic carbon accumulation and mitigation of N_2O emissions during the seasons of the year with higher mineral N content. The higher plant N content of gamba grass was related to faster N-cycling and greater microbial activity, thus AND showed greater N_2O emissions and mineral-associated organic carbon accumulation. As for guinea grass, its greater root length development (precision strategy) allowed for reduced N_2O emissions during the seasons with lower mineral N content, but it stimulated the 'priming' of both fractions of soil organic carbon.

In general, this study confirms that plant species traits affect ecosystem functioning regarding C and N-cycling and that specific combinations of species with diverging traits may result in complementarity, more efficient use of resources and mitigation of environmental impacts. Longer-term studies in different edaphoclimatic conditions are required for more broad generalization on how biodiversity and the association of specific traits may affect N_2O emissions and soil organic carbon stocks. Also, the genetic improvement of forage plants should consider the relation among plant traits, their association and how they affect ecosystem functioning in order to develop new cultivars and/or mixtures that could provide ecosystem services.