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

Association of tropical forage grasses in pastures: agronomic aspects that determine plant performance

Alex Marciano dos Santos Silva

Thesis presented to obtain the degree of Doctor in Science.
Area: Animal Science and Pastures

Piracicaba
2023

Alex Marciano dos Santos Silva
Bachelor of Animal Science

Association of tropical forage grasses in pastures: agronomic aspects that determine plant performance

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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

Thesis presented to obtain the degree of Doctor in Science.
Area: Animal Science and Pastures

Piracicaba
2023

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Silva, Alex Marciano dos Santos

Associação de gramíneas forrageiras tropicais em pastagens: aspectos agronômicos que determinam o desempenho das plantas / Alex Marciano dos Santos Silva. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2023.

119 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Competição por luz 2. Diversidade botânica 3. Identificação molecular 4. Mistura de gramíneas 5. Morfogênese 6. Sistema radicular 7. Status de nitrogênio I. Título

ACKNOWLEDGMENTS

First, I would like to express my deepest gratitude to my family, Almir Nunes da Silva, Maria Ribeiro dos Santos Silva, Marcilene dos Santos Silva, and Marcineia dos Santos Silva, for their constant encouragement and understanding. Without your love and support, I would have never made it. This thesis is dedicated to you.

To my advisor, Dr. Sila Carneiro da Silva, for his guidance and support during my doctorate program. Above the scientific insights you provided me, thank you for the example of professor and researcher, conducting all activities with professionalism, dedication, and commitment.

Thanks are also due to the University of São Paulo, "Luiz de Queiroz" College of Agriculture, and the faculty staff of the Department of Animal Science that gave me the opportunity and contributed to my formation.

To Dr. Gaëtan Louarn and Dr. Ela Frak, from the National Research Institute for Agriculture, Food and Environment. Thank you for receiving me as visiting student, for sharing your research, support in data analysis, and valuable comments towards improving my thesis. Also, to the friends who helped me during the internship: Aurélie Baquet, Junpeng Niu, Nathalie Bonnet, Zineb El Ghazzal and Zulfikar Khan.

My special thanks to Dr. André Fischer Sbrissia, Dr. Carlos Guilherme Silveira Pedreira, Dr. Luiz Lehmann Coutinho, Dr. Polyana Cristine Tizioto, Dr. Rodrigo Amorim Barbosa, and Marcela Paduam for all the help and knowledge transmitted during the research development.

My appreciation to the work friends who dedicated several years helping me in the field and laboratory: Alexandre Fameli Mammana, Caio Macret Gomes, Emanoella Karol Saraiva Otaviano, Gislaine Cristina Barro, and Larissa Fernanda Garcia Carvalho.

Finally, thanks to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (scholarship in Brazil 2019/26542-0, scholarship in France 2021/12209-8 and research project thematic 2020/06644-0), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (scholarship in Brazil 140542/2019-1), for the funding and support.

CONTENTS

RESUMO

Associação de gramíneas forrageiras tropicais em pastagens: aspectos agronômicos que determinam o desempenho das plantas

A riqueza e diversidade botânica regula muitas funções do ecossistema e oferece a oportunidade de intensificação sustentável das pastagens perenes por meio do cultivo simultâneo ou parcial de gramíneas no mesmo campo, o que pode resultar em aumento de produtividade, redução do uso de insumos como fertilizantes e pesticidas e menor influência de variações edafoclimáticas. Entretanto, as informações existentes sobre o tema são, em sua maioria, direcionadas para associações de gramíneas anuais de clima temperado ou associação de gramíneas e leguminosas, com poucas informações para associações apenas de gramíneas forrageiras perenes de clima tropical. Além disso, muitos estudos não exploram o "como e o porquê" das interações e respostas agronômicas encontradas entre as espécies de plantas componente da associação. Nesse contexto, a hipótese geral deste estudo foi que três espécies de gramíneas forrageiras tropicais perenes com estratégias complementares de exploração de nichos acima e abaixo do solo cultivadas em associação e manejadas sob regime de desfolhação moderado não competem por recursos (luz e nitrogênio) e têm produção de forragem superior à média das monoculturas. O objetivo geral foi descrever a dinâmica do desempenho agronômico de três espécies de gramíneas forrageiras tropicais perenes cultivadas em monocultura e associação, visando identificar as interações que moldam o uso de recursos (luz e nitrogênio), aspectos determinantes da proporção botânica de cada espécie na associação e desempenho produtivo nessas pastagens. O estudo foi realizado em Piracicaba, São Paulo, Brasil. Os tratamentos foram *Andropogon gayanus* cv. Planaltina (capim andropogon), *Panicum maximum* cv. Massai (capim massai) e *Brachiaria brizantha* cv. BRS Piatã (capim piata) cultivados em monocultivo e associação (as três espécies em proporções iguais com base no número de sementes viáveis) com quatro repetições. O manejo da desfolha foi comum a todos os tratamentos e correspondeu a uma alturas pré-corte de 35 cm e pós-corte 17.5 cm. Também é descrita a calibração de um método baseado em DNA-cloroplasto com objetivos de determinar se a proporção botânica de amostras de raízes de misturas artificiais dessas gramíneas pode ser estimada com precisão e o método aplicado em dados coletados em campo para determinar a proporção botânica abaixo do solo e sua relação com a correspondente proporção acima do solo. Os resultados indicaram que a associação foi moldada pela competição pela luz e pouca competição por nitrogênio, com o capim massai apresentando maior proporção botânica e desempenho produtivo. Incluindo as monoculturas, o capim piata apresentou menor número de ciclos e maior produção de forragem. Em contrapartida, o capim andropogon, o capim massai e a associação apresentaram maior número de ciclos com menor produção de forragem, resultando produção total de forragem semelhante para todos os tratamentos. Os traços funcionais das espécies em monocultivo indicaram que o capim massai possui maior ângulo de folhagem e menor índice de área foliar nos 10 cm superiores, o que resultou em maior proporção de luz no perfil vertical do dossel e favorecendo rápido alongamento foliar. O fato resultou em maior densidade populacional de perfilhos, favorecendo o sombreando das outras duas espécies na associação e causando competição por luz. Os resultados do método de calibração indicaram que a proporção botânica abaixo do solo foi estimada com precisão e que existe alta correlação com a proporção acima do solo. Como conclusão, a associação apresentou desempenho produtivo similar à média dos monocultivos e os traços funcionais das espécies foram importantes preditores para explicar as estratégias de aquisição de recursos, devendo ser considerados para a escolha de espécies que irão compor novas associações.

Palavras-chave: Competição por luz, Diversidade botânica, Identificação molecular, Mistura de gramíneas, Morfogênese, Sistema radicular, Status de nitrogênio

ABSTRACT

Association of tropical forage grasses in pastures: agronomic aspects that determine plant performance

Botanical richness and diversity regulate many ecosystem functions and offer the opportunity for sustainable intensification of perennial pastures through simultaneous or partial cultivation of grasses in the same field, which can result in increased productivity, reduced fertilizer and pesticide inputs, and less influence of edaphoclimatic variations. However, the existing information is mostly directed to associations of annual temperate grasses or association between grasses and legumes, with little information for associations of tropical perennial forage grasses. In addition, many studies do not explore the "how and why" of the interactions and agronomic responses among plant species that make up the association. In this context, the general hypothesis of this study was that three species of tropical perennial forage grasses with complementary strategies of exploration of niches, above and below ground, cultivated in association and managed under moderate defoliation regime do not compete for resources (light and nitrogen) and have greater herbage production than the monocultures individually. The general objective was to describe the dynamics of agronomic performance of the three tropical perennial forage grasses cultivated in monoculture and association aiming to identify the interactions that shape the use of resources (light and nitrogen), a determinant aspect of the botanical proportion and productive performance in these pastures. The study was carried out in Piracicaba, São Paulo, Brazil. The treatments were *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass) and *Brachiaria brizantha* cv. BRS Piatã (piata grass) cultivated as monocultures and in association (the three grass species in equal proportions based on the number of viable seeds) with four replications. Defoliation management was common to all treatments and corresponded to a pre-cutting height of 35 cm and a post-cutting height of 17.5 cm. We also described the calibration of a DNA-chloroplast based method to determine whether the botanical proportion of root samples from DNA artificial mixtures of these grasses can be accurately estimated and applied the method to data collected in the field to determine below ground botanical proportion and its relationship to the corresponding above ground botanical proportion. The results indicated that the association was shaped by competition for light (little competition for nitrogen), with massai grass showing greater botanical proportion and productive performance. Including the monocultures, piata grass had the lowest number of cycles and the greatest herbage accumulation per cycle. On the other hand, andropogon grass, massai grass, and the association showed greater number of cycles with smaller herbage accumulation per cycle, resulting in similar total herbage production for all treatments. The functional traits of the grass species in monoculture indicated that massai grass has greater foliage angle and smaller leaf area index in the upper 10 cm, which resulted in a greater proportion of light in the vertical profile of the canopy, favoring rapid leaf elongation. The fact resulted in greater tiller population density, resulting in shading on the others two grass species in the association and causing competition for light. The results of the calibration method indicated that the below ground botanical proportion was accurately estimated, and there is a high correlation between below ground and above ground botanical proportion. In conclusion, the association presented a productive performance similar to the monocultures. The grass species functional traits are important predictors to explain resource acquisition strategies and should be considered when choosing grass species that will be used to compose new associations.

Keywords: Competition for light, Botanical diversity, Molecular identification, Grass mixture, Morphogenesis. Root system, Nitrogen status

1. INTRODUCTION, OVERVIEW AND THESIS STRUCTURE

The use of biodiverse pastures, based on the increase in richness and diversity of plant species or genotypes in the same pasture, has shown interesting results in increasing the supply of ecosystem services and in reducing the influences of anthropogenic and edaphoclimatic factors (Catford et al., 2020; Tilman et al., 2020; Wright et al., 2021). On a field scale, richness and diversity of cultivated pastures are achieved by simultaneous or partial cultivation of at least two plant species or genotypes (Vandermeer, 1989; Louarn et al., 2020). These species can be spatially distributed in different ways, such as mixed rows, alternating rows, strips, or random seedings (Li et al., 2020). Many studies, including meta-analyses (Guay et al., 2018; Chen et al., 2019; Li et al., 2020) and biodiversity experiments such as the Cedar Creek Experiment (Tilman et al., 2001) and Jena Experiment (Scherber et al., 2010), provided strong evidence on the positive relationships between species richness and diversity and the provision of ecosystem services, highlighting that biodiverse grasslands generate good results of complementarity, selection or facilitation effects (Callaway, 1995; Loreau, 1998; Loreau and Hector, 2001; Wright et al., 2017; Wang et al., 2021). A major challenge in supporting the development of these biodiverse ecosystems remains and it is related to the understanding of the above- and below-ground dynamics of agronomic performance of the species that make up the association in order to identify the interactions that shape the use of resources (light and nitrogen), the determinant aspects of species diversity, and forage production of such pastures. There is growing evidence that functional diversity is more important for ecosystem functioning than the number of taxonomic individuals (Roscher et al., 2012; Li et al., 2021) and that greater dissimilarity in traits indicates less niche overlapping, therefore, more efficient capture of resources in space and time (Gross et al., 2017; Wagg et al., 2017).

Resource partitioning in time and space is seen as one of the main hypotheses to explain the success of such systems (Hopper, 1998). Resource partitioning in space occurs when forage species comprising association pastures acquire resources from different strata of the sward canopy or soil depth. As result, if association forage plants use resources from different vertical strata or can adapt to grow in association, the available resources will be used more efficiently (Barry et al., 2020). As example, there are associations of plants with contrasting characteristics, such as growth habit (erect or prostrate), morphogenetic responses (leaf lifespan, leaf elongation and senescence rate, stem elongation rate) and structural characteristics (leaf size and number of live leaves per tiller) that combined may result in better distribution of the leaf area and use of the incident light by the canopy (Duchini et al., 2016). Below ground, spatial partitioning of resources may occur for water and nutrients uptake from different soil depths, consequence of differences in root length and/or diameter that result in more uniform occupation of soil volume (Bardgett et al., 2014; Mommer et al., 2010). In this case, if below ground resources are more efficiently used, above ground sward canopy may also show better performance. The resource partitioning in time occurs when the species comprising the association stand out in different moments according to their genetics, phenological state or availability of resources, resulting in smaller intra-annual variation in herbage production, i.e. seasonality of production (Hector et al., 2010).

The balance of biodiverse ecosystems has a direct influence on two central characteristics that determine the complementarity of ecological niches and multifunctionality: (i) the richness of species present and (ii) the homogeneity of the distribution of individuals from those species (Magurran, 2004). The good relationship between species that make up an association may be related to inputs and management. In this sense, fertile environments with frequent and lenient defoliations would result in low intensity of disturbances (defoliation severity) and stresses (competition for growth factors), mainly in relation to competition for light and nutrients, providing ideal conditions for the species to coexist and persist simultaneously or to express their characteristics according to their genetic programming at different times throughout the seasons (Grime, 1977; Borer et al., 2014; Eskelinen et al., 2022). Competition for resources can occur above and below ground (Grime, 1973) or only above ground for light (Suding et al., 2005; Hautier et al., 2009). Competition for light predicts that, as productivity increases, the availability of light for understory species is reduced, leading to their exclusion by faster growing or taller species that appropriate this directionally supplied resource (Hautier et al., 2009). The hypothesis provided to explain greater competition for light and less competition for soil nutrients in the same ecosystem is based on the idea that light is a resource that comes only from above and must be used immediately, offering a limited opportunity to differentiate species space niches. On the other hand, soil resources such as macronutrients, micronutrients, microorganisms, and water can be acquired by roots in various dimensions and time scales, ranging from seconds for ions to decades for organic matter (Parton et al., 1988; Hiiesalu et al., 2012; Pärtel et al., 2012). The greater resource availability in the ground can dampen the decline of resources and favor greater richness and diversity of species below ground compared to above ground (Pärtel et al., 2012).

The choice of forage plant species or genotypes to compose an association must consider, among other factors, the characteristics linked to complementarity and or facilitation of access to resources (Volaire et al., 2014). Species can use the same or different resources at different times or places (complementarity) (Naeem et al., 1994). Similarly, some species improve adverse conditions and increase the availability of resources for other groups of species, being mechanisms inherent to a species, beneficial to the other associated species, and reducing possible effects of diseases and pests when at least one of the species in the association is tolerant or resistant (facilitation) (Wright et al., 2017; Wright et al., 2021). Therefore, species with contrasting growth strategies and different survival mechanisms and interactions with neighboring species can coexist and maintain stable populations when the adopted management controls the competition for resources. It is also important, in each ecosystem, to consider the objectives for the association, considering the functional traits of the species used and the possible ecosystem services that the association can offer. According to Hanisch et al. (2020), in a systematic review of the literature based on results with pastures in temperate climates, it was found that in 108 studies, 40 functional characteristics and 11 ecosystem services were found, showing that the functional characteristics of the species are related with the services provided by the ecosystem. However, it is necessary to highlight that, in real situations, not all ecosystem services can be improved simultaneously, and knowledge of the functional traits of species and their ecosystem services is a promising way to choose different combinations of species to compose an association that meets specific objectives.

The increase in richness and diversity of plant species or genotypes in pastures has resulted in improved ecosystem services, such as greater forage production (Grace et al., 2018; Haughey et al., 2018; Sonkoly et al., 2019), greater productive stability (Gross et al., 2014; Duchini et al., 2019), greater nutritional value (Deak et al., 2009; Nobilly et al., 2013), greater root mass and depth, and improved quality of the soil (Bresciano et al., 2019; Yang et al., 2019; Bennett et al., 2020), mitigation of environmental impacts (Cardinale, 2011; Lange et al., 2015; Yang et al., 2019; Abagandura et al., 2020), lower incidence of weeds (Finn et al., 2013) and improved animal performance (Pembleton et al., 2016; Dillard et al., 2018; Nieman et al., 2018; Jonker et al., 2018). Despite the many benefits, research involving the association of forage species is directed to pastures in temperate climates, especially with associations between forage grasses and legumes or associations including annual species. On the other hand, under tropical conditions and environments, this type of study is little explored, particularly considering the association only of well-managed perennial grasses (Figure 1).

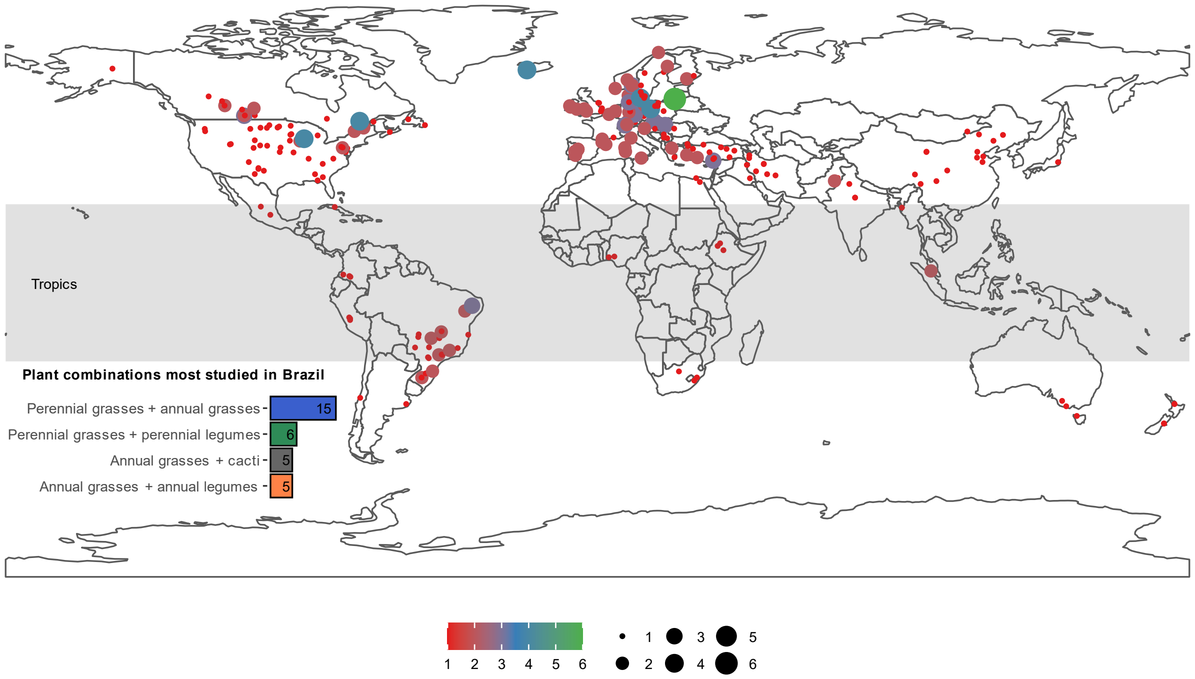


Figure 1. Global distribution of research involving associations of forage plants, and types of plant combinations. The systematic search was carried out on a data set (ISI Web of Science Core Collection) (title session) to select research articles published between 01/01/2000 and 09/17/2022. The search terms used were ("grassland*" or "pasture*", or "forage*") and ("association*" or "mixture*" or "intercropping*"). The result was a total of 571 non duplicated research articles. All titles and abstracts were read, and articles were selected that reported the association of forage plants in natural and/or cultivated pastures or multiple associations with annual crops. Subsequently, the geographic coordinates reported in the articles were extracted to assemble the database. Single studies may have collected data from different locations. The final total number of articles included in the study was 248.

In Brazil, given its edaphoclimatic and seasonal production characteristics and the great diversity of species and genotypes of forage grasses that can be combined to meet pre-defined ecosystem services, the association of forage grasses in pastures would be interesting and strategic to expand the potential for use, productivity, and sustainability of cultivated pastures in the country, which correspond to around 45% of the national territory (IBGE, 2017). In this context, the association of perennial forage grasses would have a high potential for use, favoring their dissemination in production areas, especially on those where pasture recovery is necessary. These associations are already used informally in pasture based animal production systems in tropical biomes. However, many questions still need to be answered before their use becomes widespread. For this, knowledge of the dynamics of resource use, agronomic performance, and interspecific competition among species that make up the association is a central part of the understanding of how such ecosystems work, central information to determine and define management requirements.

Perennial grasses have undergone a long evolution, adapting to grazing and expanding their storage capacity of organic reserves that ensure their resilience and perenniality, ensuring rapid regrowth and restoration of leaf area after defoliation, a process that can happen more slowly in other forage species (Hodgson, 1990). In addition, grasses are efficient in the use of resources when compared to legumes, for example, which can cause disproportionate competition among species (Da Silva et al., 2013), or greater grazing of forage legumes, reducing diversity and ecosystem success in some cases (Grace et al., 2018). In this context, associations of only well-managed perennial grasses would be an adaptable alternative, with benefits that would allow sustainable production and ensure the provision of important ecosystem services.

This thesis is the first constituent of four theses conducted concurrently to study the association of perennial tropical forage grass species in pastures. The theses were subdivided as follows: (thesis 1 - above and below ground agronomic performance determinants of resource use, proportion of grasses, and forage production); (thesis 2 - tillering dynamics, population stability and persistence of grass species populations, overyielding and underyielding); (thesis 3 - physical, chemical and microbiological characteristics of the soil, and emissions of greenhouse gases CH_4, N_2O and CO_2 by the soil), and (thesis 4 - herbage intake rate, the nutritional value of consumed herbage and emission of enteric CH_4).

The perennial tropical forage grass species studied were: *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass) and *Brachiaria brizantha* cv. BRS Piatã (piata grass) cultivated in monoculture and in association (the three grass species in equal proportions based on the number of viable seeds). Defoliation management was common to all grass species in the monoculture and in the association and corresponded to 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). The management criterion of frequent and moderate defoliation was chosen as a means to ensure low competition for light (Grime, 1977; Borer et al., 2014; Eskelinen et al., 2022).

Initially, based on data available in the literature, the grass species used were chosen considering mechanisms of niche complementarity (resource conservation and resource capture) (Loreau and Hector, 2001; Cruz et al., 2002; Pontes et al., 2012) and facilitation strategies (resistance to plant diseases and pathogens) (Callaway, 1995; Wright et al., 2017), which could result in functional coexistence of the grass species (Gross et al., 2014; Gross et al., 2017; Wang et al., 2021). Andropogon grass was chosen as a resource conservation grass species (small specific leaf area, long leaf lifespan, and cespitose growth), with antibiosis and antixenosis mechanisms that allow resistance to insects, mainly spittlebugs (*Deois flavopicta stal*) and brown root stink bug (*Scaptocoris castanea*) (Pires, 2010). Piata grass was chosen as a resource capture grass species (large specific leaf area, high leaf and tiller turnover, and semi-erect growth), and massai grass was chosen as an intermediate grass species (resource capture x resource conservation) (intermediate specific leaf area and cespitose development), with high resistance to foliar fungal diseases. The three grass species studied are drought tolerant and have a high potential for forage dry matter production (Fonseca and Martuscello, 2022).

After the experiment, part of the monoculture grass species functional traits was analyzed to verify if the grass species were functionally different and which strategies, they used for acquiring resources. Subsequently, a conceptual model was elaborated, and subsets of response variables were grouped into chapters to understand the "how and why" of the agronomic interactions and responses found among grass species when cultivated in association, forming the structure of the thesis (Figure 2).

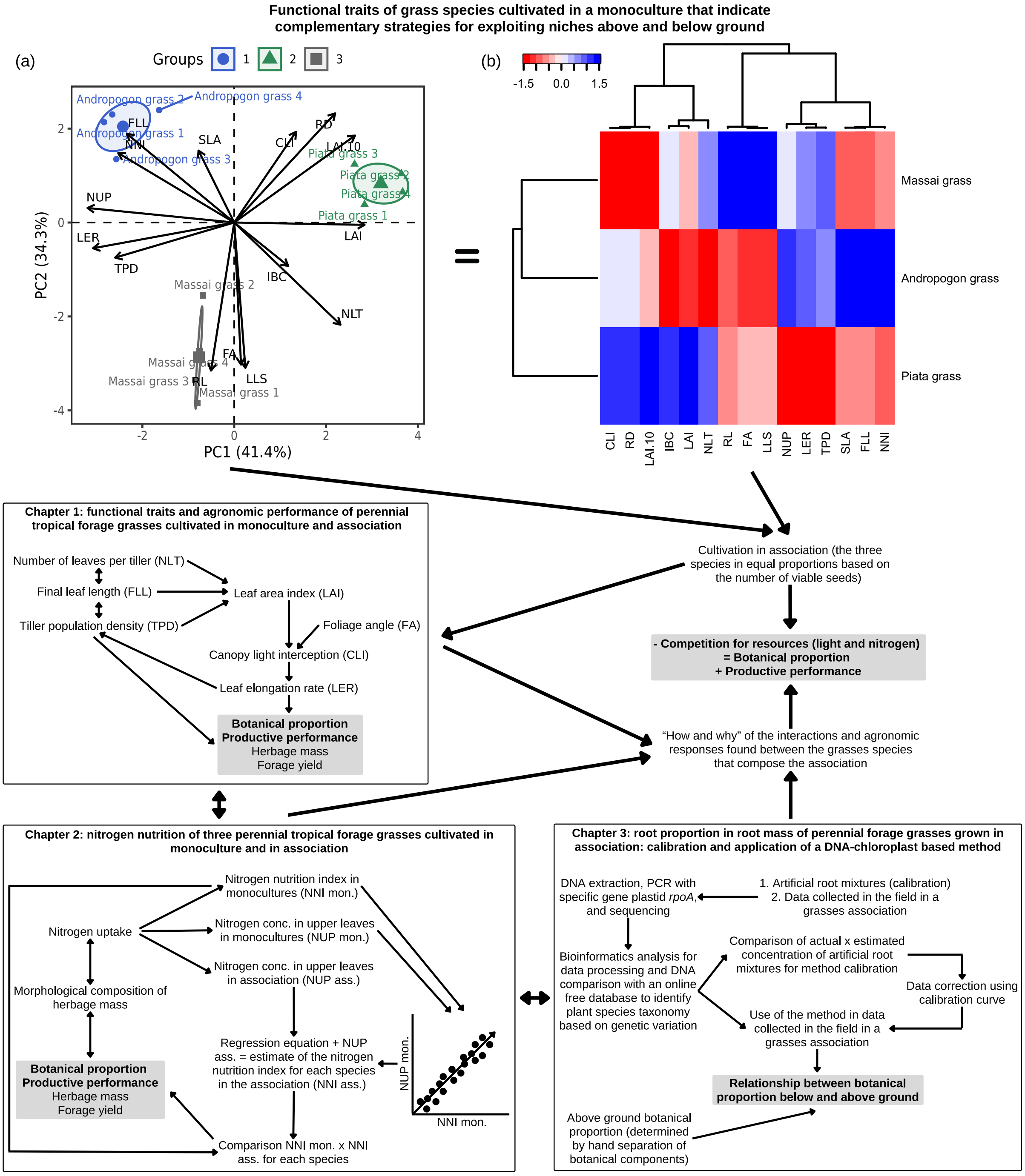


Figure 2. Conceptual model and thesis structure: biplot PC1 x PC2 in monoculture (a), and heatmap and cluster in monoculture (b) of the functional traits of grasses species. The data correspond to the average of five seasons. Legend: leaf elongation rate per tiller (cm tiller^{-1} day^{-1}) (LER); number of leaves per tiller (n leaves tiller^{-1}) (NLT); final leaf length (cm leaf^{-1}) (FLL); tiller population density (tiller^{-1} m^{2}) (TPD); canopy light interception (%) (CLI); foliage angle (º) (FA); leaf area index (m^{2} m^{-2}) (LAI); leaf area index in the upper 10 cm (m^{2} m^{-2}) (LAI.10); specific leaf area (cm^{2} g^{-1}) (SLA); leaf lifespan (days) (LLS); root diameter (mm) (RD); root length (cm m^{3}) (RL); nitrogen concentration in upper leaves (%DM) (NUP); nitrogen nutrition index (NNI); and Interval between cuttings (days) (IBC). Blocks (1, 2, 3 and 4).

1.1. Hypotheses and objectives

General hypothesis

Three species of tropical perennial forage grasses with complementary strategies of exploitation of niches above and below ground cultivated in association and managed under moderate defoliation regime do not compete for resources (light and nitrogen) and have forage production superior to the average of monocultures.

General objective

The general objective was to describe the dynamics of agronomic performance of the three tropical perennial forage grasses cultivated in monoculture and association aiming to identify the interactions that shape the use of resources (light and nitrogen), a determinant aspect of the botanical proportion and productive performance in these pastures.

Specific objectives

Chapter 1: (i) to determine whether the grass species that has the greatest productive performance in monoculture also has the greatest productive performance in the association, and (ii) to determine which underlying mechanisms shape the proportion of grasses and productive performance of grass species cultivated in monoculture and in association.

Chapter 2: (i) to determine whether tropical forage grasses cultivated as monoculture or in association have similar nitrogen uptake capacity, and (ii) to determine whether the nitrogen nutrition index of grasses grown in association differs from those of grasses grown in monocultures.

Chapter 3: (i) to determine whether botanical composition of root samples from DNA artificial mixtures of perennial forage grasses can be accurately estimated, and (ii) to apply the method to field data to determine below ground proportion of grasses and its relationship with the correspondent above ground proportion of grasses.

References

Abagandura, G.O., Sekaran, U., Singh, S., Singh, J., Ibrahim, M.A., Subramanian, S., Owens, V.N., Kumar, S., 2020. Intercropping kura clover with prairie cordgrass mitigates soil greenhouse gas fluxes. Scientific Reports. 10. https://doi.org/10.1038/s41598-020-64182-2

Bardgett, R.D., Mommer, L., Vries, F.T., 2014. Going underground: root traits as drivers of ecosystem processes. Trends in Ecology & Evolution. 29 (12), 692–699. https://doi.org/10.1016/j.tree.2014.10.006

Barry, K.E., Ruijven, J., Mommer, L., Bai, Y., Beierkuhnlein, C., Buchmann, N., Kroon, H., Ebeling, A., Eisenhauer, N., Steinicke, C.G., Hildebrandt, A., Isbell, F., Milcu, A., Neßhöver, C., Reich, P.B., Roscher, C., Sauheitl, L., Lorenzen, M.S., Schmid, B., Tilman, D., Felten, S., Weigelt, A., 2020. Limited evidence for spatial resource partitioning across temperate grassland biodiversity experiments. Ecology. 101. https://doi.org/10.1002/ecy.2905

Bennett, J.A., Koch, A.M., Forsythe, J., Johnson, N.C., Tilman, D., Klironomos, J., 2020. Resistance of soil biota and plant growth to disturbance increases with plant diversity. Ecology Letters. 23, 119–128. https://doi.org/10.1111/ele.13408

Borer, E.T., Seabloom, E.W., Gruner, D.S., Harpole, W.S., Hillebrand, H., Lind, E.M., Adler, P.B., Alberti, J., Anderson, T.M., Bakker, J.D., Biederman, L., Blumenthal, D., Brown, C.S., Brudvig, L.A., Buckley, Y.M., Cadotte, M., Chu, C., Cleland, E.E., Crawley, M.J., Daleo, P., Damschen, E.I., Davies, K.F., DeCrappeo, N.M., Du, G., Firn, J., Hautier, Y., Heckman, R.W., Hector, A., HilleRisLambers, J., Iribarne, O., Klein, J.A., Knops, J.M.H., La Pierre, K.J., Leakey, A.D.B., Li, W., MacDougall, A.S., McCulley, R.L., Melbourne, B.A., Mitchell, C.E., Moore, J.L., Mortensen, B., O'Halloran, L.R., Orrock, J.L., Pascual, J., Prober, S.M., Pyke, D.A., Risch, A.C., Schuetz, M., Smith, M.D., Stevens, C.J., Sullivan, L.L., Williams, R.J., Wragg, P.D., Wright, J.P., Yang, L.H., 2014. Herbivores and nutrients control grassland plant diversity via light limitation. Nature. 508, 517–520. https://doi.org/10.1038/nature13144

Bresciano, D., Pino, A., Borges, A., Tagera, M., Speranza, P., Astigarraga, L., 2019. Perennial C4 grasses increase root biomass and carbon in sown temperate pastures. New Zealand Journal of Agricultural Research. 62, 332–342. https://doi.org/10.1080/00288233.2018.1504089

Callaway, R.M., 1995. Positive interactions among plants. The Botanical Review. 61 (4), 306–349. https://doi.org/10.1007/BF02912621

Cardinale, B.J., 2011. Biodiversity improves water quality through niche partitioning. Nature. 472, 86–89. https://doi.org/10.1038/nature09904

Catford, J.A., Dwyer, J.M., Palma, E., Cowles, J.M., Tilman, D., 2020. Community diversity outweighs effect of warming on plant colonization. Global Change Biology. 26, 3079–3090. https://doi.org/10.1111/gcb.15017

Chen, C., Chen, H.Y.H., Chen, X., Huang, Z., 2019. Meta-analysis shows positive effects of plant diversity on microbial biomass and respirelativen. Nature Communications. 10 (1332). https://doi.org/10.1038/s41467-019-09258-y

Cruz, P., Duru, M., Therond, O., Theau, J.P.J.P., Ducourtieux, C., Jouany, C., Khaled, R.A.H., Ansquer, P., 2002. Une nouvelle approche pour caractériser les prairies naturelles et leur valeur d'usage. Fourrages. 172, 335–354

Da Silva, S.C., 2013. Desafios e perspectivas de manejo do pastejo em pastos consorciados: uma reflexão. In: Casagrande, D.R. et al. As forragens e as suas relações com o solo, o ambiente e o animal. 2 ed. Suprema Gráfica e Editora, Lavras, Minas Gerais, Brasil, p.63–75.

Deak, A., Hall, M.H., Sanderson, M.A., 2009. Grazing schedule effect on forage production and nutritive value of diverse forage mixtures. Agronomy Journal. 101, 408–414. https://doi.org/10.2134/agronj2007.0365

Dillard, S.L., Hancock, D.W., Harmon, D.D., Mullenix, M.K., Beck, P.A., Soder, K.J., 2018. Animal performance and environmental efficiency of cool-and warm-season annual grazing systems. Journal of Animal Science. 96, 3491–3502. https://doi.org/10.1093/jas/sky025

Duchini, P.G. Guzatti, G.C., Echeverria, J.R., Américo, L.F., Sbrissia, A.F., 2019. Can a mixture of perennial grasses with contrasting growth strategies compose productive and stable swards? Agronomy Journal. 111, 224–232. https://doi.org/10.2134/agronj2018.03.0218

Duchini, P.G., Guzatti, G.C., Ribeiro-Filho, H.M.N., Sbrissia, A.F., 2016. Intercropping black oat (*Avena strigosa*) and annual ryegrass (*Lolium multiflorum*) can increase pasture leaf production compared with their monocultures. Crop & Pasture Science. 67, 574–581. https://doi.org/10.1071/CP15170

Eskelinen, A., Harpole, W.S., Jessen, M.T., Virtanen, R., Hautier, Y., 2022. Light competition drives herbivore and nutrient effects on plant diversity. Nature. 611, 301–305. https://doi.org/10.1038/s41586-022-05383-9

Finn, J.A. Kirwan, L., Connolly, J., Teresa Sebastia, M., Helgadottir, A., Baadshaug, O.H., Blanger, G., Black, A., Brophy, C., Collins, R.P., op, J., Dalmannsdttir, S., Delgado, I., Elgersma, A., Fothergill, M., Frankow-Lindberg, B.E., Ghesquiere, A., Golinska, B., Golinski, P., Grieu, P., Gustavsson, A.-M., Hglind, M., Huguenin-Elie, O., Jrgensen, M., Kadziuliene, Z., Kurki, P., Llurba, R., Lunnan, T., Porqueddu, C., Suter, M., Thumm, U., Luescher, A., 2013. Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. Journal of Applied Ecology. 50, 365–375. https://doi.org/10.1111/1365-2664.12041

Fonseca, D.M., Martuscello, J.A., 2022. Plantas forrageiras, 2 ed. Editora UFV. Universidade Federal de Viçosa, 591p.

Grace, C., Boland, T.M., Sheridan, H., Lott, S., Brennan, E., Fritch, R., Lynch, M.B., 2018. The effect of increasing pasture species on herbage production, chemical composition, and utilization under intensive sheep grazing. Grass and Forage Science. 73, 852–864. https://doi.org/10.1111/gfs.12379

Grime, J.P., 1973. Competitive exclusion in herbaceous vegetation. Nature. 242, 344–347. https://doi.org/10.1038/242344a0

Grime, J.P., 1977. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. The American Naturalist. 111, 1169–1194. http://www.jstor.org/stable/2460262

Gross, K., Cardinale, B.J., Fox, J.W., Gonzalez, A., Loreau, M., Polley, H.W., Reich, P.B., Ruijven, J., 2014. Species richness and the temporal stability of biomass production: A new analysis of recent biodiversity experiments. The American Naturalist. 183 (1). https://doi.org//10.1086/673915

Gross, N., Pinguet, Y.L.B., Liancourt, P., Berdugo, M., Gotelli, N.J., Maestre, F.T., 2017. Functional trait diversity maximizes ecosystem multifunctionality. Nature Ecology & Evolution. 1 (132). https://doi.org/10.1038/s41559-017-0132

Guay, M.O.M., Paquette, A., Dupras, J., Rivest, D., 2018. The new Green Revolution: Sustainable intensification of agriculture by intercropping. Science of the Total Environment. 615, 767–772. https://doi.org/10.1016/j.scitotenv.2017.10.024

Hanisch, M., Schweiger, O., Cord, A.F., Volk, M., Knapp, S., 2020. Plant functional traits shape multiple ecosystem services, their trade-offs and synergies in grasslands. Journal of Applied Ecology. 57, 1535–1550. https://doi.org/10.1111/1365-2664.13644

Haughey, E.H., Suter, M., Hofer, D., Hoekstra, N.J., McElwain, J.C., Lüscher, A., Finn, J.A., 2018. Higher species richness enhances yield stability in intensively managed grasslands with experimental disturbance. Scientific Reports. 8. https://doi.org/10.1038/s41598-018-33262-9

Hautier, Y., Niklaus, P.A., Hector, A., 2009. Competition for light causes plant biodiversity loss after eutrophication. Science. 324, 636–638. https://doi.org/10.1126/science.1169640

Hector, Hautier, A.Y., Saner, P., Wacker, L., Bagchi, R., Joshi, J., Scherer-Lorenzen, M., Spehn, E.M., Bazeley-White, E., Weilenmann, M., Caldeira, M.C., Dimitrakopoulos, P.G., Finn, J.A., Huss-Danell, K., Jumpponen, A., Mulder, C.P.H., Palmborg, C., Pereira, J.S., Siamantziouras, A.S.D., Terry, A.C., Troumbis, A.Y., Schmid, B., Loreau, M., 2010. General stabilizing effects of plant diversity on grassland productivity through population asynchrony and overyielding. Ecology. 91, 2213–2220. https://doi.org/10.1890/09-1162.1

Hiiesalu, I., Öpik, M., Metsis, M., Lilje, L., Davison, J., Vasar, M., Moora, M., Zobel, M., Wilson, S.D., Pärtel, M., 2012. Plant species richness belowground: higher richness and new patterns revealed by next-generation sequencing. Molecular Ecology. 21, 2004–2016. https://doi.org/10.1111/j.1365-294X.2011.05390.x

Hodgson, J., 1990. Grazing management: science into practice. Essex, England, Longman Scientific & Technical, 203p.

Hopper, D.U., 1998. The role of complementarity and competition in ecosystem responses to variation in plant diversity. Ecology 79. https://doi.org/10.1890/0012-9658(1998)079[0704:TROCAC]2.0.CO;2

Instituto Brasileiro de Geografia e Estatística - IBGE. Censo agropecuário: resultados definitivos 2017. Disponível em: <https://censos.ibge.gov.br/agro/2017/templates/censo_agro/resultadosagro/estabelecimentos.html> Acesso em: 19 de julho de 2021.

Jonker, A., Farrell L., Scobie D., Dynes R., Edwards G., Hague H., McAuliffe R., Taylor A., Knight T., Waghorn G., 2019. Methane and carbon dioxide emissions from lactating dairy cows grazing mature ryegrass/white clover or a diverse pasture comprising ryegrass, legumes, and herbs. Animal Production Science. 59, 1063–1069. https://doi.org/10.1071/AN18019

Lange, M., Eisenhauer, N., Sierra, C.A., Bessler, H., Engels, C., Griffiths, R.I., Mellado-Vázquez, P.G., Malik, A.A., Roy, J., Scheu, S., Steinbeiss, S., Thomson, B.C., Trumbore, S.E., Gleixner, G., 2015. Plant diversity increases soil microbial activity and soil carbon storage. Nature communications. 6. https://doi.org/10.1038/ncomms7707

Li, W., He, S., Cheng, X., Zhang, M., 2021. Functional diversity outperforms taxonomic diversity in revealing short-term trampling effects. Scientific Reports. 11 (18889). https://doi.org/10.1038/s41598-021-98372-3

Li, X.F., Wang, Z.G., Bao, X.G., Sun, J.H., Yang, S.C., Wang, P., Wang, C.B., Wu, J.P., Liu, X.R., Tian, R.L., Wang, Y., Li, J.P., Wang, Y., Xia, H.Y., Mei, P.P., Wang, X.F., Zhao, J.H., Yu, R.P., Zhang, W.P., Che, Z.X., Gui, L.G., Callaway, R.M., Tilman, D., Li, L., 2020. Long-term increased grain yield and soil fertility from intercropping. Nature Sustainability. 4, 943–950. https://doi.org/10.1038/s41893-021-00767-7

Loreau, M., 1998. Separating sampling and other effects in biodiversity experiments. Oikos, 82, 600–602. https://doi.org/10.2307/3546381

Loreau, M., Hector, A., 2001. Partitioning selection and complementarity in biodiversity experiments. Nature. 412, 72–76. https://doi.org/10.1038/35083573

Louarn, G., Barillot, R., Combes, D., Gutiérrez, A.E., 2020. Towards intercrop ideotypes: non-random trait assembly can promote overyielding and stability of species proportion in simulated legume-based mixtures. Annals of Botany. 126, 671–685. https://doi.org/10.1093/aob/mcaa014

Magurran, A.E., 2004. Measuring biological diversity. 1 ed. Blackwell Publishing, Oxford, UK, 256p.

Mommer, L., Ruijven, J., Caluwe, W., Smit-Tiekstra, A.E., Wagemaker, C.A.M., Ouborg, N.J., Bogemann, G.M., Weerden, G.M., Berendse, F., Kroon, H., 2010. Unveiling below-ground species abundance in a biodiversity experiment: a test of vertical niche differentiation among grassland species. Journal of Ecology. 98, 1117–1127. https://doi.org/10.1111/j.1365-2745.2010.01702.x

Naeem, S., Thompson, L.J., Lawler, S.P., Lawton, J.H., Woodfin, R.M., 1994. Declining biodiversity can alter the performance of ecosystems. Nature. 368, 734–736. https://doi.org/10.1038/368734a0

Nieman, C.C., Albrecht, K.A., Schaefer, D.M., 2018. Temporal Composition of Alfalfa-Grass Pastures and Productivity Response of Holstein Steers. Crop Science. 111, 686–693. https://doi.org/10.2134/agronj2018.06.0375

Nobilly, F., Bryant, R.H., Mckenzie, B.A., Edwards, G.R., 2013. Productivity of rotationally grazed simple and diverse pasture mixtures under irrigation in Canterbury. Proceedings of the New Zealand Grassland Association. 75, 165–172. https://doi.org/10.33584/jnzg.2013.75.2920

Pärtel, M., Hiiesalu, I., Öpik, M., Wilson, S.D., 2012. Below-ground plant species richness: new insights from DNA-based methods. Functional Ecology. 26, 775–782. https://doi.org/10.1111/j.1365-2435.2012.02004.x

Parton, W., Stewart, J., Cole, C., 1988. Dynamics of C, N, P and S in grassland soils: a model. Biogeochemistry. 5, 109–131. https://doi.org/10.1007/BF02180320

Pembleton, K.G., Hills, J.L., Freeman, M.J., McLaren, D.K., French, M., Rawnsley, R.P., 2016. More milk from forage: Milk production, blood metabolites, and forage intake of dairy cows grazing pasture mixtures and spatially adjacent monocultures. Journal of Dairy Science. 99, 3512–3528. https://doi.org/10.3168/jds.2015-10542

Pires, A.V., 2010. Bovinocultura de corte. 1 ed. Editora Fealq, Piracicaba, São Paulo, Brasil, 760p.

Pontes, L.S., Maire, V., Louault, F., Soussana, J.F., Carrère, P., 2012. Impacts of species interactions on grass community productivity under contrasting management regimes. Oecologia. 168, 761–771. https://doi.org//10.1007/s00442-011-2129-3

Roscher, C., Schumacher, J., Gubsch, M., Lipowsky, A., Weigelt, A., Buchmann, N., Schmid, B., Schulze, E.D., 2012. Using plant functional traits to explain diversity–productivity relationships. PLOS ONE. 7 (5). https://doi.org/10.1371/journal.pone.0036760

Scherber, C., Eisenhauer, N., Weisser, W.W., Schmid, B., Voigt, W., Fischer, M., Schulze, E.D., Roscher, C., Weigelt, A., Allan, E., Bebler, H., Bonkowski, M., Buchmann, N., Buscot, F., Clement, L.W., Ebeling, A., Engels, C., Halle, S., Kertscher, I., Klein, A.M., Koller, R., König, S., Kowalski, E., Kummer, V., Kuu, A., Lange, M., Lauterbach, D., Middelhoff, C., Migunova, V.D., Milcu, A., Müller, R., Partsch, S., Petermann, J.S., Renker, C., Rottstock, T., Sabais, A., Scheu, S., Schumacher, J., Temperton, V.M., Tscharntke, T., 2010. Bottom-up effects of plant diversity on multitrophic interactions in a biodiversity experiment. Nature. 468, 553–556. https://doi.org/10.1038/nature09492

Sonkoly, J., Kelemen, A., Valkó, O., Deák, B., Kiss, R., Tóth, K., Miglécz, T., Tóthmérész, B., Török, P., 2019. Both mass ratio effects and community diversity drive biomass production in a grassland experiment. Scientific Reports. 9. https://doi.org/10.1038/s41598-018-37190-6

Suding, K.N., Collins, S.L., Gough, L., Clark, C., Cleland, E.E., Gross, K.L., Milchunas, D.G., Pennings, S., 2005. Functional- and abundance-based mechanisms explain diversity loss due to N fertilization. PNAS. 102, 4387–4392. https://doi.org/10.1073/pnas.0408648102

Tilman, D., 2020. Benefits of intensive agricultural intercropping. Nature Plants. 6, 604–605. https://doi.org/10.1038/s41477-020-0677-4

Tilman, D., Reich, P.B., Knops, J., Wedin, D., Mielke, T., Lehman, C., 2001. Diversity and Productivity in a Long-Term Grassland Experiment. Science. 294, 843–845. https://doi.org/10.1126/science.1060391

Vandermeer, J.H., 1989. The ecology of intercropping. Cambridge: Cambridge University Press, 237p.

Volaire, F., Barkaoui, K., Norton, M., 2014. Designing resilient and sustainable grasslands for a drier future: Adaptive strategies, functional traits, and biotic interactions. European Journal of Agronomy. 52, 81–89. https://doi.org/10.1016/j.eja.2013.10.002

Wagg, C., Ebeling, A., Roscher, C., Ravenek, J., Bachmann, D., Eisenhauer,N., Mommer, L., Buchmann, N., Hillebrand,H., Schmid,B., Weisser, W.W., 2017. Functional trait dissimilarity drives both species complementarity and competitive disparity. Functional Ecology. 31 (12), 2320–2329. https://doi.org/10.1111/1365-2435.12945

Wang, S., Isbell, F., Deng, W., Hong, P., Dee, L.E., Thompson, P., Loreau, M. 2021. How complementarity and selection affect the relationship between ecosystem functioning and stability. Ecology. e03347. https://doi.org/10.1002/ecy.3347

Wright, A.J., Mommer, L., Barry, K., Ruijven, J., 2021. Stress gradients and biodiversity: monoculture vulnerability drives stronger biodiversity effects during drought years. Ecology. 102 (1). https://doi.org/10.1002/ecy.3193

Wright, A.J., Wardle, D.A., Callaway, R., Gaxiola, A., 2017. The overlooked role of facilitation in biodiversity experiments. Trends in Ecology & Evolution. 32 (5). https://doi.org/10.1016/j.tree.2017.02.011

Yang, Y. Tilman, D., Furey, G., Lehman, C., 2019. Soil carbon sequestration accelerated by restoration of grassland biodiversity. Nature communications. 10. https://doi.org/10.1038/s41467-019-08636-w

2. FUNCTIONAL TRAITS AND AGRONOMIC PERFORMANCE OF PERENNIAL TROPICAL FORAGE GRASSES CULTIVATED IN MONOCULTURE AND ASSOCIATION

Abstract

The functional traits of plants control a series of agronomic and ecological responses that can shape terrestrial ecosystems. In an association comprised of perennial forage grasses species, understanding of their functional traits and what are the changes individual grass species undergo when compared to its monoculture can help to comprehend the dynamics of resource capture and use, the determinant aspects of grass species botanical proportion and the productive performance of such pastures. These are key information for assisting with the decision-making process regarding management and formulation of new forage grasses associations. In this context, the objectives of this study were (i) to determine whether the grass species that has the greatest productive performance when cultivated as monoculture also has the greatest productive performance when cultivated in association with other grasses, and (ii) to determine which underlying mechanisms shape the botanical proportion and productive performance of grass species cultivated in monoculture and in association. The study was conducted from Late Spring of 2020 to Summer of 2022 in Piracicaba, São Paulo, Brazil. Treatments corresponded to three perennial tropical forage grass species, *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass), and *Brachiaria brizantha* cv. BRS Piatã (piata grass) cultivated in monoculture and in association (the three grass species in equal proportions based on the number of viable seeds) with four replications. Defoliation management was common to all treatments and corresponded to a pre-cutting height of 35 cm and a post-cutting 17.5 cm. The sward herbage mass represented the description of the above ground vegetation once every season of the year and was collected at ground level. Total forage yield represents the total amount of herbage accumulated over the entire data collection period as above the post-cutting height. Among the monocultures, piata grass presented a greatest herbage mass, but in the association massai grass had greatest herbage mass. Piata grass presented a smaller number of cycles and greater herbage accumulation per cycle. In contrast, andropogon grass, massai grass, and the association presented greater number of cycles with smaller herbage accumulation per cycle, resulting in similar total forage yield for piata grass, massai grass, and the association. The association was mainly shaped by competition for light, with massai grass present in greater proportion and showing greater productive performance. The functional traits of grass species in monoculture indicated that massai grass had greater foliage angle and smaller leaf area index in the upper 10 cm of the sward canopy, which resulted in a larger proportion of light in the vertical profile of the canopy, causing fast leaf elongation per tiller, larger tiller population density, and shading of the others two grass species in the association. The smaller rate of leaf elongation per tiller observed for piata grass may have been caused by the combination of large leaf area index and smaller foliage angle, resulting in self-shading of leaves at the base of the sward canopy. Although andropogon grass presented the largest final leaf length, it presented smaller number of leaves per tiller, which explain its smaller proportion and productive performance both in the monoculture and in the association. The foliage angle, leaf elongation rate per tiller, number of leaves per tiller, and leaf area index are functional traits that shape the dynamics of the competition for light, botanical proportion, and productive performance of grass species in the association, and should be taken into account when choosing grass species for comprising new associations.

Keywords: botanical proportion, foliage angle, light competition, mixed pastures, morphogenesis

2.1. Introduction

Simultaneous or partial cultivation of two or more species or genotypes of plants in the same field is an ancient practice commonly used in productive subsistence ecosystems that normally include species with an annual cycle. These productive ecosystems provide good yields or yield stability with reduced fertilizer and pesticide inputs and are less influenced by edaphoclimatic variations (Isbell et al., 2015; Li et al., 2020; Tilman, 2020). In pastoral ecosystems of perennial forage grasses, this model of cultivation offers the opportunity for sustainable intensification through simultaneous cultivation of forage plants species or genotypes with complementary strategies to exploit ecological niches above and below ground that will coexist under frequent defoliation and interactions to capture and use available resources. These interactions and strategies, as well as the speed at which they occur, may depend on the availability of environmental factors, management, and fertilizer input (Thakur et al., 2021). A major challenge in supporting the development of such biodiverse ecosystems remains as the choice of the species that will compose the association in order to achieve multifunctionality, whose underlying mechanisms shape the botanical proportion and productive performance of the association.

Functional characteristics of plant species may reveal agronomic and ecological strategies for resource use (Hanisch et al., 2020), allowing inferences on which species will thrive and which species will suffer competition when grown in association. This can have important implications as interactions among species not only affect individual agronomic performance of each species but can also affect overall performance of the association, such as biomass production and provision of other ecosystem services (Roscher et al., 2012; Plas et al., 2020). Among the functional characteristics that may be used, the dynamics of leaf elongation per tiller stand out, since it determines the structural characteristics of the pasture (final leaf length, number of leaves per tiller, and tiller population density), factors that result in canopy leaf area index and distribution of the leaf area from each species along the vertical profile of the canopy in the association. Along with the canopy foliage angle, these functional characteristics may shape canopy light interception, water, and nutrient uptake, which ultimately affect species perenniality, botanical proportion, forage yield, and nutritional value (Reis et al., 2014).

The mechanisms underlying the coexistence of plant species comprising an association are complex and dynamic. All species need the same resources to develop; light, CO_2, water, and mineral nutrients, which are acquired through similar processes (Roscher et al., 2011). The differentiation of niches among species is one of the hypotheses used to explain their coexistence, which is defined as differences in morphological, physiological, or phenological characteristics that allow for segregation of species and spatial and temporal partition resource capture (Tilman, 1997). In fertile environments, competition for light becomes the main limitation for the coexistence of species (Stevens et al., 2004; Clark and Tilman, 2008). Light is a directional resource that comes only from above and must be used immediately, offering a limited opportunity for spatial niche differentiation (Hiiesalu et al., 2012; Pärtel et al., 2012). In plant associations, species that are capable of rapid growth, or are taller, receive and intercept greater proportion of the incoming light when compared to understory species (Weiner, 1990), increasing the likelihood of competitive exclusion due to competition for light (Grime, 1973). Plant species in the understory can respond to low light availability through plasticity in several of their characteristics as a means to adapt and maximize capture of the available light, for example, increasing the proportion of chlorophylls, presenting smaller leaf mass and nitrogen per unit of leaf area, or by modifying shoot architecture to optimize leaf exposure and reduce self-shading (Niinemets, 2007; Roscher et al., 2011). Perennial pastures comprised of forage grasses cultivated in association are considered highly regulated ecosystems where any biotic or abiotic influence determines morphophysiological adaptations of its constituents that may modify canopy structure and plant species diversity. In spite of their potential of use for conceiving sustainable pastoral systems of animal production, there is still little information about the competitive strategies under those circumstances to optimize light capture among plants comprising the association.

Currently, studies involving associations of forage species in pastures are mainly directed to pastures in temperate climates or associations between grasses and legumes. On the other hand, studies in tropical conditions and environments are practically nonexistent, especially considering the association only among well-managed perennial tropical forage grasses. In the tropics, there is a great diversity of forage grass species and genotypes (Rios and Pitman, 2000; Fonseca and Martuscello, 2022) with different possibilities of combination in different biomes, which could provide solutions to guarantee ecosystem services from biodiverse pastures, mainly in areas where the recovery of degraded pastures is necessary. In addition, perennial forage grasses have a long history of co-evolution with herbivores, which resulted in adaptation to grazing, expanding their ability to store organic reserves to ensure resilience and persistence, which favors rapid regrowth and restoration of canopy leaf area after defoliation (Hodgson, 1990). Therefore, understanding the responses of such grasses cultivated in association and comparing them with their monocultures may help to understand their growth and development strategies and provide important information to formulate and manage new forage species associations.

In this context, the hypotheses of this study were: (i) the grass species with the best productive performance when cultivated in monoculture also presents the best productive performance when cultivated in association with other grass species with complementary strategies of exploration of ecological niches, and (ii) the functional characteristics of grass species explain the underlying mechanisms that shape the their botanical proportion and productive performance when cultivated in monoculture and in association. The objectives were: (i) to determine whether the grass species with the best productive performance when cultivated in monoculture also has the best productive performance when cultivated in association and (ii) to determine which are the underlying mechanisms that shape the botanical proportion and productive performance of grass species when cultivated in monoculture and in association.

2.2. Methodology

2.2.1. Study site

The study was conducted from December 2020 to March 2022 (Late spring 2020 to Summer 2022) in an experimental area of the Department of Animal Science of the "Luiz de Queiroz" College of Agriculture, University of São Paulo (ESALQ/USP), in Piracicaba, São Paulo, Brazil (22°42'35" South Latitude, 47°38'24" West Longitude and 546 m altitude). The climate of the region is Cwa (subtropical climate with dry Winter and hot Summer) (Köppen classification) (Alvares et al., 2013; Beck et al., 2018). Climatic data were collected in a Meteorological Station located approximately 2000 m from the experimental site (Figure S.2 supplementary material).

The soil is a Red Eutroferric Nitosol with a clayey texture (FAO, 2015) with the following chemical and physical characteristics at the 0 to 20 cm depth before the implementation of the experiment: 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% and sand content = 358 g kg^{-1}; clay = 446 g kg^{-1} and silt = 196 g kg^{-1}. The results indicated the need to increase base saturation of the soil, which was carried out by using dolomitic limestone aiming at reaching 70% (Raij et al. 1996) during late Winter 2019.

2.2.2. Treatments, experimental design, and management

Treatments corresponded to three tropical perennial forage grass species: *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass) and *Brachiaria brizantha* cv. BRS Piata (piata grass) cultivated in monoculture and in association (the three grass species in equal proportions based on the number of viable seeds). These were allocated to experimental units (180 m^2 paddocks) according to a randomized complete block design with four replications. Paddocks were 12 x 15 m, and 3 m wide races separated blocks. All raceways and a 3 m wide strip around the experimental area were kept free of vegetation by frequent mowing.

The experimental area was seeded in January 2020, through broadcast sowing using a seeding rate equivalent to 300 pure-viable seeds m^{-2} (1/3 for each grass species in the association - 100 pure-viable seeds m^{-2}), followed by compaction with a roller compactor weighing approximately 100 kg. All pastures were subjected to a common defoliation regime characterized by a pre-cutting canopy height of 35 cm and a post-cutting canopy height of 17.5 cm. Forage cuts were performed using a motorized brush cutter.

Canopy heights were monitored every three days during regrowth, starting soon after each cut. As canopy heights reached values close to the pre-cutting target of 35 cm, measurements became daily until paddocks reached their targeted canopy height for cutting. Measurements were made using a sward stick on 40 points per paddock distributed along four transect lines (Table S.1 supplementary material).

The common defoliation management used to all treatments (monocultures and the association) was based on the 95% canopy light interception criterion during the regrowth and its flexibility range to define the ideal moment to interrupt regrowth, ensuring maximum leaf dry matter accumulation (Sbrissia et al., 2018; Gomes, 2019). This corresponded to the 35 cm canopy height, which was used as the pre-cutting target. The post-cutting height was equivalent to 50% of the pre-cutting canopy height in order to leave generous residual leaf area (Giacomini et al., 2009) and ensure frequent non-severe defoliations, favoring adequate conditions for growth and development of all plants in the association (low disturbance level – defoliation severity, and low-stress level – competition for light). This was expected to provide adequate conditions for grass species to express their functional characteristics. Under no soil fertility limiting conditions, competition for light becomes the main factor determining grass species botanical proportion. In this scenario, frequent non-severe defoliations may result in favorable environment for both resource capture and resource conservation type grass species since the severity of disturbance (defoliation) and stress (competition for light) is reduced, allowing for their coexistence and persistence (Grime, 1977; Borer et al., 2014; Eskelinen et al., 2022).

Nitrogen fertilization was performed only during the rainy seasons of the year (Late Spring, Summer, and Early Autumn), always at post-cutting, using ammonium nitrate. The amount of nitrogen for each application was proportional to the cutting interval of each paddock (daily rate of 1.7 kg N ha^{-1}) and calculated to result in equal amounts of nitrogen applied to all paddocks at the end of each rainy season (Table S.2 supplementary material).

2.2.3. Measurements

2.2.3.1. Canopy light interception and foliage angle

Monitoring of canopy light interception (%) and foliage angle (º) was carried out concomitantly with measurements of canopy height using a LAI 2000 canopy analyzer (LI-COR, Lincoln, Nebraska, EUA). Measurements were carried out consistently at dawn or dusk from eight reading points per paddock in areas representative of the average sward condition at the time to sampling (visual assessment of herbage mass and height). A reference reading was taken above the canopy and five at ground level, totalizing eight readings above the canopy and forty readings at ground level per paddock.

2.2.3.2. Morphogenic development, and structural characteristics

Evaluations of morphogenic responses and structural characteristics were performed once every season of the year, using the marked tiller technique (Davies, 1993). Tillers were assessed at different intervals depending on climatic/growth conditions (3 or 4 days during Spring and Summer and 7 or 14 days during Autumn and Winter), starting soon after cutting until the new cut a the pre-cutting target of 35 cm. A total of 21 tillers per paddock were marked on the monoculture treatments and 36 on the association treatment (12 for each grass species). Each tiller was evaluated for stem length, leaf blade length, and leaves were classified as expanding, expanded, senescent, or dead. Stem length was measured from ground level to the ligule of the youngest fully expanded leaf. The length of the leaves was measured according to the stage of their development. For expanded leaves, leaf length was measured from the tip of the blade to its ligule. For expanding leaves, the same procedure was adopted, however, considering the ligule of the last fully expanded leaf as reference for measurements. Leaves were classified as expanding when the ligule was not exposed, expanded when the ligule was visible, senescent when the leaf blade showed signs of senescence (necrosis and/or yellowing) on up to 50% of its area, and dead when more than 50% of the leaf blade showed signs of senescence (Duru and Ducrocq, 2000) (Figure S.3 supplementary material). On leaves with less than 50% of the leaf blade showing signs of senescence, readings were taken from the ligule to the edge between green tissue and yellow/necrose tissue.

From these data, the following response variables were derived: (1) leaf elongation rate per tiller (cm tiller^{-1} day^{-1}) - the positive change in leaf lamina length between successive measurements (Equation 1); (2) final leaf length (cm leaf^{-1}), and (3) number of live leaves per tiller (n leaves tiller^{-1}).

$$\text{LER} = \sum (F_{\text{l}} - I_{\text{l}}) / E_{\text{d}} \qquad (1)$$

Where: LER are leaf elongation rate per tiller (cm tiller^{-1} day^{-1}), F_{l} and I_{l} are the final, and initial leaf length per tiller (cm), respectively; E_{d} evaluation duration (days). The leaf elongation rate per tiller represents the average rate of leaf elongation from all tiller leaves throughout the evaluation period.

2.2.3.3. Tiller population density, herbage mass, canopy leaf area index, and forage yield

Tiller population density (tiller^{-1} m^{2}), herbage mass (kg DM ha^{-1}), and leaf area index were quantified once every season of the year at the pre-cutting condition. Two representative points were sampled per paddock (visual evaluation of herbage mass and height) using a 100 × 25 cm metal frame and cutting all the herbage inside (cutting at ground level). Herbage samples were taken to the laboratory where the population density of total tillers and from each grass species individually in the association were determined. Subsequently, samples were homogenized, and a subsample separated to determine canopy leaf area index. The remaining part of the samples were dried in a forced draught oven at 60°C until constant weight. The results were used to calculate the sward herbage mass in the monocultures and for each grass species in the association (kg DM ha^{-1}) (i.e., herbage mass without dead material). Due to the difficulty of separating dead material by grass species in the association, all dead material in the association and monocultures was not included in the herbage mass calculation.

Leaf area was determined by scanning leaf blades from the subsample in a leaf area integrator model LI-3100 (Li-Cor, Lincoln, Nebraska, USA). Subsequently, scanned leaves were dried in a forced draught oven at 60°C until constant weight and date used to calculate the leaf area index (m^2 m^{-2}) for each grass species in monocultures and in the association.

Herbage accumulation was quantified throughout the experimental period. Two representative points per paddock were sampled (visual evaluation of herbage mass and height) using a 100 × 25 cm metal frame. Samples were collected when swards reached the targeted pre-cutting height (35 cm) and the cuts were performed at the targeted post-cutting height (17.5 cm). Subsequently, they were dried in a forced draft oven at 60°C until constant weight. Data were used to calculate the number of regrowth cycles, average herbage accumulation per cycle (kg DM ha^{-1}), and total forage yield (kg DM ha^{-1}) throughout the experiment.

The relative yield, relative number of leaves per tiller, relative final leaf length, relative leaf elongation rate per tiller, and relative leaf senescence rate were estimated as the ratio between data from grass species grown in association divided by the corresponding data from grass species grown in monoculture. Values close to the dotted line indicate similarity, values above indicate superiority of plants when grown in association and values below indicate superiority of plants grown in monoculture.

2.2.3.4. Vertical distribution of grass species and leaf area index along the vertical profile of the sward canopy

The vertical distribution of grass species and leaf area index along the vertical profile of the sward canopy in the association was assessed at pre-cutting every season of the year using the inclined point quadrat method (Wilson, 1960; Laca et al., 2000). Measurements were made on areas representative of the average sward condition at the time of sampling (visual assessment of herbage mass and canopy height). A minimum of 100 touches per paddock was used as reference and results were expressed as percentage of touches in each grass species relative to the total number of touches

The vertical distribution of canopy leaf area index was calculated similarly to the botanical composition by dividing the canopy leaf area index by the total number of touches in leaves (leaf area per touch) and leaf area distribution at 5 cm interval from the canopy top estimated by multiplying the result for the number of touches in leaves at the top 5 cm strata.

2.2.3.5. Calculation of thermal time and kinetics of regrowth

Data from the ESALQ/USP meteorological station were used (Figure S.2 supplementary material) for all calculations. Thermal time was expressed as growing degree-days (°C) from the beginning of the tillers evaluation period. Cumulative growing degree-days were calculated using a base temperature of 10°C (Silva et al., 2019) according to (Equation 2).

$$\text{GDD} = \sum_{i}^{n} \left[\left(\frac{T_{\text{max}} + T_{\text{min}}}{2} \right) - T_{\text{base}} \right] \tag{2}$$

Where: GDD are degree-days (°C), T_{max} and T_{min} are the maximum and minimum daily temperatures (°C), respectively, and T_{base} represents the base temperature (°C).

The kinetics of regrowth: leaf elongation rate per tiller in monoculture and in association was calculated during the regrowth period, from post-cutting to pre-cutting.

2.2.4. Statistical analysis

Statistical analyses were performed using R software (version 4.1.2; R Core Team, 2022). First, data were tested for normal distribution (Shapiro-Wilk test, $p < 0.05$) and homoscedasticity (Bartlett test, $p < 0.05$). The canopy leaf area index in monocultures during Late Spring I was transformed into the Log scale to test statistical difference. Subsequently, analyses of variance (ANOVA "aov" procedure) were used to test significant differences among treatments and grass species in monoculture and in association. Differences were considered significant when $p < 0.05$. Finally, significant differences between means were tested by the Least Significant Difference test. Pearson correlation matrix and principal components analysis were used to evaluate the relationship among the functional traits of the plants.

2.3. Results

2.3.1. Herbage mass and forage yield in monocultures and in the association

In monoculture, herbage mass varied with treatments in Late Spring I, Summer I, Autumn/Winter/Early Spring and Summer II ($p < 0.01$). The grass species with greatest herbage mass in monoculture was not the same in the association. In Late Spring I and Summer I, piata grass and massai grass presented similar herbage mass, with smallest values recorded for andropogon grass. During Autumn/Winter/Early Spring and Summer II, greatest herbage mass was recorded for piata grass. In the association, the contribution of different grass species to sward herbage mass varied with season of the year ($p < 0.05$). In general, massai grass showed the greatest herbage mass, except during Autumn/Winter/Early Spring and Late Spring II, when there was no difference between piata grass and massai grass (Figure 1 a). Relative yield was very different for andropogon grass and piata grass. The results indicated that no grass species growing in the association presented greater herbage mass than its monoculture. However, massai grass presented closer proximity values, mainly in Late Spring I and Summer I (Figure 1 b).

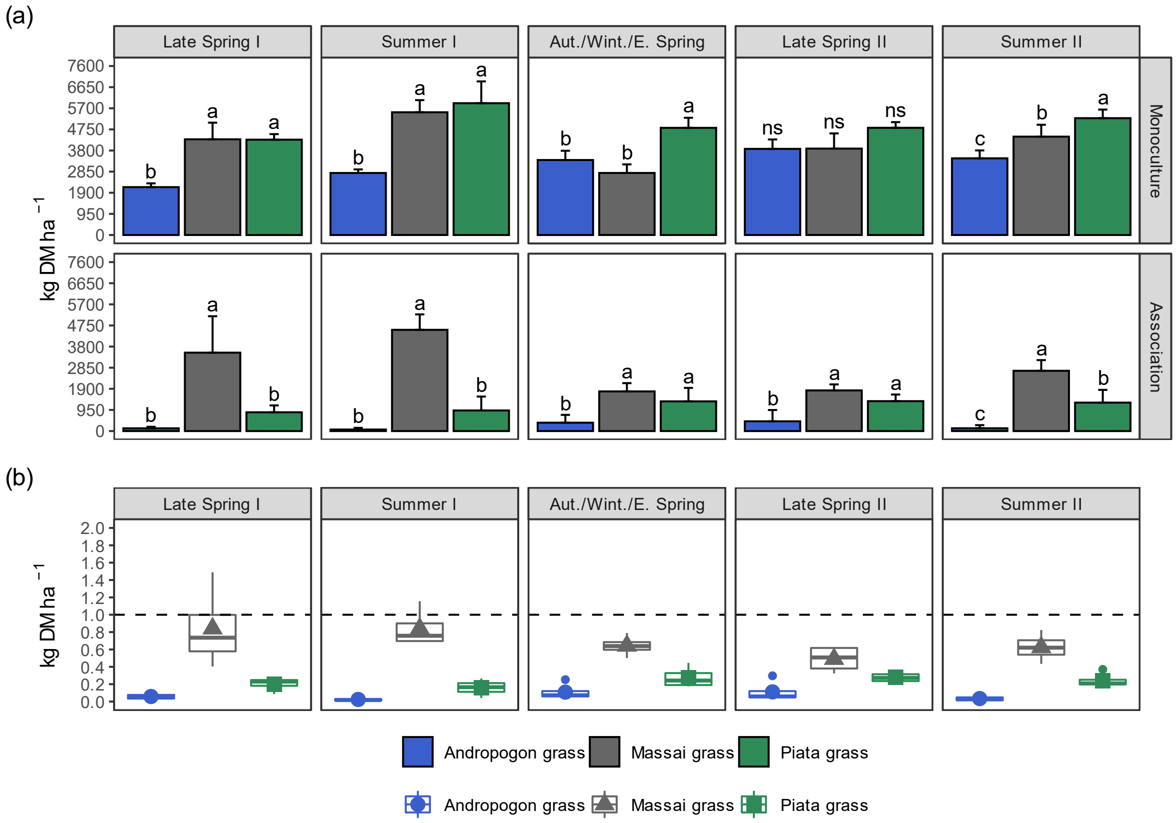


Figure 1. Herbage mass in monocultures and in the association (a), and relative yield (association/monoculture) (b) of andropogon, massai and piata grass cultivated as monocultures and in association.

The number of regrowth cycles ($p < 0.001$), herbage accumulation per regrowth cycle ($p < 0.001$) and forage yield ($p < 0.021$) varied with treatments. Piata grass had the smallest number of regrowth cycles and greatest herbage accumulation per cycle. In contrast, massai grass, the association, and andropogon grass had greater number of regrowth cycles with smaller herbage accumulation per cycle, which resulted in similar forage yield for piata grass, massai grass, and the association (Figure 2 a b c) (Figure S.5 supplementary material). In the association, the contribution of different grass species varied in herbage accumulation per cycle ($p < 0.001$) and forage yield ($p < 0.001$). With higher values for greatest for massai grass and smallest for andropogon grass (Figure 2 d e).

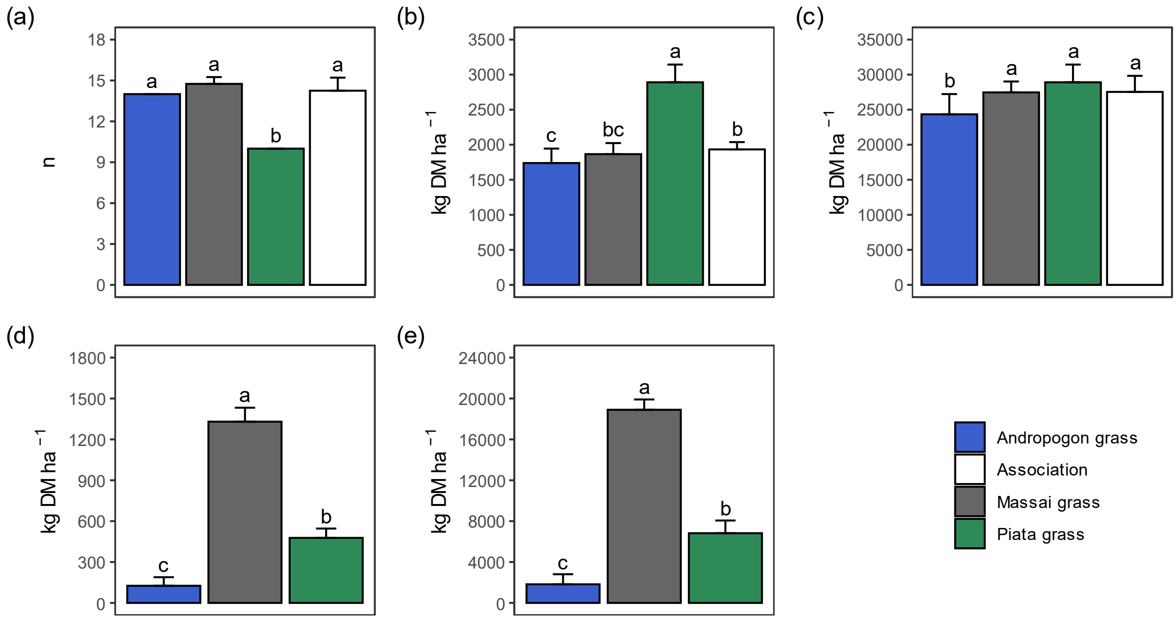


Figure 2. Number of regrowth cycles (a), herbage accumulation per regrowth cycle (b), forage yield (c), herbage accumulation per cycle for each grass species in the association (d), and forage yield for each grass species in the association (e) of andropogon, massai and piata grass cultivated as monocultures and in association (2020/11/30 to 2022/03/07).

2.3.2. Components of light interception in the monocultures and in the association

In the monocultures, canopy leaf area index varied with treatments during Late Spring I, Summer I and Autumn/Winter/Early Spring ($p < 0.05$). In Late Spring I and Summer I, canopy leaf area index was similar for massai grass and piata grass, both greater than andropogon grass. During Autumn/Winter/Early Spring, canopy leaf area index was greater for piata. In the association, the contribution of grass species to canopy leaf area index varied in Late Spring I, Summer I, Late Spring II, and Summer II ($p < 0.05$). In general, massai grass showed greater leaf area index than massai grass and andropogon grass, except during Late Spring II, when leaf area index from piata grass was similar to that of massai grass (Figure 3 a).

The distribution of the leaf area along the vertical profile of the sward canopy in the monocultures showed greater proportion of piata grass. This proportion was consistent during all seasons of the year and was more evident in the upper part of the canopy and during Autumn/Winter/Early Spring. In the association, massai grass had the greater proportion of the leaf area along the vertical profile of the canopy. During Autumn/Winter/Early Spring and Late Spring II, piata grass and massai grass showed similar proportion of leaf area (Figure 3 b).

The percentage of grass species along the vertical profile of the canopy in the association revealed a similar pattern of distribution of the leaf area, with greater proportion of massai grass, followed by piata grass and andropogon grass, with lesser differences during Autumn/Winter/Early Spring (Figure 3 c).

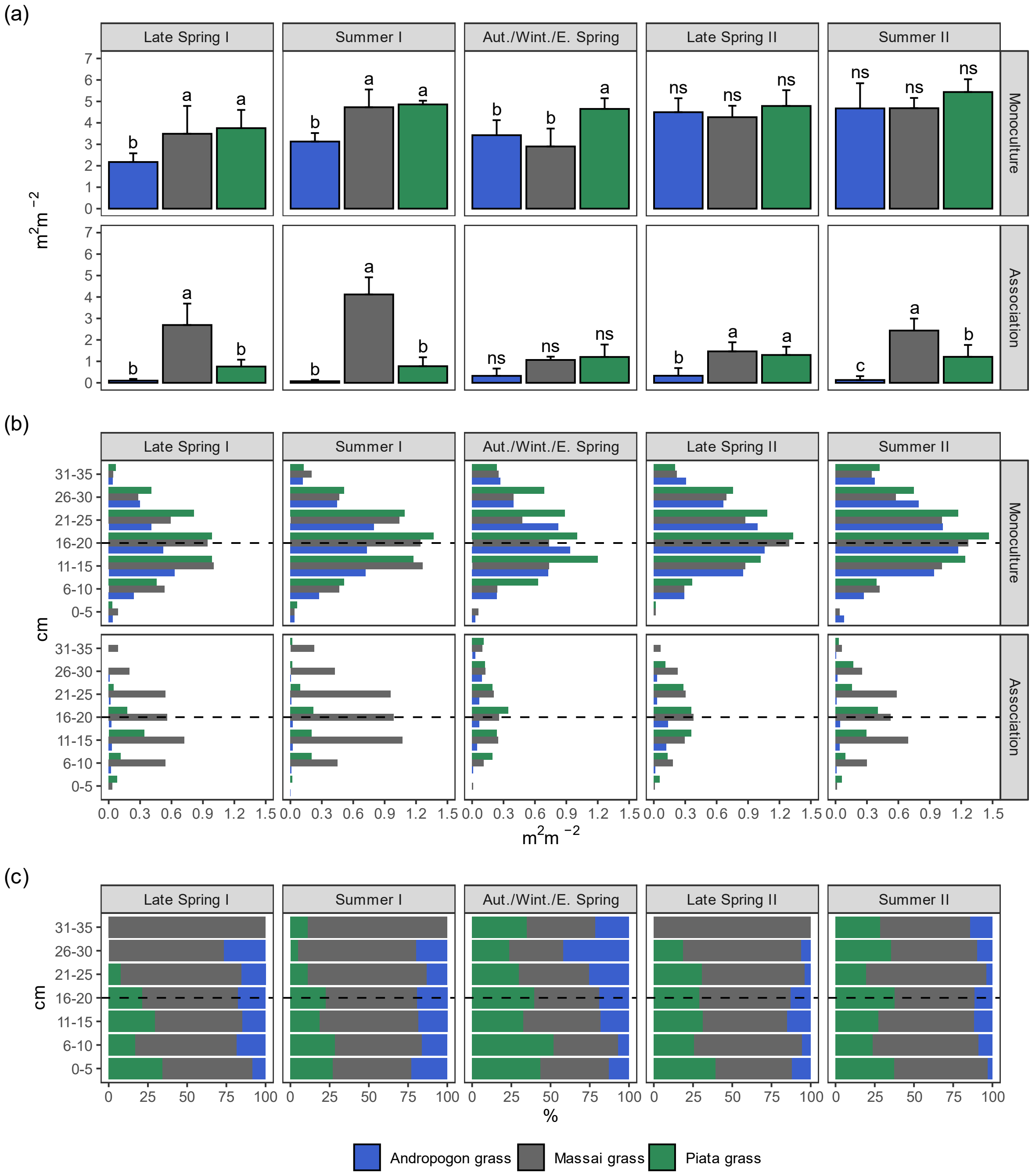


Figure 3. Canopy leaf area index in monocultures and in the association (a), vertical distribution of canopy leaf area index along the vertical profile of the canopy in monoculture and in the association (b), and percentage of grass species along the vertical profile of the canopy in the association (c) of andropogon, massai and piata grass cultivated as monocultures and in association.

Canopy foliage angle varied with treatments in Late Spring I, Summer I, Late Spring II, and Summer II ($p < 0.05$). Greater values were recorded for massai grass, followed by the association, piata grass and andropogon grass, respectively (Figure 4 a).

Canopy light interception varied with treatments during Late Spring I and Summer I ($p < 0.05$). In Late Spring I, canopy light interception was greater for piata grass compared to massai grass. In Summer I, values were greater for piata grass compared to andropogon grass, with intermediate values recorded for the association and massai grass (Figure 4 b).

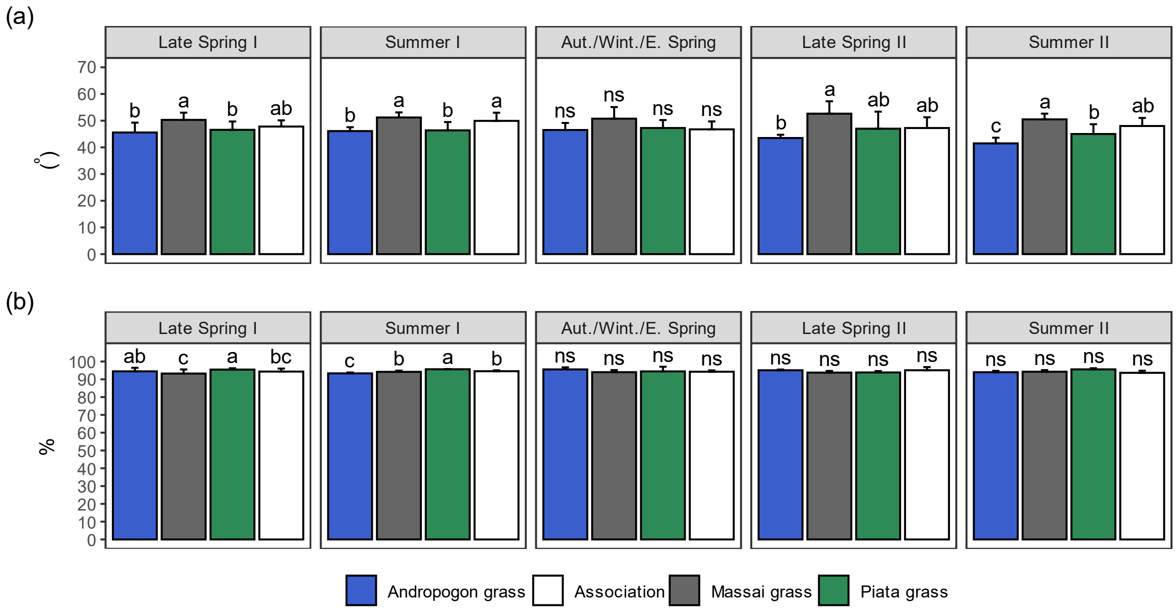


Figure 4. Canopy foliage angle (a), and canopy light interception (b) of andropogon, massai and piata grass cultivated as monocultures and in association.

2.3.3. Components of canopy leaf area index formation in the monocultures and in the association

In monoculture, the number of live leaves per tiller varied with treatments during the Late Spring I, Summer I, Late Spring II, and Summer II ($p < 0.01$). In Late Spring I, Summer I, and Late Spring II, massai grass and piata grass showed similar number of live leaves per tiller with smaller values recorded for andropogon grass. During Summer II, greater values were recorded for massai grass relative to andropogon grass, with intermediate values recorded for piata grass. In the association, the contribution of grass species varied in the number of live leaves per tiller in all year seasons ($p < 0.05$). In Late Spring I, recorded values were greater for piata grass relative to andropogon grass, with intermediate values recorded for massai grass. During Summer I, Late Spring II, and Summer II, similar values were recoded for massai grass and piata grass, both greater than those recorde for andropogon grass. During

Autumn/Winter/Early Spring, greater values were recorded for piata grass relative to andropogon grass (Figure 5 a).

The relative number of live leaves per tiller indicated greater values for andropogon grass in the association during Late Spring I and Late Spring II. A slight superiority of piata grass was also observed in the association in Late Spring I, Autumn/Winter/Early Spring, and Summer II (Figure 5 b).

In monoculture, final leaf length varied with treatments during the Late Spring I, Summer I, Late Spring II, and Summer II ($p < 0.05$). Andropogon grass showed greater final leaf length than massai and piata grass consistently throughout the experimental period. In the association, the contribution of grass species varied in final leaf length in all year seasons ($p < 0.05$). In Late Spring I, recorded values were greater for massai grass relative to piata grass. During Summer I, Late Spring II and Summer II, values were greater for andropogon grass relative to piata grass, with intermediate values recorded for massai grass. During Autumn/Winter/Early Spring, greatest values were recorded for massai grass (Figure 5 c).

Data from the relative final leaf length indicated that final leaf length of andropogon grass grown in association was smaller than those when grown as monoculture during Late Spring I, Summer I, Autumn/Winter/Early Spring, and Late Spring II. Smaller values were also recorded for piata grass growing in association in Late Spring I, Summer I, Autumn/Winter/Early Spring, and Summer II (Figure 5 d).

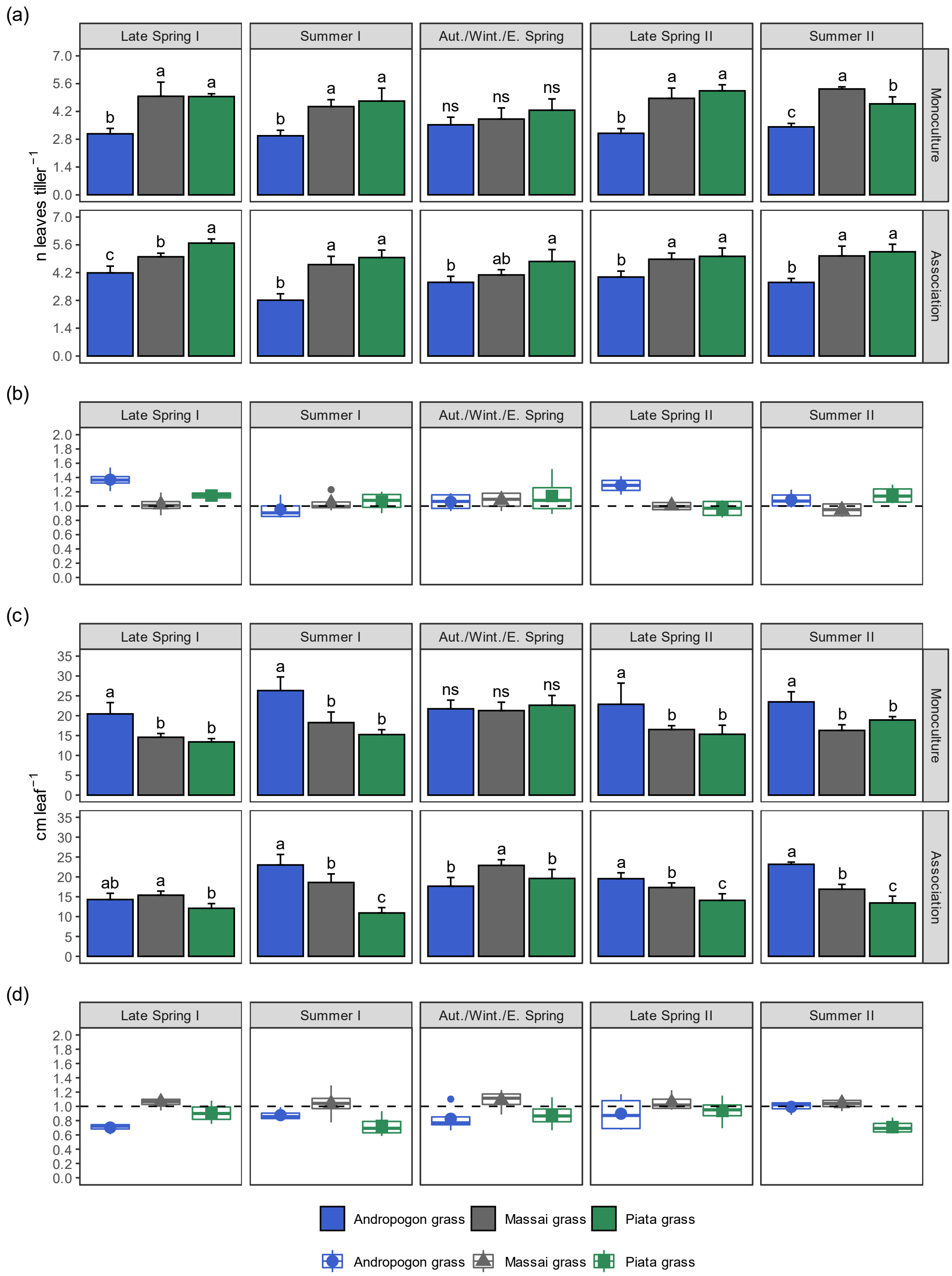


Figure 5. Number of live leaves per tiller in monoculture and in the association (a), relative number of live leaves per tiller (association/monoculture) (b), final leaf length in monoculture and in the association (c), and relative final leaf length (association/monoculture) (d) of andropogon, massai and piata grass cultivated as monocultures and in association.

In monoculture, leaf elongation rate per tiller varied with treatments during Late Spring I, Summer I, Autumn/Winter/Early Spring, and Summer II ($p < 0.01$). In Late Spring I, Summer I, and Summer II, greater values were recorded for massai grass and andropogon grass relative to andropogon grass. During Autumn/Winter/Early Spring, greater values were recorded for andropogon grass relative to massai grass, with intermediate values recorded for piata grass. In the association, the contribution of grass species varied in leaf elongation rate per tiller in the Late Spring I, Summer I, Late Spring II and Summer II ($p < 0.05$). During Late Spring I, greatest values were recorded for massai grass. During Summer I, recorded values were greater for massai grass relative to piata grass, with intermediate values recorded for andropogon grass. During Late Spring II and Summer II, recorded values for andropogon grass and massai grass were greater than those for piata grass (Figure 6 a).

The relative leaf elongation rate per tiller indicated that piata grass grown in the association had greater leaf elongation rate per tiller in Late Spring I and Late Spring II. Greater values were also observed for massai grass grown in the association during Autumn/Winter/Early Spring. Andropogon grass showed smaller values during Summer I, and Autumn/Winter/Early Spring, and greater values during Late Spring II (Figure 6 b).

In monoculture, the kinetics of regrowth followed a consistent pattern of response, with greater differences observed during Autumn/Winter/Early Spring. Overall, andropogon grass and massai grass showed similar growth kinetics, and piata grass showed slower response, indicating that this grass species has a longer interval between post-cutting and pre-cutting, a result that was confirmed by the long interval between cuttings recorded (Figure S.6 supplementary material). During Late Spring I and Late Spring II, massai grass showed a slightly superior response to andropogon grass, and during Summer I and Summer II, these two grass species showed similar growth kinetics. During Autumn/Winter/Early Spring, andropogon grass and piata grass showed faster growth kinetics relative to massai grass. In the association, the pattern of response for massai grass was similar to that of monocultures for all seasons of the year. During Late Spring I and Summer I, massai grass showed slightly faster kinetics than andropogon grass and piata grass. During Autumn/Winter/Early Spring, growth kinetics was similar for the three grass species. During Late Spring II and Summer II, andropogon grass and massai grass showed faster growth kinetics than piata grass (Figure 6 c).

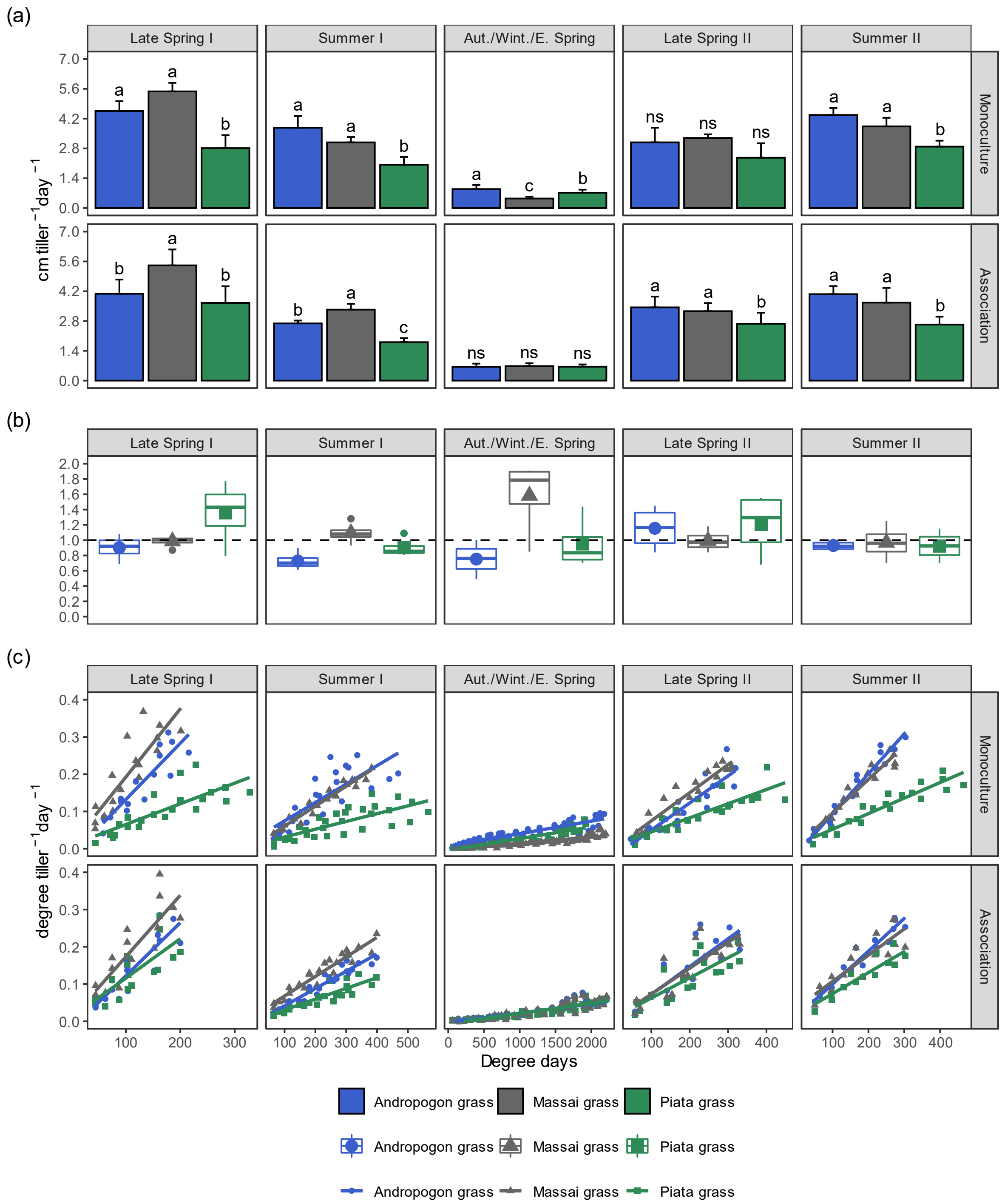


Figure 6. Leaf elongation rate per tiller in monoculture and in the association (a), relative leaf elongation rate per tiller (association/monoculture) (b), kinetics of regrowth of leaf elongation rate per tiller in monoculture and association (c) of andropogon, massai and piata grass cultivated as monocultures and in association.

In monoculture, tiller population density varied with treatments during all seasons of the year ($p < 0.05$). During Late Spring I and Autumn/Winter/Early Spring, greater values were recorded for andropogon grass and massai grass relative to piata grass. During Summer I, greater values were recorded for massai grass and piata grass. During Late Spring I, values recorded for andropogon grass greater than for piata grass. During Summer I, massai grass had greater tiller population density than piata grass. In the association, the contribution of grass species varied in tiller population density in Late Spring I, Summer I, Autumn/Winter/Early Spring, and Summer II ($p < 0.05$). Massai grass was the species with greatest tiller population density (Figure 7).

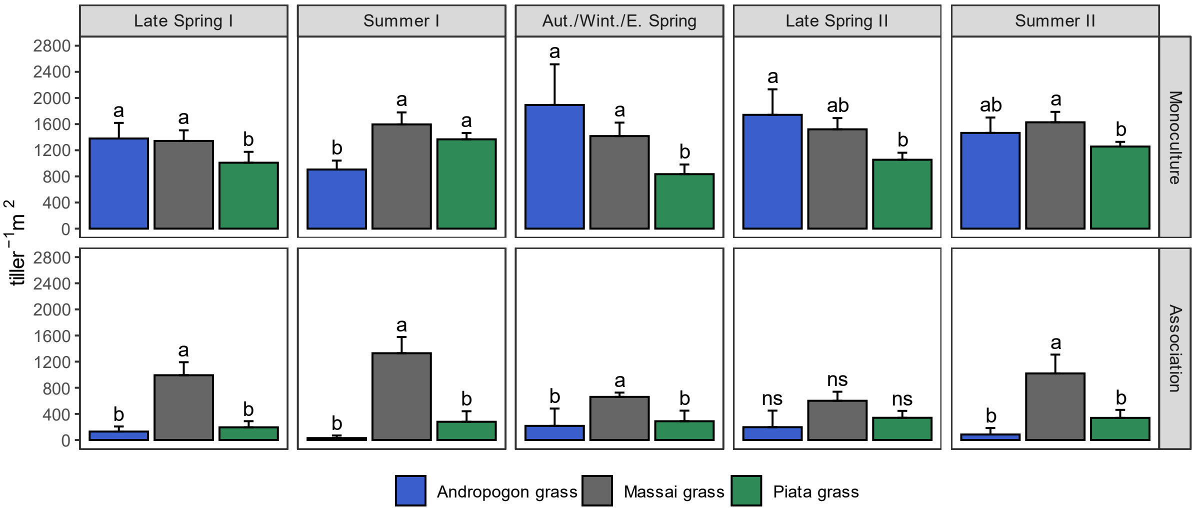


Figure 7. Tiller population density in monoculture and in the association of andropogon, massai and piata grass cultivated as monocultures and in association.

2.3.4. Multivariate analysis of functional traits determinants of the agronomic performance of grasses in monocultures and in the association

The results from the Pearson correlation analysis highlights strong correlations among functional traits (Figure 8 a b), and the principal component analysis (PCA) indicates the functional traits representing grass species (Figure 8 c d). For monocultures, the first principal component (PC1) explained 55.0% of the total variation in the dataset, and the second (PC2) explained 27.7% totaling 82.7% (Table S.3 supplementary material). The array of vectors in the PC1 × PC2 biplot show that piata grass was represented by large values of herbage mass, leaf area index, leaf area index in the upper 10 cm of the canopy, and canopy light interception, and small values of tiller population density and leaf elongation rate per tiller. Andropogon grass showed large final leaf length and small number of live leaves per tiller, herbage mass, canopy foliage angle, and leaf area index. Massai grass showed large canopy foliage angle, tiller population density, and leaf elongation rate per tiller, and small leaf area index in the upper 10 cm of the canopy, canopy light interception, and final leaf length (Figure 8 a c).

In the association, the first principal component (PC1) explained 48.9% of the total variation of the dataset, and the second (PC2) explained 26.6%, totaling 75.5% (Table S.3

supplementary material). The array of vectors in the PC1 × PC2 biplot showed that the massai grass was represented by large values of herbage mass, leaf area index, leaf area index in the upper 10 cm of the canopy, tiller population density, and leaf elongation rate per tiller. The andropogon grass showed large final leaf length and small number of live leaves per tiller. Piata grass showed small final leaf length and leaf elongation rate per tiller values. The contribution of canopy foliage angle and light interception was small (Figure 8 b d).

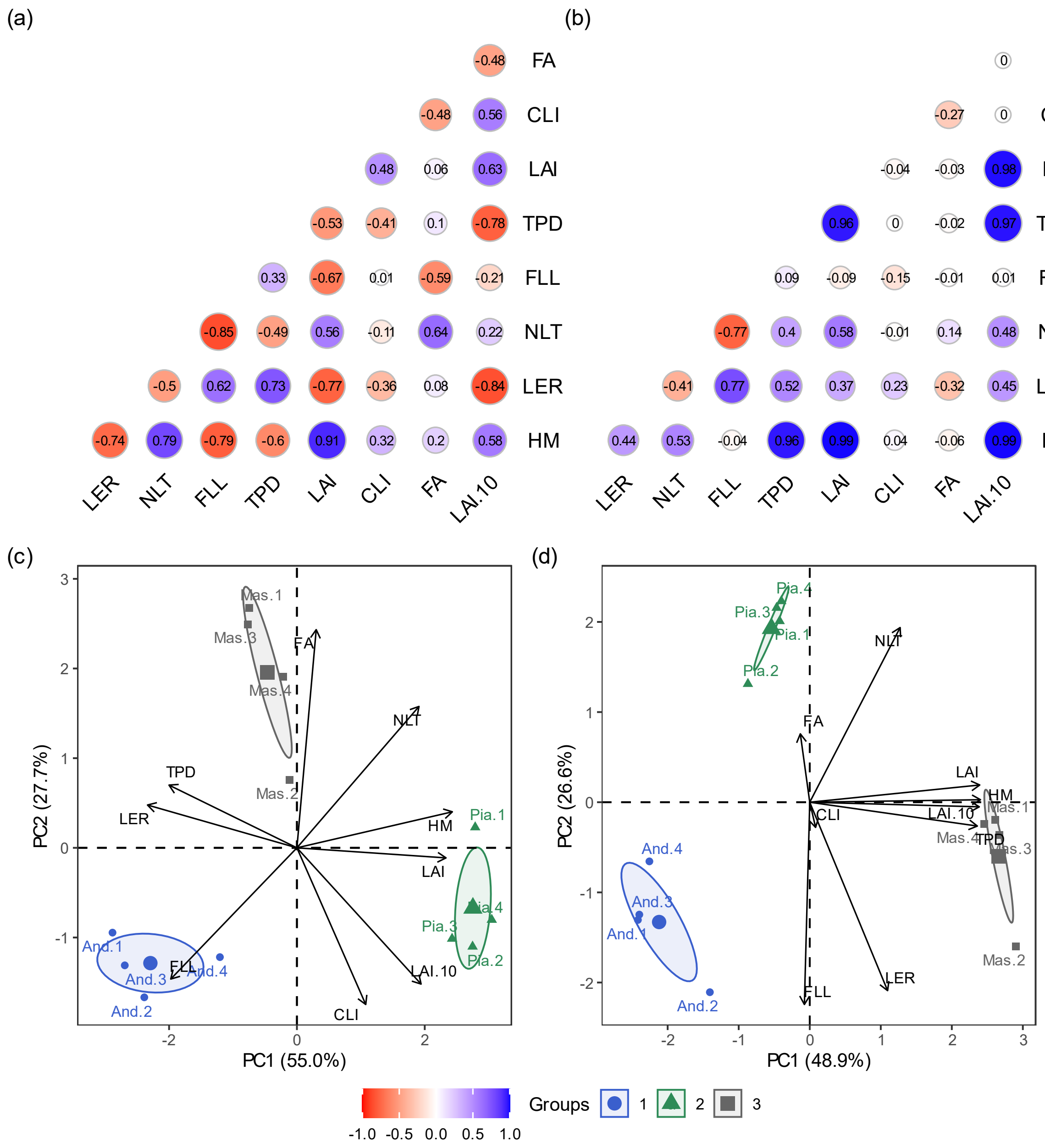


Figure 8. Pearson correlation matrix, monoculture (a), and association (b). Principal components analysis, biplot PC1 x PC2 in monoculture (c), and biplot PC1 x PC2 in association (d). The data correspond to the average of the five seasons evaluated. Legend of the functional grass species traits: leaf elongation rate per tiller (cm tiller^{-1} day^{-1}) (LER); number of leaves per tiller (n leaves tiller^{-1}) (NLT); final leaf length (cm leaf^{-1}) (FLL); tiller population density (tiller^{-1} m^{2}) (TPD); herbage mass (kg DM ha^{-1}) (HM); canopy light interception (%) (CLI); foliage

angle (º) (FA); leaf area index (m^2 m^{-2}) (LAI); and leaf area index in the upper 10 cm (m^2 m^{-2}) (LAI.10). Legend of the grass species: andropogon grass (And.); massai grass (Mas.); and piata grass (Pia.). Blocks (1, 2, 3 and 4). In the association, the variables FA, and CLI were the same for the three grass species because they are variables that it is not possible to determine for each grass species separately in the association.

2.4. Discussion

The botanical diversity or smaller competitive differences between the plant species that make up an association depends on the supply of nutrients and management of the forage harvest, where the supply of nutrients acts as a mechanism that maximizes the competition for resources between the species of plants that compose the association, and herbivores act as relievers of these competitions (Clark and Tilman, 2008; Borer et al., 2014; Eskelinen et al., 2022). The central hypothesis that explains the mechanisms and links the effects of nutrients and herbivory on botanical proportion has been competition for light (Holt et al., 2004; Borer et al., 2014; Eskelinen et al., 2022). The addition of nutrients promotes the growth of taller plants with greater canopy coverage and greater access to light, as growth increases, the availability of light for understory species is reduced, leading to their exclusion by species of faster growth or taller ones that appropriate this directionally provided resource (Hautier et al., 2009; DeMalach et al., 2017). In contrast, herbivores consuming vegetation and prioritizing mainly taller species can directly reduce canopy cover and increase light availability for plants in the understory (DeMalach et al., 2017; Eskelinen et al., 2022). In this context, the sustainable intensification of biodiverse pastoral ecosystems, aiming less competition for light, may be centered on the ideal balance between nutrient supply and management of animal forage harvesting.

In the present study, in order to reduce competition for light, a common intermittent defoliation management criterion was used for all treatments (monocultures and association) based on the criterion of 95% interception of canopy light during regrowth and its range of flexibility to define the ideal time to stop regrowth (Sbrissia et al., 2018; Gomes, 2019). In this context, considering this management criterion, the regrowth of the grass species should be interrupted when they reach 50 cm in height for andropogon grass (Souza et al., 2010), 55 cm for massai grass (Barbosa et al., 2010) and 35 cm for piata grass (Crestani et al., 2017). However, considering the pre-grazing flexibilization range, all treatments were managed at 35 cm of pre-cutting height. The results confirmed that the treatments presented light interception very close to the target (Figure 4 b). The post-cut height was equivalent to 50% of the pre-cut height to leave a generous residual leaf area (Giacomini et al., 2009). Despite these management criteria adopted, the results generally indicated that in the herbage mass (Figure 1) and the herbage accumulation (Figure 2), the grass species with the highest productive performance in the monoculture was piata grass in the association, massai grass. The greater productive performance of massai grass in the association, for example, was shaped by the greater foliage angle (Figure 4 a) and the lower leaf area index in the upper 10 cm (Figure 3 b), which resulted in a greater proportion of light in the vertical profile of the canopy and favoring rapid leaf elongation (Figure 6). This fact resulted in a higher population density of tillers (Figure 7), favoring the shading of the other two grass species in the association and causing competition for light.

The greater productive performance and botanical proportion of massai grass in the association may also be related to the criterion of the flexibility of pre-cutting height targets. Although the massai grass was the most flexibilized plant, going from 55 cm to 35 cm, this flexibility may have caused the leaf blades to always remain in the most vertical position and reach 35 cm before inflection, as observed in the results of the angle of the foliage (Figure 4a), as well as visually in (Figure S.4 supplementary material). This flexibility strategy means that the critical leaf area index is reached in lower pastures with a high population density of small tillers as long as the resistance limit of the plant is respected (Sbrissia et al., 2018; Gomes, 2019). This higher proportion of small tillers with newly expanded and expanding leaves have greater photosynthetic efficiency than mature and/or senescent leaves, responsible for approximately 75% of plant photosynthesis (Parsons et al., 1988). In addition, the greater foliage angle observed in massai grass may have modified the light environment inside the canopy, activated dormant meristems at the base of stems, and stimulated a higher population density of tillers (Figure 7). Although the light interception of the canopy, which was measured only at ground level, did not show great differences between treatments (Figure 4 b), the data indicated that the massai grass in monoculture has a lower leaf area index in the upper 10 cm of the canopy (Figure 3 b), a fact that helps to validate the hypothesis of greater penetration of light into the vertical layer of the canopy in massai grass.

The lower productive performance of andropogon grass, both in monoculture and in association, may be related to two main results observed in its functional traits and adopted management. The first is that although it had the highest final leaf length (Figure 5 c), it had the lowest number of leaves per tiller (Figure 5 a), indicating that the greater number of leaves was more important for productive performance when compared to the greatest final length of leaves. The second relates to a morphological structure called "false petiole" (Figure S.3 supplementary material). In the post-cutting, the leaves had this structure with little remaining leaf area. In response, the tiller killed these leaves and released new leaves as a strategy to seek the light. These results were confirmed in the leaf senescence rate data (Figure S.7 supplementary material). Andropogon grass also showed leaf elongation rate and regrowth kinetics of leaf elongation rate equal to or greater than massai grass (Figure 6), even so, it showed much lower productive performance. These results indicate that in addition to the ability to elongate the leaves, it is necessary to have a greater number of leaves for good productive performance and competitive capacity.

The lower leaf elongation rate per tiller observed for piata grass may have been caused by the combination of a higher leaf area index (Figure 3) and lower foliage angle (Figure 4), resulting in the self-shading of leaves at the base of the canopy. Leaves that develop in the lower portions of the canopy and are therefore adapted to shade have limited photosynthetic capacity, even when exposed to high light intensities (Woledge, 1973). In addition, the quality spectrum of visible sunlight, which ranges from violet (400nm) to red (700nm), can change as it penetrates along the canopy profile towards the ground. Thus, sunlight that reaches the lowest strata of the canopy, located closest to the ground, where most tillering takes place, is deficient in red light, and tillering is reduced (Davies, 1974; Skinner and Nelson, 1992). In piata grass, the management criterion used did not result in flexibility in the height target, and this may have shaped the higher leaf area index (Figure 3) and lower foliage angle (Figure 4), in which there was little light penetration red throughout the canopy and showed a low rate of leaf elongation (Figure 6) and low tiller population density (Figure 7).

In a general context, the management criterion adopted together with the plants' functional traits and morphophysiological characteristics shaped the dynamics of competition for light in the association. Massai grass was favored by the management criterion in which it was the most flexible grass species and caused a greater angle of the foliage and penetration of red light into the deeper layers of the canopy, which promoted a higher rate of leaf elongation (Figure 6) and greater tiller population density (Figure 7). Andropogon grass was the second most flexible grass species, however, it was the one that presented the lowest productive performance, probably caused by the lower number of leaves per tiller (Figure 5) and "false petiole" (Figure S.3 supplementary material). And finally, piata grass, because it was the grass species that was not flexible in the management adopted, a fact that resulted in a higher leaf area index (Figure 3) and a smaller foliage angle (Figure 4), in which there was little penetration of red light along the of the canopy and showed a low rate of leaf elongation (Figure 6) and low tiller population density (Figure 7).

In future studies involving grass associations and aiming at less competition for light, combining grass species with smaller differences in canopy height might be interesting, considering the management criterion based on 95% light interception. It is also interesting to choose grass species with a similar number and size of leaves per tiller, which results in the same leaf area index, to avoid competitive advantages due to morphological characteristics. Finally, test the frequency and intensity of defoliation. Generally, under frequent defoliation, usually associated with continuous stocking, competition for light is low due to the constant removal of the leaf area. In this condition, the grass species develop a photomorphogenic response to more constant light availability since, at each defoliation, only a part of the leaf area is removed, and the structure of the canopy does not undergo major changes (Mazzanti and Lemaire, 1994). On the other hand, in situations of intermittent stocking, competition for light increases continuously during the regrowth period, and with each defoliation, there is a rapid change in the quantity and quality of light absorbed (Sbrissia et al., 2007).

2.5. Conclusions

The grass species with the highest productive performance in the monoculture differ from those with the highest productive performance in the association. Among the monocultures, piata grass presented a greatest herbage mass, but in the association massai grass had greatest herbage mass. Piata grass presented a smaller number of cycles and greater herbage accumulation per cycle. In contrast, andropogon grass, massai grass, and the association presented greater number of cycles with smaller herbage accumulation per cycle, resulting in similar total forage yield for piata grass, massai grass, and the association. The association was mainly shaped by competition for light, with massai grass present in greater proportion and showing greater productive performance. The functional traits of grass species in monoculture indicated that massai grass had greater foliage angle and smaller leaf area index in the upper 10 cm of the sward canopy, which resulted in a larger proportion of light in the vertical profile of the canopy, causing fast leaf elongation per tiller, larger tiller population density, and shading of the others two grass species in the association. The smaller rate of leaf elongation per tiller observed for piata grass may have been caused by the combination of large leaf area index and smaller foliage angle, resulting in self-shading of leaves at the base of the sward canopy. Although andropogon grass presented the largest final leaf length, it presented smaller number of leaves per tiller, which explain its smaller proportion and productive performance both in the monoculture and in the association. The foliage angle, leaf elongation rate per tiller, number of leaves per tiller, and leaf area index are functional traits that shape the dynamics of the competition for light, botanical proportion, and productive performance of grass species in the association, and should be taken into account when choosing grass species for comprising new associations.

References

Alvares, C.A., Stape, J.E., Sentelhas, P.C., Gonçalves, J.L.M., Sparovek, G., 2013. Köppen's climate classification map for Brazil. Meteorologische Zeitschrift. 22 (6), 711–728. https://doi.org/10.1127/0941-2948/2013/0507

Barbosa, R.A., Rosa, P.M., Lima, G.O., 2010. Capim-massai manejado em diferentes combinações de intensidade e frequência de corte. In: Reunião Anual Da Sociedade Brasileira De Zootecnia. 47 ed. Salvador, Bahia, Brasil.

Beck, H., Zimmermann, N.E., McVicar, T.R., Vergopolan, N., Berg, A., Wood, E.F., 2018. Data Descriptor: Present and future Köppen-Geiger climate classification maps at 1-km resolution. Scientific Data. 5 (180214). https://doi.org/10.1038/sdata.2018.214

Borer, E.T., Seabloom, E.W., Gruner, D.S., Harpole, W.S., Hillebrand, H., Lind, E.M., Adler, P.B., Alberti, J., Anderson, T.M., Bakker, J.D., Biederman, L., Blumenthal, D., Brown, C.S., Brudvig, L.A., Buckley, Y.M., Cadotte, M., Chu, C., Cleland, E.E., Crawley, M.J., Daleo, P., Damschen, E.I., Davies, K.F., DeCrappeo, N.M., Du, G., Firn, J., Hautier, Y., Heckman, R.W., Hector, A., HilleRisLambers, J., Iribarne, O., Klein, J.A., Knops, J.M.H., La Pierre, K.J., Leakey, A.D.B., Li, W., MacDougall, A.S., McCulley, R.L., Melbourne, B.A., Mitchell, C.E., Moore, J.L., Mortensen, B., O'Halloran, L.R., Orrock, J.L., Pascual, J., Prober, S.M., Pyke, D.A., Risch, A.C., Schuetz, M., Smith, M.D., Stevens, C.J., Sullivan, L.L., Williams, R.J., Wragg, P.D., Wright, J.P., Yang, L.H., 2014. Herbivores and nutrients control grassland plant diversity via light limitation. Nature. 508, 517–520. https://doi.org/10.1038/nature13144

Callaway, R.M., 1995. Positive interactions among plants. The Botanical Review. 61 (4), 306–349. https://doi.org/10.1007/BF02912621

Catford, J.A., Dwyer, J.M., Palma, E., Cowles, J.M., Tilman, D., 2020. Community diversity outweighs effect of warming on plant colonization. Global Change Biology. 26, 3079–3090. https://doi.org/10.1111/gcb.15017

Chen, C., Chen, H.Y.H., Chen, X., Huang, Z., 2019. Meta-analysis shows positive effects of plant diversity on microbial biomass and respirelativen. Nature Communications. 10 (1332). https://doi.org/10.1038/s41467-019-09258-y

Clark, C.M., Tilman, D., 2008. Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. Nature. 451, 712–715. https://doi.org/10.1038/nature06503

Crestani, S., Mascheroni, J.D.C., Geremia, E.V., Carnevalli, R.A., Mourão, G.B., Da Silva, S.C., 2017. Sward structural characteristics and herbage accumulation of Piatã palisade grass (*Brachiaria brizantha*) in a crop–livestock–forest integration area. Crop and Pasture Science. 68, 859–871. https://doi.org/10.1071/CP16341

Da Silva, S.C., Sbrissia, A.F., Pereira, L.E.T., 2015. Ecophysiology of C 4 forage grasses - Understanding plant growth for optimising their use and management. Agriculture. 5, 598–625. https://doi.org/10.3390/agriculture5030598

Davies, A., 1974. Leaf tissue remaining after cutting and regrowth in perennial ryegrass. The Journal of Agricultural Science. 82, 165–172. https://doi.org/10.1017/S0021859600050334

Davies, A., 1993. Tissue turnover in the sward. In A. Davies, R. D. Baker, S. A. Grant, and A. S. Laidlaw (Eds.), Sward measurement handbook (p.183–216). London, UK: British Grassland Society.

DeMalach, N., Zaady, E., Kadmon, R., 2017. Light asymmetry explains the effect of nutrient enrichment on grassland diversity. Ecology Letters. 20, 60–69. https://doi.org/10.1111/ele.12706

Duru, M., Ducrocq, H., 2000. Growth and senescence of the suc-cessive leaves on a cocksfoot tiller. Effect of nitrogen and cutting regime. Annals of Botany. 85, 645–653. https://doi.org/10.1006/anbo.1999.1117

Eskelinen, A., Harpole, W.S., Jessen, M.T., Virtanen, R., Hautier, Y., 2022. Light competition drives herbivore and nutrient effects on plant diversity. Nature. 611, 301–305. https://doi.org/10.1038/s41586-022-05383-9

FAO IUSS Working Group., 2015. World Reference Base for Soil Resources 2014. International soil classification system for naming soils and creating legends for soil maps, Update 2015, World Soil Resources Reports 106. FAO, Rome. https://www.fao.org/3/i3794en/I3794en.pdf

Fonseca, D.M., Martuscello, J.A., 2022. Plantas forrageiras, 2 ed. Editora UFV. Universidade Federal de Viçosa, 591p.

Giacomini, A.A., Silva, S.C., Sarmento, D.O.L., Zeferino, C.V., Trindade, J.K., Souza Júnior, S.J., Guarda, V.A., Sbrissia, A.F., Nascimento Júnior, D.N., 2009. Components of the leaf area index of marandu palisadegrass swards subjected to strategies of intermittent stocking. Scientia Agricola. 66 (6), 721–732. http://dx.doi.org/10.1590/S0103-90162009000600002

Gomes, C.M. Oportunidade para flexibilização das metas pré-pastejo do manejo rotativo do capim-marandu. 2019. Dissertação (Mestrado em Ciência Animal e Pastagens) – Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, São Paulo.

Grime, J.P., 1973. Competitive exclusion in herbaceous vegetation. Nature. 242, 344–347. https://doi.org/10.1038/242344a0

Grime, J.P., 1977. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. The American Naturalist. 111, 1169–1194.

Gross, K., Cardinale, B.J., Fox, J.W., Gonzalez, A., Loreau, M., Polley, H.W., Reich, P.B., Ruijven, J., 2014. Species richness and the temporal stability of biomass production: A new analysis of recent biodiversity experiments. The American Naturalist. 183 (1). https://doi.org//10.1086/673915

Gross, N., Pinguet, Y.L.B., Liancourt, P., Berdugo, M., Gotelli, N.J., Maestre, F.T., 2017. Functional trait diversity maximizes ecosystem multifunctionality. Nature Ecology & Evolution. 1 (132). https://doi.org/10.1038/s41559-017-0132

Guay, M.O.M., Paquette, A., Dupras, J., Rivest, D., 2018. The new Green Revolution: Sustainable intensification of agriculture by intercropping. Science of the Total Environment. 615, 767–772. https://doi.org/10.1016/j.scitotenv.2017.10.024

Hanisch, M., Schweiger, O., Cord, A.F., Volk, M., Knapp, S., 2020. Plant functional traits shape multiple ecosystem services, their trade-offs and synergies in grasslands. Journal of Applied Ecology. 57, 1535–1550. https://doi.org/10.1111/1365-2664.13644

Hautier, Y., Niklaus, P.A., Hector, A., 2009. Competition for light causes plant biodiversity loss after eutrophication. Science. 324, 636–638. https://doi.org/10.1126/science.1169640

Hiiesalu, I., Öpik, M., Metsis, M., Lilje, L., Davison, J., Vasar, M., Moora, M., Zobel, M., Wilson, S.D., Pärtel, M., 2012. Plant species richness belowground: higher richness and new patterns revealed by next-generation sequencing. Molecular Ecology. 21, 2004–2016. https://doi.org/10.1111/j.1365-294X.2011.05390.x

Hodgson, J., 1990. Grazing management: science into practice. Essex, England, Longman Scientific & Technical, 203p.

Holt, R.D., Grover, J., Tilman, D., 2004. Simple rules for interspecific dominance in systems with exploitative and apparent competition. The American Naturalist. 144, 741–771. https://doi.org/10.1086/285705

Li, W., He, S., Cheng, X., Zhang, M., 2021. Functional diversity outperforms taxonomic diversity in revealing short-term trampling effects. Scientific Reports. 11 (18889). https://doi.org/10.1038/s41598-021-98372-3

Li, X.F., Wang, Z.G., Bao, X.G., Sun, J.H., Yang, S.C., Wang, P., Wang, C.B., Wu, J.P., Liu, X.R., Tian, R.L., Wang, Y., Li, J.P., Wang, Y., Xia, H.Y., Mei, P.P., Wang, X.F., Zhao, J.H., Yu, R.P., Zhang, W.P., Che, Z.X., Gui, L.G., Callaway, R.M., Tilman, D., Li, L., 2020. Long-term increased grain yield and soil fertility from intercropping. Nature Sustainability. 4, 943–950. https://doi.org/10.1038/s41893-021-00767-7

Loreau, M., 1998. Separating sampling and other effects in biodiversity experiments. Oikos, 82, 600–602. https://doi.org/10.2307/3546381

Loreau, M., Hector, A., 2001. Partitioning selection and complementarity in biodiversity experiments. Nature. 412, 72–76. https://doi.org/10.1038/35083573

Louarn, G., Barillot, R., Combes, D., Gutiérrez, A.E., 2020. Towards intercrop ideotypes: non-random trait assembly can promote overyielding and stability of species proportion in simulated legume-based mixtures. Annals of Botany. 126, 671–685. https://doi.org/10.1093/aob/mcaa014

Mazzanti, A, Lemaire, G., 1994. The effect of nitrogen fertilization upon herbage production of tall fescue swards continuously grazed with sheep. 2. Herbage growth dynamics. Grass and forage science. 49, 111–120. https://doi.org/10.1111/j.1365-2494.1994.tb02010.x

Niinemets, Ü., 2007. Photosynthesis and resource distribution through plant canopies. Plant, Cell & Environment. 30, 1052–1071. https://doi.org/10.1111/j.1365-3040.2007.01683.x

Parsons, A.J., Johnson, I.R., Harvey, A. 1988. Use of a model to optimize the interaction between frequency and severity of intermittent defoliation to provide a fundamental comparison of the continuous and intermittent defoliation of grass. Grass and Forage Science. 43, 49–59. https://doi.org/10.1111/j.1365-2494.1988.tb02140.x

Pärtel, M., Hiiesalu, I., Öpik, M., Wilson, S.D., 2012. Below-ground plant species richness: new insights from DNA-based methods. Functional Ecology. 26, 775–782. https://doi.org/10.1111/j.1365-2435.2012.02004.x

Pires, A.V., 2010. Bovinocultura de corte. 1 ed. Editora Fealq, Piracicaba, São Paulo, Brasil, 760p.

Plas, F., Georgi, T.S., Weigelt, A., Barry, K., Meyer, S., Alzate, A., Barnard, R., Buchmann, N., Kroon, H., Ebeling, A., Eisenhauer, N., Engels, C., Fischer, M., Gleixner, G., Hildebrandt, A., France, E.K., Leimer, S., Milcu, A., Mommer, L., Niklaus, P.A., Oelmann, Y., Roscher, C., Scherber, C., Lorenzen, M.S., Scheu, S., Schmid, B., Schulze, E.D., Temperton, V., Tscharntke, T., Voigt, W., Weisser, W., Wilcke, W., Wirth, C., 2020. Plant traits alone are poor predictors of ecosystem properties and long-term ecosystem functioning. Nature Ecology & Evolution. 4, 1602–1611. https://doi.org/10.1038/s41559-020-01316-9

Pontes, L.S., Maire, V., Louault, F., Soussana, J.F., Carrère, P., 2012. Impacts of species interactions on grass community productivity under contrasting management regimes. Oecologia. 168, 761–771. https://doi.org//10.1007/s00442-011-2129-3

Raij, B., Cantarella, H., Quaggio, J.A., Furlani, Â.M.C., 1997. Recomendações de adubação e calagem para o Estado de São Paulo. 2 ed. Instituto Agronômico de Campinas, São Paulo, Brazil, 285p.

Reis, R.A., Bernardes, T.F., Siqueira, G.R., 2014. Forragicultura - Ciência, tecnologia e gestão dos recursos forrageiros, 1 ed. Funep, 714p.

Rios, A.S., Pitman, W.D., 2000. Tropical forage plants, development and use, 1 ed. CRC Press., 404p.

Roscher, C., Kutsch, W.L., Kolle, O., Ziegler, W., Schulze, E.D., 2011. Adjustment to the light environment in small-statured forbs as a strategy for complementary resource use in mixtures of grassland species. Annals of Botany. 107, 965–979. https://doi.org/10.1093/aob/mcr044

Roscher, C., Schumacher, J., Gubsch, M., Lipowsky, A., Weigelt, A., Buchmann, N., Schmid, B., Schulze, E.D., 2012. Using plant functional traits to explain diversity–productivity relationships. PLOS ONE. 7 (5). https://doi.org/10.1371/journal.pone.0036760

Sbrissia, A.F., Da Silva, S.C., Nascimento Júnior, D., 2007. Ecofisiologia de plantas forrageiras e o manejo do pastejo. Simpósio sobre Manejo da Pastagem. FEALQ, Piracicaba, São Paulo, Brazil.

Sbrissia, A.F., Duchini, P.G., Zanini, G.D., Santos, G.T., Padilha, D.A., Schmitt, D., 2018. Defoliation strategies in pastures submitted to intermittent stocking method: underlying mechanisms buffering forage accumulation over a range of grazing heights. Crop Science. 58 (2), 945–954. https://doi.org/10.2135/cropsci2017.07.0447

Scherber, C., Eisenhauer, N., Weisser, W.W., Schmid, B., Voigt, W., Fischer, M., Schulze, E.D., Roscher, C., Weigelt, A., Allan, E., Bebler, H., Bonkowski, M., Buchmann, N., Buscot, F., Clement, L.W., Ebeling, A., Engels, C., Halle, S., Kertscher, I., Klein, A.M., Koller, R., König, S., Kowalski, E., Kummer, V., Kuu, A., Lange, M., Lauterbach, D., Middelhoff, C., Migunova, V.D., Milcu, A., Müller, R., Partsch, S., Petermann, J.S., Renker, C., Rottstock, T., Sabais, A., Scheu, S., Schumacher, J., Temperton, V.M., Tscharntke, T., 2010. Bottom-up effects of plant diversity on multitrophic interactions in a biodiversity experiment. Nature. 468, 553–556. https://doi.org/10.1038/nature09492

Silva, G.P., Da Silva, S.C., Guttiérrez, A.E., Lemaire, G., Louarn, G., 2019. Stem elongation in Pennisetum purpureum results from a fixed pattern of vegetative development potentially enhanced by the initiation of flowering. Grass and Forage Science. 74, 708–719. https://doi.org/10.1111/gfs.12449

Skinner, R.H., Nelson, C.J., 1992. Estimation of potential tiller production and site usage during tall fescue canopy development. Annals of Botany. 70, 493–499. https://www.jstor.org/stable/42758760

Sousa, B.M.L., Nascimento Júnior, D.N., Da Silva, S.C., Monteiro, H.C.F., Rodrigues, C.S., Fonseca, D.M., Silveira, M.C.T., Sbrissia, A.F., 2010. Morphogenetic and structural characteristics of andropogon grass submitted to different cutting heights. Revista Brasileira de Zootecnia. 39, 2141–2147. https://doi.org/10.1590/S1516-35982010001000006

Stevens, C.J., Dise, N.B., Mountford, J.O., Gowing, D.J., 2004. Impact of nitrogen deposition on the species richness of grasslands. Science. 303, 1876–1879. https://doi.org/10.1126/science.1094678

Thakur, M.P., Putten, W.H., Wilschut, R.A., Veen, G.F.C., Kardol, P., Ruijven, J., Allan, E., Roscher, C., Kleunen, M., Bezemer, T.M., 2021. Plant–Soil feedbacks and temporal dynamics of plant diversity–productivity relationships. Trends in Ecology & Evolution. 36, 651–661. https://doi.org/10.1016/j.tree.2021.03.011

Tilman D., 1997. Distinguishing between the effects of species diversity and species composition. Oikos. 80. https://doi.org/10.2307/3546532

Tilman, D., Reich, P.B., Isbell, F., 2012. Biodiversity impacts ecosystem productivity as much as resources, disturbance, or herbivory. Proceedings of the National Academy of Sciences (PNAS). 109 (26), 10394–10397. https://doi.org/10.1073/pnas.1208240109

Tilman, D., Reich, P.B., Knops, J., Wedin, D., Mielke, T., Lehman, C., 2001. Diversity and Productivity in a Long-Term Grassland Experiment. Science. 294, 843–845. https://doi.org/10.1126/science.1060391

Vandermeer, J.H., 1989. The ecology of intercropping. Cambridge: Cambridge University Press, 237p.

Volaire, F., Barkaoui, K., Norton, M., 2014. Designing resilient and sustainable grasslands for a drier future: Adaptive strategies, functional traits, and biotic interactions. European Journal of Agronomy. 52, 81–89. https://doi.org/10.1016/j.eja.2013.10.002

Wagg, C., Ebeling, A., Roscher, C., Ravenek, J., Bachmann, D., Eisenhauer,N., Mommer, L., Buchmann, N., Hillebrand,H., Schmid,B., Weisser, W.W., 2017. Functional trait dissimilarity drives both species complementarity and competitive disparity. Functional Ecology. 31 (12), 2320–2329. https://doi.org/10.1111/1365-2435.12945

Wang, S., Isbell, F., Deng, W., Hong, P., Dee, L.E., Thompson, P., Loreau, M., 2021. How complementarity and selection affect the relationship between ecosystem functioning and stability. Ecology. e03347. https://doi.org/10.1002/ecy.3347

Weiner, J., 1990. Asymmetric competition in plant populations. Trends in Ecology and Evolution. 5, 360–364. https://doi.org/10.1016/0169-5347(90)90095-U

Woledge, J., 1973. The photosynthesis of ryegrass leaves growth in a simulated sward. Annals of Applied Botany. 73, 229–237. https://doi.org/10.1111/j.1744-7348.1973.tb01329.x

Wright, A.J., Ebeling, A., Kroon, H., Roscher, C., Weigelt, A., Buchmann, N., Buchmann, T., Fischer, C., Hacker, N., Hildebrandt, A., Leimer, S., Mommer, L., Oelmann, Y., Scheu, S., Steinauer, K., Strecker, T., Weisser, W., Wilcke, W., Eisenhauer, N., 2015. Flooding disturbances increase resource availability and productivity but reduce stability in diverse plant communities. Nature Communications, 6 (6092). https://doi.org/10.1038/ncomms7092

Wright, A.J., Mommer,L., Barry, K., Ruijven, J., 2021. Stress gradients and biodiversity: monoculture vulnerability drives stronger biodiversity effects during drought years. Ecology. 102 (1). https://doi.org/10.1002/ecy.3193

Wright, A.J., Wardle, D.A., Callaway, R., Gaxiola, A., 2017. The overlooked role of facilitation in biodiversity experiments. Trends in Ecology & Evolution. 32 (5). https://doi.org/10.1016/j.tree.2017.02.011

Supplementary material

Table S.1. Pre- and post-cutting height (cm) of the experimental paddocks throughout the experimental period (mean ± standard error of the mean).

Season	Treatments				Mean
	Andropogon grass	Massai grass	Piata grass	Association	
Pre-cutting height (cm)					
Late Spring I	35.6 ± 0.10	35.4 ± 0.27	35.2 ± 0.08	35.6 ± 0.40	35.4
Summer I	35.1 ± 0.06	35.1 ± 0.06	35.3 ± 0.10	35.2 ± 0.13	35.2
Aut./Wint./E. Spring	35.1 ± 0.14	35.0 ± 0.06	32.7 ± 0.37	34.9 ± 0.21	34.4
Late Spring II	35.3 ± 0.15	35.3 ± 0.15	34.9 ± 0.11	35.1 ± 0.13	35.1
Summer II	35.4 ± 0.13	35.1 ± 0.14	35.1 ± 0.17	34.9 ± 0.06	35.1
Mean	35.3	35.2	34.6	35.1	
Post-cutting height (cm)					
Late Spring I	17.6 ± 0.20	17.2 ± 0.16	17.2 ± 0.22	17.3 ± 0.18	17.3
Summer I	17.6 ± 0.15	17.2 ± 0.14	17.5 ± 0.07	17.2 ± 0.10	17.4
Aut./Wint./E. Spring	17.6 ± 0.06	17.3 ± 0.13	17.5 ± 0.17	17.3 ± 0.13	17.4
Late Spring II	17.4 ± 0.13	17.1 ± 0.25	17.6 ± 0.24	17.5 ± 0.37	17.4
Summer II	17.5 ± 0.09	17.1 ± 0.04	17.3 ± 0.04	17.3 ± 0.06	17.3
Mean	17.5	17.2	17.4	17.3	

Table S.2. Amount of nitrogen applied until the day of data collection (mean ± standard error of the mean).

Season	Treatments				Mean
	Andropogon grass	Massai grass	Piata grass	Association	
Amount of nitrogen applied until data collection (kg ha^{-1})					
Late Spring I	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0
Summer I	80.7 ± 4.50	83.6 ± 4.47	74.8 ± 2.22	79.8 ± 4.26	79.7
Autumn	79.3 ± 4.50	76.4 ± 4.47	85.2 ± 2.22	80.2 ± 4.26	80.3
Winter/Early Spring	00.0 ± 0.00	00.0 ± 0.00	00.0 ± 0.00	00.0 ± 0.00	00.0
Late Spring II	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0
Summer II	62.9 ± 1.85	53.8 ± 5.15	47.5 ± 2.50	42.5 ± 4.22	51.7
Mean	50.5	49.0	47.9	47.1	

Table S.3. Principal Component Analysis (PCA) performed on a subset of functional traits of grass species grown in monoculture and in association.

Principal Components	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Monoculture									
LER	-0.404	0.117	0.223	0.368	-0.499	0.062	-0.018	-0.472	0.402
NLT	0.331	0.385	-0.139	0.238	-0.307	0.376	0.317	0.514	0.257
FLL	-0.341	-0.357	-0.158	0.202	-0.390	-0.435	0.344	0.374	-0.301
TPD	-0.346	0.171	0.526	-0.546	0.009	0.187	0.475	0.100	-0.068
HM	0.421	0.098	0.200	-0.061	-0.519	0.097	-0.083	-0.271	-0.641
CLI	0.186	-0.426	0.582	0.534	0.293	0.207	0.145	0.077	-0.070
FA	0.052	0.594	0.026	0.328	0.334	-0.453	0.365	-0.221	-0.192
LAI	0.404	-0.027	0.411	-0.206	-0.191	-0.610	-0.120	0.164	0.418
LAI.10	0.336	-0.371	-0.285	-0.172	-0.020	0.021	0.616	-0.456	0.224
Standard deviation	2.225	1.578	0.819	0.654	0.478	0.355	0.260	0.152	0.119
Proportion of variance	55.010	27.670	7.458	4.758	2.537	1.402	0.749	0.257	0.159
Cumulative proportion	55.010	82.680	90.138	94.896	97.434	98.836	99.585	99.841	100.000
Association									
LER	0.217	-0.560	0.087	0.082	-0.475	0.151	0.123	0.562	0.214
NLT	0.251	0.519	0.082	-0.024	-0.769	0.025	0.080	-0.238	-0.066
FLL	-0.015	-0.601	-0.302	0.060	-0.259	-0.199	-0.126	-0.594	-0.261
TPD	0.466	-0.071	-0.064	0.034	0.223	0.784	0.098	-0.309	0.054
HM	0.475	0.007	0.007	-0.006	0.105	-0.378	-0.358	-0.166	0.682
CLI	0.016	-0.075	0.711	0.661	0.062	-0.052	-0.064	-0.161	-0.123
FA	-0.026	0.203	-0.617	0.739	-0.018	0.019	-0.027	0.161	0.067
LAI	0.473	0.051	-0.040	-0.063	0.080	-0.066	-0.554	0.316	-0.592
LAI.10	0.472	-0.014	-0.056	0.031	0.210	-0.415	0.716	0.021	-0.209
Standard deviation	2.098	1.546	1.151	0.850	0.337	0.177	0.107	0.081	0.015
Proportion of variance	48.890	26.560	14.710	8.028	1.261	0.347	0.126	0.073	0.003
Cumulative proportion	48.890	75.450	90.160	98.191	99.451	99.798	99.925	99.997	100.000

Legend of the functional grass species traits: leaf elongation rate per tiller (cm tiller^{-1} day^{-1}) (LER); number of leaves per tiller (n leaves tiller^{-1}) (NLT); final leaf length (cm leaf^{-1}) (FLL); tiller population density (tiller^{-1} m^{2}) (TPD); herbage mass (kg DM ha^{-1}) (HM); canopy light interception (%) (CLI); foliage angle (º) (FA); leaf area index (m^{2} m^{-2}) (LAI); and leaf area index in the upper 10 cm (m^{2} m^{-2}) (LAI.10).

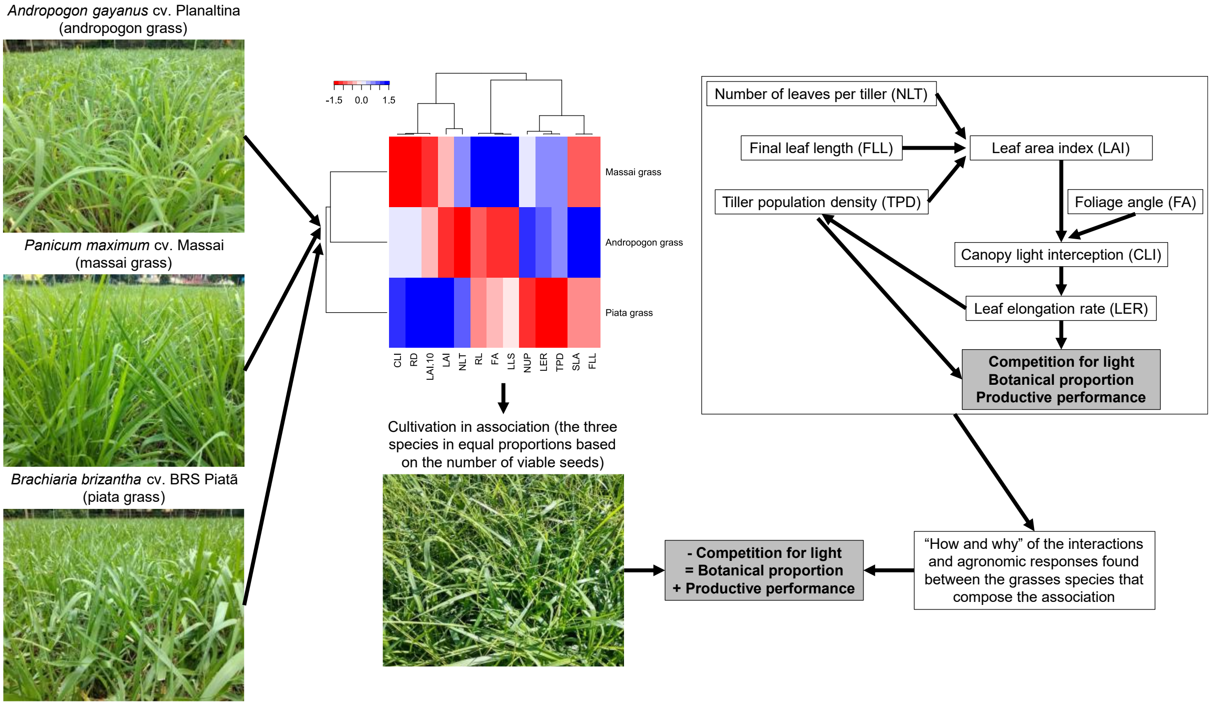


Figure S.1. Graphical abstract of chapter structure. Heatmap and cluster in monoculture of the functional traits of grass species. The data correspond to the average of five seasons. Legend: leaf elongation rate per tiller (cm tiller^{-1} day^{-1}) (LER); number of leaves per tiller (n leaves tiller^{-1}) (NLT); final leaf length (cm leaf^{-1}) (FLL); tiller population density (tiller^{-1} m^{2}) (TPD); canopy light interception (%) (CLI); foliage angle (°) (FA); leaf area index (m^{2} m^{-2}) (LAI); leaf area index in the upper 10 cm (m^{2} m^{-2}) (LAI.10); specific leaf area (cm^{2} g^{-1}) (SLA); leaf lifespan (days) (LLS); root diameter (mm) (RD); root length (cm m^{3}) (RL); and nitrogen concentration in upper leaves (%DM) (NUP).

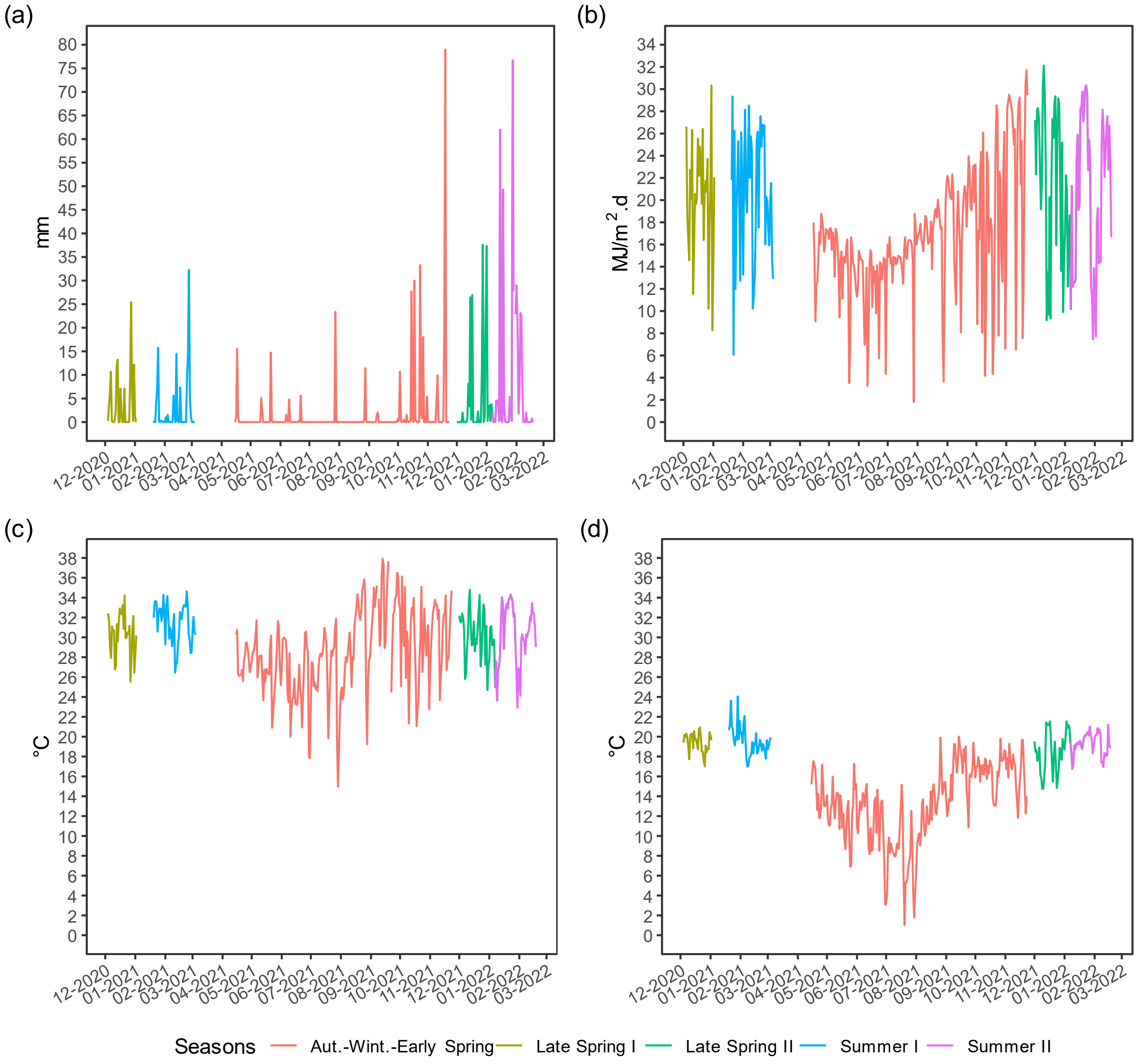


Figure S.2. Daily weather data of rainfall (mm) (a), radiation (MJ/m^2.d) (b), maximum air temperature (ºC) (c), and minimum air temperature (ºC) (d). Each color represents the period the data was collected.

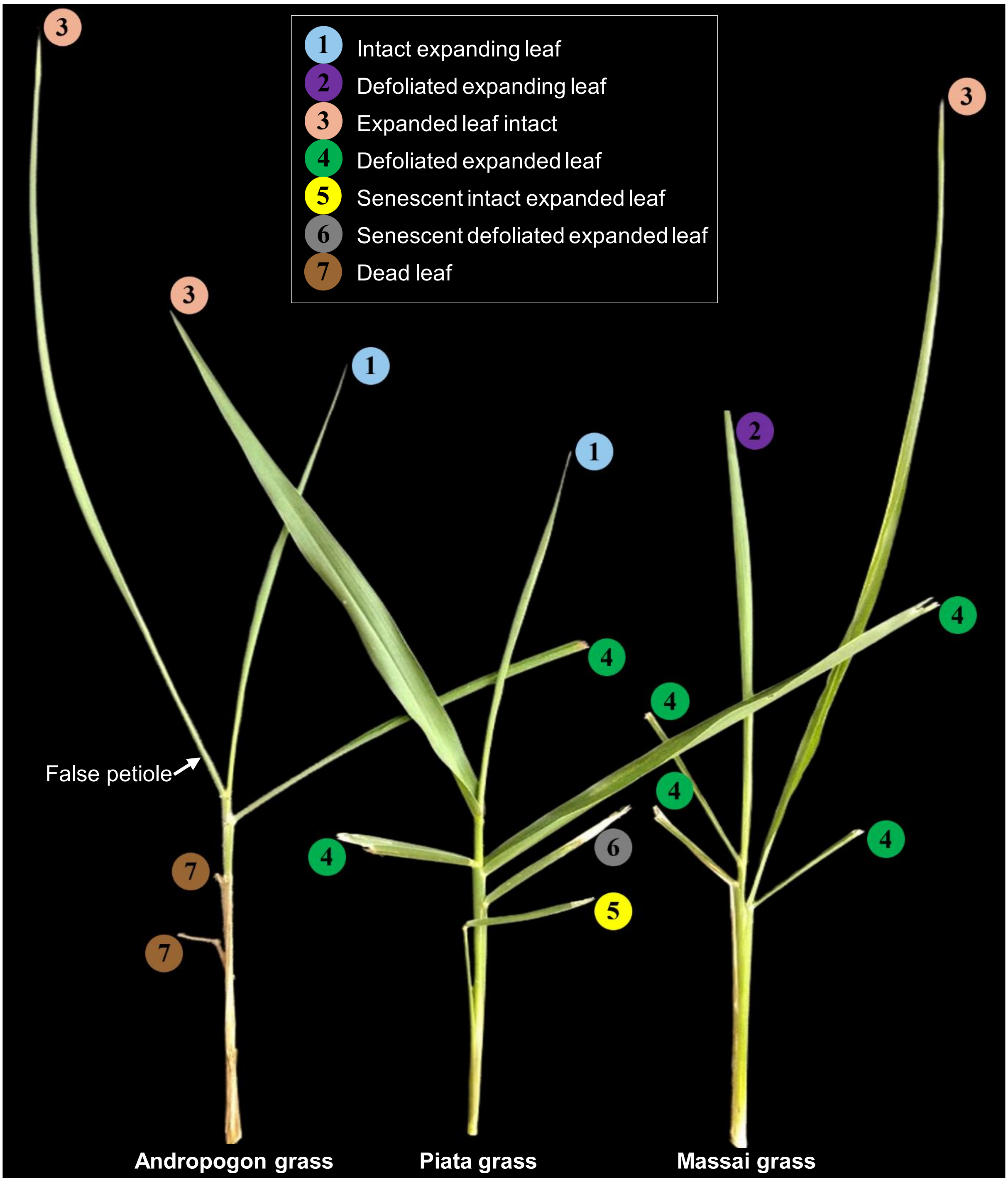


Figure S.3. Diagrammatic illustration leaf classification on marked tillers.

Figure S.4. Photos illustrating canopy foliage angle, and general grass species structure: andropogon grass (a, b), massai grass (c, d), and piata grass (e, f).

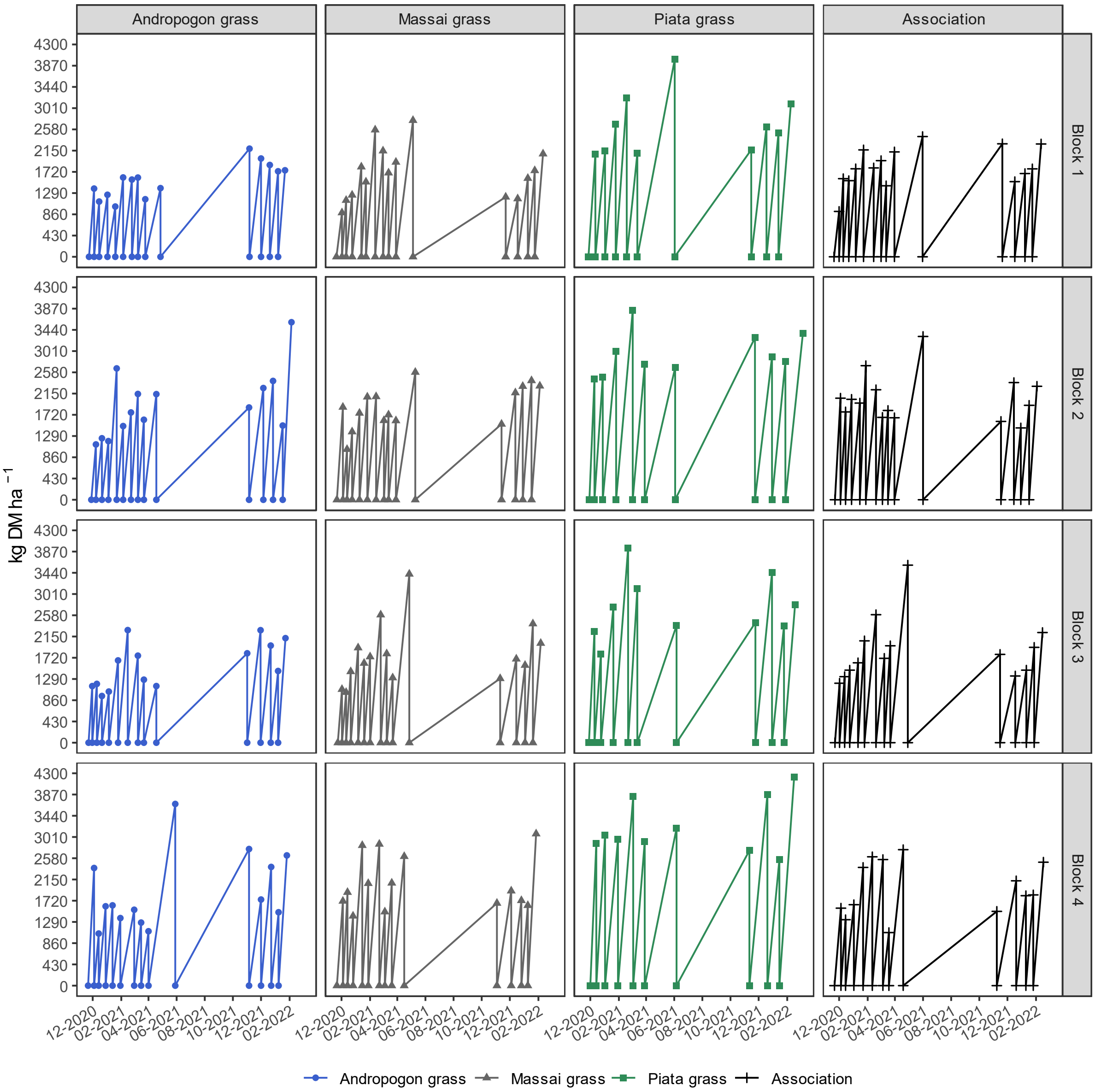


Figure S.5. Number of regrowth cycles, and herbage accumulation per cycle throughout the experiment (2020/11/30 to 2022/03/07).

Cutting interval varied with treatments in Late Spring I, Summer I, Late Spring II and Summer II ($p < 0.05$). Piata grass showed the longest cutting interval among the studied grass species (Figure S.6).

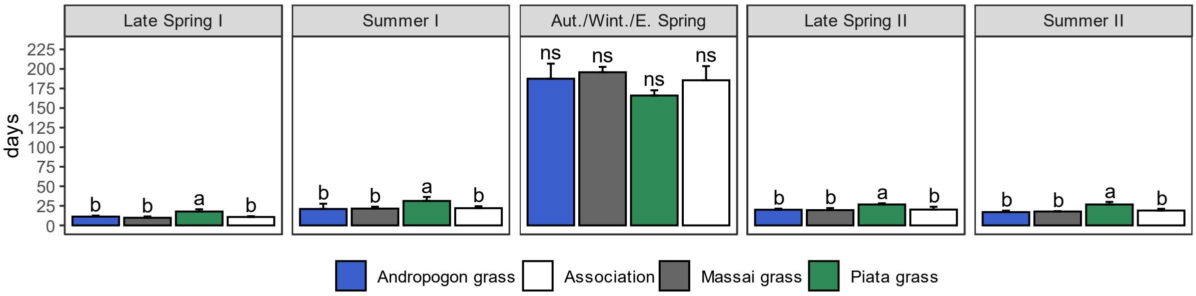


Figure S.6. Cutting interval for monocultures and the association throughout the experimental period.

In monoculture and the contribution of different grass species in the association, the of leaf senescence rate in the pasture varied in all year seasons ($p < 0.05$). However, in general, andropogon grass showed the highest of leaf senescence rate, except in Aut./Wint./E. Spring, when massai grass was the same as andropogon grass (Figure S.7 a). The relative leaf senescence rate indicates a higher leaf senescence rate in the association (Figure S.67 b).

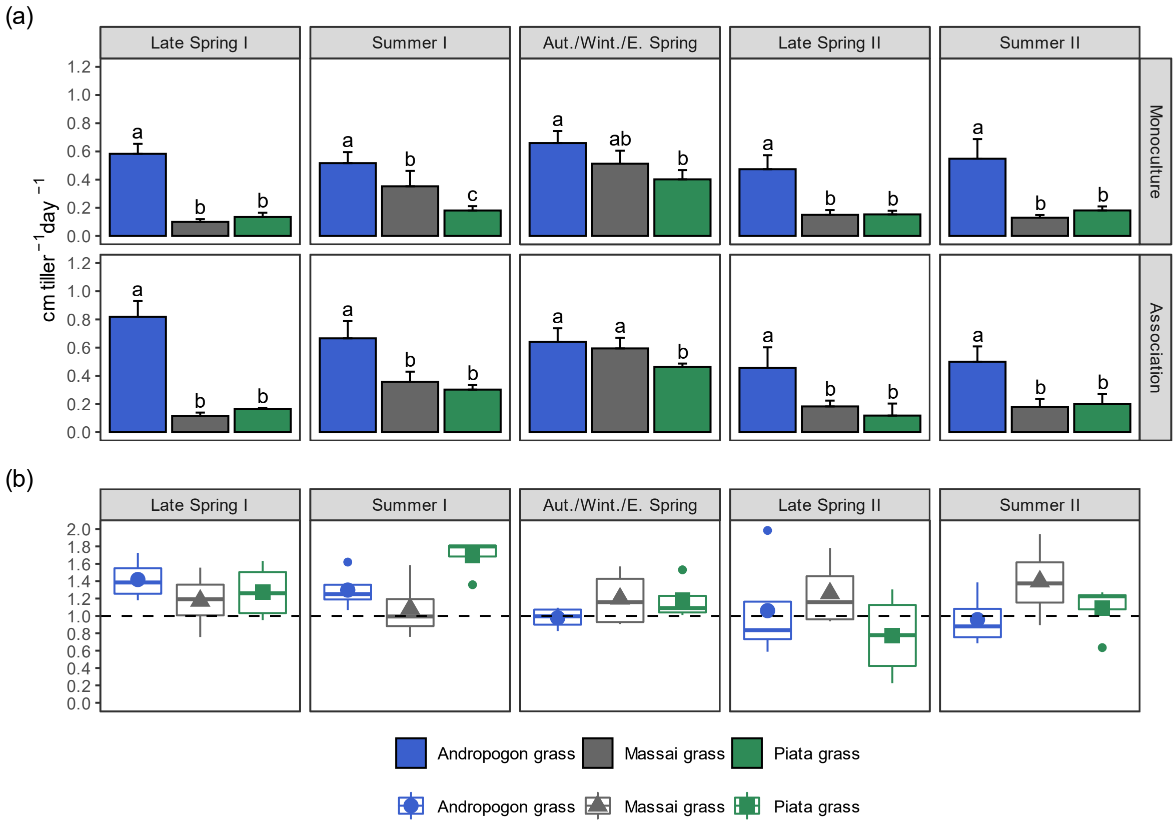


Figure S.7. Leaf senescence rate in monocultures and in the association (a), and relative leaf senescence rate (association/monoculture) (b) of andropogon, massai and piata grass cultivated as monocultures and in association.

3. NITROGEN NUTRITION OF THREE PERENNIAL TROPICAL FORAGE GRASSES CULTIVATED IN MONOCULTURE AND IN ASSOCIATION

Abstract

Studies aimed at better understanding the nitrogen nutritional status of plants have provided significant progress towards sustainable intensification of pastoral systems of animal production. Despite the advances, knowledge gaps still exist, particularly when different forage plants are cultivated in association. Knowledge regarding nitrogen nutrition status of plants comprising an association, along with information on botanical proportion of the mixture as well as distribution of plant species along the vertical profile of the canopy, may help to understand how such biodiverse swards operate, their mode of resource capture/use activities, determining forage yield and other ecosystem services. This knowledge is central for the decision-making process regarding management and formulation of new forage plant associations. The hypotheses of this study are that grass species growing in monocultures and in association have the same nitrogen nutritional status, and the association has greater forage yield than the average yield from the monocultures. The objectives were: (i) to determine whether tropical forage grasses cultivated as monoculture or in association have similar nitrogen uptake capacity, and (ii) to determine whether the nitrogen nutrition index (NNI) of tropical forage grasses grown in association differs from that of those grown as monocultures. The study was conducted from Summer 2021 to Summer 2022 in Piracicaba, São Paulo, Brazil. Treatments corresponded to three perennial tropical forage grasses: *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass), and *Brachiaria brizantha* cv. BRS Piatã (piata grass) cultivated in monoculture and in association (the three plants in equal proportions based on the number of viable seeds) with four replications. Defoliation management was common to all treatments and corresponded to a pre-cutting height of 35.0 cm and a post-cutting 17.5 cm. The herbage mass represented the description of the above ground vegetation once every season of the year, and forage yield represents all the quantified herbage accumulation throughout the experimental period. Herbage mass varied among treatments within seasons of the year, but values remained relatively stable for massai grass, piata grass, and the association. The number of regrowth cycles was smaller for piata grass, but with greaer herbage accumulation per cycle. On the other hand, andropogon grass, massai grass, and the association showed greater number of regrowth cycles, but smaller herbage accumulation per cycle, resulting in similar forage yield for all treatments. Nitrogen uptake was greater only for andropogon grass in Late Spring, indicating that monocultures and the association absorbed the same amount of nitrogen during most of the experimental period. However, the NNI varied among grass species grown in monoculture and in association. The NNI was closer to 1 (NNI considered satisfactory) during Summer I, Autumn, and Summer II; during the other seasons of the year, a slight nitrogen deficiency was observed. Overall, the NNI was greater for grass species grown in monoculture during Autumn and Winter/Early Spring, while in Late Spring it was greater for grass species grown in association. Although differences were observed for NNI within seasons of the year, recorded values were close to each other. These results indicate that there was no severe competition for nitrogen among grass species, both in monoculture and in association, even with massai grass participating in greater proportion of the herbage mass in the association. The greater proportion of massai grass in the association was likely shaped by other growth and development strategies related to competition for light. These underlying strategies did not strongly influence the dynamics of competition for nitrogen grass species.

Keywords: nitrogen nutrition index; nitrogen uptake; NUP method; resource capture; mixed tropical pastures

3.1. Introduction

In recent decades several studies have sought to understand and develop methods to describe the nitrogen nutrition status of forage plants in pastoral ecosystems, intending to maximize quality of the herbage produced and minimize bioeconomic losses (Lemaire and Gastal, 1997; Lemaire et al., 2008; Ciampitti et al., 2022). Nitrogen status represents the N dynamics of each ecosystem and shows whether the applied nitrogen is being well used or lost (Farrugia et al., 2004). Nitrogen fertilizers are finite resources and, when misused, may cause nitrate (NO_3) leaching (Di and Cameron, 2002; Fraters et al., 2015) and increase the intensity of greenhouse gases emission, such as nitrous oxide (N_2O), methane (CH_4) and carbon dioxide (CO_2) (Liu et al., 2013; Zhao et al., 2017; Luo et al., 2020), resulting in climate change with negative health implications to humans, biological diversity and environment (Vitousek et al., 1997; Clark and Tilman, 2008; Uwizeye et al., 2020). Despite advances, knowledge gaps still exist in pastoral ecosystems where forage plants are cultivated in association. The nitrogen nutrition status of forage plants grown in association may differ from that when grown as monoculture, likely consequence of competition for light and soil resources that would alter patterns of plant growth and development (Lemaire et al., 1991). Knowledge regarding the nitrogen status of plants comprising the association, along with information regarding botanical proportion and description of the vertical position of each plant in the forage canopy, may help to understand how such ecosystems operate, which are the resource capture strategies used, and what are the ecosystem services offered, as well as to assist in the decision-making process regarding management and planning of new associations.

The nitrogen nutrition index (NNI), represented by the ratio between the actual nitrogen concentration in the aerial part of the canopy and the nitrogen critical, is one of the most used methods for determining the nitrogen nutrition status of plants and provides an adequate estimate of nitrogen dynamics in pastoral ecosystems (Lemaire and Gastal, 1997; Gastal et al., 2014). Nitrogen critical is derived from the nitrogen dilution curve (exponential inverse relationship between herbage mass (t DM ha^{-1}) and actual nitrogen concentration in the aerial part (%)) and can be defined as the minimum nitrogen concentration in the shoot necessary to obtain maximum growth rate (Greenwood et al., 1990; Duru et al., 1997). The NNI can be interpreted as follows: values above 1 indicate "excessive" nitrogen luxury consumption, values close to 1 "satisfactory" and below 1 "deficiency" (Lemaire et al., 2008). NNI has been studied in many ecosystems (Gastal et al., 2014) and demonstrated to be an efficient and consistent estimate of the nitrogen nutritional status of plants across multiple crops, climates, and soil conditions, and is often used as calibration method for nitrogen status diagnostic tools (Errecart et al., 2012; Chen et al., 2015; Liu et al., 2018; Zha et al., 2020; Louarn et al., 2021; Jiang et al., 2022).

The NNI method considers the dilution of plant nitrogen based on soil area. As the botanical proportion of plants in the association is not symmetrical, and planting density does not represent the area occupied by each plant in the association, it is not possible to accurately estimate the NNI of each plant in the association (Louarn et al., 2021). In this condition, the method of nitrogen concentration in the upper leaves (NUP) (Farrugia et al., 2004) may be an alternative to estimate the NNI of each plant comprising the association. Several studies have shown a satisfactory relationship between NNI and NUP in monocultures (Farrugia et al., 2004; Américo et al., 2021) and in associations (Louarn et al., 2020; Louarn et al., 2021). The NUP method is relatively non-destructive and suitable for practical and frequent diagnoses and can be useful in pastures managed under lenient defoliation regimes, both for monocultures and for each plant comprising associations, as it is independent of structural tissues and soil area.

Currently, research on nitrogen nutritional status of plants in pastoral ecosystems is directed primarily to monocultures of temperate forage species or associations between grasses and legumes. On the other hand, under tropical conditions and environments, this type of study is practically non-existent, mainly considering a mixture of only well-managed perennial grasses. In this context, mixtures of forage grasses would have large potential for use, favoring their dissemination in production areas, especially in those where pasture recovery is necessary. In this context, the hypotheses of this study are that monocultures and association of perennial tropical forage plants have the same nitrogen nutritional status, and the association has a greater forage yield than the average for the monocultures. The objectives were: (i) to determine whether tropical forage grasses cultivated as monoculture or in association have similar nitrogen uptake capacity, and (ii) to determine whether the NNI of grasses grown in association differs from that of those grown as monocultures. The NUP method was used to estimate the NNI of each grass species in the association based on NUP and NNI data from their respective monocultures.

3.2. Methodology

3.2.1. Study site

The study was conducted from January 2021 to March 2022 (Summer 2021 to Summer 2022) in an experimental area of the Department of Animal Science of the "Luiz de Queiroz" College of Agriculture, University of São Paulo, in Piracicaba, São Paulo, Brazil (22°42'35"

South Latitude, 47°38'24" West Longitude and 546 m altitude). The climate of the region is Cwa (subtropical climate with dry Winter and hot Summer) (Köppen classification) (Alvares et al., 2013; Beck et al., 2018). Climatic data were collected in a Meteorological Station located approximately 2000 m from the experimental site (Figure S.2 supplementary material).

The soil is a Red Eutroferric Nitosol with a clayey texture (FAO, 2015) with the following chemical and physical characteristics at the 0 to 20 cm depth before the implementation of the experiment: 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% and sand content = 358 g kg^{-1}; clay = 446 g kg^{-1} and silt = 196 g kg^{-1}. The results indicated the need to increase base saturation of the soil, which was carried out by using dolomitic limestone aiming at reaching 70% (Raij et al. 1996) during late Winter 2019.

3.2.2. Treatments, experimental design, and management

Treatments corresponded to three tropical perennial forage grass species: *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass) and *Brachiaria brizantha* cv. BRS Piata (piata grass) cultivated in monoculture and in association (the three grass species in equal proportions based on the number of viable seeds). These were allocated to experimental units (180 m^2 paddocks) according to a randomized complete block design with four replications. Paddocks were 12 x 15 m, and 3 m wide races separated blocks. All raceways and a 3 m wide strip around the experimental area were kept free of vegetation by frequent mowing.

The experimental area was seeded in January 2020, through broadcast sowing using a seeding rate equivalent to 300 pure-viable seeds m^{-2} (1/3 for each grass species in the association - 100 pure-viable seeds m^{-2}), followed by compaction with a roller compactor weighing approximately 100 kg. All pastures were subjected to a common defoliation regime characterized by a pre-cutting canopy height of 35 cm and a post-cutting canopy height of 17.5 cm. Forage cuts were performed using a motorized brush cutter.

Canopy heights were monitored every three days during regrowth, starting soon after each cut. As canopy heights reached values close to the pre-cutting target of 35 cm, measurements became daily until paddocks reached their targeted canopy height for cutting.

Measurements were made using a sward stick on 40 points per paddock distributed along four transect lines (Table S.1 supplementary material).

The common defoliation management used to all treatments (monocultures and the association) was based on the 95% canopy light interception criterion during the regrowth and its flexibility range to define the ideal moment to interrupt regrowth, ensuring maximum leaf dry matter accumulation (Sbrissia et al., 2018; Gomes, 2019). This corresponded to the 35 cm canopy height, which was used as the pre-cutting target. The post-cutting height was equivalent to 50% of the pre-cutting canopy height in order to leave generous residual leaf area (Giacomini et al., 2009) and ensure frequent non-severe defoliations, favoring adequate conditions for growth and development of all plants in the association (low disturbance level – defoliation severity, and low-stress level – competition for light). This was expected to provide adequate conditions for grass species to express their functional characteristics. Under no soil fertility limiting conditions, competition for light becomes the main factor determining grass species botanical proportion. In this scenario, frequent non-severe defoliations may result in favorable environment for both resource capture and resource conservation type grass species since the severity of disturbance (defoliation) and stress (competition for light) is reduced, allowing for their coexistence and persistence (Grime, 1977; Borer et al., 2014; Eskelinen et al., 2022).

Nitrogen fertilization was performed only during the rainy seasons of the year (Late Spring, Summer, and Early Autumn), always at post-cutting, using ammonium nitrate. The amount of nitrogen for each application was proportional to the cutting interval of each paddock (daily rate of 1.7 kg N ha^{-1}) and calculated to result in equal amounts of nitrogen applied to all paddocks at the end of each rainy season (Table S.3 supplementary material).

3.2.3. Measurements

3.2.3.1. Canopy light interception

Monitoring of canopy light interception was carried out concomitantly with measurements of canopy height at pre- and post-cutting using an LAI 2000 canopy analyzer (LI-COR, Lincoln, Nebraska, EUA). Measurements were carried out consistently at dawn or dusk from eight reading points per paddock in areas representative of the average sward condition at the sampling time (visual assessment of herbage mass and height). A reference reading was taken above the canopy and five at ground level, totalizing eight readings above the canopy and forty readings at ground level per paddock (Table S.2 supplementary material).

3.2.3.2. Herbage mass and forage yield

Measurements of herbage mass (kg DM ha^{-1}) were performed once every season of the year at the pre-cutting condition. Samples were harvested on two representative areas of the paddocks (visual assessment of herbage mass and height) using a 100 × 25 cm metallic frame and cutting all herbage inside the frame at ground level. Samples were divided into two fractions of similar size. One fraction was used for hand separation of botanical and morphological components, which were dried in a forced draught oven at 60ºC until constant weight. The results were used to calculate sward herbage mass and its botanical (% andropogon grass, % massai grass, % piata grass) and morphological (% Leaves, % Stems, % Dead material, % Seedheads, and % weeds) composition. Because of the difficulty for sorting out the dead material by grass species, all dead material from the association was bulked and used to calculate green herbage accumulation (all plants together). The second fraction was further divided into two parts, and the first part was kept as it was, and the second part had all the dead material removed. These two samples were dried in a forced draught oven at 60°C until constant weight for evaluation of nitrogen nutrition index (item 3.2.3.5.).

Herbage accumulation was quantified throughout the experimental period. Two representative points from each paddock were sampled (visual evaluation of herbage mass and height) using a 100 × 25 cm metal frame. Samples were collected when the canopy reached 35 cm and cuts were performed at 17.5 cm from the ground. Subsequently, samples were dried in a forced draft oven at 60°C until constant weight, and the number of regrowth cycles, herbage accumulation per regrowth cycle (kg MS ha^{-1}), and forage yield (kg MS ha^{-1}) were determined. The expectation of herbage mass production was determined according to Loreau and Hector (2001).

3.2.3.3. Nitrogen concentration in the upper leaves

The nitrogen concentration at the tip of the upper leaves (% DM) was determined folloing the same sampling cronogram used for herbage mass and botanical/morphological composition described above (item 3.2.3.2.). About 20 g of leaves exposed to the sun were sampled from the upper layer of the canopy (top 10 cm from the tip of the leaf blades) for each grass species in the monocultures and in the association (Farruggia et al., 2004; Louarn et al., 2020) (Figure S.3 supplementary material). Samples were dried in a forced draught oven at 60°C until constant weight and ground in a "Wiley" type mill with a 1 mm sieve. The dried and ground samples were then submitted to total nitrogen concentration analysis by dry combustion at 1400°C using the Leco FP 528 system (Leco Corporation, St. Joseph, MI, USA).

3.2.3.4. Vertical distribution of botanical components in the association

The vertical distribution of botanical components along the vertical profile of the canopy in the association was assessed consistently at pre-cutting every season of the year using the inclined point quadrat method (Wilson, 1960; Laca et al., 2000). Measurements were made on areas representative of the average sward condition at the time of sampling (visual assessment of herbage mass and canopy height). A minimum of 100 touches per paddock was used as reference and results were expressed as percentage of touches in each grass species relative to the total number of touches.

3.2.3.5. Nitrogen nutrition index

Current nitrogen concentration of the shoot was quantified using herbage samples (with and without dead material) collected as described above (item 3.2.3.2.). First, samples were ground in a "Wiley" mill with a 1 mm sieve and submitted to total nitrogen concentration analysis by dry combustion at 1400°C using the Leco FP 528 system (Leco Corporation, St. Joseph, MI, USA). Then, for monoculture stands and the association, the nitrogen nutrition index (NNI with, and NNI without dead material) (Equation 1) was calculated using data of nitrogen actual concentration in the herbage mass (N_a with, and N_a without dead material) (% DM) and the nitrogen critical concentration (N_c) (% DM) (Lemaire and Gastal, 1997).

$$NNI = N_a/N_c \tag{1}$$

The N_c represents the percentage of minimum nitrogen necessary for plants to reach maximum growth rate (Equation 2) (Lemaire and Gastal, 1997).

$$N_c = a_c.DW^b \tag{2}$$

Where a_c = (3.6) N_c of plants with metabolism C_4, DW = dry herbage mass (t DM ha^{-1}), b = (-0.34) Empirical scale factor. These coefficients correspond to estimates of the C_4 grasses, *Sorghum Sudanense* (Sudan grass), *Zea mays* (maize), and *Setaria anceps* growing in either tropical or temperate situations with sufficient nitrogen supply during the vegetative period (Lemaire and Chartier, 1992).

Because of the difficulty for sorting out the dead material from grass species in the association, the NNI^{ass}_{with}, and $NNI^{ass}_{without}$ dead material for each grass species in the association were estimated from a regression equation between NUP and NNI_{with}, and $NNI_{without}$ dead material in monocultures and the NUP^{ass} of the plants in the association (Equation 3).

$$NNI^{ass} = a.NUP^{ass}+b \tag{3}$$

Where "a" with dead material = (0.2841), and "a" without dead material (0.3072), and "b" with dead material = (0.1132), and "b" without dead material (0.1819), are coefficients, correspond to the regression equation between the NUP and NNI_{with}, and $NNI_{without}$ dead material in monocultures (Figure 5 a b, in results).

3.2.3.6. Nitrogen uptake

Nitrogen uptake (NU) (kg N ha^{-1}) was calculated by multiplying sward herbage mass (HM) (kg DM ha^{-1}) by its concentration of N with dead material (%DM) (Equation 4).

$$NU = (N/100).HM \tag{4}$$

3.2.4. Statistical analysis

Statistical analyses were performed using R software (version 4.1.2; R Core Team, 2022). First, data were tested for normal distribution (Shapiro-Wilk test, $p < 0.05$) and homoscedasticity (Bartlett test, $p < 0.05$). Subsequently, analysis of variance (ANOVA "aov" procedure) was used to test significant differences among treatments and grass species in monoculture and in association. Linear regressions and Pearson correlation were used to evaluate the relationship between the response variables. Analysis of covariance (ANCOVA "lm" procedure) was also used to simultaneously test the effects of continuous and categorical variables and to compare the slopes and intercepts of the linear relationships generated. Differences were considered significant with $p < 0.05$. Finally, significant differences between means were tested by the Least Significant Difference test.

3.3. Results

3.3.1. Control of experimental conditions and nitrogen fertilization

During the experimental period, the targets of pre- and post-cutting heights of 35.0 and 17.5 cm, respectively, were maintained close to planned. However, at the end of Autumn, due to flowering of piata grass and the beginning of the dry and cold period of the year, when plant growth is severely reduced, it was necessary to cut piata grass below the planned target of 35.0 cm, with an average pre-cutting height of 30.2 cm (Table S.1 supplementary material). Canopy light interception at pre-cutting was consistently close to 95%. At post-cutting, canopy light interception was around 88% (Table S.2 supplementary material).

A total of 200 kg nitrogen ha^{-1} per year was applied, with applications carried out in installments only during the rainy seasons of the year (Late Spring, Summer, and Early Autumn), always at post-cutting. The amount of nitrogen applied on each paddock until the data collection date in each season of the year averaged 79.7 kg in Summer I, 80.3 kg in Autumn, 00.0 kg in Winter/Early Spring, 40.0 kg in Late Spring, and 51.7 kg in Summer II. Treatments within each season of the year received similar amounts of nitrogen (Table S.3 supplementary material). Seasons of the year were characterized as follows: (1) Summer I and Summer II - period of intense vegetative growth of plants due to the abundance of solar radiation, temperature, and rainfall (with nitrogen supply); (2) Autumn - time of flowering (with nitrogen supply); (3) Winter/Early Spring - period soon after flowering, with low solar radiation, low temperatures, and little rainfall (without nitrogen input); and (4) Late Spring - period of canopy renewal with large proportion of residual dead material from Autumn and Winter/Early Spring (with low nitrogen supply).

3.3.2. Number of regrowth cycles, herbage accumulation per cycle and forage yield in monocultures and in the association

The number of regrowth cycles represents the number of times plants were cut and regrew during the entire experimental period (2020/12/28 to 2022/03/07). Cuts for determining herbage accumulation were made at 17.5 cm from ground level and data used to calculate herbage accumulation per regrowths cycle and forage yield (total herbage accumulation throughout the experimental period). The number of regrowth cycles ($p < 0.001$), herbage accumulation per cycle ($p < 0.001$), and forage yield ($p = 0.039$) varied with treatment. Piata grass showed smaller number of regrowth cycles and greater herbage accumulation per cycle. On the other hand, massai grass, the association, and andropogon grass showed greater number of regrowth cycles and smaller herbage accumulation per cycle, which resulted in greater forage yield for piata grass relative to andropogon grass, and intermediate values for the association and for massai grass (Figure 1 a b c). In the association, the contribution of different grass species varied in herbage accumulation ($p < 0.001$) and forage yield ($p < 0.001$). The massai grass presented the highest herbage accumulation and forage yield (Figure 1 d e).

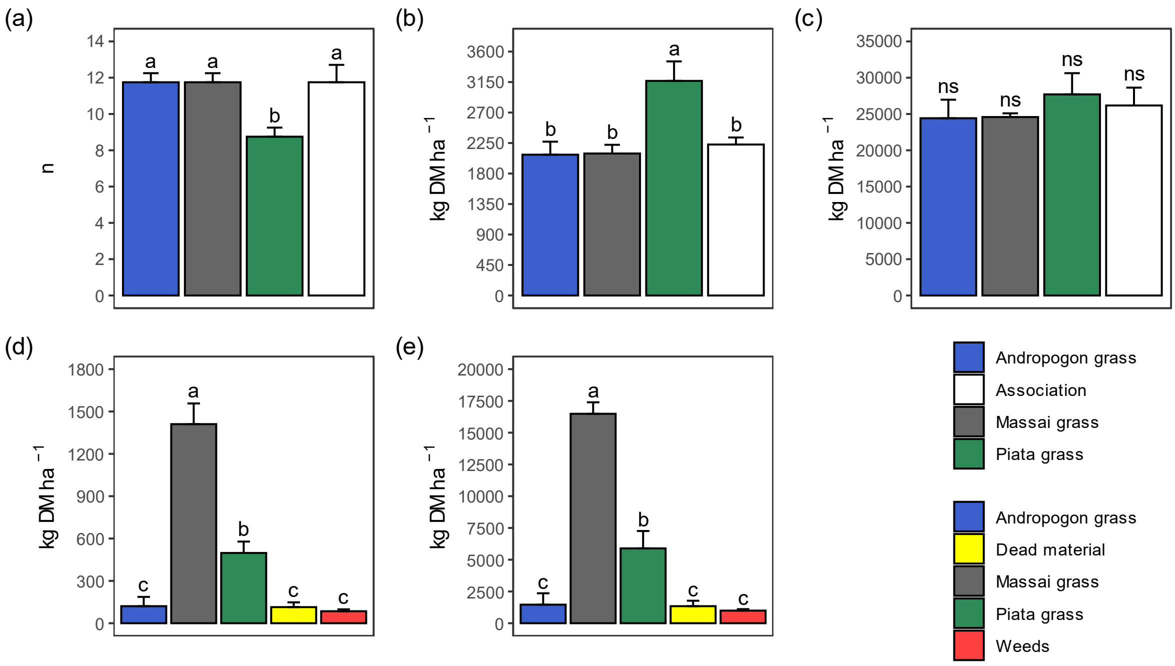


Figure 1. Number of regrowth cycles (a), herbage accumulation per cycle (b), forage yield (c), herbage accumulation per cycle for each grass species in the association (d), and yield for each grass species in the association (e) of andropogon, massai and piata grass cultivated as monocultures and in association (2020/12/28 to 2022/03/07).

3.3.3. Herbage mass, morphological and botanical composition, and vertical distribution of botanical components along the vertical profile of the grass species association canopy

Sward herbage mass represents the description of above ground vegetation at each season of the year. It varied with treatments during Summer I, Autumn, Late Spring, Summer II ($p < 0.05$). In Summer I and Autumn, greater herbage mass was recorded for piata grass, massai grass, and the association relative to andropogon grass. In Late Spring, andropogon grass presented greater herbage mass relative to massai grass, with intermediate values recorded for piata grass and the association. In Summer II, greater herbage mass was recorded for piata grass relative to the remaining grass species (Figure 2).

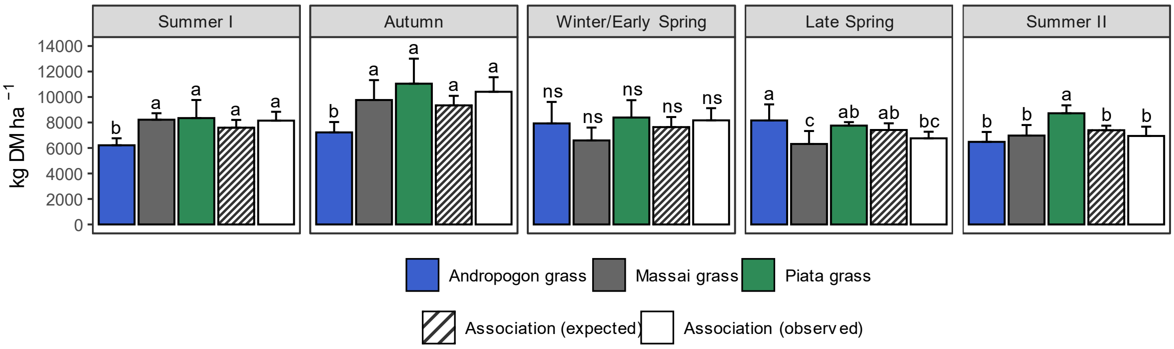


Figure 2. Herbage mass of andropogon, massai and piata grass cultivated as monocultures and in association.

The percentage of leaves varied with treatments during Summer I and Late Spring ($p < 0.05$). During Summer I, recorded values for andropogon grass were than for the remaining treatments. In Late Spring, greater values were recorded for massai grass relative to the association, with intermediate values recorded for piata grass and andropogon grass. The percentage of stems varied with treatments during Summer I, Autumn, Late Spring, and Summer II ($p < 0.01$). In general, smaller values were recorded for andropogon grass relative to the grass species and the association throughout the experiment. The percentage of dead material varied with treatments in Winter/Early Spring and Late Spring ($p < 0.05$). In Winter/Early Spring, greater values were recorded for massai grass relative to piata grass, with intermediate values recorded for the association and andropogon grass. During Late Spring, greatest values were recorded for andropogon grass. The percentage of weeds varied with treatments during Summer I, Autumn, and Summer II ($p < 0.05$), with greater values consistently recorded for andropogon grass relative to the other grass species and the association (Figure 3).

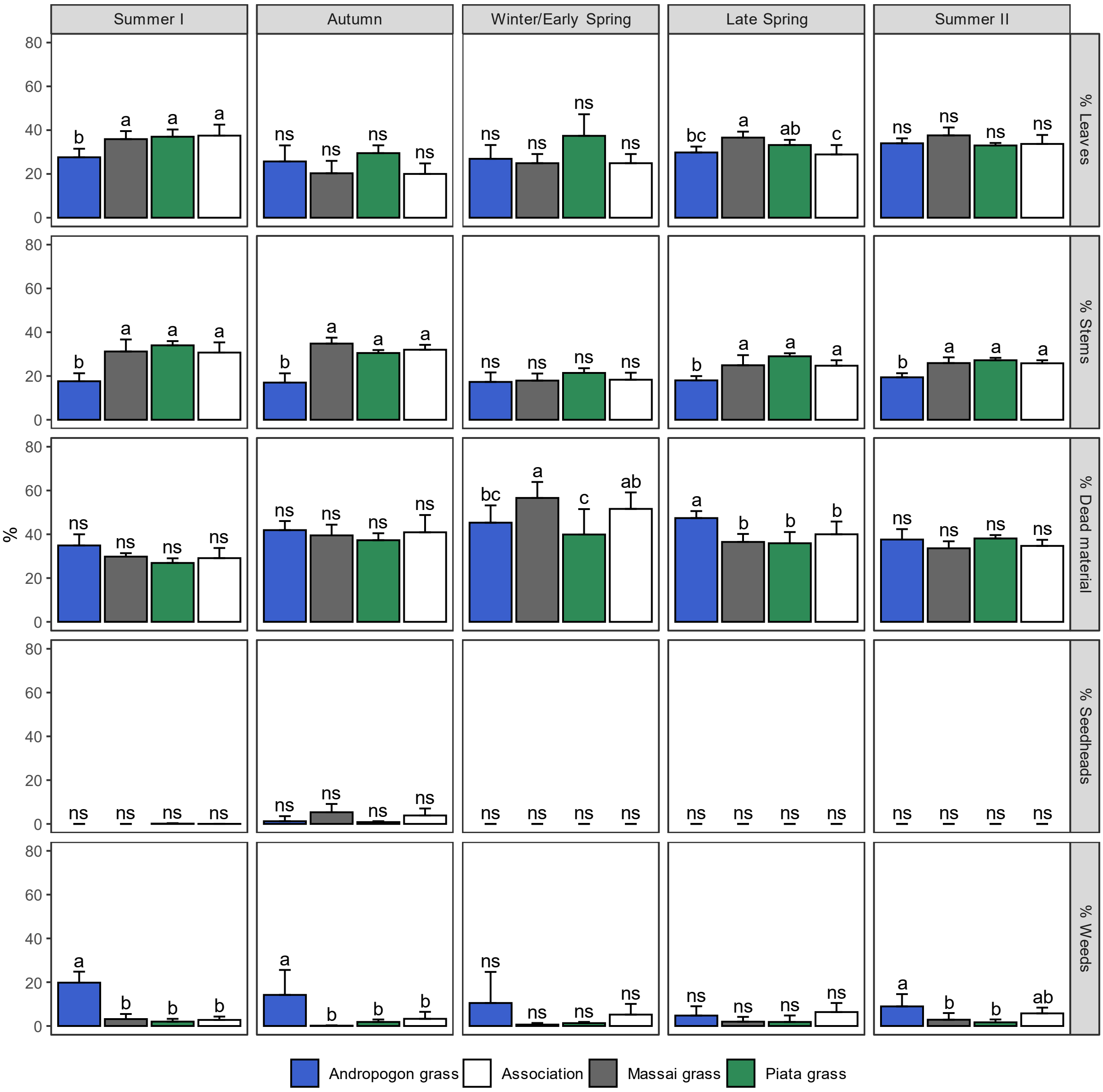


Figure 3. Percentage of leaves, stems, dead material, and weeds of andropogon, massai and piata grass cultivated as monocultures and in association.

The contribution of different grass species in the botanical proportion in the association varied in all seasons ($p < 0.05$). During Summer I and Summer II, greater proportion of the association herbage mass was represented by massai grass, followed by piata grass and andropogon grass. During Autumn, massai grass continued to represent greater proportion of the association herbage mass, with no difference between piata grass and andropogon grass. During Winter/Early Spring and Late Spring, piata grass and massai grass had similar contribuition to the association herbage mass, both greater than andropogon grass. Overall, the greatest botanical proportion in the association was observed during Winter/Early Spring and Late Spring (Figure 4 a).

The vertical distribution of grass species along the vertical profile of the canopy in the association showed similar pattern of variation to the botanical composition of the herbage mass, with greater proportion of massai grass along the vertical profile of the canopy, followed by piata grass and andropogon grass. This pattern of distribution was consistent during all seasons of the year and indicated greater proportion of massai grass in the upper part of the canopy. The three grass species participated in similar proportions during Winter/Early Spring. The dotted line represents the height corresponding to 50% of the canopy height (Figure 4 b).

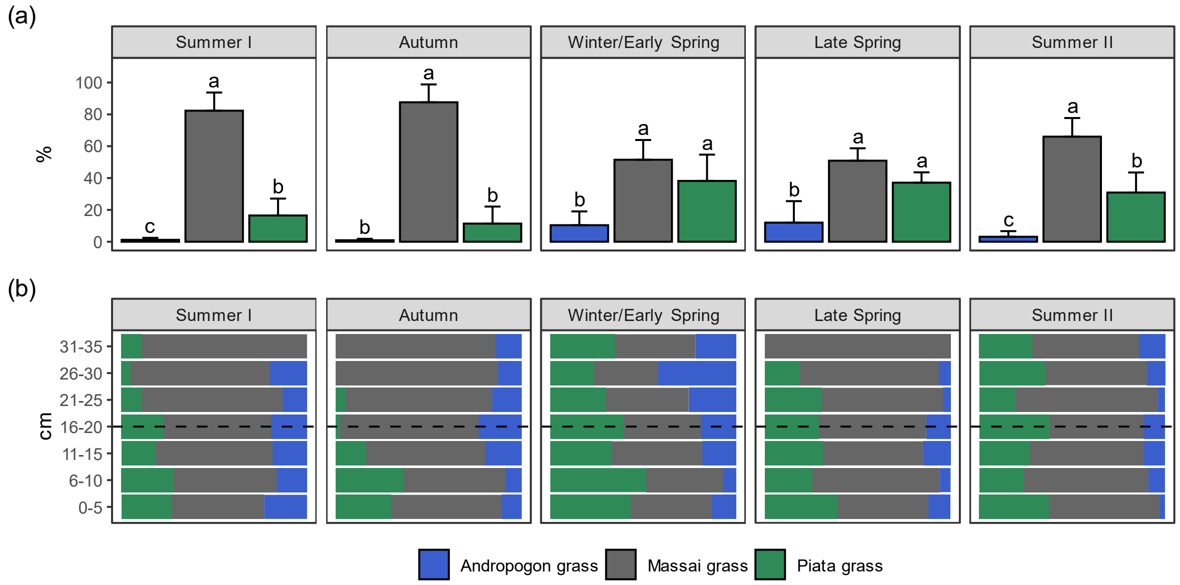


Figure 4. Botanical composition of sward herbage mass (a) and vertical distribution of botanical components along the vertical profile of the canopy in the association (b) of andropogon grass, massai grass and piata grass cultivated as monocultures and in association.

3.3.4. Nitrogen uptake

Nitrogen uptake varied with treatments during Late Spring (p = 0.021), with greater values recorded for andropogon grass relative to massai and piata (Figure 5).

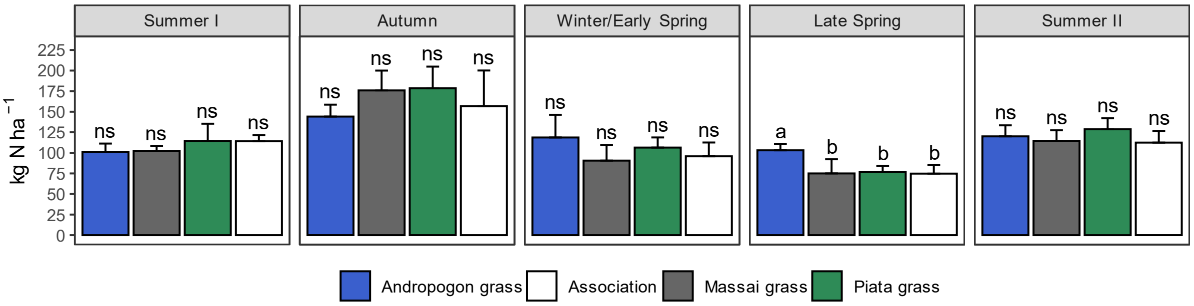


Figure 5. Nitrogen uptake of andropogon, massai and piata grass cultivated as monocultures and in association.

3.3.5. Relationship between NUP and NNI in monocultures, with and without the inclusion of dead material in samples

A linear relationship between NUP and NNI with dead material included in samples was estimated which showed a Pearson correlation of 0.67 for all grass species. The relationship had the same slope for the three grass species (ANCOVA, $p < 0.216$), but a different intercept (ANCOVA, $p < 0.001$) (Figure 6 a). The estimated linear relationship between NUP and NNI without dead material included in samples showed a Pearson correlation of 0.73 for all grass species. The relationship had the same slope (ANCOVA, $p = 0.088$) and similar intercept for all three grass species (ANCOVA, $p = 0.910$) (Figure 6 b). The slopes of the linear relationships were similar for samples including of not including the dead material component (ANCOVA, $p = 0.993$), but with different intercepts (ANCOVA, $p < 0.001$). Although both sampling methods resulted in similar linear relationships between NUP and NNI, the method that does not include dead material generated an NNI estimate 12.7% higher than the NNI estimate of the method that includes the dead material component in the samples (Figure 6 c).

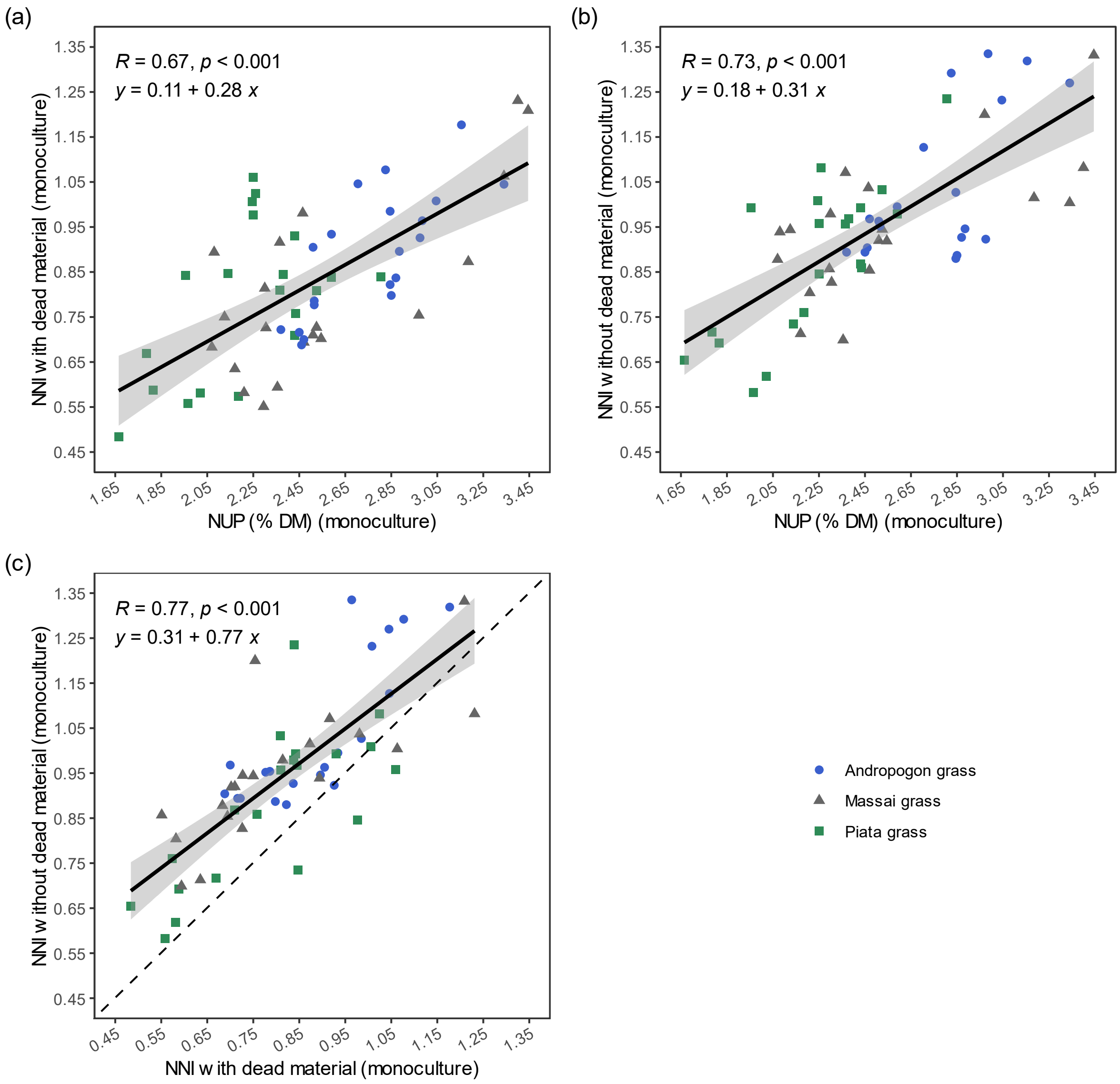


Figure 6. Relationship between nitrogen nutrition index (NNI) and nitrogen concentration in the upper leaves (NUP) of monocultures: with dead material included in herbage samples (a), without dead material included in herbage samples (b), and relationship between the NNI with and without dead material included in samples (c). *R* corresponds to Pearson correlation. The dotted line stands for the 1:1 line.

3.3.6. Nitrogen nutrition index with dead material included in herbage samples for the monocultures and the association

During Summer I, NNI with dead material include in samples was different among grass species ($p = 0.001$) and interaction cultivation x grass species ($p = 0.003$). During Autumn, there was a cultivation effect ($p < 0.001$) and interaction cultivation x grass species ($p = 0.016$). In the Winter/Early Spring, cultivation effect was observed ($p = 0.006$). In the Late Spring, the effect of plants ($p < 0.001$) and cultivation ($p < 0.001$). In Summer I, there was an effect of plants with higher NNI for andropogon grass, and the interaction with plants grown in monoculture x association indicated higher NNI for massai grass in the association compared to its monoculture. In Autumn, there was a cultivation effect with higher NNI for plants in monoculture, and there was interaction indicating that andropogon grass and massai grass in monoculture had higher NNI than their plants in the association, and piata grass showed the same NNI in both methods of cultivation. In Winter/Early Spring, there was only a cultivation effect, indicating that plants in monoculture had higher NNI than plants in the association. At Late Spring, the effect of plants and cultivation was observed, and no interaction. Among plants, andropogon grass showed higher NNI, and plants in the association had higher NNI than plants in monoculture (Figure 7).

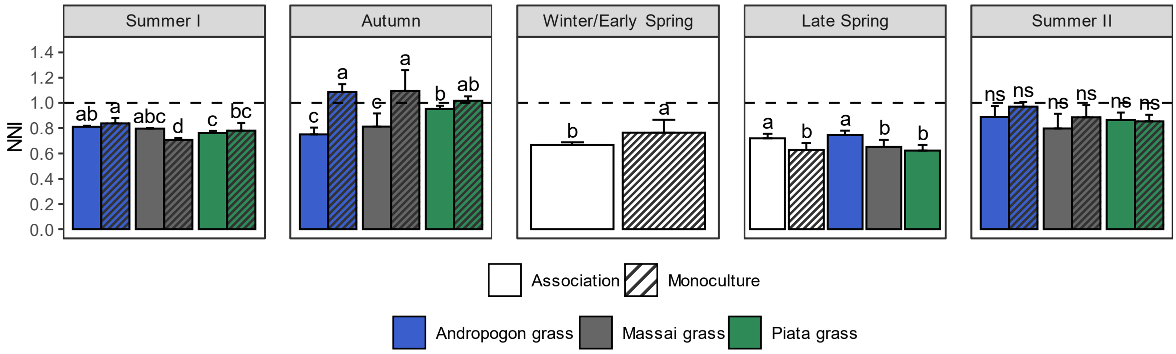


Figure 7. NNI with dead material in monocultures and association. Values close to the dotted line indicate satisfactory NNI.

3.4. Discussion

3.4.1. Herbage mass, morphological and botanical composition, nitrogen uptake, and forage yield

Sward herbage mass and its botanical and morphological composition represent the description of the aboveground vegetation and were determined once every season of the year. Herbage mass of the association was similar to that of massai grass and piata grass, with variations throughout the experimental period. During Summer I and Autumn, the association presented herbage mass similar to massai grass and piata grass (Figure 2) and botanical composition with greater percentage of massai grass (Figure 4 a b). However, in Late Spring, after a period of low solar radiation, low temperature, little rainfall and no nitrogen supply during Winter/Early Spring (Figure S.2, and Table S.3 supplementary material), massai grass showed smaller herbage mass with consequent reduce percentage in the association herbage mass.

Nitrogen uptake was also quantified once every season of the year. The results showed greater uptake for andropogon grass in Late Spring only, indicating that the three plants absorbed the same amount of nitrogen for most of the experimental period. In spite of that, piata grass presented herbage mass and forage yield similar to or greater than the other treatments. These results suggest that piata grass is a resource-competitive plant and, with the same amount of nitrogen, it can produce more forage dry matter. In addition, during Winter/Early Spring and Late Spring, piata grass increased its participation in the herbage mass of the association, balancing the negative effects on the entire plant community caused by climatic variations which affected negatively massai grass with greater intensity, the plant originaly present in greater percentage in the association (Figure 4 a b).

Forage yield correspond to total herbage accumulated during the entire experimental period. Overall, forage yield was determined by the strategies of plant growth and development. Piata grass had the smallest number of regrowth cycles and greatest herbage accumulation per cycle. On the other hand, andropogon grass, the association, and massai grass had greater number of regrowth cycles, but smaller herbage accumulation per cycle. At the end ot the experiment, forage yield was greater for piata grass relative to andropogon grass, with intermediate values recorded for the association and massai grass (Figure 1 a b c). The association had forage yield 6.79% greater than the average from the three monocultures. The results suggest a slight positive association effect relative to at least one of the grass species cultivated as monoculture. This may be explained by plants exploring resources at different times or spaces given their complementarity in terms of strategies of growth and development. The complementatiry among plant species in a mixture may facilitate resource acquisition in time and space, reducing the negative impact of competition and improving the efficiency of resource use and exploitation at both above and below ground (Loreau and Hector, 2001; Cardinale et al., 2007; Pontes et al., 2012). As benefit, when plants in the association are capable of standing out at different times of the year, there may be a more uniform distribution of herbage accumulation and lesser risk of seasonality of production, reducing risks and bringing more security for animal exclusively from pastures.

Environmental uncertainties and insecurity may increase in the coming decades due to increasing global warming (Prado et al., 2016; Bastin et al., 2019; Viciedo et al., 2019; Hein et al., 2020), and interactions among plants can occur according to the intensity of such disturbance (Pugnaire et al., 2019; Catford et al., 2020). In this context, using plant associations based on the increase of plant diversity in the same pasture may be an interesting alternative to increase productivity, improve stability of herbage production and reduce the influences of anthropogenic and edaphoclimatic factors, as well as promoting multifunctionality and more sustainable production of forage in such ecosystems (Catford et al., 2020; Li et al., 2021; Thakur et al., 2021).

3.4.2. Relationship between NUP and NNI in monocultures, with and without the dead material in the forage samples

The relationship between NUP and NNI in the monocultures was evaluated using the NNI calculated with and without dead material in the forage samples to verify the influence of the presence of dead material on estimates of NNI. According to Duru et al. (1997), the NNI should be used as a diagnostic tool only in situations of herbage accumulation after severe defoliation, where the accumulation of senescent plant material is negligible. This is because, under conditions where there are large proportions of dead material, sward herbage mass may not increase in the same proportion compared to plants regrowing after severe defoliation. Lemaire and Gastal (2016) also recommended using the nitrogen dilution curve and, consequently, the NNI in periods before flowering because, in the nitrogen dilution curve, the “b” value is constant throughout the vegetative growth, the period when plants are producing only leaves and stems, with leaf senescence occurring after the onset of flowering, so the accumulation of nitrogen in the herbage mass is mainly due to continuous absorption, recycling, and losses of nitrogen, generally leading to a decrease in coefficient "b" (acceleration of nitrogen dilution). Therefore, the use of the NNI method in the present study, in which defoliation management was moderate/lenient (condition where the senescence is inevitable) and measurements also were performed during flowering period (Autumn) or post-flowering period (Winter/Early Spring), and (Late Spring), could modify the NNI response.

It is important to highlight that the correct way to investigate the influence of lenient defoliation management and the proportion of dead material on the nitrogen dilution curve would be to follow the growth dynamics of pastures after different cutting regimes and then construct the respective nitrogen dilution curves. Once the curves are established in dynamic terms, they can be used to diagnose plant NNI (Lemaire and Gastal, 1997; Lemaire and Gastal, 2016). In the present study, as the nitrogen dilution curve was not constructed, we chose to use the coefficients "a" and "b" of the nitrogen dilution curve constructed and recommended for C_4 grasses: *Sudanese Sorghum*, *Zea mays*, and *Setaria anceps* growing in tropical or temperate situations with sufficient nitrogen supply during the growing season (Lemaire and Chartier, 1992). Further, because the percentage of visible seedheads was only 2.81% in Autumn, and large percentage of dead material was recorded during all seasons of the year (30.2% in Summer I, 39.9% in Autumn, 48.4% in Winter/Early Spring, 40.0% in Late Spring, and 36.0% in Summer II) (Figure 3), the decision was to make determinations of NNI with and without dead material in the forage samples.

The relationship between NUP and NNI was similar for both conditions, with and without dead material in forage samples (Figure 6 a b). Overall, values of NNI with dead material were 12.7% smaller than values of NNI without dead material (Figure 6 c). The smaller values may be explained by the large percentage of dead material in sward herbage mass (Figure 3), since old leaves and structural tissues typically present low nitrogen concentration. The NUP method is an alternative for monitoring the nitrogen nutrition status of plants and has good relationship with NNI in studies carried out with temperate grasses and legumes (Farrugia et al., 2004; Louarn et al., 2020; Louarn et al., 2021). The present study is one of the first to report NUP and NNI relationships for tropical perennial grasses.

Among the few studies that highlight relationships between NNI estimation methods for tropical plants, Ziadi et al. (2009) describe the relationship between leaf nitrogen concentration per unit of dry matter and NNI. We used the software (WebPlotDigitizer) (Rohatgi, 2017) and extracted the data from the figure (Figure 4 a) from the article and plotted it with the NUP and NNI relationship data with and without dead material from our study (Figure S.4 supplementary material). We observed the same response pattern for the three methods, indicating that the NUP method is a safe option for estimating NNI in plants of tropical climate.

3.4.3. Nitrogen nutrition index with dead material in monocultures and association

The NNI with dead material (Figure 7) and the NNI without dead material (Figure S.5 supplementary material) were calculated separately. We chose to discuss only the NNI results with dead material because they present large similarity with the real ecosystem in field conditions.

The NNI presented values close to 1 (NNI considered satisfactory) during Summer I, Autumn, and Summer II, with a slight nitrogen deficiency during the other seasons of the year. The results may be explained by the fact that these three seasons of the year received the greatest proportion of the nitrogen applied (79.7 kg in Summer I, 80.3 kg in Autumn, and 51.7 kg in Summer II). During the remaining two seasons of the year the amount of nitrogen applied was smaller or null (00.0 kg in Winter/Early Spring and 40.0 kg in Late Spring) (Table S.3 supplementary material).

Andropogon grass showed a slight superiority in NNI relative to the massai grass and piata grass, probably because it presented smaller % Stems (Figure 3) and, consequently, greater nitrogen content. On the other hand, Piata grass was the plant grass species with the least variation in NNI when comparing plants grown in monoculture and in association and it was the grass species that presented the largest forage yield. Interestingly, during Autumn, piata grass presented plants positioned at lowest layers of the canopy (Figure 4 b), and even then, it presented satisfactory NNI (Figure 7). Two possible hypotheses may explain these results; the first hypothesis would be that piata grass grown in monoculture, mainly during Autumn, presented a much larger cutting interval than the other two grass species (Figure S.6 supplementary material). Therefore, this plant could also have a longer cutting interval in the association. Since it was cultivated with other plants with shorter cutting interval, it was harvested earlier relative to its monoculture and presented the same NNI because it was younger. The second hypothesis would be that piata grass is a competitive plant for resources and that, even occupying a lower canopy layer, it may have the same NNI, which could have made it gain space over time in the botanical proportion of the association.

Overall, the NNI was greater for plants grown in monoculture during Autumn and Winter/Early Spring, while during Late Spring, recorded values were greater for plants grown in the association. Although differences were observed between the NNI within each season of the year, recorded values were remained close to each other. These results may indicate no severe competition for nitrogen among plants, both in monoculture and in association, even with massai grass participating in greater botanical percentage than andropogon grass and piata grass. The greater presence of massai grass in the association was probably shaped by other growth and development strategies, particularly driven by competition for light. These underlying strategies did not strongly influence the dynamics of competition for nitrogen among plants.

It is well reported in the literature that, in fertile environments, competition for light becomes the main limiting agent of species diversity (Grime, 1977; Borer et al., 2014). In the present study, the botanical proportion of plants in the association was greater during Winter/Early Spring and Late Spring in both circunstamces the absence or low nitrogen supply. These results have already been reported in studies of nutrient deposition in terrestrial ecosystems, showing that increased nitrogen availability generally increases primary productivity and decreases plant diversity (Stevens et al., 2004; Clark and Tilman, 2008). The hypotheses proposed to explain these responses focus on changes in competition, both above and below ground (Grime, 1973), or aboveground competition for light alone (Suding et al., 2005; Hautier et al., 2009). The hypothesis of increased competition for light predicts that, as productivity increases, light availability to understory plants is reduced, leading to their exclusion by faster growing or taller species that appropriate this resource that is provided directionally (Hautier et al., 2009). In the present study, the greater grass spices botanical proportion observed during Winter/Early Spring and Late Spring seems to be related to climatic variations throughout the seasons of the year, nitrogen deposition, or competition for light; however, our results are insufficient to confirm this hypothesis.

3.5. Conclusions

The results showed that herbage mass varied among treatments within seasons of the year, being during most of them similar for massai grass, piata grass, and the association. Piata grass showed the smallest number of regrowth cycles and the the greatest herbage accumulation per cycle. On the other hand, andropogon grass, massai grass, and the association showed greater number of regrowth cycles with smaller herbage accumulation per cycle, resulting in similar forage yield for all treatments at the end of the experiment. Nitrogen uptake was greater only for andropogon grass during Late Spring, indicating that monocultures and association absorbed the same amount of nitrogen during most of the experimental period. However, NNI varied between plants grown in monoculture and in association. The NNI was closer to 1 (NNI considered satisfactory) during Summer I, Autumn, and Summer II; during the other seasons of the year, a slight nitrogen deficiency was observed. Overall, the NNI was greater for plants grown in monocultures during Autumn and Winter/Early Spring, while during Late Spring, it was greater for plants grown in association. Although differences were observed in NNI within each season of the year, recorded values were close to each other. These results may indicate no severe competition for nitrogen among plants, both in monoculture and in association, even with massai grass participating with greater botanical percentage in sward herbage mass relative to andropogon grass and piata grass. The greater presence of massai grass in the association was likely shaped by other growth and development strategies, particularly those related to competition for light. These underlying strategies did not strongly influence the dynamics of competition for nitrogen between plants.

References

Alvares, C.A., Stape, J.E., Sentelhas, P.C., Gonçalves, J.L.M., Sparovek, G., 2013. Köppen's climate classification map for Brazil. Meteorologische Zeitschrift. 22 (6), 711–728. https://doi.org/10.1127/0941-2948/2013/0507

Américo, L.F., Duchini, P.G., Schmitt, D., Guzatti, G.C., Lattanzi, F.A., Sbrissia, A.F., 2021. Nitrogen nutritional status in perennial grasses under defoliation: Do stubble height and mixed cultivation matter? Journal of Plant Nutrition and Soil Science. 184 (2), 208–216. https://doi.org/10.1002/jpln.202000201

Bastin, J.F., Clark, E., Elliott, T., Hart, S., Hoogen, J., Hordijk, I., Ma, H., Majumder, S., Manoli, G., Maschler, J., Mo, L., Routh, D., Yu, K., Zohner, C.M., Crowther, T.W., 2019. Understanding climate change from a global analysis of city analogues. Plos One. 14. https://doi.org/10.1371/journal.pone.0217592

Beck, H., Zimmermann, N.E., McVicar, T.R., Vergopolan, N., Berg, A., Wood, EF, 2018. Data Descriptor: Present and future Köppen-Geiger climate classification maps at 1-km resolution. Scientific Data. 5 (180214). https://doi.org/10.1038/sdata.2018.214

Borer, E.T., Seabloom, E.W., Gruner, D.S., Harpole, W.S., Hillebrand, H., Lind, E.M., Adler, P.B., Alberti, J., Anderson, T.M., Bakker, J.D., Biederman, L., Blumenthal, D., Brown, C.S., Brudvig, L.A., Buckley, Y.M., Cadotte, M., Chu, C., Cleland, E.E., Crawley, M.J., Daleo, P., Damschen, E.I., Davies, K.F., DeCrappeo, N.M., Du, G., Firn, J., Hautier, Y., Heckman, R.W., Hector, A., HilleRisLambers, J., Iribarne, O., Klein, J.A., Knops, J.M.H., La Pierre, K.J., Leakey, A.D.B., Li, W., MacDougall, A.S., McCulley, R.L., Melbourne, B.A., Mitchell, C.E., Moore, J.L., Mortensen, B., O'Halloran, L.R., Orrock, J.L., Pascual, J., Prober, S.M., Pyke, D.A., Risch, A.C., Schuetz, M., Smith, M.D., Stevens, C.J., Sullivan, L.L., Williams, R.J., Wragg, P.D., Wright, J.P., Yang, L.H., 2014. Herbivores and nutrients control grassland plant diversity via light limitation. Nature. 508, 517–520. https://doi.org/10.1038/nature13144

Callaway, R.M., 1995. Positive interactions among plants. The Botanical Review. 61 (4), 306–349.

Cardinale, B.J., Wright, J.P., Cadotte, M.W., Carroll, I.T., Hector, A., Srivastava, D.S., Loreau, M., Weis, J.J., 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. PNAS. 104, 18123–18128. https://doi.org/10.1073/pnas.0709069104

Catford, J.A., Dwyer, J.M., Palma, E., Cowles, J.M., Tilman, D., 2020. Community diversity outweighs effect of warming on plant colonization. Global Change Biology. 26, 3079–3090. https://doi.org/10.1111/gcb.15017

Chen, P., 2015. A Comparison of two approaches for estimating the wheat nitrogen nutrition index using remote sensing. Remote Sensing. 7. https://doi.org/10.3390/rs70404527

Ciampitti, I., Versendaal, E., Rybecky, J.F., Lacasa, J., Fernandez, J., Makowski, D., Lemaire, G., 2022. A global dataset to parametrize critical nitrogen dilution curves for major crop species. Scientific Data. 9, 277. https://doi.org/10.1038/s41597-022-01395-2

Clark, C.M., Tilman, D., 2008. Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. Nature. 451, 712–715. https://doi.org/10.1038/nature06503

Da Silva, S.C., Sbrissia, A.F., Pereira, L.E.T., 2015. Ecophysiology of C 4 forage grasses - Understanding plant growth for optimising their use and management. Agriculture. 5, 598–625. https://doi.org/10.3390/agriculture5030598

Di, H.J., Cameron, K.C., 2002. Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. Nutrient Cycling in Agroecosystems. 46, 237–256. https://doi.org/10.1023/A:1021471531188

Duru, M., Lemaire, G., Cruz, P., 1997. Grasslands, in Lemaire, G. (ed.): Diagnosis of the Nitrogen Status in Crops. Springer, Heidelberg, Germany, p.59–72.

Errecart, P.M., Agnusdei, M.G., Lattanzi, F.A., Marino, M.A., 2012. Leaf nitrogen concentration and chlorophyll meter readings as predictors of tall fescue nitrogen nutrition status. Field Crops Research. 129, 46–58. https://doi.org/10.1016/j.fcr.2012.01.008

Eskelinen, A., Harpole, W.S., Jessen, M.T., Virtanen, R., Hautier, Y., 2022. Light competition drives herbivore and nutrient effects on plant diversity. Nature. 611, 301–305. https://doi.org/10.1038/s41586-022-05383-9

FAO IUSS Working Group., 2015. World Reference Base for Soil Resources 2014. International soil classification system for naming soils and creating legends for soil maps, Update 2015, World Soil Resources Reports 106. FAO, Rome. https://www.fao.org/3/i3794en/I3794en.pdf

Farrugia, A., Gastal, F., Scholefield, D., 2004. Assessment of nitrogen status of grassland. Grass and Forage Science. 59, 113– 120. https://doi.org/10.1111/j.1365-2494.2004.00411.x

Fonseca, D.M., Martuscello, J.A., 2022. Plantas forrageiras, 2 ed. Editora Universidade Federal de Viçosa, 591p.

Fraters, D., Leeuwen, T., Boumans, L., Reijs, J., 2015. Use of long-term monitoring data to derive a relationship between nitrogen surplus and nitrate leaching for grassland and arable land on well-drained sandy soils in the Netherlands. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science. 65, 144–154, 2015. https://doi.org/10.1080/09064710.2014.956789

Gastal, F., Lemaire, G., Durand, J-L., Louarn, G., 2014. Quantifying crop responses to nitrogen and avenues to improve nitrogen-use efficiency. In Sadras V.O., Calderini, D.F. (eds) Crop physiology: applications for genetic improvement and agronomy. Elsevier, Amsterdam, p.161–206. https://doi.org/10.1016/B978-0-12-417104-6.00008-X

Giacomini, A.A., Da Silva, S.C., Sarmento, D.O.L., Zeferino, C.V., Trindade, J.K., Souza Júnior, S.J., Guarda, V.A., Sbrissia, A.F., Nascimento Júnior, D.N., 2009. Components of the leaf area index of marandu palisadegrass swards subjected to strategies of intermittent stocking. Scientia Agricola. 66 (6), 721–732. http://dx.doi.org/10.1590/S0103-90162009000600002

Gomes, C.M. Oportunidade para flexibilização das metas pré-pastejo do manejo rotativo do capim-marandu. 2019. Dissertação (Mestrado em Ciência Animal e Pastagens) – Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, São Paulo.

Greenwood, D.J., Lemaire, G., Gosse, G., Cruz, P., Draycott, A., Neeteson, J.J., 1990. Decline in percentage N of C_3 and C_4 crops with increasing plant mass. Annals of Botany. 66, 425–436. https://doi.org/10.1093/oxfordjournals.aob.a088044

Grime, J.P., 1973. Competitive exclusion in herbaceous vegetation. Nature. 242, 344–347. https://doi.org/10.1038/242344a0

Grime, J.P., 1977. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. The American Naturalist. 111, 1169–1194.

Gross, K., Cardinale, B.J., Fox, J.W., Gonzalez, A., Loreau, M., Polley, H.W., Reich, P.B., Ruijven, J., 2014. Species richness and the temporal stability of biomass production: A new analysis of recent biodiversity experiments. The American Naturalist. 183 (1). https://doi.org//10.1086/673915

Gross, N., Pinguet, Y.L.B., Liancourt, P., Berdugo, M., Gotelli, N.J., Maestre, F.T., 2017. Functional trait diversity maximizes ecosystem multifunctionality. Nature Ecology & Evolution. 1 (132). https://doi.org/10.1038/s41559-017-0132

Hautier, Y., Niklaus, P.A., Hector, A., 2009. Competition for light causes plant biodiversity loss after eutrophication. Science. 324, 636–638. https://doi.org/10.1126/science.1169640

Hein, C.J., Usman, M., Eglinton, T.I., Haghipour, N., Galy, V.V., 2020. Millennial-scale hydroclimate control of tropical soil carbon storage. Nature. 581, 63–66. https://doi.org/10.1038/s41586-020-2233-9

Jiang, J., Atkinson, P.M., Zhang, J., Lu, R., Zhou, Y., Cao, Q., Tian, Y., Zhu, Y., Cao, Y., Liu, X., 2022. Combining fixed-wing UAV multispectral imagery and machine learning to diagnose winter wheat nitrogen status at the farm scale. European Journal of Agronomy. 138, 126537. https://doi.org/10.1016/j.eja.2022.126537

Lemaire, G., 1997. Diagnosis of the Nitrogen Status in Crops. 5 ed. Springer, Heidelberg, Germany, 240p.

Lemaire, G., Chapman, D., 1996. Tissue fluxes in grazing plant communities. In The Ecology and Management of Grazing Systems, 1st ed.; Hodgson, J., Illius, A.W., Eds.; CABI Publishing: Wallingford, UK, p.3–36.

Lemaire, G., Chartier, M., 1992. Relationships between growth dynamics and nitrogen uptake for individual sorghum plants growing at different plant densities. Proc 2nd Congr. of European Society of Agronomy, Warwick University, p.98–99.

Lemaire, G., Gastal, F., 2016. Improved estimation of nitrogen uptake in grasslands using the nitrogen dilution curve (Reyes et al. 2015), 35:1561–1570. Agronomy for Sustainable Development. 36. https://doi.org/10.1007/s13593-016-0388-4

Lemaire, G., Gastal, F.N., 1997. Uptake and distribution in plant canopies. in Lemaire, G. (ed.): Diagnosis of the Nitrogen Status in Crops. Springer, Heidelberg, Germany, p.3–43.

Lemaire, G., Jeuffroy, M.H., Gastal, F., 2008. Diagnosis tool for plant and crop N status in vegetative stage Theory and practices for crop N management. European Journal of Agronomy. 28, 614–624. https://doi.org/10.1016/j.eja.2008.01.005

Lemaire, G., Onillon, B., Gosse, G., Chartier, M., Allirand, J.M., 1991. Nitrogen distribution within a lucerne canopy during regrowth: relation with light distribution. Annals of Botany. 68 (6), 483–488. https://doi.org/10.1093/oxfordjournals.aob.a088286

Lemaire, G., Tang, L., Belanger, G., Zhu, Y., Jeuffroy, M.H., 2021. Forward new paradigms for crop mineral nutrition and fertilization towards sustainable agriculture. European Journal of Agronomy. 125, 126248. https://doi.org/10.1016/j.eja.2021.126248

Li, X.F., Wang, Z.G., Bao, X.G., Sun, J.H., Yang, S.C., Wang, P., Wang, C.B., Wu, J.P., Liu, X.R., Tian, X.L., Wang, Y., Li, J.P., Wang, Y., Xia, H.Y., Mei, P.P., Wang, X.F., Zhao, J.H., Yu, R.P., Zhang, W.P., Che, Z.X., Gui, L.G., Callaway, R.M., Tilman, D., Li, L., 2021. Long-term increased grain yield and soil fertility from intercropping. Nature Sustainability. 4, 943–950. https://doi.org/10.1038/s41893-021-00767-7

Liu, X., Cao, Q., Yuan, Z., Liu, X., Wang, X., Tian, Y., Cao, W., Zhu, Y., 2018. Leaf area index-based nitrogen diagnosis in irrigated lowland rice. Journal of Integrative Agriculture. 17 (1), 111–121. https://doi.org/10.1016/S2095-3119(17)61714-3

Liu, X., Zhang, Y., Han, W., Tang, A., Shen, J., Cui, Z., Vitousek, P., Erisman, J.W., Goulding, K., Christie, P., Fangmeier A., Zhang, F., 2013. Enhanced nitrogen deposition over China. Nature. 494, 459–462. https://doi.org/10.1038/nature11917

Loreau, M., Hector, A., 2001. Partitioning selection and complementarity in biodiversity experiments. Nature. 412, 72–76. https://doi.org/10.1038/35083573

Louarn, G., Bedoussac, L., Gaudio, N., Journet, E.P., 2021. Moreau, D., Jensen, E.S., Justes, E. Plant nitrogen nutrition status in intercrops– a review of concepts and methods. European Journal of Agronomy. 124, 126229. https://doi.org/10.1016/j.eja.2021.126229

Louarn, G., Chabbi, A., Gastal, F., 2020. Nitrogen concentration in the upper leaves of the canopy is a reliable indicator of plant N nutrition in both pure and mixed grassland swards. Grass Forage Science. 75, 127–133. https://doi.org/10.1111/gfs.12466

Luo, C., Wang, S., Zhang, L., Wilkes, A., Zhao, L., Zhao, X., Xu S., Xu, B., 2020. CO_2, CH_4 and N_2O fluxes in an alpine meadow on the Tibetan Plateau as affected by N-addition and grazing exclusion. Nutrient cycling in agroecosystems. 117, 29–42. https://doi.org/10.1007/s10705-020-10062-0

Martins, C.D.M., Schmittm, D., Duchini, P.G., Miqueloto, T., Sbrissia, A.F., 2021. Defoliation intensity and leaf area index recovery in defoliated swards: implications for forage accumulation. Scientia Agricola. 78, 1–8. https://doi.org/10.1590/1678-992X-2019-0095

Pires, A.V., 2010. Bovinocultura de corte. 1 ed. Editora Fealq, Piracicaba, São Paulo, Brasil, 760p.

Pontes, L.S., Maire, V., Louault, F., Soussana, J.F., Carrère, P., 2012. Impacts of species interactions on grass community productivity under contrasting management regimes. Oecologia. 168, 761–771. https://doi.org//10.1007/s00442-011-2129-3

Prado, C.H.B.A., Bortolin, L.H.G.C., Castro, É., Martinez, C.A., 2019. Leaf dynamics of Panicum maximum under future climatic changes. Plos One. 11. https://doi.org/10.1371/journal.pone.0149620

Pugnaire, F.I., Morillo, J.A., Peñuelas, J., Reich, P.B., Bardgett, R.D., Gaxiola, A., Wardle, D.A., Putten, W.H., 2019. Climate change effects on plant-soil feedbacks and consequences for biodiversity and functioning of terrestrial ecosystems. Science Advances. 5, eaaz1834. https://doi.org/10.1126/sciadv.aaz1834

Raij, B., Cantarella, H., Quaggio, J.A., Furlani, Â.M.C., 1997. Recomendações de adubação e calagem para o Estado de São Paulo. 2 ed. Instituto Agronômico de Campinas, São Paulo, Brazil, 285p.

Rohatgi, A., 2017. WebPlotDigitizer. https://automeris.io/WebPlotDigitizer/

Sbrissia, A.F., Duchini, P.G., Zanini, G.D., Santos, G.T., Padilha, D.A., Schmitt, D., 2018. Defoliation strategies in pastures submitted to intermittent stocking method: underlying mechanisms buffering forage accumulation over a range of grazing heights. Crop Science. 58 (2), 945–954. https://doi.org/10.2135/cropsci2017.07.0447

Silva, G.P., Da Silva, S.C., Guttiérrez, A.E., Lemaire, G., Louarn, G., 2019. Stem elongation in Pennisetum purpureum results from a fixed pattern of vegetative development potentially enhanced by the initiation of flowering. Grass and Forage Science. 74, 708–719. https://doi.org/10.1111/gfs.12449

Stevens, C.J., Dise, N.B., Mountford, J.O., Gowing, D.J., 2004. Impact of nitrogen deposition on the species richness of grasslands. Science. 303, 1876–1879. https://doi.org/10.1126/science.1094678

Suding, K.N., Collins, S.L., Gough, L., Clark, C., Cleland, E.E., Gross, K.L., Milchunas, D.G., Pennings, S., 2005. Functional- and abundance-based mechanisms explain diversity loss due to N fertilization. PNAS. 102, 4387–4392. https://doi.org/10.1073/pnas.0408648102

Thakur, M.P., Putten, W.H., Wilschut, R.A., Veen, G.F., Kardol, P., Ruijven, J., Allan, E., Roscher, C., Kleunem, M., Bezemer, T.M., 2021. Plant–Soil feedbacks and temporal dynamics of plant diversity–productivity relationships. Trends in Ecology & Evolution. 36, 651–661. https://doi.org/10.1016/j.tree.2021.03.011

Uwizeye, A., Boer, I.J.M., Opio, C.I., Schulte, R.P.O., Falcucci, A., Tempio, G., Teillard, F., Casu, F., Rulli, M., Galloway, J.N., Leip, A., Erisman, J.W., Robinson, T.P., Steinfeld, H., Gerber, P.J., 2020. Nitrogen emissions along global livestock supply chains. Nature food. 1, 437–446, 2020. https://doi.org/10.1038/s43016-020-0113-y

Viciedo, D.O., Prado, R.M., Martínez, C.A., Habermann, E., Piccolo, M.C., 2019. Short-term warming and water stress affect Panicum maximum Jacq. stoichiometric homeostasis and biomass production. Science of The Total Environment. 681, 267–274. https://doi.org/10.1016/j.scitotenv.2019.05.108

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D., 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecological Applications. 7, 737–750. https://doi.org/10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2

Volaire, F., Barkaoui, K., Norton, M., 2014. Designing resilient and sustainable grasslands for a drier future: Adaptive strategies, functional traits, and biotic interactions. European Journal of Agronomy. 52, 81–89. https://doi.org/10.1016/j.eja.2013.10.002

Wang, S., Isbell, F., Deng, W., Hong, P., Dee, L.E., Thompson, P., Loreau, M. 2021. How complementarity and selection affect the relationship between ecosystem functioning and stability. Ecology. e03347. https://doi.org/10.1002/ecy.3347

Zha, H., Miao, Y., Wang, T., Li, Y., Zhang, J., Sun, W., Feng, Z., Kusnierek, K., 2020. Improving unmanned aerial vehicle remote sensing-based rice nitrogen nutrition index prediction with machine learning. Remote Sensing, 12. https://doi.org/10.3390/rs12020215

Zhao, Z., Dong, S., Jiang, X., Liu, S., Ji, H., Li, Y., Han, Y., Sha, W., 2017. Effects of warming and nitrogen deposition on CH_4, CO_2 and N_2O emissions in alpine grassland ecosystems of the Qinghai-Tibetan Plateau. Science of The Total Environment. 592, 565–572. https://doi.org/10.1016/j.scitotenv.2017.03.082

Ziadi, N., Bélanger, G., Gastal, F., Claessens, A., Lemaire, G., Tremblay, N., 2009. Leaf nitrogen concentration as an indicator of corn nitrogen status. Agronomy Journal. 101, 947–957. https://doi.org/10.2134/agronj2008.0172x

Supplementary material

Table S.1. Pre- and post-cutting canopy height (mean ± standard error of the mean).

Season	Treatments				Mean
	Andropogon grass	Massai grass	Piata grass	Association	
Pre-cutting height (cm)					
Summer I	35.1 ± 0.06	35.0 ± 0.05	35.3 ± 0.10	35.2 ± 0.13	35.2
Autumn	34.9 ± 0.29	35.1 ± 0.06	30.2 ± 0.74	34.8 ± 0.30	33.7
Winter/Early Spring	35.3 ± 0.13	34.9 ± 0.15	35.2 ± 0.13	35.1 ± 0.12	35.1
Late Spring	35.3 ± 0.15	35.3 ± 0.15	34.9 ± 0.11	35.1 ± 0.13	35.1
Summer II	35.4 ± 0.13	35.1 ± 0.14	35.1 ± 0.17	34.9 ± 0.06	35.1
Mean	35.2	35.1	34.1	35.0	
Post-cutting height (cm)					
Summer I	17.6 ± 0.15	17.2 ± 0.14	17.5 ± 0.07	17.2 ± 0.10	17.4
Autumn	17.5 ± 0.13	17.1 ± 0.09	17.3 ± 0.29	17.3 ± 0.18	17.3
Winter/Early Spring	17.7 ± 0.09	17.4 ± 0.21	17.6 ± 0.18	17.4 ± 0.09	17.5
Late Spring	17.4 ± 0.13	17.1 ± 0.25	17.6 ± 0.24	17.5 ± 0.37	17.4
Summer II	17.5 ± 0.09	17.1 ± 0.04	17.3 ± 0.04	17.3 ± 0.06	17.3
Mean	17.5	17.2	17.5	17.3	

Table S.2. Pre- and post-cutting canopy light interception (mean ± standard error of the mean).

Season	Treatments				Mean
	Andropogon grass	Massai grass	Piata grass	Association	
Pre-cutting canopy light interception (%)					
Summer I	93.3 ± 0.26	94.2 ± 0.35	95.6 ± 0.05	94.5 ± 0.24	94.4
Autumn	93.8 ± 0.44	94.0 ± 0.60	95.6 ± 0.29	94.2 ± 0.66	94.4
Winter/Early Spring	95.6 ± 0.58	94.0 ± 0.58	94.4 ± 1.31	94.3 ± 0.37	94.6
Late Spring	95.1 ± 0.19	93.8 ± 0.47	93.9 ± 0.39	95.1 ± 0.89	94.4
Summer II	93.9 ± 0.39	94.2 ± 0.46	95.6 ± 0.33	96.6 ± 0.58	94.3
Mean	94.3	94.0	95.0	94.9	
Post-cutting canopy light interception (%)					
Summer I	84.2 ± 1.77	87.3 ± 0.69	88.1 ± 0.94	87.0 ± 0.42	86.6
Autumn	84.5 ± 1.54	88.8 ± 0.85	89.3 ± 1.18	88.2 ± 1.49	87.7
Winter/Early Spring	89.8 ± 0.88	89.7 ± 0.93	92.4 ± 0.83	92.1 ± 0.64	91.0
Late Spring	86.8 ± 1.42	84.4 ± 2.94	87.9 ± 0.94	85.6 ± 2.76	86.2
Summer II	83.6 ± 1.56	86.6 ± 0.77	89.1 ± 0.71	87.5 ± 1.22	86.7
Mean	85.8	87.4	89.4	88.1	

Table S.3. Amount of nitrogen applied (mean ± standard error of the mean).

Season	Treatments				Mean
	Andropogon grass	Massai grass	Piata grass	Association	
Amount of nitrogen applied until data collection (kg ha^{-1})					
Late Spring I	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0
Summer I	80.7 ± 4.50	83.6 ± 4.47	74.8 ± 2.22	79.8 ± 4.26	79.7
Autumn	79.3 ± 4.50	76.4 ± 4.47	85.2 ± 2.22	80.2 ± 4.26	80.3
Winter/Early Spring	00.0 ± 0.00	00.0 ± 0.00	00.0 ± 0.00	00.0 ± 0.00	00.0
Late Spring II	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0 ± 0.00	40.0
Summer II	62.9 ± 1.85	53.8 ± 5.15	47.5 ± 2.50	42.5 ± 4.22	51.7
Mean	50.5	49.0	47.9	47.1	

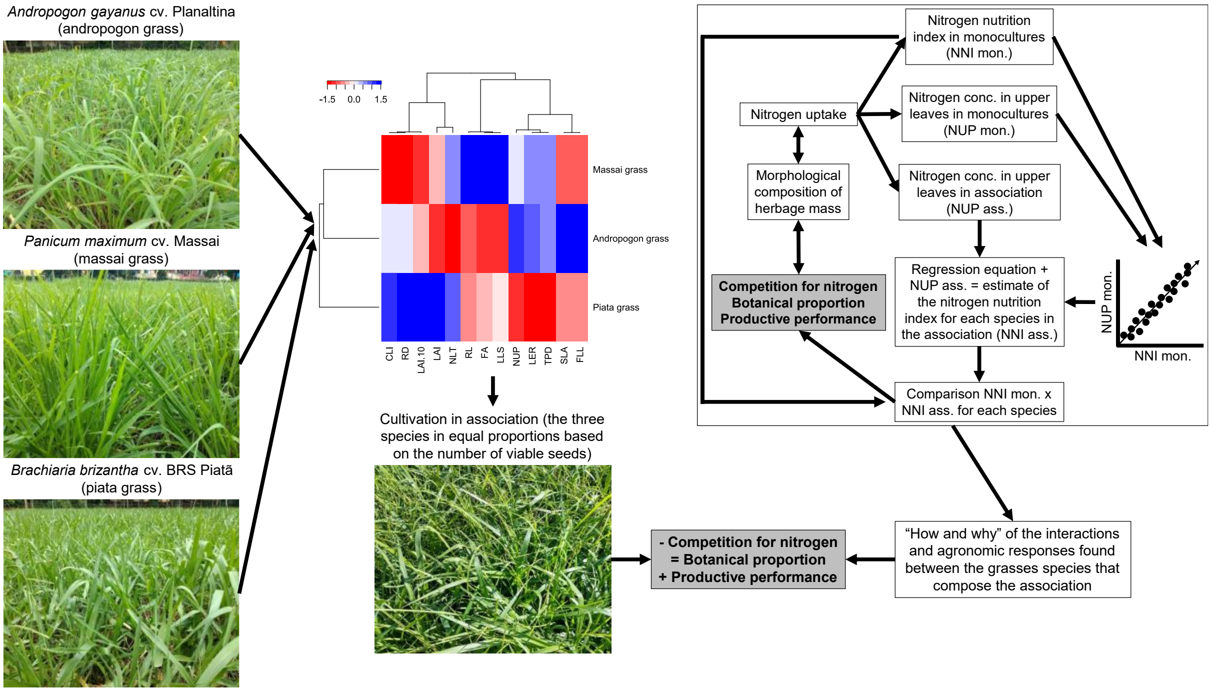


Figure S.1. Graphical abstract of chapter structure. Heatmap and cluster in monoculture of the functional traits of grasses species. The data correspond to the average of five seasons. Legend: leaf elongation rate per tiller (cm tiller^{-1} day^{-1}) (LER); number of leaves per tiller (n leaves tiller^{-1}) (NLT); final leaf length (cm leaf^{-1}) (FLL); tiller population density (tiller^{-1} m^{2}) (TPD); canopy light interception (%) (CLI); foliage angle (°) (FA); leaf area index (m^{2} m^{-2}) (LAI); leaf area index in the upper 10 cm (m^{2} m^{-2}) (LAI.10); specific leaf area (cm^{2} g^{-1}) (SLA); leaf lifespan (days) (LLS); root diameter (mm) (RD); root length (cm m^{3}) (RL); and nitrogen concentration in upper leaves (%DM) (NUP).

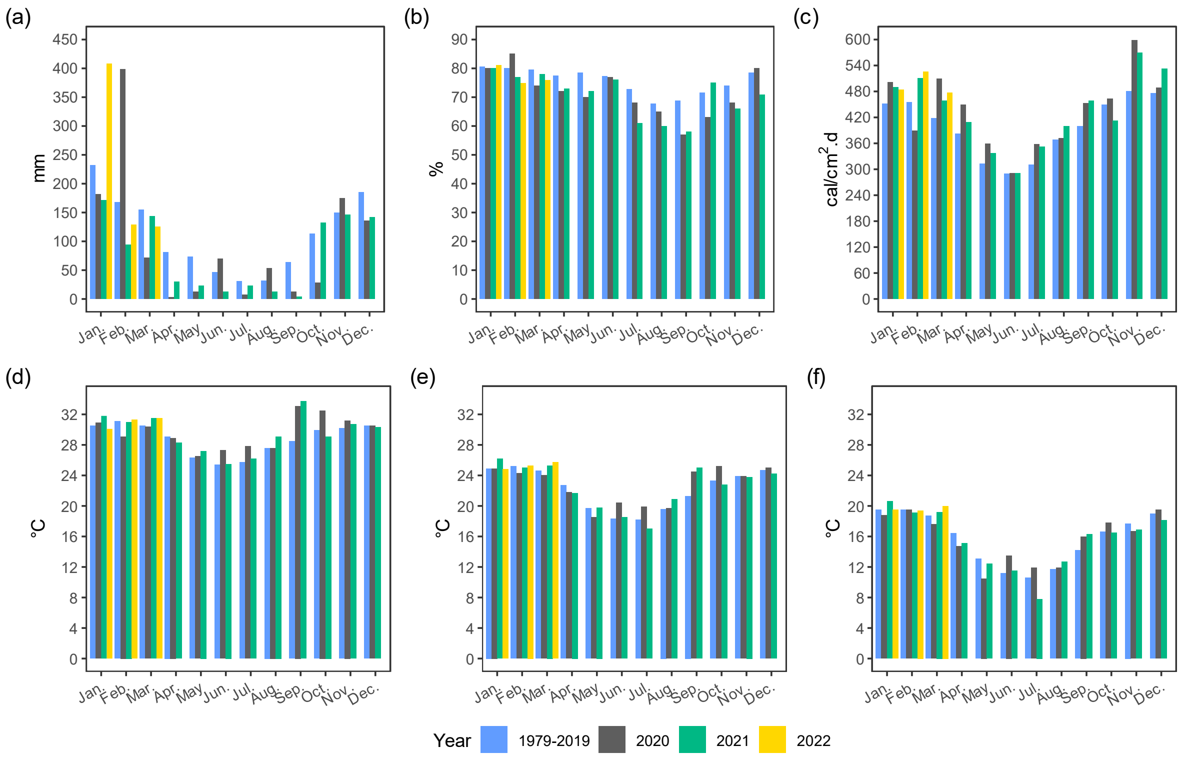


Figure S.2. Monthly weather data: 40-year average (1979-2019) and years of experiment implementation (2020) and data collection (2021-2022). Accumulated rainfall (a), average air humidity (b), average radiation (c), maximum (d), mean (e), and minimum (f) air temperature.

Figure S.3. Upper leaves of forage grasses: andropogon grass (a), massai grass (b), and piata grass (c).

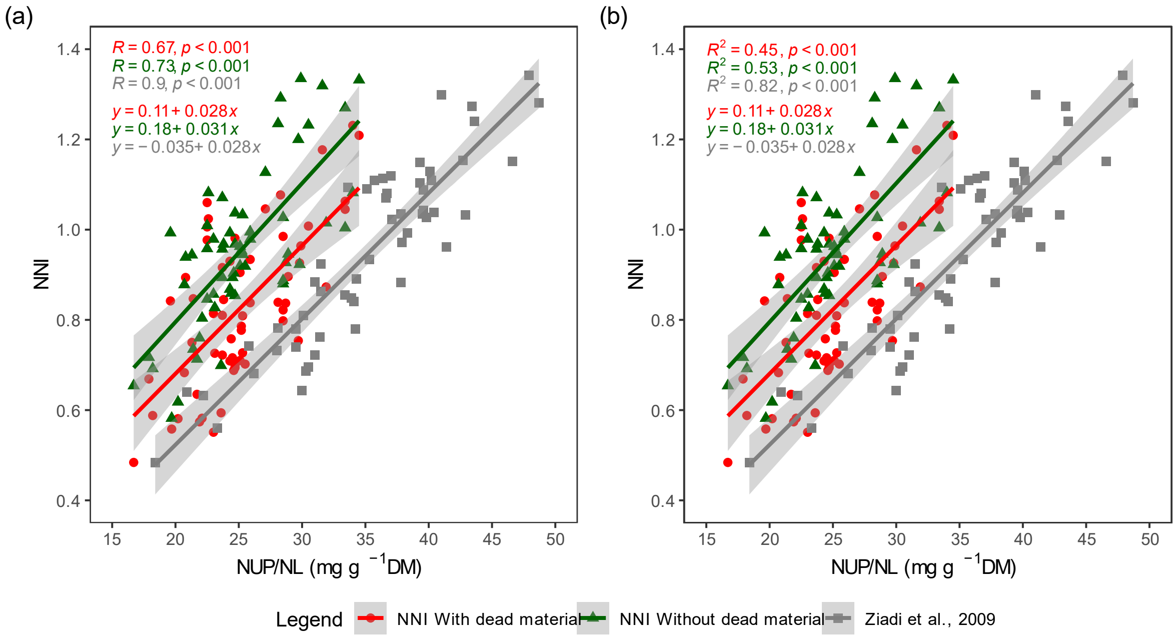


Figure S.4. Relationship between NNI with and without dead material and NUP (plants: andropogon grass, massai grass, and piata grass), and the relationship between NNI and nitrogen concentration in the leaf (NL) (plant: corn) (Ziadi et al., 2009). *R* equivalent to Pearson correlation (a), and R^2 equivalent to determination coefficient (b). The data from (Ziadi et al., 2009 – Figure 4 a) was extracted with the software (WebPlotDigitizer) (Rohatgi, 2017).

During Autumn, the NNI without dead material was affected by cultivation method ($p = 0.002$) and interaction cultivation x grass species ($p < 0.001$). During Winter/Early Spring, cultivation effect was observed ($p = 0.014$). In the Late Spring, plant effect was observed ($p < 0.001$) and interaction cultivation x grass species ($p = 0.006$). In the Summer II, cultivation effect was observed ($p = 0.037$). In Autumn, there was a cultivation effect with higher NNI for plants in monoculture, and there was interaction indicating that andropogon grass and massai grass in monoculture had higher NNI than their plants in the association, and piata grass showed the same NNI in both methods of cultivation. In Winter/Early Spring, there was only a cultivation effect, indicating that plants in monoculture had higher NNI than plants in the association. In Late Spring, plants with higher NNI for andropogon grass were affected, and the interaction indicated higher NNI for piata grass in the association compared to its monoculture (Figure S.5 a b).

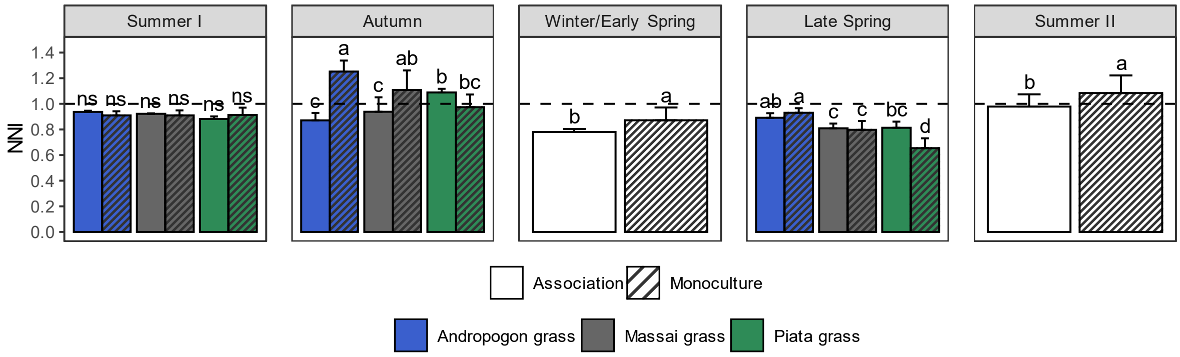


Figure S.5. NNI with dead material in monocultures and association. Values close to the dotted line indicate satisfactory NNI.

Cutting interval varied with treatments during Summer I, Autumn, Late Spring, and Summer II ($p < 0.05$). During Summer I, Autumn, and Summer II piata grass showed the longest cutting interval. In Late Spring piata grass presented longer cutting interval than andropogon grass (Figure S.6).

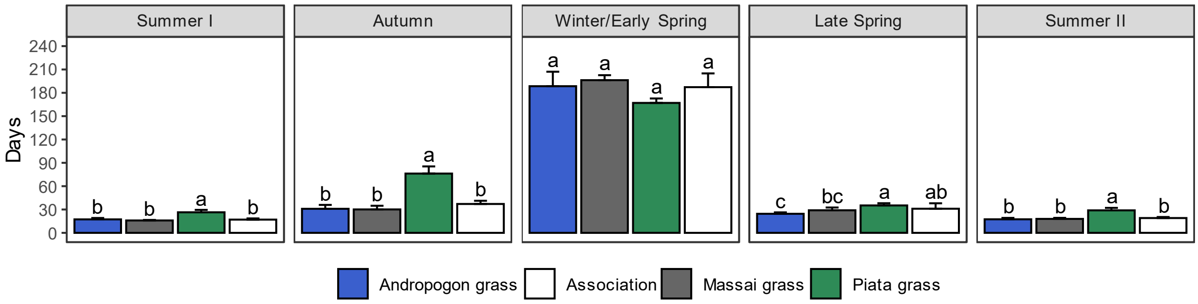


Figure S.6. Cutting interval throughout the experimental period.

References

Rohatgi, A., 2017. WebPlotDigitizer. https://automeris.io/WebPlotDigitizer/

Ziadi, N., Bélanger, G., Gastal, F., Claessens, A., Lemaire, G., Tremblay, N., 2009. Leaf nitrogen concentration as an indicator of corn nitrogen status. Agronomy Journal. 101, 947–957. https://doi.org/10.2134/agronj2008.0172x

4. ROOT PROPORTION IN ROOT MASS OF PERENNIAL FORAGE GRASSES GROWN IN ASSOCIATION: CALIBRATION AND APPLICATION OF A DNA-CHLOROPLAST BASED METHOD

Abstract

Above ground botanical proportion can be easily attributed to different species in biodiverse ecosystems using methods of taxonomic and morphological identification. However, below ground identification can be challenging when no assignable parts are available. Here, we describe the calibration of a DNA-chloroplast based method with two objectives: (i) to determine whether botanical composition of root samples from DNA artificial mixtures of perennial forage grasses can be accurately estimated, and (ii) to apply the calibrated method to field data to determine below ground botanical proportion and its relationship with the correspondent above ground botanical proportion. The study was carried out following three consecutive steps: (i) DNA extraction, PCR with specific gene plastid *rpoA*, and sequencing, (ii) bioinformatics analysis for data processing and DNA comparison with an online free database to identify grass species taxonomy based on genetic variation, and (iii) application of the method to a data set harvested from a two-year field study to compare below and above ground botanical proportion. The calibration method treatments were four DNA artificial mixtures of DNA known proportions of three tropical perennial forage grass species: *Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass) and *Brachiaria brizantha* cv. BRS Piatã (piata grass). The DNA artificial mixtures were, mixture 1 = 33%, 33%, 33%, mixture 2 = 60%, 20%, 20%, mixture 3 = 20%, 60%, 20%, and mixture 4 = 20%, 20%, 60% respectively. Field data were collected once every season of the year during two consecutive years in a pasture established with 33% of each of the three grass species, with four replications. Above ground botanical proportion was determined by hand separation of herbage mass botanical components. The results from the calibration method indicated that differences between the actual and the estimated percentage were 0.10±0.01 for andropogon grass, -8.97±0.99 for massai grass, and 11.9±0.29 for piata grass. Field data were corrected using calibration curves for each forage grass species. Correlation between below and above ground botanical proportion was $R = 0.88$. The pros and cons of this method and future challenges and opportunities regarding its use are discussed throughout the manuscript.

Keywords: botanical proportion, molecular identification, plant root, plant taxonomy, species abundance

4.1. Introduction

Botanical diversity regulates many ecosystem functions and offers the opportunity for sustainable agricultural intensification through simultaneous or partial cultivation of plant species in the same field, which can result in increased forage yield, reduced fertilizer and pesticide inputs, and smaller influence of edaphoclimatic variations (Isbell et al., 2015; Hanisch et al., 2020; Tilman, 2020). However, the mechanisms that highlight these relationships are almost entirely explained by the diversity of above ground plants, with little information directed to the root system (Bardgett et al., 2014; Wagemaker et al., 2021). More than half of the global plant biomass corresponds to below ground tissue (Jackson et al., 1996), and it is unknown whether the richness patterns described for above ground vegetation are also valid for the large below ground portion. In this context, understanding of plant-plant interactions below ground and their relationship with the above ground vegetation may unravel important knowledge and information for the better understanding of such biodiverse ecosystems. It is expected that in species-rich pastures, resource capture below ground can happen at different scales in time and space, from the uptake of water and nutrients at different soil depths, as consequence of differences in root length and/or diameter that result in a more uniform filling of the occupied soil volume (Mommer et al., 2010; Bardgett et al., 2014). In this case, if below ground resources are used more efficiently, the forage canopy may perform better above ground. Therefore, it becomes important to differentiate the proportion of roots of each species in biodiverse pastures for better understanding the functioning of such ecosystems.

Traditionally, taxonomic classification and root proportions of each species in samples with several species are determined through hand separation, considering morphological differences such as color, diameter, branching order, and root tip (Freschet et al., 2021). However, hand separation may be impractical when the species that make up the association have similar root characteristics, making botanical differentiation indistinguishable and resulting in underestimation of diversity.

Several techniques have been developed to differentiate botanical proportions of roots from different species without the need for hand separation. For example, near-infrared spectroscopy (NIRS) provides relative estimates based on species-specific chemical composition (Roumet et al., 2006). Carbon isotope signatures distinguish roots from C_3 and C_4 grasses (Hobbie et al., 2004). And biochemical evaluations of cuticular wax compositions, since alkanes and fatty alcohol compositions of these waxes are different in specific plant parts, also make it possible to differentiate them (Dawson et al., 2000; Soussana et al., 2005). All these methods provide accurate estimates; however, they may be limited by the fact that the chemical properties of plant tissue may change under different management and edaphoclimatic conditions, such as frequent cutting (Dawson et al., 2000) and changing atmospheric CO_2 conditions (Soussana et al., 2005).

DNA-based detection techniques, from classical PCR amplification, chloroplast, or mitochondrial barcode loci, and in some cases, combined with Sanger sequencing or restriction fragment length polymorphism (Bobowski et al., 1999; Jackson et al., 1999; Brunner et al., 2001; Ridgway et al., 2003; Wildová, 2004; McNickle et al., 2008; Mommer et al., 2011), promoted great advances in this field of research, mainly because they are less dependent on management and edaphoclimatic conditions. As of 2008, Mommer et al. (2008) introduced the quantitative polymerase chain reaction (qPCR) in studies regarding plant species root distributions. Wagemaker et al. (2021) described a new methodology based on multiple species genotyping by sequencing (msGBS) as the next step to address challenges generated in previous studies. Despite the advances, the results suggest that further studies must be conducted to make the methods more efficient and create methods that can accurately identify all species in mixed samples.

Here, we describe the calibration of a DNA-chloroplast based method with two objectives: (i) to determine whether botanical composition of root samples from DNA artificial mixtures of perennial forage grasses can be accurately estimated, and (ii) to apply the calibrated method to field data to determine below ground botanical proportion and its relationship with the correspondent above ground botanical proportion. The hypotheses are: (i) the method recovers the same proportions of plants in artificially mixed root mass samples, and (ii) the botanical proportion below and above ground is similar. The study was carried out following three consecutive steps: (i) DNA extraction, PCR with specific gene plastid *rpoA*, and sequencing, (ii) bioinformatics analysis for data processing and DNA comparison with an online free database to identify grass species taxonomy based on genetic variation, and (iii) application of the method to a data set harvested from a two-year field study to compare below and above ground botanical proportion.

4.2. Methodology

4.2.1. Grass species, growth conditions, and sampling

Three species of tropical perennial forage grasses (*Andropogon gayanus* cv. Planaltina (andropogon grass), *Panicum maximum* cv. Massai (massai grass) and *Brachiaria brizantha* cv. BRS Piatã (piata grass)) were cultivated in an experimental area of the Department of Animal Science of the "Luiz de Queiroz" College of Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil (22°42'35" South Latitude, 47°38'24" West Longitude and 546 m altitude). All grass species were managed intermittently with a pre-cutting height of 35.0 cm and a post-cutting height of 17.5 cm. Root samples were collected in Late Spring 2020 immediately after cutting at the midpoint of the distance between tussocks at 32 representative locations (visual mass assessment of canopy forage and height) for each grass species using a 6.3 cm diameter steel auger/probe at a depth of 0-20 cm. Subsequently, samples were pooled into a group sample per grass species, frozen for storage at -35 °C, thawed washed in running water, dried in a forced draught oven at 60°C until constant weight, and ground in a "Wiley" type mill with a 1 mm sieve. Illustrations of the experimental area, forage grass species, and root samples are provided as supplementary material (Figures S.1 and S.2).

4.2.2. Treatments (DNA artificial mixtures)

Treatments corresponded to four DNA artificial mixtures of root mass from three grass species prepared with known proportions on a dry matter basis. The grass species were andropogon grass, massai grass, and piata grass, and the DNA artificial mixtures corresponded to: mixture 1 = 33%, 33%, 33%; mixture 2 = 60%, 20%, 20%; mixture 3 = 20%, 60%, 20%; and mixture 4 = 20%, 20%, 60%, respectively, prepared in triplicates.

4.2.3. Root samples collection for application of the method and comparisons between below and above ground botanical proportion

Root mass samples were collected from a mixed pasture of the three grass species cultivated in the same experimental area as monocultures. Grasses, experimental site, and management were the same as described in item 2.1. The mixed pasture was sown in January 2020, with 1/3 for each grass species in the association (100 viable pure seeds m^{-2}). Root samples were harvested once every season of the year as follows: Autumn 2020, Winter/Early Spring 2020, Late Spring 2020, Summer 2020/2021, Autumn 2021, Winter/Early Spring 2021, Late Spring 2021, Summer 2021/2022, with four replicates. Sampling was performed always at post-cutting, at the midpoint of the distance between tussocks on eight representative locations (visual assessment of canopy forage mass and height) per paddock using a steel auger/probe (6.3 cm in diameter, and 30 cm depth) at three soil depths: 0–10, 10–20, 20–30 cm. The eight samples per paddock were grouped by soil depth, frozen for storage at -35 °C, thawed washed in running water, dried in a forced draught oven at 60°C until constant weight, and ground in a "Wiley" type mill with a 1 mm sieve.

The botanical proportion above ground was also performed once every season of the year (see above) at pre-cutting. Samples of herbage mass were collected from two representative areas of the paddocks (visual assessment of canopy forage mass and height) using a 100 × 25 cm metallic frame and cutting all the forage inside the frame at ground level. Subsequently, botanical components were hand separated and dried in a forced draught oven at 60°C until constant weight. The results were used to calculate the botanical percentage (% andropogon grass, % massai grass, % piata grass, % dead material overall, and % weeds) with four replications. Due to the difficulty of separating dead material by grass species and identifying weeds, all dead material and weeds were measured separately and not included in the botanical percentage.

4.2.4. DNA extraction and genome sequencing

Approximately 0.0300 mg of root sample was placed in 2 ml microtubes and subsequently sprayed with 2.3 mm chrome steel beads (BioSpec Products, Inc., Bartlesville, OK, USA) in a Bead Rupter (OMNI International, PerkinElmer Company). DNA extraction was performed with the MagMaxTM Plant DNA Kit (Applied Biosystems). Initially, PCR was performed for the three grass species in monoculture. The PCRs were tested on two primers in different regions of the chloroplast: *rpoA*, and *psbH*. Silico analysis, such as multiple alignments of the flanking region for the three grass species, showed that the primers would be efficient in separating the three grass species (Figures S.5 and S.6 complementary material). The PCR reaction conditions were: 1µL of DNA, 0.2 µM of each primer (forward/reverse), 12.5 µL of 2X PCRBio Ultra Mix (PCR Biosystems) and ultrapure water elution in a final volume of 25 µL. The reactions were carried out in a BioRad T100 thermocycler (BioRad Laboratories Inc, CA – USA) under the following conditions: 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes. PCR products were separated by 1.5% agarose gel electrophoresis and PCR products were purified with AMPure XP beads (Beckman Coulter, Brea, USA). Then, the purified DNA was subjected to another PCR reaction to link barcodes according to the Nextera XT Kit (Illumina, San Diego, USA). The PCR reaction comprised 12.5µL of 2x PCR Ultra Mix (PCR BioSystems), 2.5µL of each adapter (Nextera XT Index 1 Primers (barcode N7XX and Nextera XT Index 2 Primers (S5XX), 2.5µL of product of the previous reaction and ultrapure water for a final volume of 25 µL, which was carried out under the following conditions: 95°C for 3 minutes, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds ; followed by a final extension at 72°C for 5 minutes. At the end of the reaction, amplification of DNA purified with AMPure XP beads and was evaluated by agarose gel electrophoresis. The libraries were measured using Nanodrop and Qubit DNA HS.

All purified amplicons were pooled in equal molar amounts and the final concentration of the library was determined using a SYBR green quantitative PCR assay with specific primers for Illumina adapters (KAPA Biosystems, Roche).

The library pool was denatured with 0.1N NaOH, then diluted and combined with 20% denatured PhiX prepared according to Illumina guidelines. Samples were sequenced on the MiSeq (Illumina, San Diego - CA), sequencing platform, using a 2 x 250 cycle V2 kit.

4.2.5. Bioinformatics and statistical analyses

All bioinformatics and statistical analyzes were performed in the R language (version 4.1.2; R Core Team, 2022). First, multiplexed reads were assigned to biological samples. Then, the DADA2 program (version 1.22.0; Callahan et al., 2016) was used for modeling and error correction of amplicons. The DADA2 package has a complete pipeline to transform the Amplicon Sequencing Variants (ASV) fastq files into inferred, disassembled, and chimera-free sample ASVs.

Filtering of fastq files was performed to cut the PCR primer ASVs and filter the 3' ends of the reads due to quality decay (Q<30). After filtering, the reads had a size of 2 x 235 bp; due to the size of the generated amplicon, the readings were only concatenated. The DADA2 algorithm uses a parametric error model, and each amplicon dataset has different error rates. The learnErrors method learns this error model from the data, alternating between estimating error rates and inferring sample composition until they converge on a consistent solution. As with many machine learning problems, the algorithm must start with an initial guess, for which the maximum possible error rates on that data are used. Then, the denoising step is performed to obtain a detailed list of unique ASVs and abundances and produce consensus position quality scores for each unique ASV, averaging the positional qualities of the component reads.

After the initial processing of the sequencing data by DADA2, that totaled 50 ASVs, including the validation test and field data, taxonomies were assigned to each ASV using the blast algorithm of the National Center for Biotechnology Information (NCBI), NR database (O’Leary et al., 2016). The taxonomy assigned to each ASV resulted in separations by genus and grass species, which made it possible to adjusted taxonomic nomenclature based on prior knowledge of above ground vegetation. Subsequently, ASV merging was performed for each adjusted taxonomy (Table S.1 supplementary material). Finally, after merging the ASVs, where each adjusted taxonomy represented number of ASVs, it was possible to observe the results for each sample. The results of the calibration method were expressed as a taxonomic percentage of each grass species found in each DNA artificial mixture.

A second-order polynomial regression of the proportion of grass species, maximum at proportion = 50% and zero at proportions 0% and 100% was used to determine the relationship between the actual percentage and the estimated percentage of each grass species. Equations of regression were used to adjust the estimated percentage of each grass species (Equations 1 and 2).

$$y = d–y.100 \tag{1}$$

$$d = a.y(y/100–1) \tag{2}$$

Where "y" = represents the estimate recovered by the method and value to be corrected, and "d" = represents the deviation what is a measure of the difference between the estimated and actual value, and the parameters "a" estimated in polynomial regression for each grass species were: andropogon grass (-0.00005), massai grass (0.00573), and piata grass (-0.00543) (Figure 1 b). The difference between actual and estimated percentages was calculated by the mean of the derivation.

Field data also were expressed as taxonomic percentage and shown as two-year average at each season of the year. First, we tested the normal distribution (Figure S.3 supplementary material). Subsequently, analysis of variance (ANOVA "aov" procedure) was used to test for significant differences among treatments within seasons of the year. The least significant difference test (LSD test) tested significant differences between means. Differences were considered significant with $p < 0.05$. Relative botanical proportion was estimated from above ground botanical proportion divided by below ground botanical proportion. Values close to the dotted line indicate similarity, values above indicate greater percentage above ground, and values below indicate a greater percentage below ground. Finally, Pearson correlation was used to evaluate the relationship between below and above ground botanical percentages and the relationships between the three soil depths.

4.3. Results

4.3.1. Calibration method

The taxonomic classification in the mixtures indicated the presence of artificially mixed grass species; andropogon grass, massai grass, and piata grass, in addition to a small percentage of plants from the *Miscanthus* and *Paspalum* genus, which are common weeds in the region. The difference between actual and estimated percentage presence was 0.10±0.01 for andropogon grass, -8.97±0.99 for massai grass, and 11.9±0.29 for piata grass (Figure 1 a b). Polynomial regression equations were used to adjust the estimated percentage of each grass species (Figure 1 b). After correction, values for estimated percentage presence were very close to values of actual percentage presence, indicating that the calibration improved the method's precision (Figure 1 c).

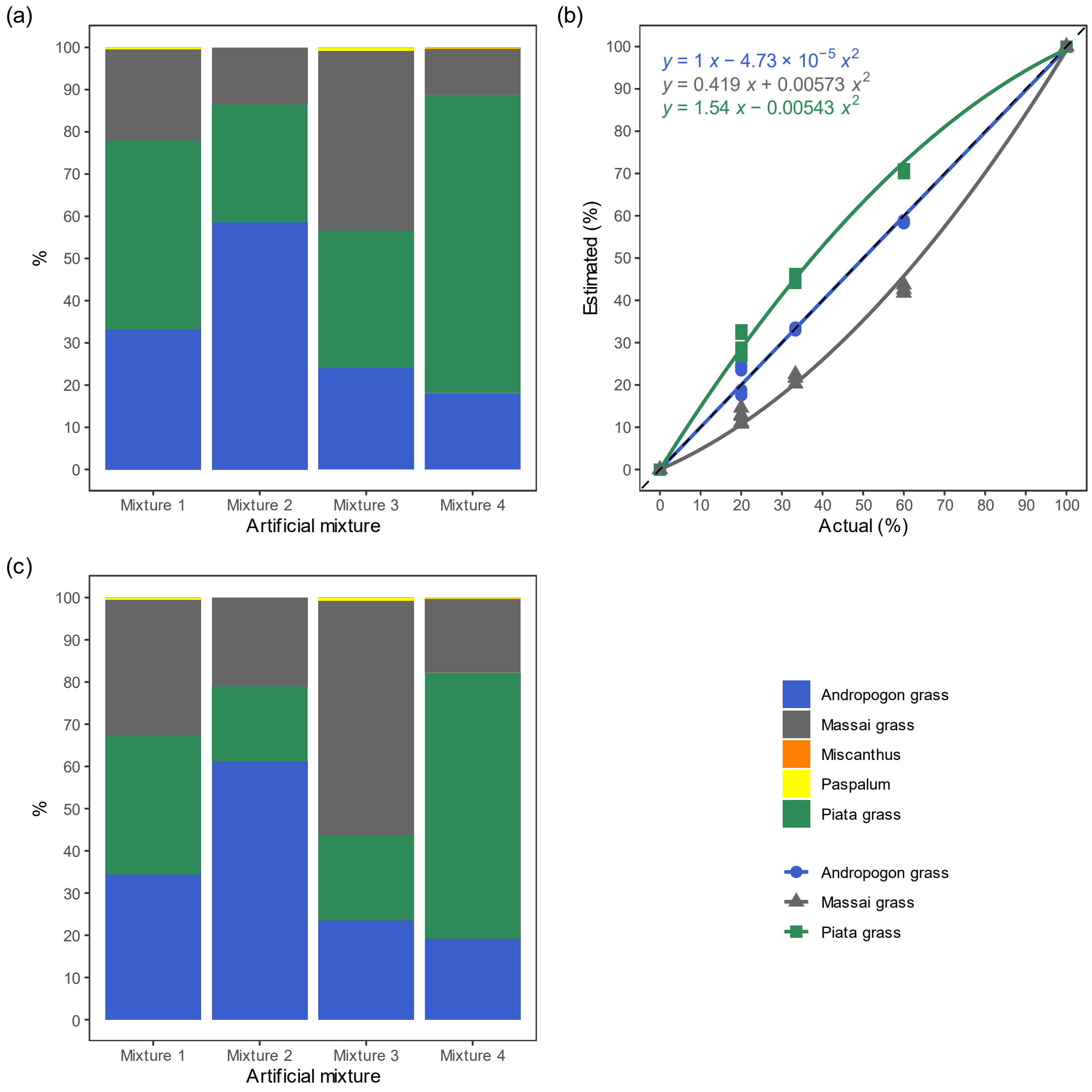


Figure 1. Botanical proportion of each grass species in DNA artificial mixture before correction (a), regression of actual percentage presence and estimated percentage presence before correction (b), and botanical proportion of each grass species in DNA artificial mixture after correction (c). Legend of grass species and DNA artificial mixtures: grass species = andropogon grass, massai grass, and piata grass, DNA artificial mixtures = mixture 1 = 33%, 33%, 33%, mixture 2 = 60%, 20%, 20%, mixture 3 = 20%, 60%, 20%, and mixture 4 = 20%, 20%, 60% of each grass species, respectively. The dotted line stands for the 1:1 line.

4.3.2. Botanical proportion in field data

The botanical composition of canopy herbage mass showed greater percentage of massai grass, dead material, piata grass, weeds, and andropogon grass, respectively, except during

Winter/Early Spring and Late Spring, when the percentage of dead material was greater than percentage of massai grass (Figure 2 a).

The application of the method DNA-chloroplast on a field data set indicated that the taxonomic classification resulted in greater values of percentage presence for massai grass, piata grass, and andropogon grass, respectively, in addition to plant species from the *Eleusine*, *Digitaria*, *Malvastrum*, and *Paspalum* genus, common weeds of the experimental area. The botanical percentage at different soil depths showed the same pattern of response, indicating that botanical proportion was similar at the three depths studied, with a slightly greater percentage of *Paspalum* at the deepest soil layer (20-30 cm) relative to 0-10 and 10-20 cm. Over the seasons of the year, piata grass and andropogon grass showed a slight increase in percentage presence relative to massai grass, with maximum percentage of piata grass recorded in Late Spring and of andropogon grass in Summer. The maximum percentage presence of massai grass was recorded in Autumn (Figure 2 b).

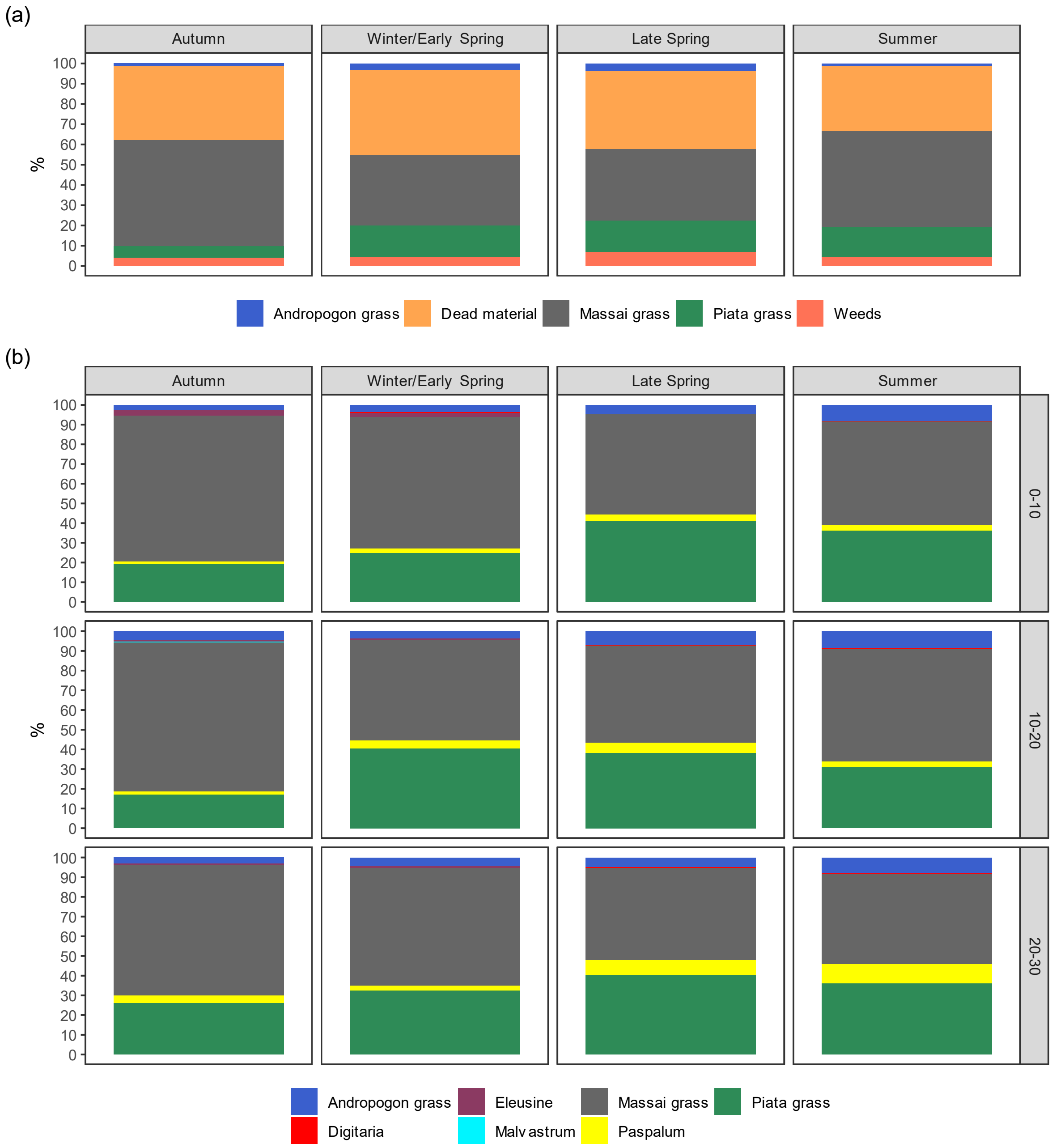


Figure 2. Botanical proportion, dead material, and weeds in canopy herbage mass (a), and botanical proportion in root mass at 0-10 cm, 10-20 cm, and 20-30 cm soil depth (b).

The above ground and below ground botanical proportion varied throughout the seasons of the year ($p < 0.01$). At establishment, the plan was to have an initial percentage presence of 33.3% for each grass species, but over time, as the experiment progressed, the recorded values of percentage presence varied, resulting in greater presence of massai grass and a smaller presence of andropogon grass, except for above ground during Autumn, when andropogon grass and piata grass showed the same percentage presence, and below ground during Late Spring and Summer, when massai grass and piata grass showed the same percentage presence. It is possible to observe variations throughout seasons of the year, with the smallest difference among grass species above ground recorded during Winter/Early Spring and Late Spring, and below ground recorded during Late Spring and Summer (Figure 3 a b). The relative botanical proportion indicated that massai grass, the grass species with greatest percentage presence, showed less variation between above ground and below ground botanical proportion, with a slight indication of greater above ground percentage. Andropogon grass showed greater below ground percentage presence during Autumn and Summer, and greater above ground percentage presence in Winter/Early Spring and Late Spring. Piata grass had the same percentage presence during Winter/Early Spring, and during the other seasons of the year, it had a greater percentage presence below ground (Figure 3 c).

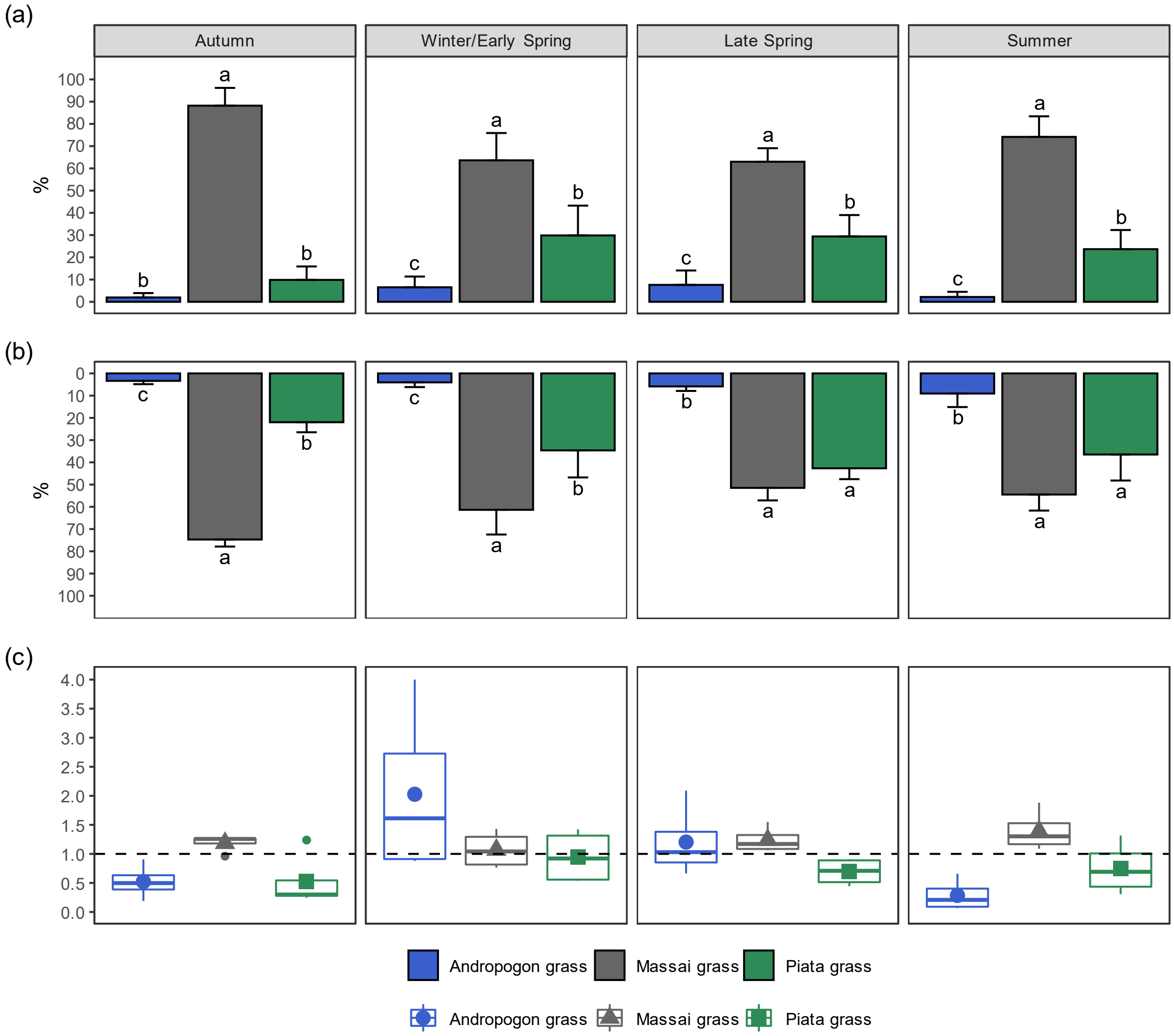


Figure 3. Botanical proportion in above ground (a), below ground (b), and relative botanical proportion (c). Averages followed by the same letter within each year season do not differ from each other (p <0.05).

Pearson correlation between above ground and below ground botanical percentage presence indicated a high positive association, with an *R* of 0.88 (Figure 4 a). Pearson correlation matrix also indicated high correlation, with *R* above 0.83, indicating a high correlation between above ground and below ground botanical proportion at different soil depths and among soil depths (Figure 4 b).

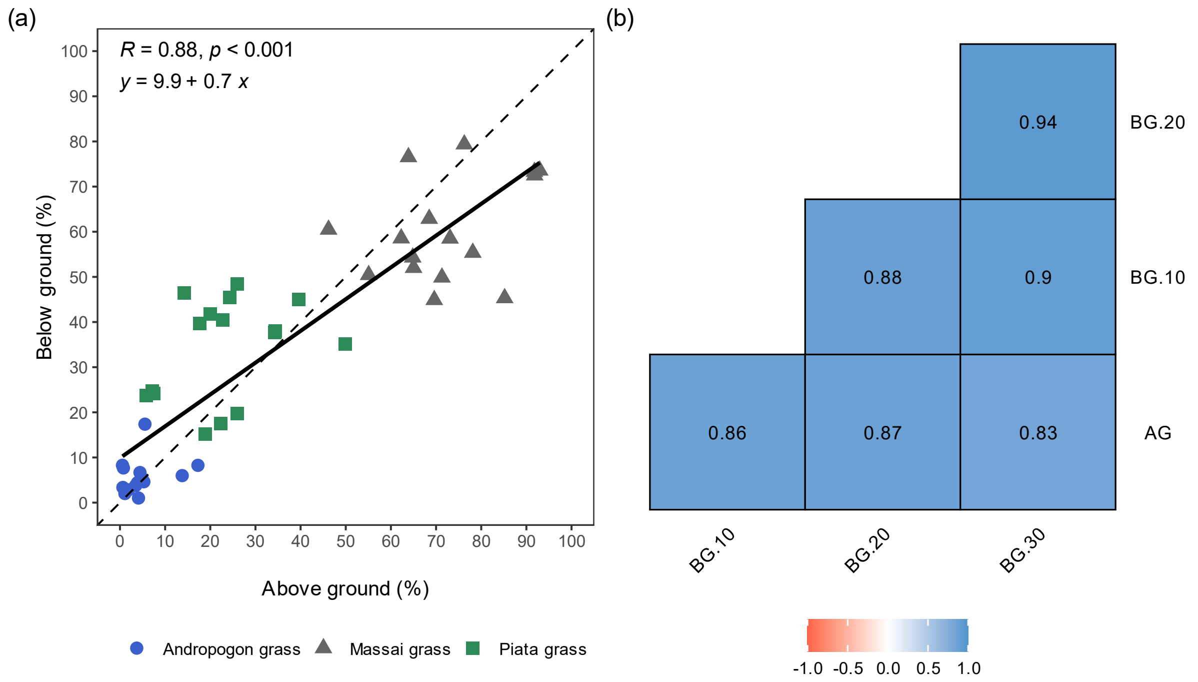


Figure 4. Pearson correlation (a), and Pearson correlation matrix between below and above ground per soil depth botanical proportion (b). Legend: botanical proportion in above ground (AG), and below ground per soil depth (soil depth 0-10 cm = BG.10), (soil depth 10-20 cm = BG.20), and (soil depth 20-30 cm = BG.30). The dotted line stands for the 1:1 line.

4.4. Discussion

4.4.1. Calibration method

As in many studies of development and calibration of methods to differentiate the taxonomy and taxonomic proportion of roots below ground in biodiverse ecosystems, challenges and interesting results emerged that may contribute to advances towards better understanding of plant-plant interactions below ground and their relationship with above ground vegetation.

Among the challenges, the first was choosing the most appropriate DNA barcode markers. DNA barcoding involves sequencing a standard region of DNA as a tool for plant species identification. And based on the proportions of DNA barcodes in a sample with several plant species, it is possible to define the quantitative proportion of each species (Pennisi, 2019). In animal studies, large-scale standardized sequencing of the mitochondrial *COI* gene has made

DNA barcoding an efficient tool for identifying species in groups of animals (Hebert et al., 2003). In plant studies, however, mitochondrial genes have not shown promising results because they have low nucleotide substitution rates (Cowan et al., 2006; Mower et al., 2007). Other studies based on multilocus approaches of plastid genes (chloroplasts) have shown interesting results for the taxonomic identification of plants (Kesanakurti et al., 2011; Mommer et al., 2011; Rewald et al., 2012). However, the practical application of barcoding chloroplast genes in plants is limited because there is no consensus on the most suitable DNA region or regions for all plants (Pennisi, 2007; Fazekas et al., 2008). In the last two decades, many studies have focused on the use of universal regions of the genome as an internal transcribed spacer (ITS) and have concluded that these regions have the potential to be used for a more general application, making it possible to differentiate plants at the level of genus and some at the level of species (Linder et al., 2000; Kesanakurti et al., 2011; Wang et al., 2022). The studies described different recommendations. Kress and Erickson (2007) recommended a combination of the non-coding *trnH-psbA* spacer region and a portion of the *rbcL* coding gene as a global two-locus land plant barcode provides the necessary universality and species discrimination. The CBOL Plant Working Group (2009) recommended the combination of the *rbcL* + *matK* loci. And Wang et al. (2022) recommended *matK* and ITS for presenting greater discriminatory power of plant species. In the present study, we focused on the region of the *rpoA* RNA polymerase gene. The *rpoA* region was chosen based on the results (Figures S.5 and S.6 supplementary material) because it had greater proportion of different regions and because of the interest in studying this coding region, since only few studies are available in the literature.

A second challenge was choosing a reference database to compare ASVs and assign the taxonomy of tropical grasses. Several databases are available online; however, they are directed to specific regions or types of vegetation (Tnah et al., 2019; Banchi et al., 2020; Gostel et al., 2022). We used the NCBI database, which addresses global data on general characteristics (O'Leary et al., 2016).

A third challenge was the results found after comparison with the reference database and taxonomic attribution. According to Ledford (2008), DNA barcodes for plants can vary among species, but they hardly vary within them. As consequence, the results (Table S.1 supplementary material) showed classifications of grass species such as *Andropogon*, *Megathyrsus maximus*, *Panicum*, and *Urochloa brizantha*, in addition to other species and genera classified as weeds that were present in the experimental area. Therefore, it was not possible to accurately differentiate the cultivars we were looking for (*Andropogon gayanus* cv.

Planaltina, *Panicum maximum* cv. Massai, and *Brachiaria brizantha* cv. BRS Piatã). From the initial taxonomic attribution, we adjusted the nomenclatures. *Andropogon* grass species were classified as andropogon grass, *Megathyrsus maximus* and *Panicum* were classified as massai grass, and *Urochloa brizantha* was classified as piata grass. In addition, some initial taxonomic classifications indicated different cultivars within grass species but based on the knowledge of the existing above ground vegetation where some cultivars mentioned were not common in the studied region, and because they corresponded to nomenclatures that in some situations were not described correctly, we chose to merge the ASVs of these cultivars by grass species (Table S.1 supplementary material). The fusion of the ASVs was also necessary, mainly because grass species showed genetic variation and, therefore, different ASVs resulted in the same taxonomic classification, and because the taxonomic classification nomenclature of some ASVs in the database may have been registered without the cultivar nomenclatures. Interestingly, each grass species had an ASV that most represented it. Further, the grass species that presented the most different ASVs were piata grass and massai grass, possibly because they are grass species that were more genetically improved because they are widely used in tropical pastures (Resende et al., 2008; Fonseca and Martuscello, 2022).

As a fourth and final challenge, grass species showed differences in actual and estimated percentage presence of 0.10±0.01 for andropogon grass, -8.97±0.99 for massai grass, and 11.9±0.29 for piata grass with a low standard error of the mean (Figure 1 a). These results indicate that some consistent influence caused massai grass to be slightly underestimated and piata grass slightly overestimated, however, our data were not sufficient to explain this variation. We applied a correction method based on the construction of a calibration curve (Figure 1 b), and we used polynomial regression equations, resulting in estimated percentages values very close to the actual percentages (Figure 1 c d). The possibility of calibrating the data is something interesting that can improve the response of this methodology. In microbiology studies, for example, which use a methodology similar to the methodology described in this study, it is difficult to perform accurate calibration from known proportions. For plants, this calibration is possible and has been used previously in a study that showed that estimates were improved after calibration (Wagemaker et al., 2021).

4.4.2. Botanical proportion below and above ground

The application of the method in the calibrated mode associated with the previous knowledge of the vegetation above ground provided a good estimate of the taxonomy and quantitative proportion of grass species below ground, in addition to indicating the presence of common weeds in the experimental area. A correlation between below and above ground proportion was observed with $R = 0.88$, indicating that below ground proportion is similar to above ground proportion. The greater percentage presence of massai grass, both above ground and below ground, may be related to the competitive growth strategy of this grass species (Figure 2 and 3). Unpublished data collected in the same study indicated that massai grass has rapid leaf elongation, which resulted in greater tiller population density and shaded the other two grass species in the association, causing competition for light. Overall, the above ground response was very similar to the below ground response, with massai grass showing less variation in relative botanical proportion (Figure 3 c). The smaller variation of percentage presence of massai grass can be explained by its greater botanical presence, which results in less disturbance and greater stability in both above ground and below ground botanical composition. Andropogon grass and piata grass showed variations throughout seasons of the year, probably related to climatic variations (Figure S.4 supplementary material) and competition for light, as they were in present in smaller percentage in the mixture.

A high correlation was observed among the three soil depths (Figures 2 and 4 b), indicating that the three grass species had the same botanical percentage presence at the three depths. Possibly the grass species present niche differentiation at deeper layers; however, as evaluations were made up to 30 cm only, the results did not indicate significant differences. The slight indication of greater presence of *Paspalum* at the 20-30 cm layer relative to 0-10 and 10-20 cm layers (Figure 2), may be related to the coexisting roots and rhizomes of this grass species that has a deep root system (Carvalho et al., 2020). The experimental area was established in January 2020 and original vegetation had a large proportion of *Paspalum*. After soil preparation, from harrows up to 20 cm deep, part of the roots and rhizomes of these grass species may have been excluded, a fact that may not have happened at the deepest layer used in this study (20-30 cm).

The theoretical background suggests that below ground botanical proportion typically exceeds above ground proportion because the morphological parts of plants below ground, such as roots and rhizomes, can be more widely dispersed in space and time than above ground morphological parts (Deyn and Putten, 2005). Below ground, there is greater opportunity for the availability of resources such as water, macro and micronutrients, and microorganisms, while above ground, the main available resources are light and CO_2. Soil resources can be acquired by roots in various dimensions and time scales, as they can be absorbed immediately or stored for decades, while light, for example, comes only from above and must be utilized immediately, offering a limited opportunity for spatial niche differentiation. Above ground botanical proportion may decrease in biodiverse pastures due to competition for light and edaphoclimatic variations. In contrast, below ground botanical proportion varies less due to dormancy and phenotypic plasticity of grass species and by physical protection and resource availability. High disturbances, however, limit richness above ground and below ground (Hiiesalu et al., 2012; Pärtel et al., 2012). In our study, the same botanical proportion pattern observed above ground and below ground may have being related to the high intensity of disturbance due to competition for light.

4.4.3. Future challenges and opportunities

It is estimated that competition for light is more likely to cause competitive exclusion when compared to competition for soil nutrients (Suding et al., 2005; Hautier et al., 2009; Pärtel et al., 2012). In this context, it is important to integrate plant responses seeking a better understanding of competitive strategies for light above ground and to relate them to competitive strategies for nutrients below ground to generate ecosystems with less competition and greater coexistence in space and time. The results are based on above ground plant responses, the "tip of the iceberg" when we think about the myriad interactions below ground. There are few studies on below ground species proportion in temperate grasslands (Frank et al., 2010; Mommer et al., 2010; Kesanakurti et al., 2011; Mommer et al., 2011; Rewald et al., 2012; Oram et al., 2017), and other types of ecosystems have also been poorly studied.

DNA-based techniques offer opportunities to explore these underlying responses, allowing responses beyond species proportion, such as niche distribution along soil spatial gradients and under edaphoclimatic variations, taxonomy and proportion of roots and rhizomes coexisting in the soil, dormancy and phenotypic plasticity, root behavioral ecology and interspecific competition. Using appropriate taxon-specific primers, the same samples for plant species identification can also be used to explore root biota (invertebrates, fungi, and bacteria). This will allow understanding beyond plant-plant interactions, such as providing insights into plant-microorganism competition for nutrients (Wardle et al., 2004; Pärtel et al., 2012). The responses generated from a better understanding of below ground interactions and their relationship to above ground vegetation also have the potential to review coexistence theories that were developed based only on above ground vegetation.

4.5. Conclusions

The results of the calibration method indicated that differences between the actual and estimated presence percentages were 0.10±0.01 for andropogon grass, -8.97±0.99 for massai grass, and 11.9±0.29 for piata grass. The correlation between below and above ground botanical proportion in the calibrated data was R = 0.88, indicating that below ground botanical proportion is similar to above ground botanical proportion. As in the results of studies with other regions of the chloroplast genes, the method using the *rpoA* region showed consistent taxonomic classification at the genus level and grass species, but it was not able to differentiate at the level of plant cultivars. Our results suggest that using this method in the calibrated mode associated with previous knowledge of the grass species present above ground offers the opportunity to investigate the quantitative responses of roots when multiple grass species are present, allowing for a better understanding of plant-plant interactions below ground and their relationship with above ground vegetation for better use and conservation of ecosystem biodiversity.

References

Banchi, E., Ametrano, C.G., Greco, S., Stanković, D., Muggia, L., Pallavicini, A., 2020. PLANiTS: a curated sequence reference dataset for plant ITS DNA metabarcoding. Database. 2020. https://doi.org/10.1093/database/baz155

Bardgett, R.D., Mommer, L., Vries, F.T., 2014. Going underground: root traits as drivers of ecosystem processes. Trends in Ecology & Evolution. 29 (12), 692–699. https://doi.org/10.1016/j.tree.2014.10.006

Bobowski, B., Hole, D., Wolf, P., Bryant, L., 1999. Identification of roots of woody species using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Molecular Ecology. 8, 485–491. http://www.ncbi.nlm.nih.gov/pubmed/10199009

Brunner, I., Brodbeck, S., Büchler, U., Sperisen, C., 2001. Molecular identification of fine roots of trees from the Alps: Reliable and fast DNA extraction and PCR-RFLP analyses of plastid DNA. Molecular Ecology. 10, 2079–2087. https://doi.org/10.1046/j.1365-294X.2001.01325.x

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods. 13, 581–583. https://doi.org/10.1038/nmeth.3869

Carvalho, A.M., Santos, L.D.V., Holanda, F.S.D., Pedrotti, A., Antonio, G.M., 2020. Digital image processing for evaluation of *Paspalum millegrana* schrad root system. Revista Caatinga. 33, 100–107. https://doi.org/10.1590/1983-21252020v33n111rc

CBOL Plant Working Group., 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences of the United States of America. 106, 31, 12794– 12797. https://doi.org/10.1073/pnas.0905845106

Cowan, R.S., Chase, M.W., Kress, W.J., Savolainen, V., 2006. 300,000 species to identify: problems, progress, and prospects in DNA bar-coding of land plants. Taxon. 55, 611–616. https://doi.org/10.2307/25065638

Dawson, L.A., Mayes, R.W., Elston, D.A., Smart, T.S., 2000. Root hydrocarbons as potential markers for determining species composition. Plant, Cell, and Environment. 23, 743–750. https://doi.org/10.1046/j.1365-3040.2000.00592.x

Deyn, G.B., Putten, W.H.V., 2005. Linking aboveground and belowground diversity. Trends in Ecology and Evolution. 20, 625–633. https://doi.org/10.1016/j.tree.2005.08.009

Eskelinen, A., Harpole, W.S., Jessen, M.T., Virtanen, R., Hautier, Y., 2022. Light competition drives herbivore and nutrient effects on plant diversity. Nature. 611, 301–305. https://doi.org/10.1038/s41586-022-05383-9

FAO IUSS Working Group., 2015. World Reference Base for Soil Resources 2014. International soil classification system for naming soils and creating legends for soil maps, Update 2015, World Soil Resources Reports 106. FAO, Rome. https://www.fao.org/3/i3794en/I3794en.pdf

Fazekas, A.J., Burgess, K.S., Kesanakurti, P.R., Graham, S.W., Newmaster, S.G., Husband, B.C., Percy, D.M., Hajibabaei, M., Barrett, S.C.H., 2008. Multiple Multilocus DNA Barcodes from the Plastid Genome Discriminate Plant Species Equally Well. Plos One. 3, e2802. https://doi.org/10.1371/journal.pone.0002802

Fonseca, D.M., Martuscello, J.A., 2022. Plantas forrageiras, 2 ed. Editora Universidade Federal de Viçosa, 591p.

Frank, D.A., Pontes, A.W., Maine, E.M., Caruana, J., Raina, R., Raina, S., Fridley, J.D., 2010. Grassland root communities: species distributions and how they are linked to aboveground abundance. Ecology. 91, 3201–3209. https://doi.org/10.1890/09-1831.1

Freschet, G.T., Pagès, L., Iversen, C.M., Comas, L.H., Rewald, B., Roumet, C., Klimešová, J., Zadworny, M., Poorter, H., Postma, J.A., Adams, T.S., Bagniewska-Zadworna, A., Bengough, A.G., Blancaflor, E.B., Brunner, I., Cornelissen, J.H.C., Garnier, E., Gessler, A., Hobbie, S.E., Meier, I.C., Mommer, L., Picon-Cochard, C., Rose, L., Ryser, P., Scherer-Lorenzen, M., Soudzilovskaia, N.A., Stokes, A., Sun, T., Valverde-Barrantes, O.J., Weemstra, M., Weigelt, A., Wurzburger, N., York, L.M., Batterman, S.A., Moraes, M.G., Janeček, S., Lambers, H., Salmon, V., Tharayil, N., McCormack, M.L., 2021. A starting guide to root ecology: strengthening ecological concepts and standardising root classification, sampling, processing and trait measurements. New Phytologist. 232, 973–1122. https://doi.org/10.1111/nph.17572

Gostel, M.R., Carlsen, M.M., Devine, A., Barker, K.B., Coddington, J.A., Steier, J., 2022. Data Release: DNA Barcodes of Plant Species Collected for the Global Genome Initiative for Gardens (GGI-Gardens) II. Diversity. 14, 234. https://doi.org/10.3390/d14040234

Gross, K., Cardinale, B.J., Fox, J.W., Gonzalez, A., Loreau, M., Polley, H.W., Reich, P.B., Ruijven, J., 2014. Species richness and the temporal stability of biomass production: A new analysis of recent biodiversity experiments. The American Naturalist. 183 (1). https://doi.org//10.1086/673915

Hanisch, M., Schweiger, O., Cord, A.F., Volk, M., Knapp, S., 2020. Plant functional traits shape multiple ecosystem services, their trade-offs and synergies in grasslands. Journal of Applied Ecology. 57, 1535–1550. https://doi.org/10.1111/1365-2664.13644

Hautier, Y., Niklaus, P.A., Hector, A., 2009. Competition for light causes plant biodiversity loss after eutrophication. Science. 324, 636–638. https://doi.org/10.1126/science.1169640

Hiiesalu, I., Öpik, M., Metsis, M., Lilje, L., Davison, J., Vasar, M., Moora, M., Zobel, M., Wilson, S.D., Pärtel, M., 2012. Plant species richness belowground: higher richness and new patterns revealed by next-generation sequencing. Molecular Ecology. 21, 2004–2016. https://doi.org/10.1111/j.1365-294X.2011.05390.x

Hobbie, E.A., Werner, R.A., 2004. Intramolecular, compound-specific, and bulk carbon isotope patterns in C_3 and C_4 plants: a review and synthesis. New Phytologist. 161, 271–385. https://doi.org/10.1111/j.1469-8137.2004.00970.x

Isbell, F., Craven, D., Connolly, J., Loreau, M., Schmid, B., Beierkuhnlein, C., Bezemer, T.M., Bonin, C., Bruelheide, H., Luca, E., Ebeling, A., Griffin, J.N., Guo, Q., Hautier, Y., Hector, A., Jentschl, A., Kreyling, J., Lanta, V., Manning, P., Meyer, S.T., Mori, A.S., Naeem, S., Niklaus, P.A., Polley, H.W., Reich, P.B., Roscher, C., Seabloom, E.W., Smith, M.D., Thakur, M.P., Tilman, T., Tracy, B.F., Putten, W.H., Ruijven, J., Weigelt, A., Weisser, W.W., Wilsey, B., Eisenhauer, N., 2015. Biodiversity increases the resistance of ecosystem productivity to climate extremes. Nature. 526, 574–577. https://doi.org/10.1038/nature15374

Jackson, R.B., Canadel J., Ehleringer, J.R., Mooney, H.A., Sala, O.E., Schulze, E.D., 1996. A global analysis of root distributions for terrestrial biomes. Oecologia. 108, 389–411. https://doi.org/10.1007/BF00333714

Kesanakurti, P.R., Fazekas, A.J., Burgess, K.S., Percy, D.M., Newmaster, S.G., Graham, S.W., Barrett, S.C.H., Hajibabaei, M., Husband, B.C., 2011. Spatial patterns of plant diversity below-ground as revealed by DNA barcoding. Molecular Ecology. 20, 1289–1302. https://doi.org/10.1111/j.1365-294X.2010.04989.x

Kress, W.J., Erickson, D.L., 2007. A Two-Locus Global DNA Barcode for Land Plants: The Coding *rbcL* Gene Complements the Non-Coding *trnH-psbA* Spacer Region. Plos One. 6, e508. https://doi.org/10.1371/journal.pone.0000508

Ledford, H., 2008. Botanical identities: DNA barcoding for plants comes a step closer. Nature. 451, 616. https://doi.org/10.1038/451616b

Linder, C.R., Moore, L.A., Jackson, R.B., 2000. A universal molecular method for identifying underground plant parts to species. Molecular Ecology, 9, 1549–1559. https://doi.org/10.1046/j.1365-294x.2000.01034.x

McNickle, G.G., Cahill, J.F., Deyholos, M.K., 2008. A PCR-based method for the identification of the roots of 10 co-occurring grassland species in mesocosm experiments. Botany-Botanique. 86, 485–490. https://doi.org/10.1139/B08-014

Mommer, L., Dumbrell, A.J., Wagemaker, C.A.M., Ouborg, N. J., 2011. Belowground DNA-based techniques: Untangling the network of plant root interactions. Plant and Soil. 348, 115–121. https://doi.org/10.1007/s11104-011-0962-0

Mommer, L., Ruijven, J., Caluwe, W., Smit-Tiekstra, A.E., Wagemaker, C.A.M., Ouborg, N.J., Bogemann, G.M., Weerden, G.M., Berendse, F., Kroon, H., 2010. Unveiling below-ground species abundance in a biodiversity experiment: a test of vertical niche differentiation among grassland species. Journal of Ecology. 98, 1117–1127. https://doi.org/10.1111/j.1365-2745.2010.01702.x

Mommer, L., Wagemaker, C.A.M., De Kroon, H., Ouborg, N.J., 2008. Unravelling below-ground plant distributions: A real-time polymerase chain reaction method for quantifying species proportions in mixed root samples. Molecular Ecology Resources. 8, 947–953. https://doi.org/10.1111/j.1755-0998.2008.02130.x

O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., Farrell, C.M., Goldfarb, T., Gupta, T., Haft, D., Hatcher, E., Hlavina, W., Joardar, V.S., Kodali, V.K., Li, W., Maglott, D., Masterson, P., McGarvey, K.M., Murphy, M.R., O'Neill, K., Pujar, S., Rangwala, S.H., Rausch, D., Riddick, L.D., Schoch, C., Shkeda, A., Storz, S.S., Sun, H., Thibaud-Nissen, F., Tolstoy, I., Tully, R.E., Vatsan, A.R., Wallin, C., Webb, D., Wu, W., Landrum, M.J., Kimchi, A., Tatusova, T., DiCuccio, M., Kitts, P., Murphy, T.D., Pruitt, K.D., 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Research. 44, 733–745. https://doi.org/10.1093/nar/gkv1189

Oram, N.J., Ravenek, J.M., Barry, K.E., Weigelt, A., Chen, H., Gessler, A., Gockele, A., Kroon, H., van der Paauw, J.W., Scherer-Lorenzen, M., Smit-Tiekstra, A., van Ruijven, J., Mommer, L., 2018. Below-ground complementarity effects in a grassland biodiversity experiment are related to deep-rooting species. Journal of Ecology. 106, 265–277. https://doi.org/10.1111/1365-2745.12877

Pärtel, M., Hiiesalu, I., Öpik, M., Wilson, S.D., 2012. Below-ground plant species richness: new insights from DNA-based methods. Functional Ecology. 26, 775–782. https://doi.org/10.1111/j.1365-2435.2012.02004.x

Pennisi, E., 2007. Wanted: A barcode for plants. Science. 318, 190–191. https://doi.org/10.1126/science.318.5848.190

Pennisi, E., 2019. DNA barcodes jump-start search for new species. Science, 364, 920–921. https://doi.org/10.1126/science.364.6444.920

Resende, R.M.S., Valle, C.B., Jank, L., 2008. Melhoramento de forrageiras tropicais. 1 ed. Campo Grande: Embrapa Gado de Corte, 293p.

Rewald, B., Meinen, C., Trockenbrodt, M., Ephrath, J.E., Rachmilevitch, S., 2012. Root taxa identification in plant mixtures – current techniques and future challenges. Plant and Soil. 359, 165–182. https://doi.org/10.1007/s11104-012-1164-0

Ridgway, K.P., Duck, J.M., Young, J.P.W., 2003. Identification of roots from grass swards using PCR-RFLP and FFLP of the plastid trnL (UAA) intron. BMC Ecology. 3, 6–11. https://doi.org/10.1186/1472-6785-3-8

Roumet, C., Picon-Cochard, C., Dawson, L.A., Joffre, R., Mayes, R., Blanchard, A., Brewer, M.J., 2006. Quantifying species composition in root mixtures using two methods: near-infrared reflectance spectroscopy and plant wax markers. New Phytologist. 170, 631–638. https://doi.org/10.1111/j.1469-8137.2006.01698.x

Soussana, J.F., Teyssonneyre, F., Picon-Cochard, C., Dawson, L., 2005. A trade-off between nitrogen uptake and use increases responsiveness to elevated CO_2 in infrequently cut mixed C_3 grasses. New Phytologist, 166, 217–230. https://doi.org/10.1111/j.1469-8137.2005.01332.x

Suding, K.N., Collins, S.L., Gough, L., Clark, C., Cleland, E.E., Gross, K.L., Milchunas, D.G., Pennings, S., 2005. Functional- and abundance-based mechanisms explain diversity loss due to N fertilization. PNAS. 102, 4387–4392. https://doi.org/10.1073/pnas.0408648102

Tilman, D., 2020. Benefits of intensive agricultural intercropping. Nature Plants. 6, 604–605. https://doi.org/10.1038/s41477-020-0677-4

Tnah, L.H., Lee, S.L., Tan, A.L., Lee, C.T., Ng, K.K.S., Ng, C.H., Farhanah, Z.N., 2019. DNA barcode database of common herbal plants in the tropics: a resource for herbal product authentication. Food Control. 95, 318–326. https://doi.org/10.1016/j.foodcont.2018.08.022

Wagemaker, C.A.M., Mommer, L., Visser, E.J.W., Weigelt, A., Gurp, T.P., Postuma, M., Smit-Tiekstra, A.E., Kroon, H., 2021. msGBS: A new high-throughput approach to quantify the relative species abundance in root samples of multispecies plant communities. Molecular Ecology Resources. 21, 1021–1036. https://doi.org/10.1111/1755-0998.13278

Wang, J., Yan, Z., Zhong, P., Shen, Z., Yang, G., Ma, L., 2022. Screening of universal DNA barcodes for identifying grass species of Gramineae. Frontiers in Plant Science. https://doi.org/10.3389/fpls.2022.998863

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., Putten, W.H.V.D., Wall, D.W., 2004. Ecological linkages between aboveground and belowground biota. Science. 304, 1629–1633. https://doi.org/10.1126/science.1094875

Wildová, R., 2004. Below-ground spatial pattern of rhizomes in a grassland community and its relevance to above-ground spatial pattern. Plant Ecology Formerly Vegetation. 174, 321–338. https://doi.org/10.1023/b:vege.0000049111.477

Supplementary material

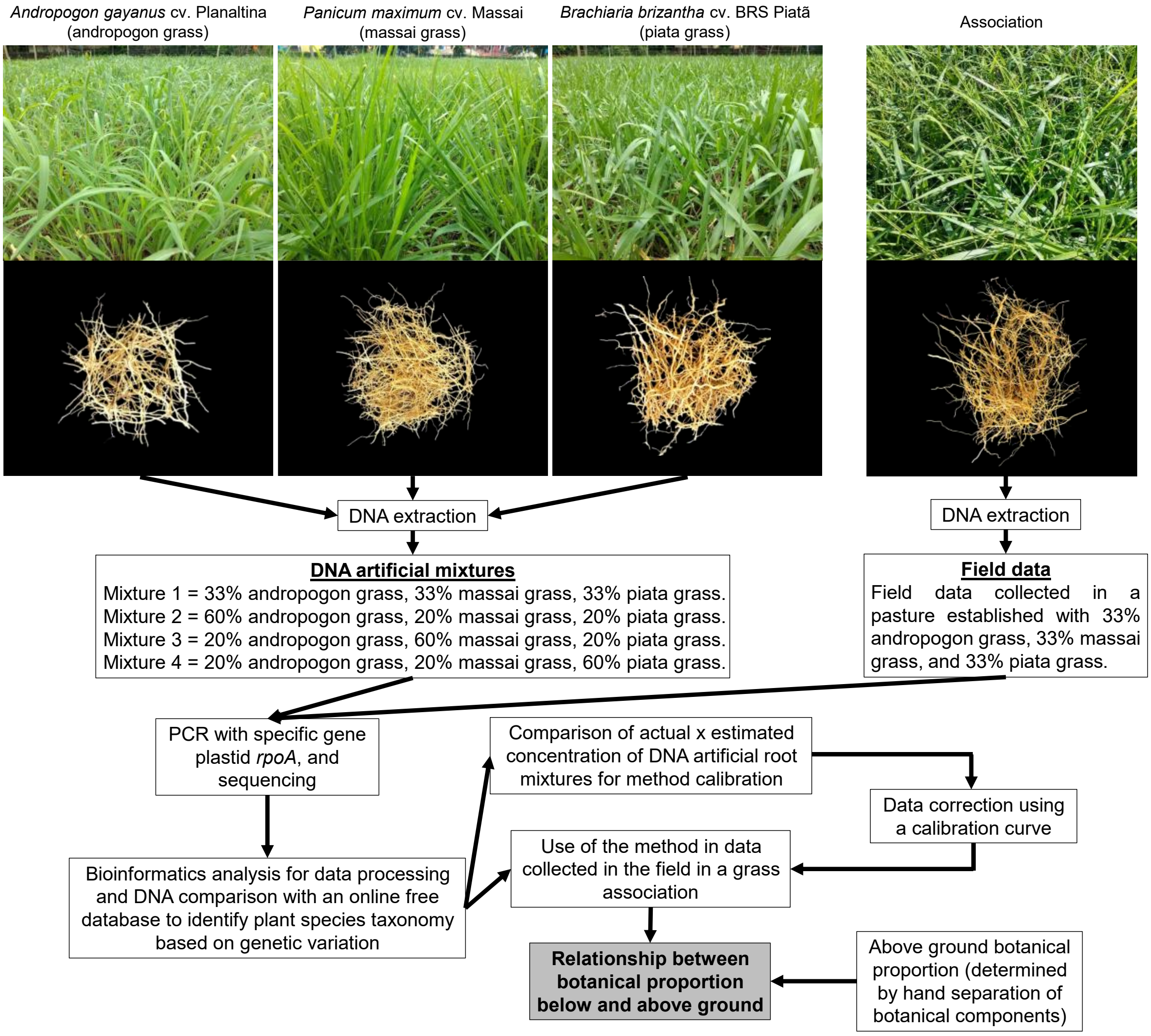


Figure S.1. Graphical abstract of chapter structure.

Figure S.2. Experimental area.

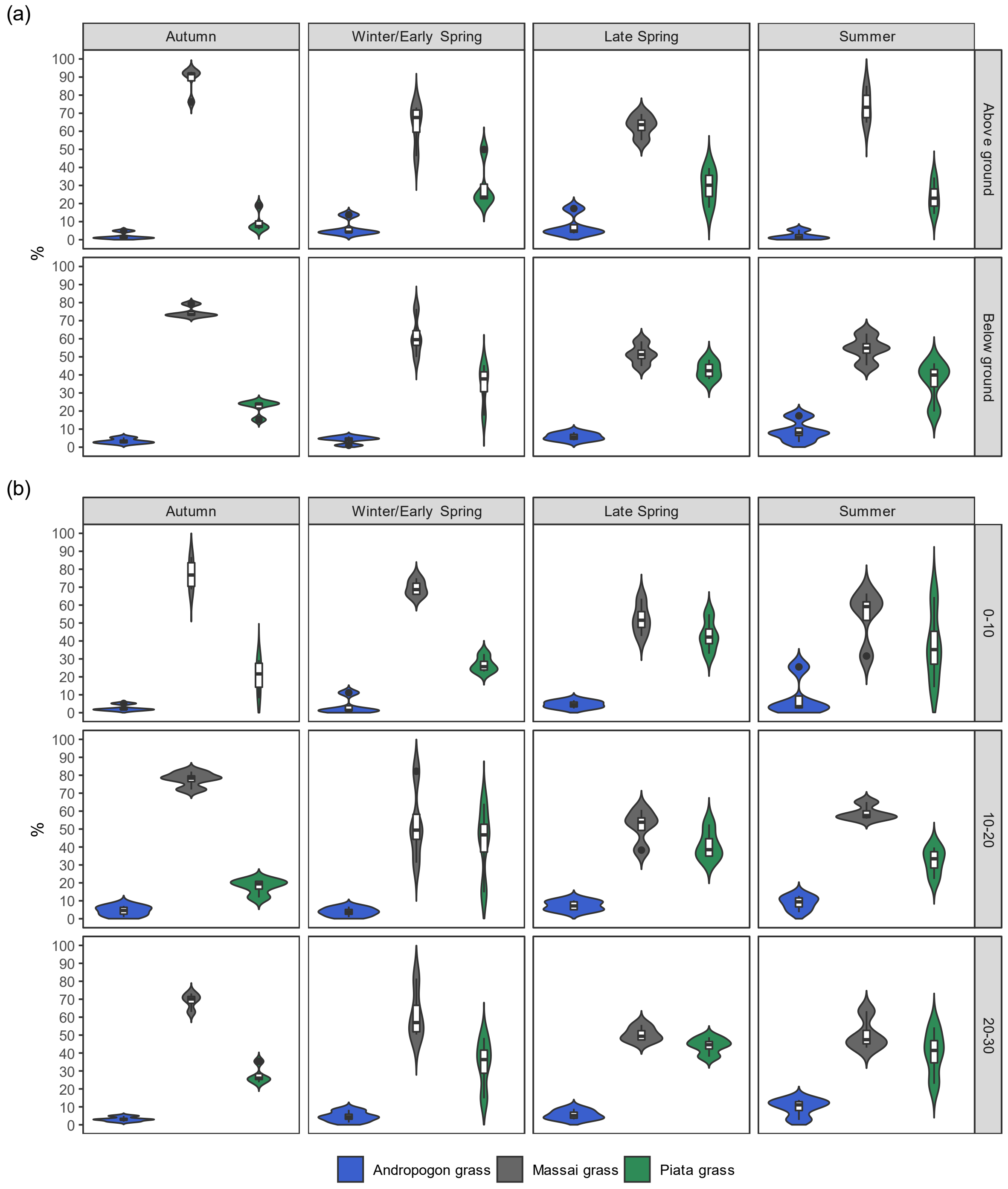


Figure S.3. Violin plots represent the normal distribution of the data. Botanical proportion in above ground (a), and below ground, and botanical proportion in below ground per soil depth (b). Data that are not normally distributed were not transformed because the difference is large between treatments.

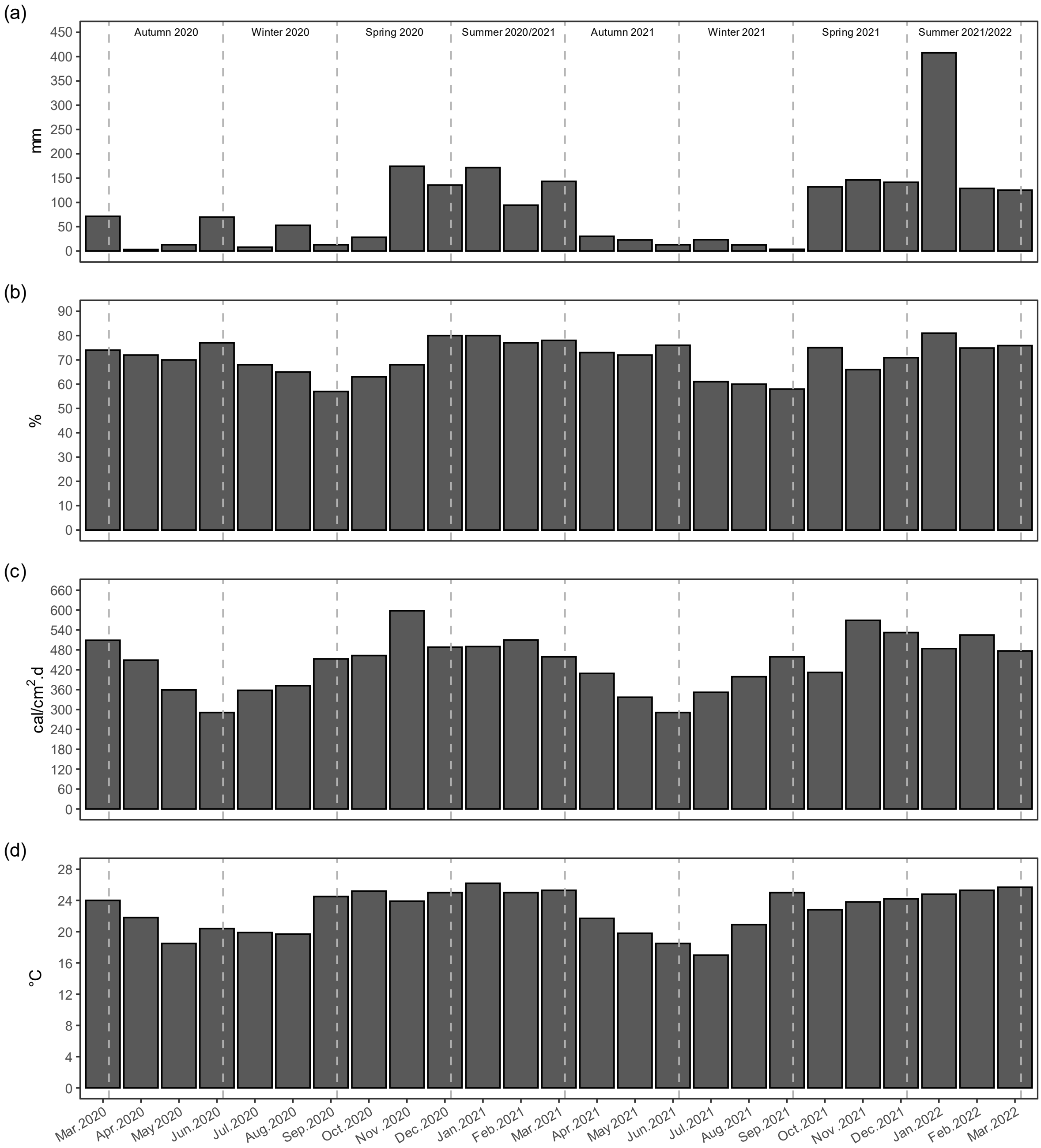
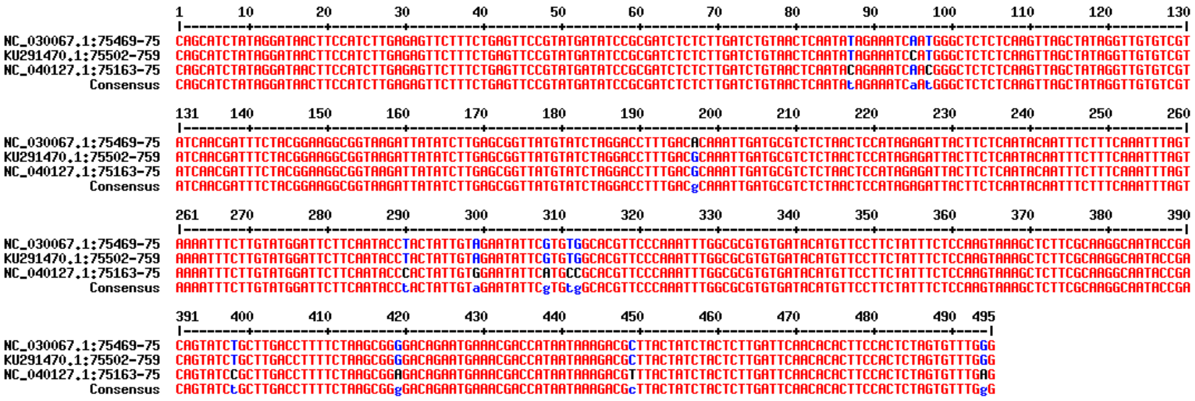


Figure S.4. Monthly meteorological data in the experimental period. Data were collected in a Meteorological Station located approximately 2000 m from the experimental area. Accumulated rainfall (a), average air humidity (b), average radiation (c), and mean air temperature (d).



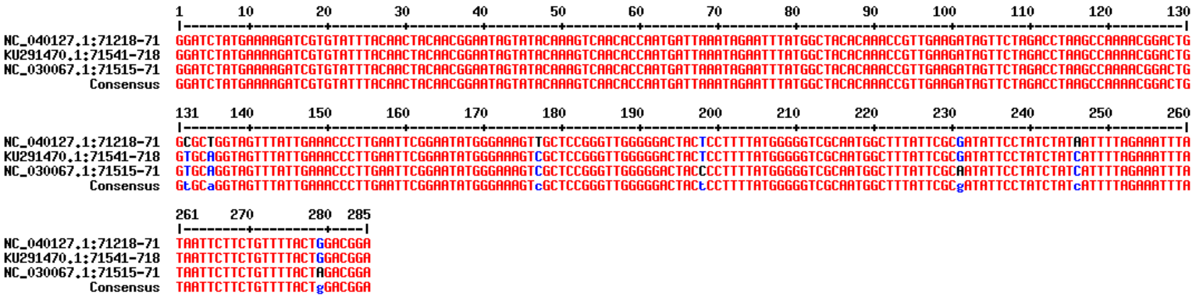
```
                     1       10        20        30        40        50        60        70        80        90       100       110       120       130
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
NC_030067.1:75469-75 CAGCATCTATAGGATAACTTCCATCTTGAGAGTTCTTTCTGAGTTCCGTATGATATCCGCGATCTCTCTTGATCTGTAACTCAATATAGAAATCAATGGGCTCTCTCAAGTTAGCTATAGGTTGTGTCGT
KU291470.1:75502-759 CAGCATCTATAGGATAACTTCCATCTTGAGAGTTCTTTCTGAGTTCCGTATGATATCCGCGATCTCTCTTGATCTGTAACTCAATATAGAAATCCATGGGCTCTCTCAAGTTAGCTATAGGTTGTGTCGT
NC_040127.1:75163-75 CAGCATCTATAGGATAACTTCCATCTTGAGAGTTCTTTCTGAGTTCCGTATGATATCCGCGATCTCTCTTGATCTGTAACTCAATACAGAAATCAACGGGCTCTCTCAAGTTAGCTATAGGTTGTGTCGT
           Consensus CAGCATCTATAGGATAACTTCCATCTTGAGAGTTCTTTCTGAGTTCCGTATGATATCCGCGATCTCTCTTGATCTGTAACTCAATAtAGAAATCaAtGGGCTCTCTCAAGTTAGCTATAGGTTGTGTCGT

                     131    140       150       160       170       180       190       200       210       220       230       240       250       260
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
NC_030067.1:75469-75 ATCAACGATTTCTACGGAAGGCGGTAAGATTATATCTTGAGCGGTTATGTATCTAGGACCTTTGACACAAATTGATGCGTCTCTAACTCCATAGAGATTACTTCTCAATACAATTTCTTTCAAATTTAGT
KU291470.1:75502-759 ATCAACGATTTCTACGGAAGGCGGTAAGATTATATCTTGAGCGGTTATGTATCTAGGACCTTTGACGCAAATTGATGCGTCTCTAACTCCATAGAGATTACTTCTCAATACAATTTCTTTCAAATTTAGT
NC_040127.1:75163-75 ATCAACGATTTCTACGGAAGGCGGTAAGATTATATCTTGAGCGGTTATGTATCTAGGACCTTTGACGCAAATTGATGCGTCTCTAACTCCATAGAGATTACTTCTCAATACAATTTCTTTCAAATTTAGT
           Consensus ATCAACGATTTCTACGGAAGGCGGTAAGATTATATCTTGAGCGGTTATGTATCTAGGACCTTTGACgCAAATTGATGCGTCTCTAACTCCATAGAGATTACTTCTCAATACAATTTCTTTCAAATTTAGT

                     261    270       280       290       300       310       320       330       340       350       360       370       380       390
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
NC_030067.1:75469-75 AAAATTTCTTGTATGGATTCTTCAATACCTACTATTGTAGAATATTCGTGTGGCACGTTCCCAAATTTGGCGCGTGTGATACATGTTCCTTCTATTTCTCCAAGTAAAGCTCTTCGCAAGGCAATACCGA
KU291470.1:75502-759 AAAATTTCTTGTATGGATTCTTCAATACCTACTATTGTAGAATATTCGTGTGGCACGTTCCCAAATTTGGCGCGTGTGATACATGTTCCTTCTATTTCTCCAAGTAAAGCTCTTCGCAAGGCAATACCGA
NC_040127.1:75163-75 AAAATTTCTTGTATGGATTCTTCAATACCCACTATTGTGGAATATTCATGCCGCACGTTCCCAAATTTGGCGCGTGTGATACATGTTCCTTCTATTTCTCCAAGTAAAGCTCTTCGCAAGGCAATACCGA
           Consensus AAAATTTCTTGTATGGATTCTTCAATACCtACTATTGTaGAATATTCgTGtgGCACGTTCCCAAATTTGGCGCGTGTGATACATGTTCCTTCTATTTCTCCAAGTAAAGCTCTTCGCAAGGCAATACCGA

                     391    400       410       420       430       440       450       460       470       480       490  495
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+----|
NC_030067.1:75469-75 CAGTATCTGCTTGACCTTTTCTAAGCGGGGACAGAATGAAACGACCATAATAAAGACGCTTACTATCTACTCTTGATTCAACACACTTCCACTCTAGTGTTTGGG
KU291470.1:75502-759 CAGTATCTGCTTGACCTTTTCTAAGCGGGGACAGAATGAAACGACCATAATAAAGACGCTTACTATCTACTCTTGATTCAACACACTTCCACTCTAGTGTTTGGG
NC_040127.1:75163-75 CAGTATCCGCTTGACCTTTTCTAAGCGGAGACAGAATGAAACGACCATAATAAAGACGTTTACTATCTACTCTTGATTCAACACACTTCCACTCTAGTGTTTGAG
           Consensus CAGTATCtGCTTGACCTTTTCTAAGCGGgGACAGAATGAAACGACCATAATAAAGACGcTTACTATCTACTCTTGATTCAACACACTTCCACTCTAGTGTTTGgG
```

Figure S.5. Differences between the three grass species for the primer *rpoA*, NC_030067.1 = piata grass chloroplast, complete genome; KU291470.1 = massai grass voucher PI:12181 chloroplast, complete genome; and NC_040127.1 = andropogon grass isolate TK480 chloroplast, complete genome.



```
                     1       10        20        30        40        50        60        70        80        90       100       110       120       130
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
NC_040127.1:71218-71 GGATCTATGAAAAGATCGTGTATTTACAACTACAACGGAATAGTATACAAAGTCAACACCAATGATTAAATAGAATTTATGGCTACACAAACCGTTGAAGATAGTTCTAGACCTAAGCCAAAACGGACTG
KU291470.1:71541-718 GGATCTATGAAAAGATCGTGTATTTACAACTACAACGGAATAGTATACAAAGTCAACACCAATGATTAAATAGAATTTATGGCTACACAAACCGTTGAAGATAGTTCTAGACCTAAGCCAAAACGGACTG
NC_030067.1:71515-71 GGATCTATGAAAAGATCGTGTATTTACAACTACAACGGAATAGTATACAAAGTCAACACCAATGATTAAATAGAATTTATGGCTACACAAACCGTTGAAGATAGTTCTAGACCTAAGCCAAAACGGACTG
           Consensus GGATCTATGAAAAGATCGTGTATTTACAACTACAACGGAATAGTATACAAAGTCAACACCAATGATTAAATAGAATTTATGGCTACACAAACCGTTGAAGATAGTTCTAGACCTAAGCCAAAACGGACTG

                     131    140       150       160       170       180       190       200       210       220       230       240       250       260
                     |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
NC_040127.1:71218-71 GCGCTGGTAGTTTATTGAAACCCTTGAATTCGGAATATGGGAAAGTTGCTCCGGGTTGGGGGACTACTCCTTTTATGGGGGTCGCAATGGCTTTATTCGCGATATTCCTATCTATAATTTTAGAAATTTA
KU291470.1:71541-718 GTGCAGGTAGTTTATTGAAACCCTTGAATTCGGAATATGGGAAAGTCGCTCCGGGTTGGGGGACTACTCCTTTTATGGGGGTCGCAATGGCTTTATTCGCGATATTCCTATCTATCATTTTAGAAATTTA
NC_030067.1:71515-71 GTGCAGGTAGTTTATTGAAACCCTTGAATTCGGAATATGGGAAAGTCGCTCCGGGTTGGGGGACTACCCCTTTTATGGGGGTCGCAATGGCTTTATTCGCAATATTCCTATCTATCATTTTAGAAATTTA
           Consensus GtGCaGGTAGTTTATTGAAACCCTTGAATTCGGAATATGGGAAAGTcGCTCCGGGTTGGGGGACTACtCCTTTTATGGGGGTCGCAATGGCTTTATTCGCgATATTCCTATCTATcATTTTAGAAATTTA

                     261    270       280  285
                     |--------+---------+----|
NC_040127.1:71218-71 TAATTCTTCTGTTTTACTGGACGGA
KU291470.1:71541-718 TAATTCTTCTGTTTTACTGGACGGA
NC_030067.1:71515-71 TAATTCTTCTGTTTTACTAGACGGA
           Consensus TAATTCTTCTGTTTTACTgGACGGA
```

Figure S.6. Differences between the three grass species for the primer *psbH*, NC_030067.1 = piata grass chloroplast, complete genome; KU291470.1 = massai grass voucher PI:12181 chloroplast, complete genome; and NC_040127.1 = andropogon grass isolate TK480 chloroplast, complete genome.

Table S.1. Assigned taxonomy for each sequence, adjusted taxonomic nomenclature based on prior knowledge of above ground vegetation, and sequences merger for each adjusted taxonomy.

Sequences	Access number NCBI	Score	Taxonomy NCBI	Adjusted taxonomy
1	gi|1025807325|ref|NC_030067.1|	702	Urochloa brizantha	Piata grass
2	gi|1040154941|gb|KU291470.1|	702	Megathyrsus maximus	Massai grass
3	gi|1524089141|gb|MH660710.1|	702	Andropogon ivorensis isolate TK716	Andropogon grass
4	gi|1025807325|ref|NC_030067.1|	398	Urochloa brizantha	Piata grass
5	gi|1488178801|ref|NC_039461.1|	702	Paspalum simplex	Paspalum
6	gi|1025807325|ref|NC_030067.1|	697	Urochloa brizantha	Piata grass
7	gi|1025807325|ref|NC_030067.1|	575	Urochloa brizantha	Piata grass
8	gi|1040154941|gb|KU291470.1|	691	Megathyrsus maximus	Massai grass
9	gi|1040154941|gb|KU291470.1|	691	Megathyrsus maximus	Massai grass
10	gi|1040154941|gb|KU291470.1|	551	Megathyrsus maximus	Massai grass
11	gi|1040154941|gb|KU291470.1|	686	Megathyrsus maximus	Massai grass
12	gi|347824800|gb|JF804852.1|	680	Panicum millegrana rps11-rpoA intergenic spacer	Massai grass
13	gi|1025807325|ref|NC_030067.1|	564	Urochloa brizantha	Piata grass
14	gi|1196593969|gb|KY596167.1|	664	Andropogon burmanicus	Andropogon grass
15	gi|1025807325|ref|NC_030067.1|	675	Urochloa brizantha	Piata grass
16	gi|1040154041|gb|KU833246.1|	702	Eleusine indica	Eleusine
17	gi|1025807417|ref|NC_030068.1|	675	Urochloa ruziziensis	Piata grass
18	gi|1025807325|ref|NC_030067.1|	658	Urochloa brizantha	Piata grass
19	gi|1834970347|ref|NC_047337.1|	702	Arachis duranensis voucher Yi14725-KUN	Arachis
20	gi|1025807325|ref|NC_030067.1|	686	Urochloa brizantha	Piata grass
21	gi|2154023596|gb|MT083940.1|	691	Digitaria californica plastid	Digitaria
22	gi|1488179053|ref|NC_039464.1|	702	Paspalum ionanthum	Paspalum
23	gi|1535790001|ref|NC_040129.1|	686	Andropogon ascinodis isolate TK547	Andropogon grass
24	gi|1025807325|ref|NC_030067.1|	697	Urochloa brizantha	Piata grass
25	gi|1040154941|gb|KU291470.1|	697	Megathyrsus maximus	Massai grass
26	gi|930158884|gb|KR232942.1|	675	Urochloa brizantha	Piata grass
27	gi|1025807325|ref|NC_030067.1|	610	Urochloa brizantha	Piata grass
28	gi|1025807325|ref|NC_030067.1|	697	Urochloa brizantha	Piata grass
29	gi|1025807325|ref|NC_030067.1|	664	Urochloa brizantha	Piata grass
30	gi|1025807325|ref|NC_030067.1|	569	Urochloa brizantha	Piata grass
31	gi|1025807325|ref|NC_030067.1|	664	Urochloa brizantha	Piata grass
32	gi|1025807325|ref|NC_030067.1|	686	Urochloa brizantha	Piata grass
33	gi|1025807325|ref|NC_030067.1|	691	Urochloa brizantha	Piata grass
34	gi|1025807325|ref|NC_030067.1|	669	Urochloa brizantha	Piata grass
35	gi|1025807325|ref|NC_030067.1|	575	Urochloa brizantha	Piata grass
36	gi|1025807325|ref|NC_030067.1|	636	Urochloa brizantha	Piata grass
37	gi|1040154941|gb|KU291470.1|	697	Megathyrsus maximus	Massai grass
38	gi|1811259483|tpg|BK010675.1|	619	Miscanthus floridulus	Miscanthus
39	gi|1025807325|ref|NC_030067.1|	675	Urochloa brizantha	Piata grass
40	gi|1509841013|ref|NC_039619.1|	603	Panicum incomtum	Massai grass
41	gi|577698966|gb|KF810540.1|	702	Paspalum saccharoides voucher	Paspalum
42	gi|1025807325|ref|NC_030067.1|	675	Urochloa brizantha	Piata grass
43	gi|1834999326|gb|MK860037.1|	702	Malvastrum coromandelianum	Malvastrum
44	gi|1509841013|ref|NC_039619.1|	608	Panicum incomtum	Massai grass
45	gi|1488178801|ref|NC_039461.1|	680	Paspalum simplex	Paspalum
46	gi|1488178801|ref|NC_039461.1|	686	Paspalum simplex	Paspalum
47	gi|347824800|gb|JF804852.1|	691	Panicum millegrana rps11-rpoA intergenic spacer	Massai grass
48	gi|1025807325|ref|NC_030067.1|	616	Urochloa brizantha	Piata grass
49	gi|1025807325|ref|NC_030067.1|	580	Urochloa brizantha	Piata grass
50	gi|1509841013|ref|NC_039619.1|	608	Panicum incomtum	Massai grass

Sequences merger	Adjusted taxonomy
3, 14, 23	Andropogon grass
2, 8, 9, 10, 11, 12, 25, 37, 40, 44, 47, 50	Massai grass
1, 4, 6, 7, 13, 15, 17, 18, 20, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 39, 42, 48, 49	Piata grass
5, 22, 41, 45, 46	Paspalum
21	Digitaria
16	Eleusine
43	Malvastrum
38	Miscanthus

5. GENERAL CONCLUSION

The interactions and competitive strategies by resources between the grass species that make up an association are dynamic and complex. Furthermore, these responses can be influenced by biotic and abiotic factors, which can modify the grass species functional traits and the association's overall response. Therefore, giving conclusive ideas about the functioning of these ecosystems cannot always be affirmed as an absolute truth. This study found some response patterns that can help understand grass species resource acquisition strategies. However, there is still a need to seek more knowledge on the subject.

The results indicated that the association was shaped by competition for light and little competition for nitrogen, with massai grass showing higher botanical proportions and productive performance. Including the monocultures, piata grass had the lowest number of cycles and the highest average forage productivity. On the other hand, andropogon grass, massai grass, and association showed a higher number of cycles with lower average forage productivity, resulting in similar total forage yield for all treatments. The functional traits of grass species in monoculture indicated that massai grass has a higher foliage angle and lower leaf area index in the upper 10 cm, which resulted in a higher proportion of light in the vertical profile of the canopy and caused rapid leaf elongation rate per tiller, and resulted in higher tiller population density, shading the others two grass species in the association, and causing competition for light. The results of the calibration method indicated that the below ground botanical proportion was accurately estimated, and there is a high relationship between below ground and above ground botanical proportion.

In general, the association presented a productive performance similar to the average of the monocultures and indicated that the competition for light was explained mainly by the functional traits: foliage angle, leaf elongation rate, number of leaves per tiller, and leaf area index, suggesting that are important predictors to explain light acquisition strategies and should be considered when choosing grass species that will compose new associations. Furthermore, the results indicated that nitrogen was not a limiting factor, and that competition was mainly for light.

6. FUTURE STUDIES

Based on the results of the present study, new questions were generated for future studies, seeking to obtain more diverse associations (i.e., less difference between the botanical proportions of the component grass species) and with less competition for resources.

At the first moment, it would be interesting to carry out fieldwork to cultivate several grass species or forage genotypes of tropical climate under different edaphoclimatic simulations, fertility, and management. Then, based on the data obtained, group the grass species into functional groups (resource conservers and resource competitors), verify whether the functional trait changes under different manipulations, and relate grass species that best adapt in different biomes under different technological levels, as well as and the ecosystem services they can provide when grouped. This functional grouping can also be performed based on data from works already published in the literature through a meta-analysis relating traits that describe characteristics of conservative plants and resource competitors. Therefore, the main information to be extracted would be what are the main functional traits present in the grass species that should be taken as decisive for choosing the grass species to be implanted in an association, as well as the management strategies (defoliation intensity, or nutrient replacement, between others) that best adapt, ensuring long-term persistence and multifunctionality of these pastures.

It is also important to evaluate the methods of implementing these associations. For example: sowing the different grass species by broadcast, planting in mixed lines, or planting in interspersed lines. In this context, is it possible that in an association of grass species planted in interspersed lines, interactions and competitions take place at the same level of an association by casting or mixed lines, or will the selection by animals be greater, and this may compromise the perpetuity of the grass species in the association. It is also important to investigate the proportion of seeds of each grass species, the germination rate, the dormancy level, the time each grass species takes to develop, the seeding/planting fertilization level, and other questions. These answers would make it possible to understand better the dynamics and complexity of an association of tropical forage grasses and would make it possible to formulate more functional multispecific pastoral ecosystems.