



Increasing diversity of warm- and cool-season species enhances aboveground and root carbon and nitrogen accumulation in subtropical forage mixtures

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Abstract

Background and aims This study evaluated how increasing forage species diversity affects herbage and root mass, associated C and N accumulation, and soil particulate organic matter (POM).

Methods Over three years a field experiment compared a baseline system of warm-season perennial bahiagrass (*Paspalum notatum*) annually overseeded with black oat (*Avena strigosa*) to eight mixtures containing 3 to 6 species, including legumes and grasses, differing in seasonal growth (warm vs. cool season) and life cycle (annual vs. perennial). Added legumes included warm-season forage

peanut (*Arachis pintoi*) and cool-season white clover (*Trifolium repens*), while added grasses included warm-season limpograss (*Hemarthria altissima*) and cool-season annual ryegrass (*Lolium multiflorum*).

Results Increasing diversity from the two-species baseline to mixtures containing 3–6 species increased herbage accumulation, root mass and respective C and N accumulation under cool and warm seasons. The addition of legume species consistently increased herbage and root N accumulation. The addition of warm-season species increased annual herbage accumulation, while cool-season species increased both herbage and root mass during the cool-season. Only annual ryegrass contributed to greater soil POM-C and POM-N after three years.

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Conclusions Increasing diversity in forage mixtures from two to 3–6 species had a positive effect on herbage, roots and C and N accumulation in a subtropical environment in Southern Brazil. The contribution of species varied depending on whether they were cool or warm-season species, or whether they belonged or not to the legume family. Selecting complementary species based on functional characteristics is therefore key to obtaining benefits from diverse forage swards.

Keywords Soil carbon sequestration · Particulate organic matter · Grass-legume mixtures · Root mass · Functional diversity

Introduction

Grasslands store one-third of the soil organic carbon (SOC) on earth, making them critical components of the global biogeochemical C cycle (Bai and Cotrufo 2022; White et al. 2000). However, these ecosystems are highly sensitive to management practices and environmental disturbances and may easily shift from C sinks to C sources. Intensive grazing is consistently associated with SOC loss across diverse soil types and climates (Abdalla et al. 2018; McSherry and Ritchie 2013; Zhou et al. 2017), but management strategies that enhance net primary production, particularly through increased root mass and rhizodeposition, can promote SOC sequestration over time (Fornara and Tilman 2008; Yang et al. 2019). Among these strategies, increasing plant functional diversity through the inclusion of complementary species is widely recommended to maximize both C sequestration potential and broader ecosystem functions (Isbell et al. 2017; Mi et al. 2022; Nabe-Nielsen et al. 2017).

The functional identity of forage species defined by dominant characteristics such as leaf area, morphology, root depth, and biological N fixation (BNF) dictates niche partitioning across spatial and temporal scales and the resultant ecological functioning of the community (Husse et al. 2017; Paredes et al. 2018). Effective species combinations leverage trait complementarity to enhance resource use efficiency and stabilize ecosystem productivity. For example, the inclusion of legume forages in mixtures can transfer 10 to 75 kg N ha⁻¹ year⁻¹ to neighboring grass plants, improving overall yields and nutritive value (Pirhofer-Walzl

et al. 2012). Low availability of N often limits grass tillering, leaf elongation, and productivity (Whitehead 1995), yet legumes, such as *Trifolium pratense* L., can supply 6–29% of the N taken up by grasses in a mixture (Frankow-Lindberg and Dahlin 2013). In a long-term grassland restoration with up to 16 forage species, Yang et al. (2019) found SOC stocks to be up to 70% higher in diversified grass and legume mixtures than in grass monocultures; and this was attributed to increased forage productivity (14–86%) and root mass (40–100%), highlighting the positive interactions between grasses and legumes in mixtures.

In establishing diversified grassland swards, the inclusion of different root architectures and rooting depths also increase niche complementarity, and can enhance root mass in deeper soil layers and improve soil structure, as well as nutrient uptake and cycling (Gould et al. 2016; Husse et al. 2017). Roots also play a crucial role in building up SOC in grasslands, since most of the aboveground mass is mowed for hay or grazed (Poeplau et al. 2018) and only a small fraction returns to soil as grazing animals' feces (Chiavegato et al. 2015; Ribeiro et al. 2020). Thus, decomposition of belowground mass is a key pathway for SOC formation. Fine roots and root exudates decay rapidly and stabilize C by binding to soil minerals, whereas coarse roots decompose more slowly because of their higher C:N ratio and higher concentration of lignin and suberin (Cotrufo et al. 2013; Jackson et al. 2017; Rasse et al. 2005). As root-derived inputs break down, much of the material initially becomes part of the soil particulate organic matter (POM), a fraction of sand-sized particles highly responsive to environmental and management changes (Cambardella and Elliott 1992; Lavalée et al. 2020). Although increases in root mass may not immediately change total soil C stocks, they can affect POM (Lavalée et al. 2020; Ribeiro et al. 2023). Moreover, greater root mass is also associated with greater rhizodeposition (Bolinder et al. 2012; Mueller et al. 2024), an important labile pool for microbial use that is stabilized in the mineral soil fraction (Cotrufo et al. 2013; Sokol et al. 2019).

Although forage mixtures are expected to increase herbage, root mass and POM (Fornara & Tilman 2008; Ribeiro et al. 2023; Yang et al. 2019), establishing a productive and persistent mixture sward is challenging. The performance of forage mixtures depends not only on the inclusion of different species, but also on the ability of these species to coexist over time (Hernandez and

Picon-Cochard 2016; Høgh-Jensen et al. 2006; Ribeiro et al. 2023). As highlighted by Sanderson (2010) and Tracy and Sanderson (2004), simply increasing the number of forage species in a mixture does not necessarily lead to higher herbage yield or stability. Drawing on the preceding discussion, effective mixtures rely on complementary functional traits, such as differences in growth phenology, nutrient acquisition strategies, and canopy structure, which together enhance resource use efficiency and sustain productivity.

In subtropical regions, warm-season perennial pastures typically enter dormancy during the cool season, resulting in pronounced seasonal gaps in forage availability. To mitigate this limitation, producers commonly overseed cool-season annual forages into established warm-season perennial stands, a practice widely adopted in environments in southern Brazil (Barreta et al. 2023; Flores et al. 2025) and in the southeastern United States (Santos et al. 2020; Trumpp et al. 2025). When implemented under no-till or reduced-disturbance conditions, overseeding exploits temporal niche complementarity between dormant perennials and actively growing annuals, increasing annual forage supply while maintaining perennial persistence. This seasonal integration of species with contrasting life cycles provides a practical framework for evaluating how mixture complexity and species composition influence forage productivity and soil C and N dynamics under subtropical conditions. Thus, this study tested the hypothesis that herbage and root production, C and N accumulation, and soil POM respond differently to the addition of species with contrasting functional roles in warm- and cool-season forage mixtures in a subtropical environment. The objective of this study was to determine whether increasing species diversity with contrasting functional traits, specifically nitrogen fixation capacity, life cycle (annual vs. perennial), and seasonal growth patterns, differ in their contribution to herbage and root production, C and N accumulation, and soil POM in subtropical forage mixtures combining warm- and cool-season species.

Material and methods

Field experiment

A field experiment was carried out at the Agrarias Campus of the Federal University of Parana, in Curitiba, Brazil (25° 24' 47" S; 49° 14' 59" W;

916 m altitude). The climate is classified as temperate oceanic (Cfb), according to the Köppen classification, within a region broadly considered subtropical (Alvares et al. 2013). Mean air temperature of 12.8 °C in the coldest month of July and 20.4 °C in the warmest month of February, while the mean annual precipitation is 1480 mm (averages from 1976–2015) (Nitsche et al. 2019). Information on daily precipitation and mean daily temperature during the six growing seasons from May 2020 to May 2023 (Fig. 1) was obtained from a meteorological station located 8 km away from the experiment site (National Institute of Meteorology- Instituto Nacional de Meteorologia, INMET).

The soil in the experimental area was clayey and was classified as *Cambissolo Haplico*, according to the Brazilian soil classification (H. G. d. Santos et al. 2018a, b), or as Cambisol, according to the international classification (WRB 2015). Some of the soil characteristics at the start of the experiment in February 2020 are presented in Table 1.

The experimental design was a randomized block, with nine treatments, four replicates, for a total of 36 plots of 2 × 2 m. Each plot received a predefined forage mixture that combined specific warm-season and cool-season species and remained as one experimental unit throughout the study. Treatments consisted of gradual increment in the number of species by introducing season-specific species with contrasting functional groups and life cycles, including warm-season (grown from November to April) or cool-season (grown from May to October) species (Table 2). In this subtropical environment, where warm-season perennials are dormant during the cool period, overseeding cool-season annuals into established plots is a practical approach to enhance total forage productivity without affecting the perennials. The choice of species was based on their functional roles, life cycles, spatial-temporal variability, as well as their local adaptability. Within each growing season, forage mixtures consisted of: (i) a grass species alone, (ii) a grass species and a legume species mixture, or (iii) two grasses and a legume.

Warm-season species were all perennial and included bahiagrass (*Paspalum notatum* Flueggé) (V.N.S), forage peanut (*Arachis pintoii* Krapov. & W.C. Greg) (var. Amarillo) and limpograss (*Hemarthra altissima* [Poir.] Stapf & C.E. Hubbard.) (var.

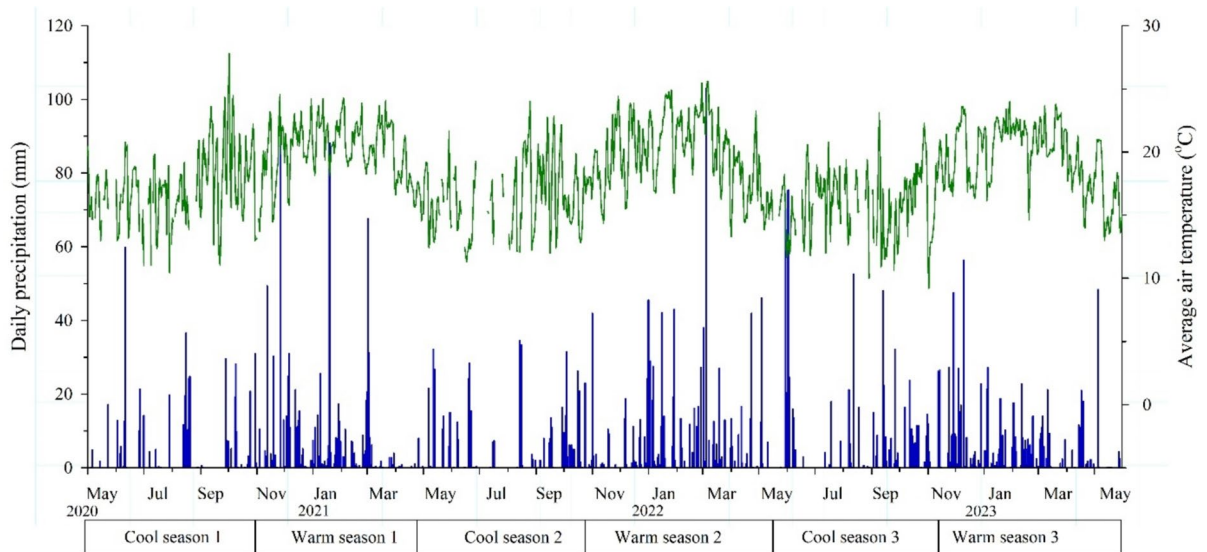


Fig. 1 Daily precipitation (blue vertical bars) and mean daily air temperature (green line) during the six growing seasons in this study from May 2020 to May 2023. Curitiba-PR, Brazil. Data obtained from INMET

Table 1 Chemical and physical characteristics of the Cambisol of this study, at the establishment of the experiment in February 2020, Curitiba, Brazil

Parameters	0–5 cm	5–10 cm	10–20 cm	20–30 cm
pH	5.4 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.7 ± 0.1
K, cmol _c kg ⁻¹	1.0 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Ca, cmol _c kg ⁻¹	14.1 ± 2.1	9.8 ± 1.4	9.1 ± 2.0	6.5 ± 0.3
Mg, cmol _c kg ⁻¹	15.0 ± 1.2	12.8 ± 1.1	12.4 ± 1.4	9.5 ± 1.7
Al, cmol _c kg ⁻¹	0.0	0.0	0.0	0.0
H + Al, cmol _c kg ⁻¹	6.7 ± 0.6	6.5 ± 0.5	6.0 ± 0.4	5.2 ± 0.4
P, mg kg ⁻¹	67.1 ± 11.6	49.5 ± 10.0	52.2 ± 8.3	33.3 ± 7.6
SOM, g kg ⁻¹	41.4 ± 4.3	39.0 ± 1.9	40.1 ± 2.0	29.9 ± 1.7
Clay, g kg ⁻¹	625	663	638	688
Silt, g kg ⁻¹	238	200	238	163
Sand, g kg ⁻¹	137	137	124	149
Bulk density, kg dm ⁻³	0.96 ± 0.03	0.98 ± 0.03	1.02 ± 0.02	1.03 ± 0.02
Total porosity, m ³ m ⁻³	0.64 ± 0.01	0.68 ± 0.01	0.65 ± 0.01	0.63 ± 0.01
Microporosity, m ³ m ⁻³	0.46 ± 0.02	0.54 ± 0.02	0.51 ± 0.01	0.46 ± 0.01
Macroporosity, m ³ m ⁻³	0.18 ± 0.03	0.14 ± 0.03	0.14 ± 0.01	0.17 ± 0.01

pH CaCl₂ 0.01 mol L⁻¹ at a soil:solution rate of 1:2.5;

Ca⁺², Mg²⁺ and Al⁺³ extracted with KCl 1 mol L⁻¹;

K and P extracted with Mehlich-1;

H + Al determined by SMP method;

SOM (soil organic matter) determined by Walkey-Black method;

Particle size determined by Bouyoucos density method;

Values are average of 4 samples ± SE (standard error of the mean), but particle size was determined in a composite sample from 4 samples.

Table 2 Nine forage mixture treatments composed of two to six species created from the combination of two perennial warm-season grasses, one warm-season and one cool-season

perennial legume and two cool-season annual grass species. In each plot, warm- and cool-season species were established sequentially according to treatment assignment

Treatment ID	Warm-season species established	Cool-season species established	Number of species
B-O	Bahiagrass	Oat	2
B-O + W	Bahiagrass	Oat, clover	3
B-O + W + R	Bahiagrass	Oat, clover, ryegrass	4
B + P-O	Bahiagrass, forage peanut	Oat	3
B + P-O + W	Bahiagrass, forage peanut	Oat, clover	4
B + P-O + W + R	Bahiagrass, forage peanut	Oat, clover, ryegrass	5
B + P + L-O	Bahiagrass, forage peanut, limpograss	Oat	4
B + P + L-O + W	Bahiagrass, forage peanut, limpograss	Oat, clover	5
B + P + L-O + W + R	Bahiagrass, forage peanut, limpograss	Oat, clover, ryegrass	6

B=Bahiagrass (*Paspalum notatum* Flueggé), P=forage peanut (*Arachis pintoi* Krapov. & W.C. Greg), L=limpograss (*Hemarthra altissima* [Poir.] Stapf & C.E. Hubbard.), O=black oat (*Avena strigosa* Schreb.), W=white clover (*Trifolium repens* L.) and R=annual ryegrass (*Lolium multiflorum* Lam.). “-” denotes sequential establishment of species across seasons within the same plot, and “+” denotes simultaneous establishment within a season

Florida); while cool-season species were annual black oat (*Avena strigosa* Schreb.) (var. Embrapa 139), perennial white clover (*Trifolium repens* L.) (var. Yi) and annual ryegrass (*Lolium multiflorum* Lam.) (var. BRS Ponteio). The baseline control combination was bahiagrass in the warm-season period, followed by black oat in the cool season. From this baseline, species were gradually added by including legume species, forage peanut in the warm season, and white clover in the cool season; followed by the inclusion of the additional grass species limpograss in the warm season and annual ryegrass in the cool season, as shown in Table 2.

The experiment was established in February of 2020 during the summer period. Evaluations were carried out over the subsequent three years, from May 2020 to May 2023, and were subdivided into six growing periods: cool season 1, warm season 1, cool season 2, warm season 2, cool season 3, and warm season 3. The cool-season period was defined as May to October, and warm-season period as November to April. The specific dates of the experimental activities performed from the establishment of the experiment until the end of the evaluations are shown in Table 3.

Prior to establishment, the field had been under bermudagrass (*Cynodon dactylon* L. Pers.) for at least four years. Initially, bermudagrass was mowed and the mass removed in September 2019. After 30 days

of regrowth, bermudagrass was sprayed with glyphosate herbicide (2 kg a.i. ha⁻¹) and, two months later, it was sprayed with a tank mixture of glyphosate (1.8 kg a.i. ha⁻¹) plus metsulfuron methyl (2.5 g a.i. ha⁻¹).

Prior to establishment, plots were assigned to complete forage mixture treatments and remained as one experimental unit throughout the study. The perennial warm-season species were established first. Bahiagrass seeds were manually broadcast and incorporated into 2 cm depth in February 8, 2020, at a rate of 60 kg ha⁻¹. After plots were demarcated, seedlings of forage peanut alone or forage peanut plus limpograss were manually transplanted in their corresponding plots, in 20×20 cm spacing; species were interspersed in the case of forage peanut with limpograss treatments. Seedlings of forage peanut were produced in a greenhouse using commercially obtained seed. Seeds were sown in trays filled with a potting substrate (locally sourced at 40% soil compost, 30% coconut coir and 30% vermiculite) approximately 30 days prior to field transplanting. Seedlings were maintained under greenhouse conditions and irrigated as needed to ensure uniform emergence and growth. Forage peanut seedlings were approximately 30 days old at the time of transplanting. Limpograss propagules were obtained from a long-term established monoculture stand located at the Canguiri Experimental Farm of the Federal University of Parana, Brazil (25°23'30" S and 49°07'30" W, approximately

Table 3 Timeline of the experimental activities performed since the establishment of the experiment until the end of evaluations. Dates in DD-MM

Activity	Establishment 2019–20 ^a	Cool season 1 2020 ^b	Warm season 1 2020–21 ^c	Cool season 2 2021	Warm season 2 2021–22	Cool season 3 2022	Warm season 3 2022–23
Cleaning	Sep-Dec	-	-	-	-	-	-
Planting or replanting	08–02	11–05	27–10	04–05	03–11	12–05	07–11
N application	14–03	23–06	08–01	06–07	15–12	11–07	17–01
Aboveground clipping-mowing	25–03 08–05	23–06 03–07 10–07 23–07 30–07 05–08 13–08 27–08 07–09 17–09 01–10 27–10	08–01 21–01 22–02 18–03 09–04 27–04	06–07 02–08 15–08 30–08 13–09 25–09 22–10	15–12 05–01 22–01 10–02 25–02 15–03 14–04 12–05	11–07 26–07 12–08 26–08 09–09 04–10 17–10 04–11	26–12 16–01 31–01 01–03 17–03 29–03 26–04 30–05
Root sampling	-	23–10	29–04	22–10	10–05	-	-
Soil mineral N ^d	-	23–06 07–09	08–01 29–03	06–07 01–09	15–12 04–04	-	-
SOC, TN, POM ^d	-	-	-	-	20–04	-	13–05
Penetration resist. ^d	-	-	-	30–09	01–05	-	-
Bulk density ^d	-	-	-	-	-	-	30–05–12–06

^aThe 2019–20 season was only for the establishment of the perennial warm-season species, and no evaluation was performed. Aboveground mowing was used for homogenization and to allow for a better pasture establishment

^bCool-season species were black oat (*Avena strigosa* Schreb.), white clover (*Trifolium repens* L.) and annual ryegrass (*Lolium multiflorum* Lam.)

^cWarm-season species were bahiagrass (*Paspalum notatum* Flueggé), forage peanut (*Arachis pintoi* Krapov. & W.C. Greg) and limpograss (*Hemarthria altissima* [Poir.] Stapf & C.E. Hubbard.)

^dSoil evaluations: mineral N refers to ammonium and nitrate; SOC, soil organic carbon; TN, total nitrogen; POM, particulate organic matter; penetration resistance; bulk density

920 m asl). Sod sections were excavated using shovels to a depth of approximately 10 cm and manually separated into individual planting units measuring approximately 5×5 cm. These planting units were immediately transplanted into the experimental plots. Transplanting of both species occurred on the same date as field establishment of the warm-season treatments. Due to plant mortality and to fill stand gaps, forage peanut and limpograss were replanted during the first two months to maintain the stand.

Lime was applied on January 10, 2020, 19 days prior to forage seeding/transplanting, at a rate of 4 Mg ha⁻¹ and incorporated into the soil to a depth of 20 cm in the entire experimental area. At planting of warm-season forages, 21.8 kg ha⁻¹ of P and

41.5 kg ha⁻¹ of K were broadcasted manually in all plots. Nitrogen applied as urea was manually broadcast to all plots 35 days after planting at a rate of 50 kg N ha⁻¹. For the subsequent years, after the last mowing of each cool-season period, warm-season fertilizer was broadcast at 25 kg N ha⁻¹, 45.3 kg P ha⁻¹, and 56.4 kg K ha⁻¹, followed 54–77 days later by sidedress N at 50 kg N ha⁻¹.

Cool-season species were established 0–7 days after the last cut of the warm season period (mowed at 5 cm height) simulating a no-till drill over the established stand (Dates on Table 3). A hoe was used to carve furrows 2 cm deep with 20 cm spacing. Seeds of cool-season species were manually distributed along the furrows and covered. Seed rates were

80 kg ha⁻¹ for black oat, 5 kg ha⁻¹ for white clover and 50 kg ha⁻¹ for ryegrass. Species were sown simultaneously within each plot according to treatment assignment, with white clover alone or white clover + ryegrass sown in the same row as black oat. For subsequent years, only the annuals, black oat and ryegrass were sown.

Annually, at planting of each year of the cool-season period, fertilizer was manually broadcast in all plots at a rate of 25 kg N ha⁻¹, 40.1–45.3 kg P ha⁻¹ and 30.7–56.4 kg K ha⁻¹. Sidedress N was applied annually 43–63 days after sowing the cool-season species (Table 3), at a rate of 50 kg N ha⁻¹. Fertilization rates were applied to support a high yielding system for each growing season according to regional fertilizer recommendations (Pauletti & Motta 2019).

During the first year of warm-season planting and at all planting times of cool-season forages, the plots were irrigated twice a week with 10 mm of water until full emergence of plants (~20 days). In late March and early May 2020, the warm-season plants were mowed at a height of 5 cm for sward homogenization. No measurements were made during this initial season, which was dedicated exclusively to the establishment of the experiment. After the last cut of the cool-season grasses (late October), mowing was at 5 cm to allow the warm-season forages to regrow. At the end of each warm-season period (late April to early May), the stand was mowed to 5 cm to prepare the plots for planting the cool-season species.

Determination of herbage accumulation and root mass, and their C and N concentrations

Herbage accumulation was evaluated by summing up the dry mass of 6–12 clippings from each cool and warm season periods per year (dates in Table 3), presented as the herbage accumulation for each period and year, and expressed as Mg DM ha⁻¹. In the central area of each plot, plants were clipped in two quadrats of 0.5 × 0.5 m. In the warm-season period, forages were clipped to 10 cm whenever they reached a height of 20 cm. These cutting heights were used to simulate a moderate removal intensity for bahiagrass (Bresciano et al. 2018). In the cool-season period, forages were clipped to 20 cm whenever they reached 30 cm, which represent a moderate removal intensity for black oats (Ribeiro et al. 2020). During the cool-season

period, warm-season perennials were dormant and therefore did not contribute to harvested biomass. All clippings from both periods were collected as bulk samples representing total herbage mass within each plot.

The clipped mass was oven-dried at 60 °C until constant mass and weighed to determine dry mass. After each evaluation, the entire plot was mowed with a string trimmer to the respective seasonal height, and the mass was removed with a rake. At the final evaluation of each growing season (Table 3), plants were clipped to 5 cm height, and the whole plot was mowed to the same height to remove biomass and facilitate the establishment or regrowth of forages in the subsequent growing season cycle (Santos et al. 2020).

Root mass was determined at the end of each of the first four growing seasons (Table 3). Soil cores from the 0–5, 5–10, 10–20 and 20–30 cm layers were taken from two sampling points per plot, one in the row and the other in the inner row, with a soil corer of 8 cm diameter and 10 cm height (Schuurman and Goedewaagen 1971). Each depth was sampled sequentially from the same hole, starting from the surface and moving downward in 10 cm increments. For the top 0–10 cm, the first 10 cm core was split into two subsamples representing the 0–5 cm and 5–10 cm layers. Sampling holes were filled with soil from the border of the plot. Cores from the two sampling points were stored together as a composite sample and frozen at -4 °C. During processing, the cores were thawed for 12 h, gently broken, and flushed with water jets on a 1.0-mm mesh sieve mounted on top of a 0.5-mm mesh sieve in order to retain roots. Roots retained on the sieves were washed and, after removal of non-root debris, transferred to a 70% ethanol solution. When all core samples were processed, roots were filtered from the ethanol solution, oven-dried at 45 °C until constant mass and weighed to determine the root mass in each layer. The total root mass at 30 cm depth at the end of each season was then calculated by summing up the root mass from each layer and expressed as Mg DM ha⁻¹ (Schuurman and Goedewaagen 1971).

Herbage and root samples were ground in a mortar to pass through 250 µm mesh. A subsample of ~15 mg was analyzed by dry combustion (Vario EL III Elementar®) to determine C and N concentrations (expressed as g kg⁻¹) (Jimenez and Ladha

1993). In the case of roots, samples of the four layers were combined before milling, to obtain a single sample and C and N concentrations for the whole 30-cm-profile. For herbage mass, only three samplings per season (early, mid, and late season) were selected for C and N analysis.

Determination of soil organic C, total N and particulate organic matter

Soil was sampled at the end of warm season 2, in the 0–5 and 5–10 cm layers, and at the end of warm season 3, in the 0–5, 5–10, 10–20 and 20–30 cm layers; to determine SOC, total N and particulate organic matter (POM) (sampling dates can be found in Table 3). Soil samples were collected from two points per plot using the same corer employed for root sampling. These samples were collected and processed separately, and air-dried and sieved through <4.0 mm.

A subsample was sieved through <2.0 mm, ground through <250 μm , and ~35 mg was analyzed by dry combustion (Vario EL III Elementar®) to determine SOC and total N concentrations in the whole soil, and were expressed as g kg^{-1} (Nelson and Sommers 1996). Another subsample was physically fractionated to obtain the POM fraction (Cambardella and Elliott 1993). For fractionation, 20 g of dried soil (<4.0 mm) was weighed and added to 70 mL of hexametaphosphate solution (5 g L^{-1}). After 16 h of dispersion in a horizontal shaker (180 oscillations per minute), the suspension was passed through a 53- μm sieve to retain POM and sand particles, which were then oven-dried at 45 °C, weighed, and ground and sieved through <250 μm for C and N determination by dry combustion, expressed as g kg^{-1} of POM. Concentrations of C and N in the POM fraction were multiplied by the proportion of POM mass relative to the total soil mass to calculate C and N concentrations on a whole-soil basis, expressed as g kg^{-1} soil.

Determination of soil mineral nitrogen

Soil ammonium and nitrate concentrations were determined in the top 5 cm of the soil at two moments in each of the first four growing seasons: at the beginning of the season (soon after the first aboveground

clipping and before N application) and at the end of the season (sampling dates, Table 3). For the end-of-season sampling, the 5–10, 10–20 and 20–30 cm layers were also included. Three cores per plot were collected using a push probe (2.5 cm in diameter), and sampling holes were filled with soil from the plot border. The samples were homogenized and stored at -4 °C until analysis. After thawing for 12 h, a subsample of 5 g of moist soil was extracted with 1 mol L^{-1} KCl (Mulvaney 1996) to determine ammonium and nitrate concentrations by spectrophotometry in a flow injection analysis system (FIA), using the salicylate method for ammonium and a cadmium column for nitrate (Pasquali et al. 2007). Soil moisture was determined after drying samples until reaching constant weight at 105 °C to correct mineral N concentrations on a dry-soil basis and expressed as mg kg^{-1} .

Determination of soil physical characteristics

Soil penetration resistance was measured to 40 cm depth in the last third of the cool season 2 and of the warm season 2, at three points per plot using a manual penetrometer equipped with 30° angle cone and 12.83 mm diameter (Falker®, PLG 1020 model) (Cavaliere-Polizeli et al. 2022). The results obtained for each centimeter depth were averaged for the 0–5, 5–10, 10–20 and 20–30 cm layers and expressed as Kpa.

Soil bulk density was determined in the 0–5, 5–10, 10–20 and 20–30 cm layers at the end of warm season 3. Undisturbed samples were collected with the excavation method (Blake and Hartge 1986) at two points per plot using metal rings of 5 cm diameter and 5 cm height, and expressed as g cm^{-3} .

Statistical analysis

Statistical analyses were performed using the R software (v. 4.4.1) (R Core Team 2024). Data of herbage and root mass and their C and N concentration, SOC, total N, POM, soil mineral N and soil physical characteristics were subjected to analysis of normality (Shapiro–Wilk) and homogeneity of variances (Bartlett). Some sampling times for NH_4^+ and NO_3^- did not meet the assumptions for data distribution ($P < 0.05$), and a Box-Cox optimum power transformation was applied (MASS package). A linear model was fitted for each variable and each growing-season period

using the `lm()` function in R, with treatment and block as fixed effects.

Treatment means were estimated using the `emmeans` package and orthogonal contrasts were tested to evaluate (1) the mixture effect (grass-only versus all other mixtures), and the individual contributions of (2) forage peanut inclusion, (3) white clover inclusion, (4) limpograss inclusion, and (5) annual ryegrass inclusion. Effects were considered significant when $P < 0.05$.

Results

Herbage accumulation

On average over the three years, the mixtures contrast (B-O treatment versus all other mixtures) revealed an increase in annual herbage accumulation by 15.3% with diversified mixtures from 3 to 6 species ($P < 0.001$) (Fig. 2). This effect was generally consistent across seasons and years, with larger differences observed in the first year of establishment (Supplementary Figure S1). Regarding the specific contribution of each species, the inclusion of forage peanut (contrast between treatments B-O, B-O+W, B-O+W+R vs. B+P-O, B+P-O+W, B+P-O+W+R) showed the most consistent effects, improving herbage accumulation in both cool (by 6.4%, $P = 0.001$) (Fig. 2a) and warm seasons (by 11.6%, $P = 0.012$) (Fig. 2b) and therefore increasing annual herbage accumulation by 9.0% (Fig. 2c). The inclusion of white clover (B-O, B+P-O, B+P+L-O vs. B-O+W, B+P-O+W, B+P+L-O+W) increased herbage accumulation only during the cool season (+4.9%, $P = 0.007$).

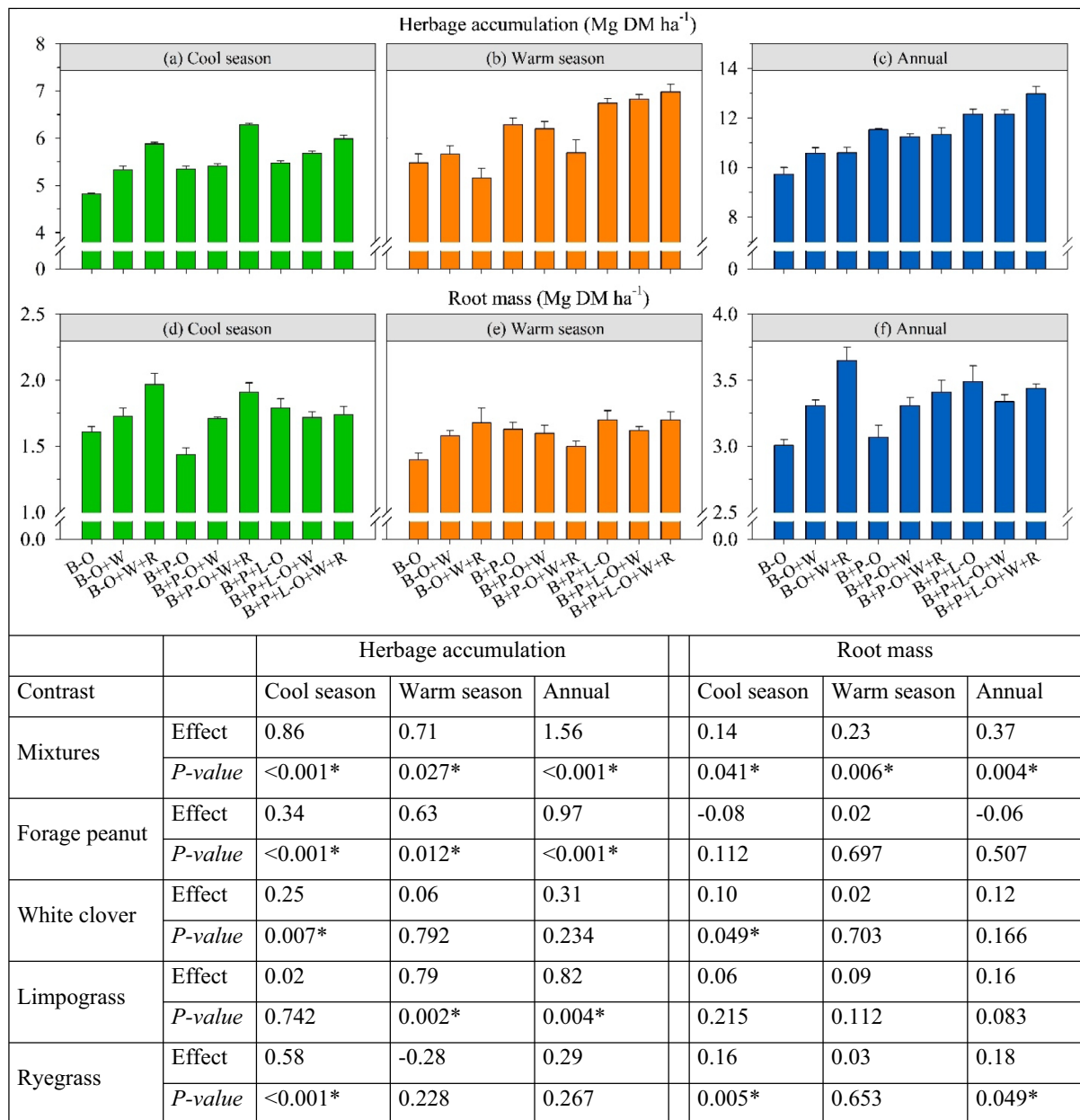
The limpograss inclusion (contrasting B+P-O, B+P-O+W, B+P-O+W+R vs. B+P+L-O, B+P+L-O+W, B+P+L-O+W+R) increased warm-season herbage accumulation by 13.1% ($P = 0.002$) (Fig. 2b) and annual herbage accumulation by 7.3% ($P = 0.004$) (Fig. 2c), but had no effect during the cool season ($P = 0.742$) (Fig. 2a). The inclusion of limpograss enhanced herbage accumulation rates, particularly later in the warm season, during autumn (April to June of each year; Supplementary Figure S2). Meanwhile the annual ryegrass inclusion (B-O+W, B+P-O+W, B+P+L-O+W vs. B-O+W+R, B+P-O+W+R, B+P+L-O+W+R) increased herbage accumulation by 10.7% only during the cool season ($P < 0.001$) (Supplementary Figure S1).

Root mass

On average, over the two years of root evaluation, the mixtures (3–6 species) increased the annual root mass by 12.2% ($P = 0.004$) compared to the B-O baseline scenario (Fig. 2f). This effect was evident in both the cool season (by 8.6%, $P = 0.041$) (Fig. 2d) and warm season (by 16.3%, $P = 0.006$) (Fig. 2e), indicating that diverse mixtures enhanced root mass accumulation throughout the year, especially at the 5–10 cm depth (Supplementary Figure S3). Regarding the contribution of each species, forage peanut and limpograss did not result in changes in root mass (Fig. 2). However, white clover inclusion increased root mass during the cool season (6.4%, $P = 0.049$), while annual ryegrass inclusion resulted in increases in the same season (9.0%, $P = 0.005$) and on an annual basis (5.5%, $P = 0.049$).

Carbon and nitrogen concentration and accumulation in diversified forage mixtures

In general, C concentrations in herbage and root tissue ranged from 391 to 429 and 363 to 425 g C kg⁻¹, respectively, and were not affected by the different mixtures (Supplementary Table S1). The inclusion of a warm-season species (forage peanut or limpograss), however, resulted in a greater root C concentration in warm season 1 ($P < 0.001$), and the inclusion of annual ryegrass resulted in a lower root C concentration in warm season 2 ($P = 0.035$). Conversely, N concentration in herbage mass increased by the effect of a diversified mixture, in warm season 3 ($P = 0.017$) and for roots in warm season 1 ($P < 0.001$) (Supplementary Table S2). The addition of a legume species had an overall positive effect on N concentration, while the addition of grass species generally decreased N concentration. More specifically, forage peanut increased N concentration in herbage during multiple periods, with notable effects in the first ($P < 0.001$) and third warm seasons ($P = 0.005$). In the roots, forage peanut also elevated N concentrations during the first cool ($P = 0.036$) and warm seasons ($P < 0.001$). Similarly, white clover enhanced herbage N concentration in the second ($P = 0.006$) and third cool seasons ($P = 0.049$) and increased root N concentration during the second cool season ($P = 0.009$) and warm season ($P = 0.032$). In contrast, limpograss



and annual ryegrass inclusions generally reduced N concentrations in herbage and root mass (Supplementary Table S2). Limpogress lowered herbage N concentration in the first ($P < 0.001$) and third warm seasons ($P = 0.001$) and decreased root N in the first cool ($P = 0.044$) and warm seasons ($P < 0.001$). Similarly, annual ryegrass reduced herbage N in the first ($P = 0.004$), second ($P = 0.002$), and third cool seasons ($P = 0.012$) and roots during the second cool season ($P = 0.008$).

On average across the study period, diversified mixtures with 3–6 species increased C (Fig. 3a) and N accumulation (Fig. 3b) in herbage ($P < 0.001$) and roots mass ($P = 0.009$ and $P < 0.001$, respectively). Among the individual species, the inclusion of a legume species, forage peanut or white clover, increased N accumulation in both herbage and roots. Forage peanut inclusion, but not white clover, enhanced C accumulation in herbage ($P = 0.001$) and in total mass ($P = 0.005$), Limpogress inclusion, however increased

Fig. 2 Mean herbage accumulation (a, b and c—averaged over three years, 2020–2023) and root mass at 0–30 cm depth (d, e and f—averaged over two years, 2020–2022) (Mg DM ha⁻¹) of nine forage mixtures consisting of gradual species increases by introducing warm and cool-season grass and legumes in subtropical Brazil. Values are means $\pm$ standard error of the mean (SEM) (n=4). Orthogonal contrasts tested all the mixtures and the individual contributions of forage peanut, white clover, limpograss, and annual ryegrass. B=Bahia-grass (*Paspalum notatum* Flueggé), P=forage peanut (*Arachis pintoi* Krapov. & W.C. Greg), L=limpograss (*Hemarthria altissima* [Poir.] Stapf & C.E. Hubbard.), O=black oat (*Avena strigosa* Schreb.), W=white clover (*Trifolium repens* L.) and R=annual ryegrass (*Lolium multiflorum* Lam.). “–” denotes sequential establishment of species across seasons within the same plot, and “+” denotes simultaneous establishment within a season. The mixtures contrast compares the treatment B-O against all other mixtures. The forage peanut inclusion contrasts treatments without peanut (B-O, B-O+W, B-O+W+R) against treatments with peanut but without limpograss (B+P-O, B+P-O+W, B+P-O+W+R). The white clover inclusion contrasts treatments without clover (B-O, B+P-O, B+P+L-O) with those containing clover but without ryegrass (B-O+W, B+P-O+W, B+P+L-O+W). The limpograss inclusion contrasts treatments without limpograss but with peanut (B+P-O, B+P-O+W, B+P-O+W+R) against those containing limpograss (B+P+L-O, B+P+L-O+W, B+P+L-O+W+R). The ryegrass inclusion contrasts treatments without ryegrass but with clover (B-O+W, B+P-O+W, B+P+L-O+W) against those with ryegrass (B-O+W+R, B+P-O+W+R, B+P+L-O+W+R). * indicates significant ($P < 0.05$) contrast effects

C accumulation ($P=0.005$), driven mainly by increases in herbage C ($P=0.006$), whereas its effect on root C was near significance ($P=0.051$). Annual ryegrass had no effect on either C accumulation in herbage or roots ($P > 0.05$).

Soil mineral nitrogen

At 0–5 cm depth, diverse forage mixtures increased the soil ammonium concentration in the late cool season ($P=0.028$) and late warm season ($P=0.003$) and nitrate in early cool ($P=0.013$) and early warm seasons ($P=0.001$) (Fig. 4). Regarding the inclusion of species, legumes occasionally increase soil ammonium concentrations, but only forage peanut increased nitrate. The inclusion of limpograss increased nitrate in the early warm season ($P=0.014$), whereas ryegrass decreased nitrate in the early warm season ($P=0.015$), followed by an increase in the late warm season ($P=0.015$).

At depths from 5 to 30 cm, the forage mixtures showed minimal consistent effects on soil ammonium

and nitrate concentrations across seasons (Supplementary Figure S4). Overall, no differences were observed among forage mixtures; however, some species tended to occasionally increase their soil N concentrations. For example, peanut and clover additions sometimes resulted in slight increases in ammonium or nitrate in certain soil layers and seasons, although these effects were sporadic and inconsistent throughout the experimental period.

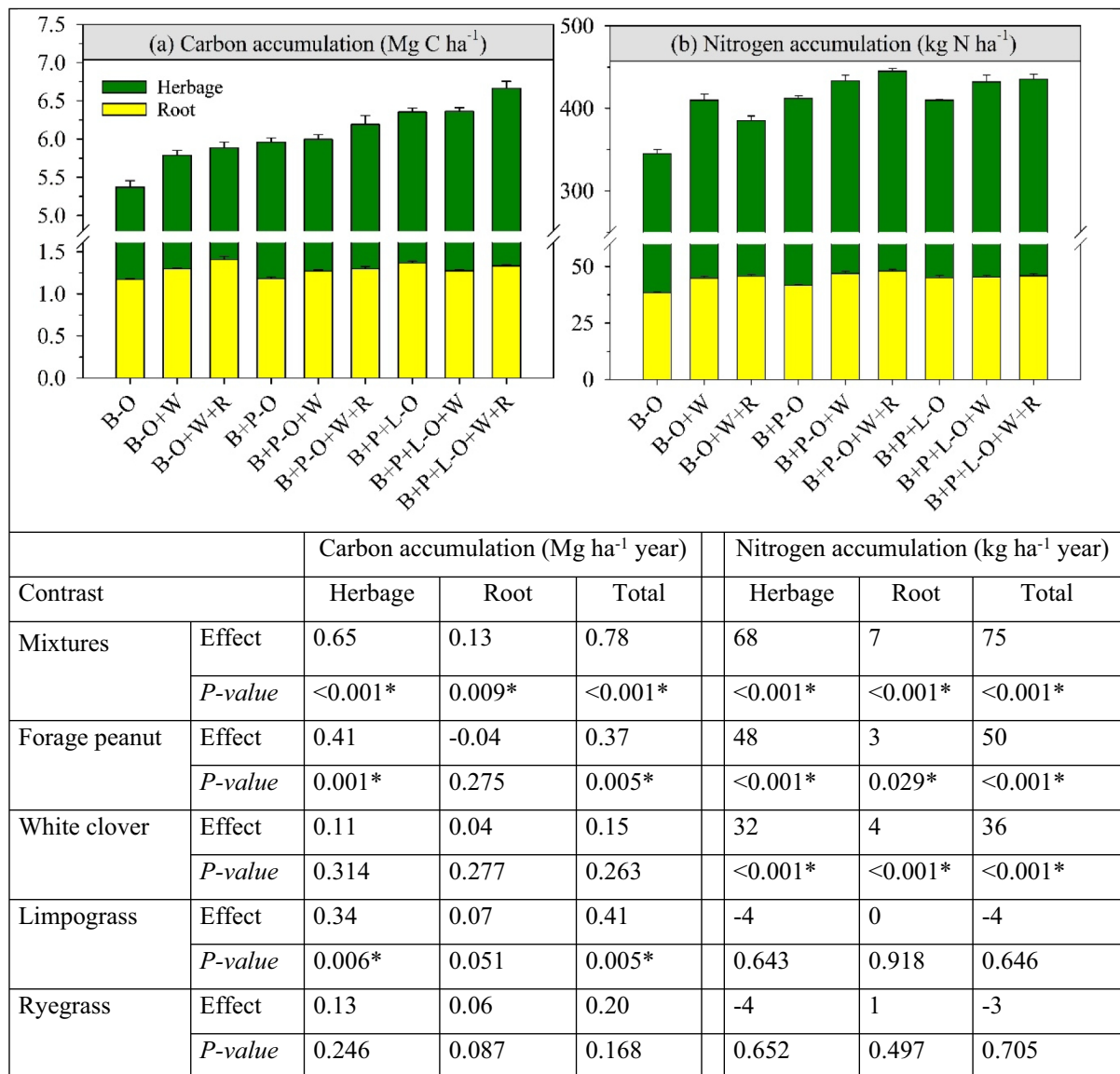
Carbon and nitrogen in whole soil and particulate organic matter fraction

Organic C and total N concentrations in the whole soil were generally not affected by the forage mixtures and different species inclusions, after two or three years of experiment establishment (Supplementary Table S3). Although in warm season 2, white clover and limpograss inclusion slightly reduced whole soil C and N concentration at 5–10 cm depth, this effect was not evident in the following year.

Similarly, the mass proportion of particulate organic matter (POM) plus sand (POM+sand) fraction retrieved after the physical functioning of the whole soil sample was not affected by the different mixtures. POM+sand mass proportion averaged 16.7% in the top 5 cm, 14.5% in the 5–10 cm, and 14.4% from 10 to 30 cm (Supplementary Table S4). The forage mixtures from 3–6 species increased the C and N concentrations in the soil POM fraction (Supplementary Table S5), and C concentration in POM as a whole soil basis (Table 4) at the 0–5 cm soil layer during warm season 2, but these effects did not persist into the third year. However, the inclusion of annual ryegrass increased both C and N concentrations in the POM fraction (Supplementary Table S5) and increased their concentration in POM on a whole soil basis at the 0–5 cm soil layer during warm season 3 ($P=0.022$ and $P=0.019$, respectively) (Table 4).

Soil physical characteristics

Soil penetration resistance (PR) after two years was generally not affected by the effect of mixtures or by the inclusion of specific species (Supplementary Table S6). Overall, PR was lower in the top 5 cm (approximately 200 kPa) and peaked at around 1500 kPa in the 20–30 cm layer. Soil bulk density (BD) at the end of warm season 3 also was generally



not affected by the effect of mixtures or inclusion of species ($P > 0.05$), and averaged 0.93 g dm^{-3} in the 0–5 cm and gradually increased to a mean of 1.11 g dm^{-3} at 20–30 cm (Supplementary Table S6).

Discussion

Effects of diversified mixtures on forage systems

Increasing species diversity from the two-species baseline (bahiagrass-black oat) to mixtures containing

three to six species enhanced herbage accumulation, root mass, and associated C and N accumulation across warm and cool seasons. In this subtropical system, characterized by alternating seasonal growth cycles, forage production is often constrained by temporal gaps between warm- and cool-season species. Incorporating species with contrasting seasonal growth patterns likely improved temporal niche occupation and resource capture throughout the year, contributing to greater annual productivity and reduced seasonal variability.

Beyond seasonal complementarity, functional differences among species further explain the

Fig. 3 Mean annual carbon accumulation (a— $\text{Mg ha}^{-1} \text{ year}^{-1}$) and nitrogen accumulation (b— $\text{kg ha}^{-1} \text{ year}^{-1}$) in the herbage, roots (to 30 cm depth) and total mass of nine forage mixtures that consisted of gradual species increases by introducing warm and cool-season grass and legumes in subtropical Brazil. Warm-season species included bahiagrass (B), forage peanut (P), and limpograss (L), while cool-season species included black oat (O), white clover (C), and annual ryegrass (R). Values are means $\pm$ standard error of the mean (SEM) ($n=4$). Orthogonal contrasts tested all the mixtures and the individual contributions of forage peanut, white clover, limpograss, and annual ryegrass. B=Bahiagrass (*Paspalum notatum* Flueggé), P=forage peanut (*Arachis pintoi* Krapov. & W.C. Greg), L=limpograss (*Hemarthria altissima* [Poir.] Stapf & C.E. Hubbard.), O=black oat (*Avena strigosa* Schreb.), W=white clover (*Trifolium repens* L.) and R=annual ryegrass (*Lolium multiflorum* Lam.). “–” denotes sequential establishment of species across seasons within the same plot, and “+” denotes simultaneous establishment within a season. The mixtures contrast compares the treatment B-O against all other mixtures. The forage peanut inclusion contrasts treatments without peanut (B-O, B-O+W, B-O+W+R) against treatments with peanut but without limpograss (B+P-O, B+P-O+W, B+P-O+W+R). The white clover inclusion contrasts treatments without clover (B-O, B+P-O, B+P+L-O) with those containing clover but without ryegrass (B-O+W, B+P-O+W, B+P+L-O+W). The limpograss inclusion contrasts treatments without limpograss but with peanut (B+P-O, B+P-O+W, B+P-O+W+R) against those containing limpograss (B+P+L-O, B+P+L-O+W, B+P+L-O+W+R). The ryegrass inclusion contrasts treatments without ryegrass but with clover (B-O+W, B+P-O+W, B+P+L-O+W) against those with ryegrass (B-O+W+R, B+P-O+W+R, B+P+L-O+W+R). * indicates significant ($P<0.05$) contrast effects

observed gains. Legumes such as forage peanut and white clover contributed biological N fixation (Burchill et al. 2014; Enriquez-Hidalgo et al. 2016), increasing N availability and promoting greater herbage and root N accumulation. Differences in canopy structure, growth phenology, and rooting characteristics among species may also have enhanced spatial resource partitioning. For example, the upright and late-season growth of limpograss likely improved light capture during peak summer conditions (Sollenberger et al. 1988), whereas the dense tillering (Duchini et al. 2017, 2014) and finer root system of annual ryegrass may have enhanced soil exploration and particulate organic matter formation during cooler periods.

Our findings also suggest that species selection plays a central role in optimizing forage mass and N accumulation in subtropical mixtures. Including one or two complementary species, particularly

functionally distinct, such as a N-fixing legume or a late-growing grass, can substantially improve productivity and forage quality (assuming N accumulation as a proxy for crude protein). In managed forage systems, Fornara and Tilman (2008), Sanderson (2010) and Tracy and Sanderson (2004), demonstrated that productivity is often driven by the presence of a few functionally complementary species rather than species richness per se. Consistent with this, our results indicate that a small number of strategically selected species can already generate substantial and stable gains in productivity and N accumulation.

While further increases in diversity did not linearly enhance mass or N availability in our study, as found in restoration (Yang et al. 2019) and semi-natural grasslands (Schaub et al. 2020), they did not hinder performance and may provide additional ecosystem services not captured in this study. However, greater diversity may result in increased establishment and management costs, highlighting the importance of aligning species choices with specific production and sustainability objectives. It is important to note that swards in this study were not grazed but rather cut at a uniform height. In a grazed system, grazing could influence species composition and herbage accumulation, potentially altering the observed trends (Herrero-Jáuregui and Oesterheld 2018).

Although increasing diversity beyond the baseline system showed positive effects, this study was not designed to test a linear diversity–productivity relationship. Instead, it enabled evaluation of how individual species with contrasting functional traits influenced forage mass, root development, and soil responses. The following sections therefore examine the contributions of specific species additions to system performance.

Legume addition effects on forage systems

The inclusion of leguminous species, such as forage peanut and white clover, into the B-O mixture had a pronounced effect on both herbage and N accumulation. Forage peanut consistently increased annual herbage mass across warm and cool seasons, suggesting both an in-season growth benefit and a legacy effect that enhanced productivity in subsequent cool seasons. These findings align with a well-documented effect in

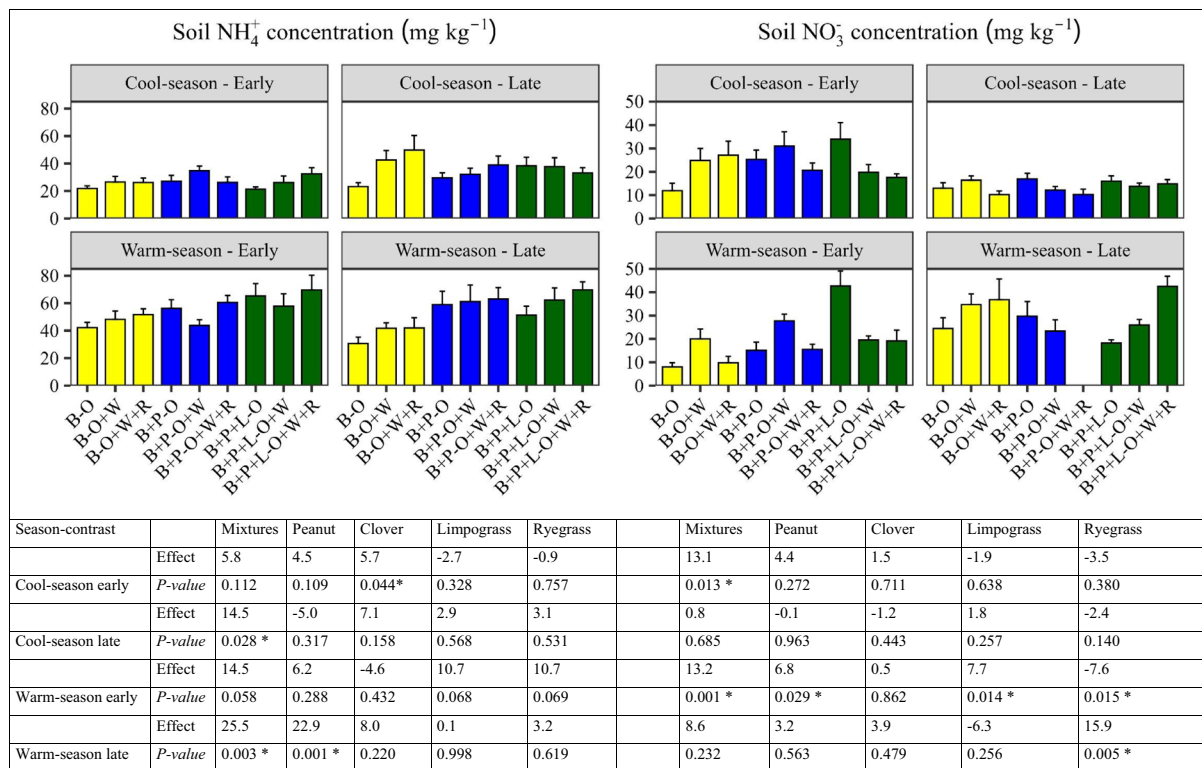


Fig. 4 Soil ammonium ($\text{g N-NH}_4^+ \text{ kg}^{-1}$ soil) and nitrate ($\text{g N-NO}_3^- \text{ kg}^{-1}$ soil) concentrations in the top 5 cm soil of nine forage mixtures that consisted of gradual species increases by introducing warm and cool-season grass and legumes in subtropical Brazil. B=Bahiagrass (*Paspalum notatum* Flueggé), P=forage peanut (*Arachis pintoi* Krapov. & W.C. Greg), L=limpograss (*Hemarthria altissima* [Poir.] Stapf & C.E. Hubbard.), O=black oat (*Avena strigosa* Schreb.), W=white clover (*Trifolium repens* L.) and R=annual ryegrass (*Lolium multiflorum* Lam.). “–” denotes sequential establishment of species across seasons within the same plot, and “+” denotes simultaneous establishment within a season. Values are means $\pm$ standard error of the mean (SEM) and represent the average of two years for each season (cool and warm) ($n=8$). Orthogonal contrasts tested all the mixtures and the individual contributions of forage peanut, white clover, lim-

pograss, and annual ryegrass. The mixtures contrast compares the treatment B-O against all other mixtures. The forage peanut inclusion contrasts treatments without peanut (B-O, B-O+W, B-O+W+R) against treatments with peanut but without limpograss (B+P-O, B+P-O+W, B+P-O+W+R). The white clover inclusion contrasts treatments without clover (B-O, B+P-O, B+P+L-O) with those containing clover but without ryegrass (B-O+W, B+P-O+W, B+P+L-O+W). The limpograss inclusion contrasts treatments without limpograss but with peanut (B+P-O, B+P-O+W, B+P-O+W+R) against those containing limpograss (B+P+L-O, B+P+L-O+W, B+P+L-O+W+R). The ryegrass inclusion contrasts treatments without ryegrass but with clover (B-O+W, B+P-O+W, B+P+L-O+W) against those with ryegrass (B-O+W+R, B+P-O+W+R, B+P+L-O+W+R). * indicates significant ($P < 0.05$) contrast effects

grass+legume mixtures (Husse et al. 2017; Lüscher et al. 2014; Pirhofer-Walzl et al. 2012) where biological N fixation by legumes enhances soil N availability and promotes higher productivity in companion grasses (Burchill et al. 2014; Enriquez-Hidalgo et al. 2016).

Soil mineral N dynamics provide additional evidence for the contribution of biological N fixation to enhanced forage productivity. Legume mixtures occasionally

increased mineral N availability, particularly in those containing forage peanut during both the late cool and warm seasons, indicating active N inputs to the system. The legacy effects observed with forage peanut likely result from accelerated N cycling through litter decomposition, which continues to elevate soil mineral N availability in subsequent seasons (Frankow-Lindberg and Dahlin 2013).

Table 4 Carbon and nitrogen concentrations in the particulate organic matter (POM) fraction on a whole soil basis of a Cambisol of nine forage mixtures consisting of gradual species increases by introducing warm and cool-season grass and legumes in subtropical Brazil

Forage mixture		Warm season 2		Warm season 3		10–20 cm	20–30 cm
		0–5 cm	5–10 cm	0–5 cm	5–10 cm		
<i>C in POM, g C kg^{−1} of whole soil</i>							
B-O		9.9±0.7	6.2±0.5	8.3±0.6	4.5±0.3	2.9±0.1	2.5±0.2
B-O + W		12.1±0.3	6.0±0.4	8.4±0.3	4.4±0.3	3.2±0.1	2.5±0.2
B-O + W + R		10.9±0.9	6.9±0.5	10.2±0.4	5.4±0.2	3.0±0.2	2.6±0.1
B + P-O		11.1±0.4	8.4±0.9	9.3±0.5	5.0±0.4	3.9±0.4	2.7±0.1
B + P-O + W		11.6±0.4	7.2±0.8	8.6±0.5	4.8±0.9	3.5±0.3	2.8±0.1
B + P-O + W + R		11.5±0.4	7.0±0.7	10.0±0.7	5.6±0.4	3.3±0.2	2.7±0.1
B + P + L-O		11.7±1.1	6.1±0.4	8.0±0.1	3.9±0.2	2.8±0.1	2.5±0.2
B + P + L-O + W		11.4±0.5	7.1±1.0	8.5±1.0	5.8±0.7	3.5±0.2	2.9±0.1
B + P + L-O + W + R		11.9±0.7	7.9±0.6	8.9±0.5	5.1±0.4	3.6±0.5	2.9±0.4
Legume	Effect	1.6	0.9	0.7	0.5	0.5	0.2
	<i>P-value</i>	0.042*	0.247	0.293	0.408	0.211	0.369
Forage peanut	Effect	0.4	1.1	0.3	0.3	0.5	0.2
	<i>P-value</i>	0.465	0.063	0.529	0.475	0.081	0.251
White clover	Effect	0.8	−0.1	−0.1	0.5	0.2	0.1
	<i>P-value</i>	0.196	0.868	0.920	0.285	0.385	0.452
Limpograss	Effect	0.3	−0.5	−0.8	−0.2	−0.3	0.1
	<i>P-value</i>	0.628	0.411	0.120	0.728	0.356	0.827
Ryegrass	Effect	−0.3	0.5	1.2	0.4	−0.1	0.0
	<i>P-value</i>	0.647	0.443	0.022*	0.435	0.783	0.883
<i>N in POM, g N kg^{−1} of whole soil</i>							
B-O		0.6±0.1	0.4±0.0	0.6±0.1	0.3±0.0	0.2±0.0	0.2±0.0
B-O + W		0.8±0.0	0.4±0.0	0.6±0.0	0.3±0.0	0.2±0.0	0.2±0.0
B-O + W + R		0.7±0.1	0.4±0.0	0.7±0.0	0.3±0.0	0.2±0.0	0.2±0.0
B + P-O		0.7±0.0	0.5±0.1	0.6±0.0	0.3±0.0	0.3±0.0	0.2±0.0
B + P-O + W		0.7±0.1	0.4±0.1	0.6±0.1	0.3±0.0	0.2±0.0	0.2±0.0
B + P-O + W + R		0.78±0.0	0.4±0.1	0.7±0.0	0.3±0.0	0.2±0.0	0.2±0.0
B + P + L-O		0.7±0.1	0.4±0.0	0.5±0.0	0.3±0.0	0.2±0.0	0.2±0.0
B + P + L-O + W		0.7±0.1	0.4±0.1	0.6±0.1	0.4±0.0	0.2±0.0	0.2±0.0
B + P + L-O + W + R		0.7±0.1	0.5±0.0	0.6±0.0	0.3±0.0	0.2±0.0	0.2±0.0
Mixtures	Effect	0.11	0.05	0.06	0.04	0.02	0.01
	<i>P-value</i>	0.104	0.381	0.212	0.397	0.390	0.498
Forage peanut	Effect	0.04	0.08	0.02	0.01	0.03	0.01
	<i>P-value</i>	0.450	0.106	0.551	0.793	0.106	0.458
White clover	Effect	0.05	−0.01	0.01	0.05	0.01	0.01
	<i>P-value</i>	0.331	0.932	0.750	0.185	0.587	0.482
Limpograss	Effect	−0.01	−0.05	−0.06	−0.01	−0.01	0.00
	<i>P-value</i>	0.818	0.317	0.072	0.905	0.418	0.700
Ryegrass	Effect	−0.02	0.01	0.09	0.01	−0.01	−0.01
	<i>P-value</i>	0.714	0.797	0.019*	0.995	0.399	0.761

Table 4 (continued)

B = Bahiagrass (*Paspalum notatum* Flueggé), P = forage peanut (*Arachis pintoii* Krapov. & W.C. Greg), L = limpograss (*Hemarthra altissima* [Poir.] Stapf & C.E. Hubbard.), O = black oat (*Avena strigosa* Schreb.), W = white clover (*Trifolium repens* L.) and R = annual ryegrass (*Lolium multiflorum* Lam.). “–” denotes sequential establishment of species across seasons within the same plot, and “+” denotes simultaneous establishment within a season. The mixtures contrast compares the treatment B-O against all other mixtures. The forage peanut inclusion contrasts treatments without peanut (B-O, B-O+W, B-O+W+R) against treatments with peanut but without limpograss (B+P-O, B+P-O+W, B+P-O+W+R). The white clover inclusion contrasts treatments without clover (B-O, B+P-O, B+P+L-O) with those containing clover but without ryegrass (B-O+W, B+P-O+W, B+P+L-O+W). The limpograss inclusion contrasts treatments without limpograss but with peanut (B+P-O, B+P-O+W, B+P-O+W+R) against those containing limpograss (B+P+L-O, B+P+L-O+W, B+P+L-O+W+R). The ryegrass inclusion contrasts treatments without ryegrass but with clover (B-O+W, B+P-O+W, B+P+L-O+W) against those with ryegrass (B-O+W+R, B+P-O+W+R, B+P+L-O+W+R). * indicates significant ($P < 0.05$) contrast effects.

Warm-season species included bahiagrass (B), forage peanut (P), and limpograss (L), while cool-season species included black oat (O), white clover (C), and annual ryegrass (R). Values are means $\pm$ standard error of the mean (SEM) ($n = 4$). Orthogonal contrasts tested all the mixtures and the individual contributions of forage peanut, white clover, limpograss, and annual ryegrass

The beneficial effects of growing forage peanut and white clover in grass stands aligns with findings from other subtropical studies. Olivo et al. (2019) demonstrated that the inclusion of forage peanut to annual ryegrass and Bermuda grass mixtures increased productivity primarily during the warm season, consistent with our results. Similarly, Trumpp et al. (2025) observed increased herbage accumulation and improved nutritive value when legumes (clovers and forage peanut) were incorporated into bahiagrass-oat-ryegrass mixtures. The authors attributed the improvements to positive grass-legume synergies, particularly through biological N fixation, which promotes long-term stability in forage production systems.

Additional grasses effects on forage systems

The inclusion of additional grass species, alongside legumes, produced season-specific responses that highlight the importance of species identity. Limpograss primarily increased warm-season herbage accumulation, consistent with its adaptation to high temperatures and extended summer growth (Sollenberger et al. 1989). In contrast, annual ryegrass enhanced cool-season herbage accumulation and root mass and was the only species associated with increased soil POM-C and POM-N after three years. The occasional positive effects of these additional grasses partially support our hypothesis regarding species identity. These outcomes likely reflect the distinct growth characteristics of each species, possibly,

though not measured, creating a new botanical balance among the species.

Both limpograss and annual ryegrass tended to reduce tissue N concentrations, likely because of a reduction in the proportion of legumes in the sward (Sanderson et al. 2005). The reductions were offset by slight gains in herbage accumulation, resulting in no net loss in N accumulation compared with the inclusion of legumes only. Although annual ryegrass reduced soil nitrate availability in the early warm season, likely due to higher root metabolic activity throughout the season (Malcolm et al. 2015; Maxwell et al. 2019), this was followed by soil nitrate increase later in the season, possibly driven by the decomposition of its late-growing tillers. However, the timing and dynamics of this shift from N immobilization to mineralization remain unclear and warrant further investigation to better understand their implications for N cycling in mixed-species forage systems.

Limited effect of forage mixtures on soil particulate organic matter

Unlike aboveground responses, soil C and N concentrations, and POM dynamics showed less consistent effects from mixtures or from the inclusion of different species. Similar to their effect on herbage and N accumulation, forage peanut and white clover also increased N accumulation in roots, although only clover led to higher root mass. This may be attributed to its shallow rooting system and the presence of stolons, which likely concentrated root mass in the topsoil compared to the fibrous roots of black oats or

the rhizomatous growth of bahiagrass (Heuermann et al. 2019; Santos et al. 2019). Despite greater root N accumulation, neither legume altered POM-C or POM-N concentrations on a whole soil basis, suggesting limited short-term stabilization of root-derived inputs. However, their N-rich root inputs, can be more microbially accessible and increase microbial C use efficiency, potentially favoring the gradual formation of more stable mineral-associated organic matter (MAOM) (Cotrufo et al. 2013). Although this remains a hypothesis, it points to a pathway through which legumes might contribute to longer-term soil C stabilization even if short-term POM responses are minimal.

The addition of limpograss, a perennial forage grass, increased herbage mass (with a trend for root mass), but did not affect root N accumulation, or soil POM-C and POM-N concentrations, possibly due to its longer root-turnover rates, compared to annual species. In contrast, the addition of annual ryegrass increased root mass and higher POM-C and POM-N concentrations in the 0–5 cm soil layer by the third year. These findings suggest that annual ryegrass may enhance stabilization of root-derived organic matter in particulate soil fractions, possibly through increased root turnover, fine root proliferation, or rhizodeposition (Haynes and Francis 2006; Reid et al. 2015). This pattern is consistent with the idea that annual species, because of their rapid root turnover, tend to contribute more to POM formation, whereas perennial species with longer-lived roots may favor pathways leading to MAOM (Mueller et al. 2024; Witzgall et al., 2024).

Additionally, annual ryegrass' deeper, finer, and more branched fibrous roots likely contributed to greater vertical soil exploration compared to legumes, such as clover and peanut (Gould et al. 2016; Quesenberry et al. 2004). The inclusion of annual ryegrass in the mixture may have improved the spatial complementarity for root growth, although roots were not separated by species, and this remains an hypothesis. Importantly, the soil conditions in this study, with low resistance to penetration, optimal bulk density, and adequate microporosity, favored root development and elongation (Bengough et al. 2011; Wendel et al. 2022).

The short-term duration of this trial, coupled with mild temperatures and the initially high SOC concentration, might have limited the magnitude of

treatment effects on POM. However, long-term studies in similar environments have shown that C inputs from shoot and root mass can eventually increase the POM stocks. For example, in a study with 10 years of an annual mixture of black oat and annual ryegrass pasture under moderate grazing, POM-C increased down to a depth of 40 cm (Assmann et al. 2014; Silva et al. 2014). Similarly, Santos et al. (2019) found that bahiagrass–peanut mixtures increased C and N stocks in sandy soils compared to peanut monocultures after four years, although not compared to heavily fertilized bahiagrass monocultures. In all cases, increases in root mass were key factors associated with improved POM accumulation. These findings support the idea that changes in root mass, observed with the addition of white clover and ryegrass, and in root composition, observed with most individual species, may precede long-term changes in soil C and N pools and other soil properties in our study.

Future grazing trials could evaluate whether higher herbage accumulation, combined with trampling and manure deposition, accelerates organic matter incorporation into the soil (Lavalley et al. 2020; Ribeiro et al. 2020; Santos et al. 2018a, b; Wei et al. 2021). The same studies could also evaluate the effects of these mixtures on stand persistence, animal weight gain, and the year-round supply of nutritive forage. Additional studies could explore how mixtures may influence soil microbial diversity and function, contributing to nutrient cycling and ecosystem services (Yan et al. 2022). Finally, future studies could quantify the extent to which legume-derived N can replace synthetic N fertilizers (Lüscher et al. 2014), reducing environmental burdens related to nitrous oxide emissions (Bracken et al. 2022), or nitrate leaching to groundwater (Eriksen et al. 2015).

Conclusion

This three-year field study demonstrated that increasing species diversity beyond the two-species baseline consistently enhanced herbage accumulation, root mass, and associated C and N accumulation in subtropical forage systems combining warm- and cool-season species. Diversified mixtures improved productivity across seasons, suggesting that incorporating species with contrasting functional traits can reduce temporal production gaps and enhance

resource use throughout the year. These findings highlight the importance of strategic species selection in designing forage systems that optimize both agro-economic performance and soil health.

Within these diversified systems, species with contrasting functional traits contributed differently to system responses. Forage peanut consistently increased herbage mass and N accumulation, with smaller but positive effects from white clover, consistent with their capacity for biological N fixation. Additional grasses such as limpgrass and annual ryegrass influenced seasonal production patterns, with limpgrass contributing primarily to herbage accumulation and annual ryegrass enhancing root mass and increasing POM-C and POM-N. These patterns indicate that nitrogen fixation capacity, seasonal growth dynamics, and root characteristics shape specific above- and belowground outcomes within mixtures.

Despite greater root C and N accumulation in most mixtures, soil POM remained largely unchanged after three years. Significant increases were detected primarily in the surface layer and were most evident in mixtures including annual ryegrass. The absence of consistent changes across depths likely reflects the relatively short experimental duration and high initial SOM. Nonetheless, increased root C and N accumulation in some mixtures suggest potential long-term changes in soil C and N pools if sustained over time. Finally, our results can guide the choice of more win–win mixture combinations towards more resilient and productive forage ecosystems.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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