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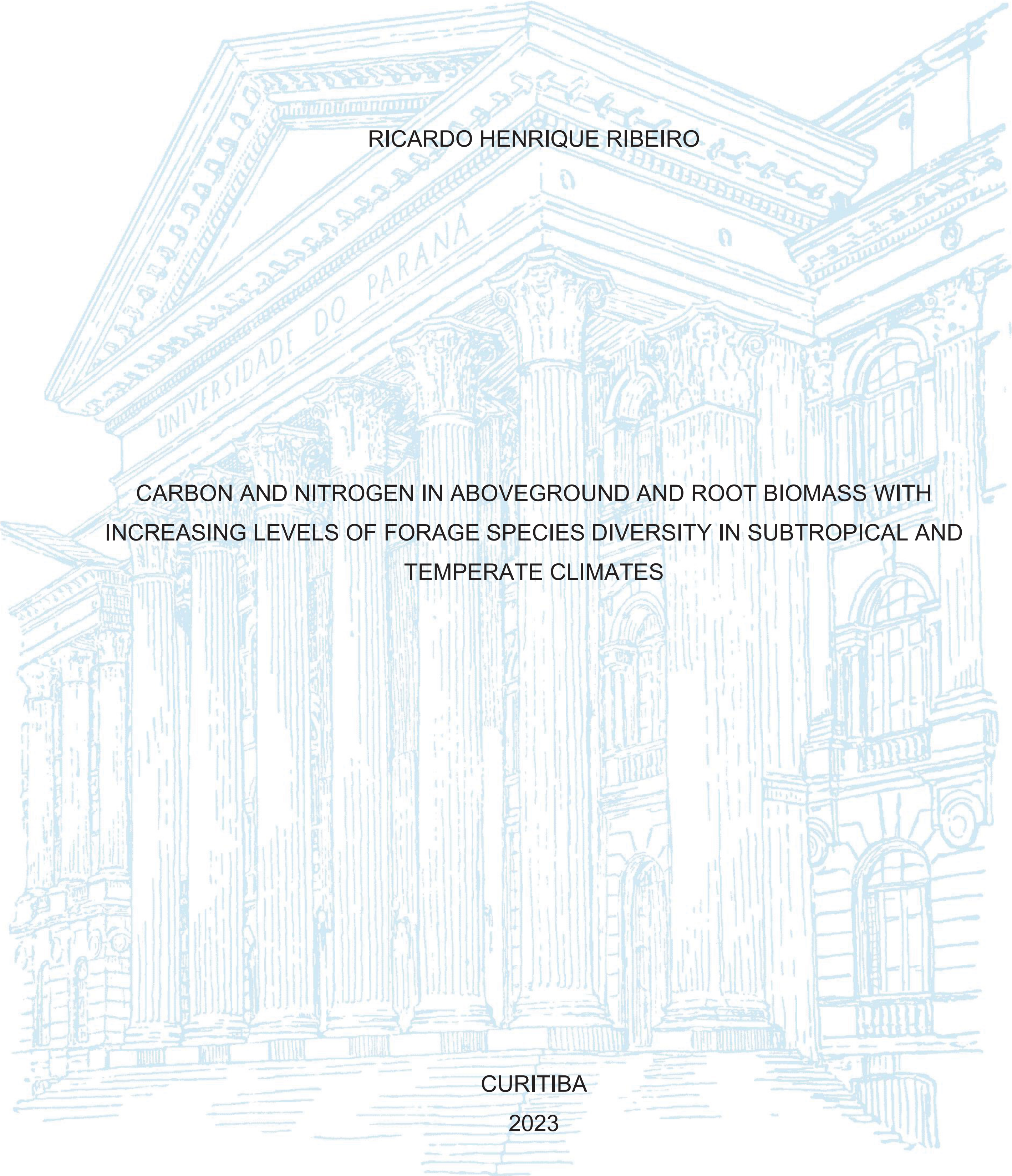

RICARDO HENRIQUE RIBEIRO

CARBON AND NITROGEN IN ABOVEGROUND AND ROOT BIOMASS WITH INCREASING LEVELS OF FORAGE SPECIES DIVERSITY IN SUBTROPICAL AND TEMPERATE CLIMATES

CURITIBA
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Tese apresentada ao curso de Pós-Graduação em Ciência do Solo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciência do Solo.

Orientador: Prof. Dr. Jeferson Dieckow

Coorientadora: Profa. Dra. Marilia Barbosa Chiavegato

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIA DO SOLO da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **RICARDO HENRIQUE RIBEIRO** intitulada: **CARBON AND NITROGEN IN ABOVEGROUND AND ROOT BIOMASS WITH INCREASING LEVELS OF FORAGE SPECIES DIVERSITY IN SUBTROPICAL AND TEMPERATE CLIMATES**, sob orientação do Prof. Dr. JEFFERSON DIECKOW, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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RESUMO

Em áreas de pastagens, as raízes representam a principal forma de entrada de resíduos para formação da matéria orgânica do solo (MOS), pois esses resíduos radiculares estão em contato direto com as particulas de solo e microrganismos, aumentado a eficiência de sua conversão para MOS em relação ao resíduo de parte aérea. O uso de consórcios entre espécies forrageiras com características complementares pode aumentar a produção de raízes e consequentemente na produção de forragem. Espécies com arquitetura radicular e profundidade de enraizamento diferentes podem explorar diferentes nichos no solo, gerando maior acesso aos recursos naturais e promovendo maior resiliência da pastagem às mudanças climáticas. O objetivo dessa pesquisa foi avaliar o efeito de pastagens consorciadas com diferentes composições de espécies sobre a produção e acúmulo de C e N na biomassa de parte aérea e raízes e seus impactos na MOS em clima subtropical e temperado. No clima subtropical foram avaliados nove tratamentos com incremento no número de espécies, intercalando gramíneas e leguminosas de estação fria e quente. No clima temperado, foram avaliados três pastagens com composição botânica diferentes: monocultura de gramínea perene, consórcio entre gramíneas e leguminosas perenes de estação fria, e a inclusão de anuais de inverno à mistura das perenes de estação fria. Um segundo fator foi avaliado em clima temperado, correspondendo à ocorrência de eventos de inundação natural do solo após chuvas intensas. A produção de forragem e raízes até 30 cm de profundidade em ambos locais foi avaliada por dois anos. A matéria orgânica particulada (MOP) foi avaliada ao final dos experimentos. Os resultados indicaram que pastagens com maior diversidade de espécies aumentaram a produção de forragem e de raízes em ambos locais, mas aumentos na MOP ocorreram apenas em clima temperado. As condições iniciais do solo sob clima subtropical com elevado teor de MOS podem ter reduzido os efeitos positivos da maior adição de C e N pelas raízes dos consórcios. A complementariedade entre as espécies avaliadas, a entrada de N adicional através da fixação biológica pelas leguminosas e as diferenças temporais no crescimento e na morfologia da parte aérea e radicular pelas gramineas foram fatores determinantes para o sucesso das misturas. Contudo, o consórcio entre perenes e anuais em clima temperado resultou em baixa população de plantas e alta pressão por daninhas, reduzindo a produção de parte aérea e raízes e consequentemente levando à perdas na MOP. As condições de inundação também causaram redução na produção de parte aérea e raízes e na MOP em clima temperado, contudo o consórcio entre perenes de estação fria mostrou um maior potencial de resiliência à inundação, em comparação à monocultura. Concluindo, misturas diversas de espécies complementares podem ser usadas para elevar o potencial produtivo das pastagens e aumentar as adições de C e N por raízes, podendo elevar o sequestro de C no solo à longo prazo.

Palavras-chave: Consórcios; Inundação; Leguminosas; Matéria orgânica do solo; Sustentabilidade.

ABSTRACT

In grasslands, roots are the main point of entry of plant residue to form soil organic matter (SOM), because they are in direct contact with soil particles and microorganisms, increasing its conversion efficiency to SOM, in relation to the aboveground residue. The use of mixtures of forage species with complementary characteristics can increase root production and consequently forage mass. Species with different root architecture and rooting depth can explore different soil niches, improving access to natural resources promoting a higher grassland resilience to climatic change. The objectives of this research were to evaluate the effects of forage mixtures with different species composition on aboveground and root production and C and N accumulation, and its impacts on SOM in a subtropical and temperate climate. In the subtropical climate nine treatments were evaluated by increasing the number of species in the mixture, combining grasses and legumes from cool and warm-season. Under temperate climate, three grasslands with different botanical composition were evaluated: a perennial grass monoculture, a mixture of perennial grasses and legumes from cool-season, and the inclusion of winter annuals to the perennial cool-season mixture. A second factor was evaluated under temperate climate, consisting of the natural occurrence of soil inundation events after heavy rains. Forage and root production, up to 30 cm depth, were evaluated during two years at both locations, and soil particulate organic matter (POM) evaluated at the end of the experiments. The results showed that grasslands with higher species diversity increased forage and root production in both sites, but POM was only increased under temperate climate. The initial soil conditions from the subtropical site, with high MOS concentration, might have reduced the positive effects of higher root C and N input by the mixtures. Complementarity among the species evaluated, with additional N input from biological fixations by legumes and different temporal and morphological growth patterns for aboveground and roots, were decisive factors for the success of mixtures. Although the mixture among perennials and annuals in temperate climate resulted in poor plant stand and high weed pressure, reducing aboveground and root production, and consequent loss of POM. Soil inundation also reduced aboveground and root production and POM in temperate climate, however the cool-season perennial mixture presented a higher resilience potential to inundation, compared to the grass monoculture. In conclusion, diverse mixtures among complementary species can be used to increase the potential of forage production in grasslands and increase C and N inputs belowground, which could lead to soil C sequestration in the long-term.

Keywords: Mixtures; Inundation; Legumes; Soil organic matter; Sustainability.

SUMMARY

GENERAL INTRODUCTION

Soil organic carbon (SOC) results from the balance between C inputs (CO_2 uptake by plant photosynthesis) and losses from soil (microbial decomposition and erosion) (LIU et al., 2022). However, in the scenario of climate change, SOC losses may increase and cause an overall decline in global SOC storage, triggering positive feedbacks to climate change (GARCÍA-PALACIOS et al., 2021; YAN et al., 2022). Yet, initiatives that support increases in SOC sequestration, mainly in agricultural soils, are being ascribed as important tools for climate change mitigation (CHENU et al., 2019; MINASNY et al., 2017). Increases in SOC will also improve other soil attributes, such as water infiltration and storage, and erosion control, which are vital for climate change adaptation (MONDAL et al., 2020; SOUSSANA et al., 2019).

Grassland ecosystems have a great potential to store and sequester SOC (WHITE et al., 2000). It is estimated that globally grasslands account for 25-34% of terrestrial C stocks (BAI and COTRUFO, 2022). The high potential to sequester SOC in grasslands is due to increased input of organic material through aboveground and belowground residues, which are transformed by soil macro and microorganisms into organic matter (LAL, 2004). However, grasslands are traditionally managed as monocultures of grass species and under intensive grazing, demanding high supply of external nutrients and causing environmental burdens such as increased greenhouse gases emissions (SCHMEER et al., 2014), soil carbon loss (ABDALLA et al., 2018; ZHOU et al., 2017) and nutrient loss (DIJKSTRA et al., 2007). In this regard, it is estimated that almost half of global grasslands are degraded to some extent, posing a risk for food, environment, and cultural values (BARDGETT et al., 2021; BENGTSSON et al., 2019).

Planned diversification of forage species with complementary traits can be an alternative strategy to promote resilience and sustainability of grasslands (ISBELL et al., 2017; LÜSCHER et al., 2014). Benefits of species mixtures in grasslands include increased forage production, yield stability, soil fertility, presence of pollinators, weed and pest suppression and decreased external nutrient demand (ISBELL et al., 2017). The inclusion of plant communities of high diversity in grasslands also accelerates soil carbon accrual, mainly due to higher proportion of roots and the low decomposition of these roots when compared to a monoculture (YANG et al., 2019). Belowground inputs play a key role in this sense, since roots, due to its higher chemical recalcitrance and physical protection against microbial activity, contribute with 2-3 times more than aboveground residues to soil organic matter formation (BOLINDER et al., 2012; KÄTTERER et al., 2011; RASSE et al., 2005). Root fragments and exudates also represent an important input to the labile pool of organic matter (PAUSCH and KUZYAKOV, 2017). Although the effects of species mixtures on forage production is well known, there is still a lack on research evaluating roots and their impact on SOC.

Results from a survey on 112 publications from 2000 to 2020 all over the globe (Figure 1), showed that adding species diversity has a positive effect on increasing forage yield by 22.7% (Table 1). This increase occurred in most of studies and most of climates surveyed (Supplementary Table 1). The effect was higher when legumes were mixed with grasses, increasing forage yield by 26.8%, in contrast to only grass species mixes, which result in 6.3% higher forage yield compared to a grass monoculture (Table 1). There was predominance in the use of mixtures of perennial species, which presented a higher gain in yield (27.9%) compared to annual mixtures (13.0%), in relation to their respective monocultures. Forage mixtures are usually composed by a binary combination of species (58% of studies), while mixtures with 3 and 4 species account to 23.8 and 9.9%, respectively (Supplementary Table 2). Mixtures with more than 5 forage species are less common, although there is research combining 11 to 16 species.

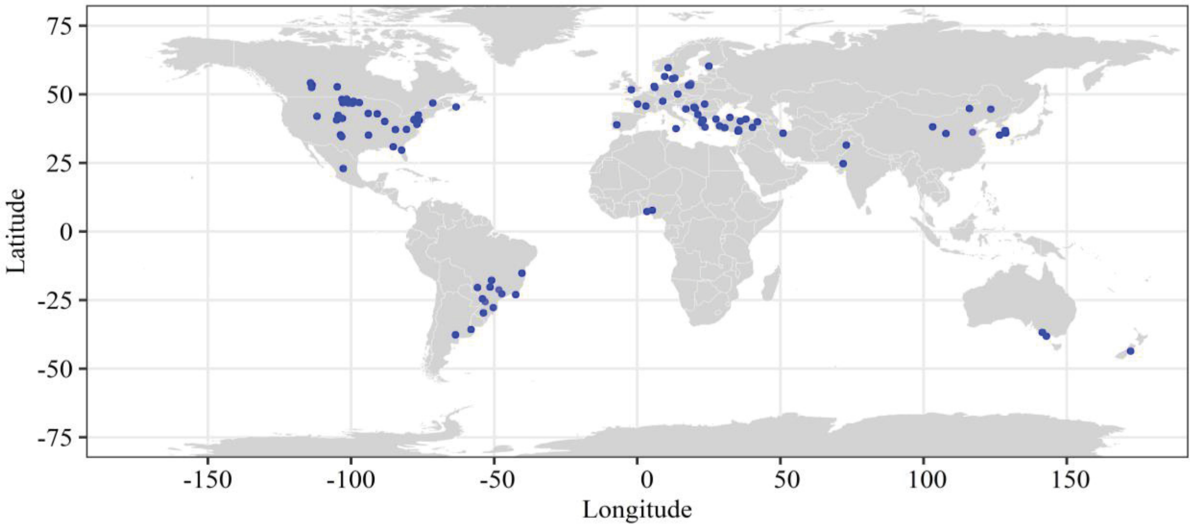

Figure 1. Global distribution of experimental sites from 112 studies comparing forage yield and root production from grassland monocultures and mixtures. The survey included publications from 2000 to 2020, indexed in Web of Science main collection and Scholar Google database. List of references and details about the search are described in Supplementary Material.

SOURCE: The author.

Table 1. Literature survey results comparing forage yield and root production (dry mass [DM] basis, means ± SD) from grassland monocultures and mixtures and their breakdown into species cycle and inclusion of legumes in the mixtures. The survey included publications from 2000 to 2020, indexed in Web of Science main collection and Scholar Google database. List of references in Supplementary Material.

	Monoculture	Mixture	Observations	Studies
	Mg DM ha^{-1}			
Forage yield				
All results	7.9±5.5	9.7±6.2	2483	112
Legume inclusion to grass monoculture	7.8±5.5	9.9±5.8	2051	91
Only grasses in monoculture and mix	8.2±4.1	8.7±5.3	329	12
Perennial species	7.9±5.2	10.2±6.2	1574	60
Annual species	7.9±5.9	8.9±6.0	867	47
Root production				
All results	5.6±2.8	8.3±3.8	47	7
Legume inclusion to grass monoculture	5.4±2.6	8.5±4.2	27	3

LEGEND: List of references and details about the search are described in Supplementary Material.

SOURCE: The author

As shown in the literature survey (Table 1), the inclusion of legume species to a grass stand has positive effects on both forage yield and root production. Legumes, due to its capacity of biologically fixing nitrogen, provide additional N supply to the grasses growing in the mix (BURCHILL et al., 2014; ENRIQUEZ-HIDALGO et al., 2016). In this type of mix, the grasses will uptake N, reducing its content in soil, favoring the legumes to fix and release more N to the soil (CHRISTOPHER and LAL, 2007; VIERA-VARGAS et al., 1995). The positive interaction between these different functional groups (grass and legumes), and the complementary of production, result in forage yield gains and stability of production from diverse species grasslands (ISBELL et al., 2017). Other complementary characteristics among species that promote better resource utilization should also be considered in designing effective mixtures. Mixtures of species varying in rooting depth and on asynchrony of nutrient uptake can increase vertical and temporal complementarity and result in a higher capture of nutrients and water, maintaining high productivity stands (CONG et al., 2017; HUSSE et al., 2017; PIRHOFER-WALZL et al., 2013).

It is expected that increases on forage production by mixtures will also increase root production, but in a smaller extent (FORNARA and TILMAN, 2008; RUTLEDGE et al., 2017; WANG et al., 2021). However, the literature survey (Table 1) reports an increase of 47% (ranging from 4 to 71%) on root biomass from mixtures compared to monocultures, which is higher than the effect on aboveground (22.7%). Although, the survey only found 7 publications with evaluation on root production, which comprised only perennial species and few environments (Supplementary Table 3 and 4). Therefore, this limited amount of information, locations and species combinations represents a gap on understanding the effects of forage mixtures on root production and further impacts on SOC.

Although increased C and N input by a higher root mass is expected to increase SOC, short-term experiments (<5 years) usually do not reflect in changes in bulk SOC but can affect labile fractions that compose it. Most of the SOM is composed of two distinct physical fractions, the particulate organic matter (POM) and the mineral-associated organic matter (MAOM) (CAMBARDELLA and ELLIOTT, 1993; COTRUFO et al., 2019; LAVALLEE et al., 2020). POM is a fraction mainly consisting of plant origin material and it is more vulnerable to disturbance, and it cycles faster than MAOM. For this reason, POM is considered to rapidly respond to short-term changes in above and belowground inputs, and it can be used as an indicator for SOC building up in the long-term (MITCHELL et al., 2018). Research with cover crops has shown increases in POM fraction under mix of species compared to monocultures (ZHANG et al., 2022), but these results still need to be checked for forage mixtures.

We hypothesized that forage mixtures increase forage mass accumulation and maintain a more stable production throughout the growing season in relation to forage monocultures. The increase on aboveground mass will also result in a greater input of C and N to soil by roots, increasing SOC in the particulate fraction. Therefore, the objective of this research was to evaluate the effects of different levels of forage species diversity, varying from one to six species, on aboveground and root production and its impact on POM under two contrasting climate conditions.

1 CHAPTER I: CARBON AND NITROGEN IN ABOVEGROUND AND ROOT BIOMASS OF FORAGE MIXTURES WITH INCREASING LEVELS OF SPECIES DIVERSITY

1.1 ABSTRACT

The increase of forage species diversity in grasslands is known to increase biomass yield due to complementarity among species, but results of impacts on root mass and in soil organic carbon fractions are lacking. The objective of this study was to evaluate effects of increasing diversity of cool and warm-season forage mixes on carbon and nitrogen accumulation in aboveground and roots, and their impacts on soil particulate organic matter (POM). A field experiment was stablished in a Cambisol in Curitiba, Brazil, under subtropical climate. Starting from a control mixture of bahiagrass (B), growing in warm-season, and oats (O), growing in cool-season, additional mixtures were established by adding species one by one up to six species in the mixture. Species added were forage peanut (P) and limpograss (L), during the warm-season, and white-clover (C) and ryegrass (R) in the cool-season. Cumulative forage yield and root mass were evaluated during 2 years in each of the two growing seasons. Soil POM was evaluated at the end of the 2 years. Increasing diversity to 3-6 forage species increased forage yield and C and N accumulation ($p<0.05$) by 16-84% compared to B/O across the seasons. Forage yields from each season were higher when triple-mixture of species from the respective season was present, regardless of the number of species from the previous season. Root mass and its C and N accumulation also increased with mixtures, mainly at 5-10 cm layer ($p<0.05$), regardless of the species combination compared to B/O. Although mixtures increased belowground C and N input to soil, no changes ($p>0.05$) were found in POM at 0-5 and 5-10 cm depth after those 2 experimental years. The results of this two years research showed that diverse forage mixtures can increase forage yield and root C and N inputs to the soil in the early years after establishment and are an indicator that changes in soil POM may occur after a longer period of continuous increased C and N input to the soil.

Keywords: Complementarity. Grasslands. Particulate organic matter. Regenerative agriculture. Soil carbon sequestration.

1.2 RESUMO

O aumento na diversidade de espécies forrageiras em pastagens aumenta a produção de biomassa devido à complementariedade entre espécies, mas resultados sobre massa radicular e frações da matéria orgânica ainda são incipientes. O objetivo desse estudo foi avaliar os efeitos do aumento na diversidade de misturas de forragem de estação fria e quente sobre o acúmulo de carbono e nitrogênio na parte aérea e raízes, e seus impactos na matéria orgânica particulada (MOP) no solo. Um experimento de campo foi estabelicido em um Cambissolo em Curitiba, Brasil, sob um clima subtropical. As misturas partiram de uma combinação entre pensacola (P), com desenvolvimento na estação quente, e aveia (A), na estação fria, sendo nas demais misturas adicionado espécies uma a uma até uma mistura com seis espécies. As espécies adicionadas foram amendoim forrageiro

(Am) e hemartria (H), na estação quente, e trevo-branco (T) e azevém (Az), na estação fria. A produção acumulada de parte aérea e a massa de raiz foram avaliadas durante 2 anos em cada estação. A MOP do solo foi avaliada ao final dos 2 anos. O aumento na diversidade da mistura com 3-6 espécies aumentou a produção de forragem e o acúmulo de C e N (p<0,05) em 16-84% em relação à mistura entre P/A, ao longo das estações. Os rendimentos de forragem de cada estação foram maiores quando a mistura de 3 espécies da mesma estação estava presente, independentemente do número de espécies da estação anterior. A massa de raízes e seu acúmulo de C e N também aumentou com as misturas, principalmente na camada 5-10 cm do solo (p<0,05), independentemente do número de espécies em comparação à P/A. Apesar do aumento nas adições de C e N abaixo do solo pelo uso das misturas, não houveram alterações na MOP (p>0,05) nas camadas de 0-5 e 5-10 cm de profundidade após os 2 anos de avaliação. Os resultados dessa pesquisa de dois anos mostraram que o uso de misturas forrageiras diversas aumentaram a produção de forragem e as adições de C e N por raízes nos primeiros anos após seu estabelecimento, e são indicadores de que mudanças na MOP podem ocorrer após um período de tempo maior com a contínua e elevada adição de C e N ao solo.

Palavras-chave: Complementariedade. Pastagens. Matéria orgânica particulada. Agricultura regenerativa. Sequestro de carbono no solo.

1.3 INTRODUCTION

Grasslands store one third of total soil organic carbon stocks (SOC) globally but are very vulnerable to biotic disturbances that affect its potential as C sink or source (BAI and COTRUFO, 2022; WHITE et al., 2000). Intensive grazing and low forage species diversity have been often related to losses on SOC in a wide range of soil types and climates (ABDALLA et al., 2018; MCSHERRY and RITCHIE, 2013; ZHOU et al., 2017). However, management practices that improve net primary production in grasslands, mainly by increasing belowground C inputs, are reported to increase SOC sequestration over time (FORNARA and TILMAN, 2008; YANG et al., 2019). In this sense, the increase of plant diversity with different functional groups is ascribed to enhance the carbon sink potential of grasslands, as well as many other ecosystem functions (ISBELL et al., 2017; MI et al., 2022; NABE-NIELSEN et al., 2017).

Forage species from different functional groups explore distinct spatial and temporal niches in an ecosystem, improving resource use and production stability via species complementarity (HUSSE et al., 2017; PAREDES et al., 2018). Legumes for example, provide extra N by biological N fixation (BNF), which is a nutrient often limiting for grasses tillering, leaf elongation and productivity (WHITEHEAD, 1995).

The inclusion of legumes can transfer 10 to 75 kg N ha^{-1} year^{-1} to the grass species growing in mix, improving yields and quality (PIRHOFER-WALZL et al., 2012). Frankow-Lindberg and Dahlin (2013) observed that *Trifolium pratense* L., a BNF legume, can contribute to supply 6-29% of N uptake by grasses cultivated in mix. In a long-term study with grassland mixtures with multiple C3 and C4 grasses and legume species, YANG et al. (2019) found soil C storage to increase up to 70% when compared to grass monocultures. According to the authors, this was a result of increased forage productivity (14-86%) and a higher increase on root biomass (40-100%) in the mix, reflecting the positive interaction and complementary among species in relation to monocultures.

In establishing diverse grassland stands, contrasting root architectures and rooting depths can add niche complementarity to belowground, enhance root density in deeper soil layers and improve soil structure and nutrient uptake and cycling (GOULD et al., 2016; HUSSE et al., 2017). Nonetheless, roots play a crucial role in building up soil carbon in grasslands as the most important input to deeper layers, since aboveground residues and feces will be only incorporated in the first centimeters of soil (CHIAVEGATO et al., 2015; RIBEIRO et al., 2020). Belowground inputs are also more efficiently stabilized as soil organic matter than aboveground residues, due to higher chemical recalcitrance and lower decomposition rates of roots (JACKSON et al., 2017; RASSE et al., 2005). Most of root residue is a part of the soil particulate organic matter (POM), a fraction of sand-sized particles which is highly responsive to environmental and management changes (CAMBARDELLA and ELLIOTT, 1992; LAVALLEE et al., 2020). Changes in root mass usually do not reflect in total soil C stocks in the short-term, but can affect POM (LAVALLEE et al., 2020). In addition, an increase in root mass is expected to increase rhizodeposition (BOLINDER et al., 2012), which represents constant C input to the soil as plant grows, and an important labile pool for microbial use stabilized into the mineral soil fraction (SOKOL et al., 2019).

Although forage mixtures are expected to increase root mass and therefore soil C stocks (FORNARA and TILMAN, 2008; YANG et al., 2019), establishing a successful mixture stand is not an easy task. Successful forage mixtures depend not only on the number of species with different functional groups, but also on the ability of species to coexist without harmful competition (HERNANDEZ and PICON-

COCHARD, 2016; HØGH-JENSEN et al., 2006). For this reason, binary mixtures of grass-legume species are often used due to relatively easier establishment, lower cost with seeds and positive interaction between species (COX et al., 2017; ELGERSMA and SØEGAARD, 2016; OLIVO et al., 2019; SANTOS et al., 2018a). However, studies with higher level of species diversity with complementarity traits that increase niche exploitation and provide forage allowance throughout the year are still lacking. In subtropical Brazil, grasslands are usually divided into cool-season annual species, being part of integrated crop-livestock systems with cash grains cultivated in summer (CARVALHO et al., 2010; MORAES et al., 2014), and warm-season perennial grasslands (MORAES et al., 2019). The latter is often nonproductive during the colder months (May-August), presenting an opportunity to introduce cool-season annuals for this period, keeping forage production stable throughout the year (MORAES et al., 2019; ROCHA et al., 2007) and contributing to a higher root input to the soil.

Despite the significant amount of research on forage mixtures' effects on grassland productivity in a wide range of environments, information about the effects of such mixtures on root production and on soil organic matter fractions are still scarce. The hypothesis of this research is that increasing forage stands diversity results in higher forage production and N accumulation by plants due to complementary production among species as well as N fixation and input by the legumes. The increase on forage yield is expected to be reflected in higher root mass, which therefore contributes to C and N additions to form the particulate pool of the soil organic matter in the short-term. Thus, the objectives were to determine the effects of increasing diversity up to six species of cool and warm-season forage mixtures on: i) forage yield and root mass; ii) C and N accumulation by aboveground and roots; and iii) soil labile organic C and N pools.

1.4 MATERIAL AND METHODS

1.4.1 Field experiment

A field experiment was conducted in the Agrarias Campus of the Federal University of Parana, in Curitiba, Brazil (25°24'47" S and 49°14'59" W, 916 m altitude). The climate was classified as humid subtropical, Cfb (Köppen) (ALVARES et al., 2013). The mean air temperature in the coldest month (July) was 12.8 °C and in the hottest (February) was 20.4 °C, while mean annual precipitation was 1480 mm (WREGE et al., 2012). Soil was classified as Cambissolo Haplico, according to the Brazilian soil classification (SANTOS et al., 2018b) or a Cambisol, according to international classification (WRB, 2015). Soil characteristics by the time the experiment was established in 2020 are presented in table 1.

Table 1. Some chemical and physical characteristics of the Cambisol of this study, by the establishment of the experiment. Curitiba, Brazil.

Layer	pH	K	Ca	Mg	Al	H+Al	P	SOM
cm		------------------------------cmol$_c$ dm^{-3}----------------------------------					mg dm^{-3}	g dm^{-3}
0-5	5.4±0.1	1.0±0.2	14.1±2.1	15.0±1.2	0.0	6.7±0.6	67.1±11.6	41.4±4.3
5-10	5.6±0.1	0.7±0.1	9.8±1.4	12.8±1.1	0.0	6.5±0.5	49.5±10.0	39.0±1.9
10-20	5.7±0.1	0.6±0.1	9.1±2.0	12.4±1.4	0.0	6.0±0.4	52.2±8.3	40.1±2.0
20-30	5.7±0.1	0.4±0.1	6.5±0.3	9.5±1.7	0.0	5.2±0.4	33.3±7.6	29.9±1.7
	Clay	Silt	Sand	Bulk density	Total porosity	Macroporosity	Microporosity	
	----------------%----------------			kg dm^{-3}	----------------------m^3 m^{-3}------------------------			
0-5	62.5	23.8	13.8	0.96±0.03	0.64±0.01	0.46±0.02	0.18±0.03	
5-10	66.3	20.0	13.8	0.98±0.03	0.68±0.01	0.54±0.02	0.14±0.03	
10-20	63.8	23.8	12.5	1.02±0.02	0.65±0.01	0.51±0.01	0.14±0.01	
20-30	68.8	16.3	15.0	1.03±0.02	0.63±0.01	0.46±0.01	0.17±0.01	

LEGEND: pH: CaCl$_2$ (0.01 mol L^{-1}) at a rate 1:2.5 (soil:solution); Ca^{+2}, Mg^{2+} and Al^{+3} extracted with KCl (1 mol L^{-1}); K and P extracted with Mehlich-1; H+Al extracted with SMP; SOM determined by Walkey-Black method; Particle size determined by Bouyoucos density method. Values are an average of 4 points ± SE; for particle size, a composite sample of 4 points per layer was analyzed.

SOURCE: The author

The experiment was established during the warm-season of 2019/2020 (November to April), in an area that had been previously under bermudagrass (*Cynodon dactylon* L. Pers.) over the past 4 years. The bermudagrass was mowed in September 2019, when aboveground biomass was removed from the area, and terminated with a first herbicide application after 30 days of regrowth (glyphosate, 2.0 kg a.i. ha^{-1}) and a second herbicide application two months afterwards (glyphosate, 1.8 kg a.i. ha^{-1}; plus metsulfuron methyl, 2.5 g a.i. ha^{-1}). Lime (RTNP of 70%) was applied on January 10th, 2020, at a rate of 4 Mg ha^{-1} and incorporated to 20 cm depth.

Treatments consisted of nine forage mixtures of increasing diversity levels arranged in a randomized block design and four replications. Mixtures were established by the combination of up to three warm-season species and up to three cool-season species. The warm-season forage species were bahiagrass (*Paspalum notatum* Flueggé cv. Pensacola), forage peanut (*Arachis pintoi* Krapov. & W.C. Greg, cv Amarillo) and limpograss (*Hemarthria altissima* [Poir.] Stapf & C.E. Hubbard. Cv Florida), while the cool-season species were black oat (*Avena strigosa* Schereb., cv Embrapa 139), white clover (*Trifolium repens* L. cv Zapican) and annual ryegrass (*Lolium multiflorum* Lam. cv Ponteio). The simplest combination, used as control, was bahiagrass in warm-season succeeded by black oat in cool-season; and increasing diversity levels to that combination were obtained by gradually including the other four species, as shown in Table 2.

Table 2. Forage mixtures of increasing diversity levels as obtained from the combination of warm- and cool-season species.

Warm-season	Cool-season	Number of species
Bahiagrass	Oat	2
Bahiagrass	Oat - Clover	3
Bahiagrass	Oat - Clover - Ryegrass	4
Bahiagrass - Forage peanut	Oat	3
Bahiagrass - Forage peanut	Oat - Clover	4
Bahiagrass - Forage peanut	Oat - Clover - Ryegrass	5
Bahiagrass - Forage peanut - Limpograss	Oat	4
Bahiagrass - Forage peanut - Limpograss	Oat - Clover	5
Bahiagrass - Forage peanut - Limpograss	Oat - Clover - Ryegrass	6

SOURCE: The author

The different species were chosen based on their complementary characteristics, such as temporal, spatial and functional traits. First, bahiagrass and oats, both grasses, were chosen as a control treatment due its lack of complementarity in the same season. Secondly, forage peanut and white clover, were chosen by their different functional characteristics, such as the ability to biologically fix N, increasing crude protein in the forage, and by its root system characterized by shallower thicker roots compared to only grasses. And thirdly, limpograss and ryegrass were chosen to provide an extended forage supply within each season of growth (temporal complementarity). Both grasses are known to have an extended growth cycle, producing additional forage when other grasses, such as bahiagrass and oats, are declining its production.

Bahiagrass was sown on February 8th, 2020, at a rate of 60 kg of seed ha^{-1} after basal fertilization with 25 kg ha^{-1} of N, 50 kg ha^{-1} of P_2O_5 and 50 kg ha^{-1} of K_2O. Seeds were manually broadcast and incorporated into 2 cm depth in the entire pasture area. Plots of 2 × 2 m were then established and arranged in blocks. Seedlings of forage peanut, or forage peanut plus limpograss (depending on the treatment), were planted in rows (20 cm between rows; and 20 cm between seedlings, which were interspersed in the case of peanut plus limpograss). Plots were irrigated twice a week until the full emergence of bahiagrass. Additional seedlings of forage peanut and limpograss were planted when necessary. On March 14th, N was applied to all plots at a rate of 50 kg N ha^{-1}. Due to high variability in the stand, this first season was not evaluated, and the forage was mowed at 5 cm height in late March and again in early May 2020. Table 3 shows the timeline of the experiment with dates of each management applied.

Table 3. Timeline of the main management practices during establishment and conduction of the experiment.

Management	Implementation 2020*	Cool-season 1 2020	Warm-season 1 2020/21	Cool-season 2 2021	Warm-season 2 2021/22
Planting/ replanting	2/08	5/11	10/27	5/4	11/3
N application	3/14	6/23	1/8	7/6	12/15
Aboveground cuts	3/25, 5/8	6/23, 7/3, 7/10, 7/23, 7/30, 8/5, 8/13, 8/27, 9/7, 9/17, 10/1, 10/27	1/8, 1/21, 2/22, 3/18, 4/9, 4/27	7/6, 8/2, 8/15, 8/30, 9/13, 9/25, 10/21	12/15, 1/5, 1/22, 2/10, 2/25, 3/15, 4/14, 5/12
Root sampling	-	10/23	4/29	10/22	5/10

LEGEND: *No evaluations were performed during the implementation phase, aboveground cuts were for homogenization and to allow a better establishment.

SOURCE: The author.

Cool-season species were sown on May 11th, 2020, in between the rows of the previous season. Basal fertilizer was broadcasted in all plots at a rate of 25 kg N ha^{-1}, 92 kg P_2O_5 ha^{-1} and 37 kg K_2O ha^{-1}. Rows with 2 cm depth and spaced in 20 cm were drawn in the plots with a row planter. Seeds were manually deposited in each row, using seeding rates of 80 kg ha^{-1} for black oats, 5 kg ha^{-1} for white clover and 50 kg ha^{-1} for ryegrass. White clover, or white clover plus ryegrass, were sown simultaneously to oat, in the same row. Plots were irrigated twice a week, when necessary, until full emergence. Sidedress N was applied 43 days after sowing, at a rate of 50 kg N ha^{-1}. At the end of the cool-season growth cycle, 170 days after sowing (by October 27th), plants were mowed to 5 cm height in order to allow the regrowth of the warm-season species established the year before. Whenever necessary, bahiagrass was resown and forage peanut and limpograss were replanted. Fertilizer was broadcasted at a rate of 25 kg N ha^{-1}, 104 kg P_2O_5 ha^{-1} and 68 kg K_2O ha^{-1}, on October 27th, 2020. A sidedress N application occurred in all plots on January 8th, 2021, at 50 kg N ha^{-1}. Total N fertilization in each season (basal + sidedress) was 75 kg ha^{-1}, which was used as half of N rate recommendation (150 kg N ha^{-1}/season). This management choice was to not compromise N fixation by the legumes, which can be reduced when N application exceeds 120 kg ha^{-1} for white clover (ENRIQUEZ-HIDALGO et al., 2016) and over 80 kg ha^{-1} for forage peanut (ARYAL et al., 2021).

The second cool-season was established at the same day of warm-season species mowing to 5 cm height (on April 27th, 2021). Seeds of black oats, ryegrass and clover were drilled according to the same method adopted in the previous year and described above. Basal fertilizer was broadcasted at 25 kg N ha^{-1}, 104 kg P_2O_5 ha^{-1} and 68 kg K_2O ha^{-1}, while sidedress N was applied 70 days later (July 6th, 2021), at a rate of 50 kg N ha^{-1}. Plants were mowed to 5 cm height at the end of the cool-season (Nov 3rd, 2021), and then the second warm-season started. Seedlings of forage peanut and limpograss were planted where needed for a homogeneous stand, followed by a broadcast fertilization of 25 kg N ha^{-1}, 104 kg P_2O_5 ha^{-1} and 68 kg K_2O ha^{-1}. A sidedress N application was performed 42 days after (Dec 15th, 2021) at a rate of 50 kg N ha^{-1}.

1.4.2 Aboveground evaluations

Forage biomass was evaluated in two square spots (0.5 m × 0.5 m) per plot (Table 3 for clipping dates). Warm-season species were cut to 10 cm, when reached

20 cm. According to TEJERA et al. (2015), this cutting height maximizes the forage allowance increasing the number of grazing cycles in *Paspalum* spp stands. Cool-season species were cut to 20 cm, when reached 30 cm, representing a moderate removal intensity of oats/ryegrass pastures in the region (CARVALHO et al., 2010). The remaining area of the plot was mowed at 10 and 20 cm, according to the respective season, and the forage biomass was removed from the plots. In addition, by the end of each growing season, plants for biomass evaluation were clipped at 5 cm and the remaining plants of the plot were mowed at the same 5 cm height and removed to allow regrowth during upcoming season. The clipped biomass was oven-dried at 60ºC and weighted to determine the aboveground biomass yield. By summing the yields of all clipping events of the season, we obtained cumulative aboveground biomass yield.

1.4.3 Root evaluations

Soil cores were sampled at the end of each season (Table 3) to determine root biomass. Samples from the 0-5, 5-10, 10-20 and 20-30 cm layers were taken from two points per plot with a soil corer of 8 cm diameter and 10 cm height (SCHUURMAN and GOEDEWAAGEN, 1971). For each layer, collected core samples of the two sampling points were frozen (– 4ºC) and stored together as a composite sample. At the time of processing, core samples were thawed for 12h, gently broken and washed with water jets on a 1.0 mm mesh sieve mounted on top of a 0.5 mm mesh sieve to retain the roots. Roots retained on the sieves were washed and, after debris removal with tweezers, transferred to a 70% ethanol solution. After all samples were washed, roots were filtered from the ethanol solution and oven-dried at 45ºC to determine the root biomass concentration in each layer. The total root biomass to 30 cm depth by the end of each season was then calculated.

1.4.4 C and N concentration of aboveground and root tissue

After weighted, aboveground and root samples were ground in a mortar and sieved to <250 µm. Then a subsample (~15 mg) was weighted and analyzed by dry combustion (Vario EL III Elementar®) to determine C and N concentrations. Before grinding, root samples across layers were combined to analyze the average concentrations in the soil profile. For aboveground samples, three sampling periods per season/year (early, mid and late season) were selected for C and N analysis.

1.4.5 Soil resistance to penetration

During the second cool-season (September 2021) and second warm-season (May 2022), soil resistance to root penetration (RP) was measured to 40 cm depth in three points per plot using a manual penetrometer (Falker®, PLG 1020 model) equipped with 30° angle cone and 12.83 mm diameter. Results were obtained for each centimeter depth, but were averaged to 0-5, 5-10, 10-20, 20-30 and 30-40 cm layers and expressed as kPa.

1.4.6 Soil particulate organic matter

Undisturbed soil samples were taken at two points per plot with a soil corer with 8 cm of internal diameter at 0-5 and 5-10 cm layer on March 20th, 2022, to determine particulate organic matter (POM). Samples were air-dried and sieved to <4.0 mm and a ¼ sub-sample was selected to the physical particle-size fraction method for separation of POM and mineral associated organic matter (MAOM) (CAMBARDELLA and ELLIOTT, 1993). Firstly, 20 g of dried soil were weighted and added to a 70 mL hexametaphosphate solution (5 g L^{-1}). After 16 hours of dispersion in a horizontal shaker (180 rpm) the suspension was passed through a 53-μm sieve to retain soil particles and organic matter particles greater than silt. Sand + POM was the material retained in the sieved and it was oven-dried at 45°C until constant mass.

Samples of sand+POM were grounded to <250 μm sieve and a subsample of ~35 mg was analyzed by dry combustion (Vario EL III Elementar®) for total C (TC) and total N (TN) concentrations.

1.4.7 Statistical analysis

Data from seasonal dry mass (DM) forage yield, root DM density and their C and N concentrations, from both years, soil resistance to root penetration (RP) from the second year of each season as well as POM, TC and TN at the end of the second year of experiment, were submitted to analysis of normality (Shapiro-Wilk) and homogeneity of variances (Bartlett). When the assumptions were not significant ($p<0.05$) Box-Cox optimum potency transformation was applied. Then, data were submitted to ANOVA and when significant ($p<0.05$) means were compared by Scott-

Knott test ($p<0.05$). The analyses were performed with R software (v.4.0.5) and packages MASS and ExpDes (R CORE TEAM, 2022).

1.5 RESULTS

1.5.1 Weather conditions

During the two years of experiment rain was somewhat well distributed, but with some periods longer than 15 days without rain during both cool-seasons (Figure 1). Overall, rain precipitation was >50% lower than the 30-year average (WREGE et al., 2012) on the months May, July and September 2020 and in February, April, July and September 2021. Total precipitation from May to October was 491 and 538 mm for cool-season 1 and 2, respectively, and from November to April was 830 and 1063 mm for warm-season 1 and 2, respectively. Mean air temperature followed the 30-year average, with an average of 16.5 ºC for the cool-seasons and 20.0 ºC for the warm-seasons.

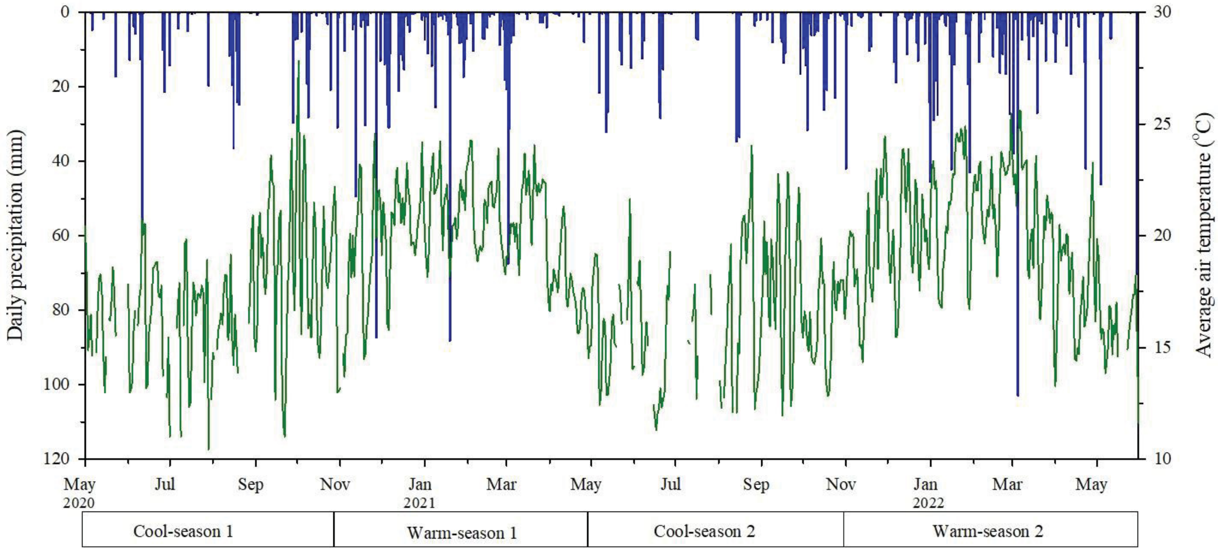

Figure 1. Daily precipitation (vertical bars) and mean daily air temperature (line) during the cool and warm-season through 2020-2022. Curitiba-PR, Brazil. Data obtained from INMET.

SOURCE: The author.

1.5.2 Aboveground forage mass

The cumulative yield of aboveground forage mass increased by 16-84% with increasing species diversity in relation to the control bahiagrass/oats (B/O), regardless of the season and year ($p<0.05$) (Figure 2). In general, higher species diversity within the season resulted in higher yields than diversity across seasons. For example, in both cool-seasons, adding clover (C) or clover+ryegrass (C-R) to oat increased yield by 9 to 14%, while adding forage peanut (P) or forage peanut+limpograss (P-L) as warm-season species increase yields by only 4 to 7% during the cool-season. In both warm-seasons, a similar pattern was found, with increases of 15 to 84% with P or P-L, while increasing diversity with cool-season species (O-C or O-C-R) led to changes of -8% to 3% during the warm-season, in relation to B/O.

For the two cool-seasons, O-C-R mixture increased yield by ~14% in relation to the single use of black oats ($p<0.05$), regardless of the warm-season mixture (Figure 2). The O-C mixture also resulted in higher yields during cool-season 1, but without significant effects on cool-season 2 compared to single oats, regardless of the warm-season mixture. During the two warm-seasons, the triple mixture (B-P-L) increased yield by 84% (year 1) and 30% (year 2) compared to the single bahiagrass, regardless of the cool-season mixture. In general, the summer mixture of bahiagrass and forage peanut did not affect forage yield relative to single bahiagrass, except in the warm-season 2 and treatment B-P/O and B-P/O-C, which increased yields by 20-24% compared to B/O.

The cumulative forage yields varied among seasons and years (Figure 2). In the first cool-season, yields ranged from 6.3 to 7.9 Mg ha^{-1} and were lower in in the second cool-season, 4.5 to 5.5 Mg ha^{-1}. For the warm-season a different pattern was observed, with lower yields in the first season, ranging from 1.7 to 3.9 Mg ha^{-1}, but reaching 6.5 to 8.9 Mg ha^{-1} in the second warm-season.

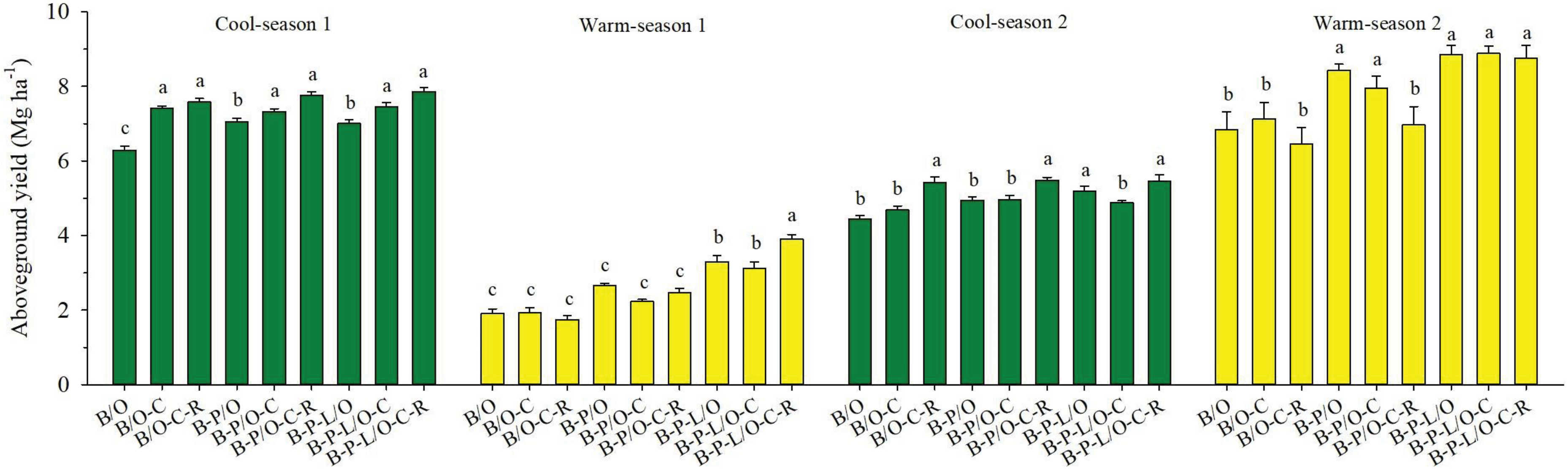

Figure 2. Cumulative aboveground biomass yield (Mg DM ha^{-1}) of forage mixtures with increasing diversity levels in cool and warm seasons over two years in subtropical Brazil. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters for each season/year represents statistical difference among treatments according to Scott-Knott test ($p<0.05$). Vertical lines represent the SEM (n=4).

SOURCE: The author.

The forage accumulation rates of aboveground biomass in the cool-season 1 peaked 80 kg DM ha^{-1} day^{-1} on mid-July and decreased to 40 kg DM ha^{-1} day^{-1} on mid-August, and so remained until the end of the season in November; while in cool-season 2 it peaked 60 kg DM ha^{-1} day^{-1} in September and a rapidly decreased to 30-40 kg DM ha^{-1} day^{-1}, remained constant until November (Figure 3). In general, forage accumulation rates throughout the season were similar across treatments, but during the peak the more diverse stands (i.e. 4 to 6 species) presented higher rates. The less diverse treatment (B/O) presented the lowest forage accumulation rates during the final sampling periods in both years.

During the warm-season 1, low forage accumulation rates were found in all treatments, with a peak of 38 kg DM ha^{-1} day^{-1} in mid-January and another peak in April (Figure 3). For warm-season 2, higher forage accumulation rates were observed, and peaks of 65 kg DM ha^{-1} day^{-1} occurred in mid-February and mid-March. For both years, the stands containing the triple combination of warm-season species presented the highest forage accumulation rates, mainly at the end of the season when less diverse stands decreased it to 15-20 kg DM ha^{-1} day^{-1} while B-P-L remained above 20 kg DM ha^{-1} day^{-1}.

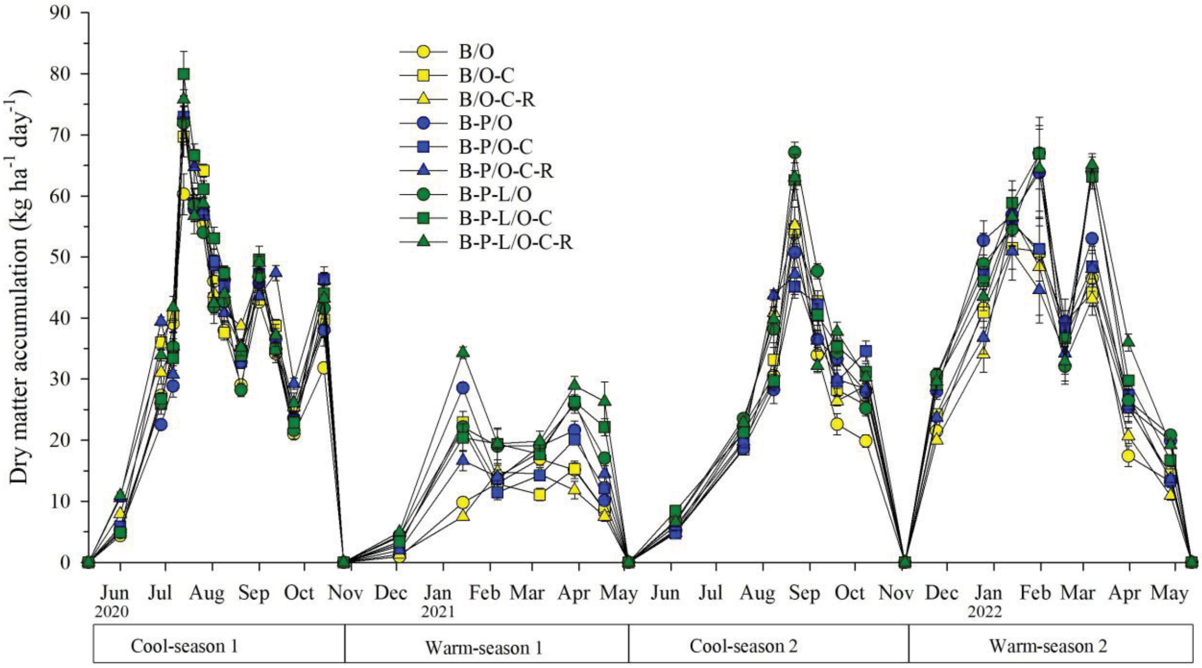

Figure 3. Accumulation rate of aboveground biomass (kg DM ha^{-1} day^{-1}) of forage mixtures of increasing diversity levels in cool and warm seasons over two years in subtropical Brazil. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Vertical lines for each symbol represent the SEM (n=4).

SOURCE: The author.

1.5.3 Root density

Root density ranged from 1.0 to 1.8 g kg^{-1} in the 0-5 cm layer and decreased to 0.2 to 0.4 g kg^{-1} in the deeper layer 20-30 cm (Figure 4). Root density in cool and warm seasons of both years were affected in some depths by the increase in forage species diversity (*p*<0.05). During cool-seasons, at 5-10 cm in the first year and at 5-10 and 10-20 cm in the second, treatments with a mix of either O-C or O-C-R increased root density compared to single oats, regardless of warm-season mixtures. In both cool-seasons, treatment B/O resulted in the lowest root densities at the 5-10 cm layer. During warm-season 1, at 0-5 and 5-10 cm, generally treatments with 4 species or more had a higher root density, regardless of the seasonal combination of species. For the warm-season 2, however, root density was only affected at 5-10 cm layer, in which B/O presented the lowest root density.

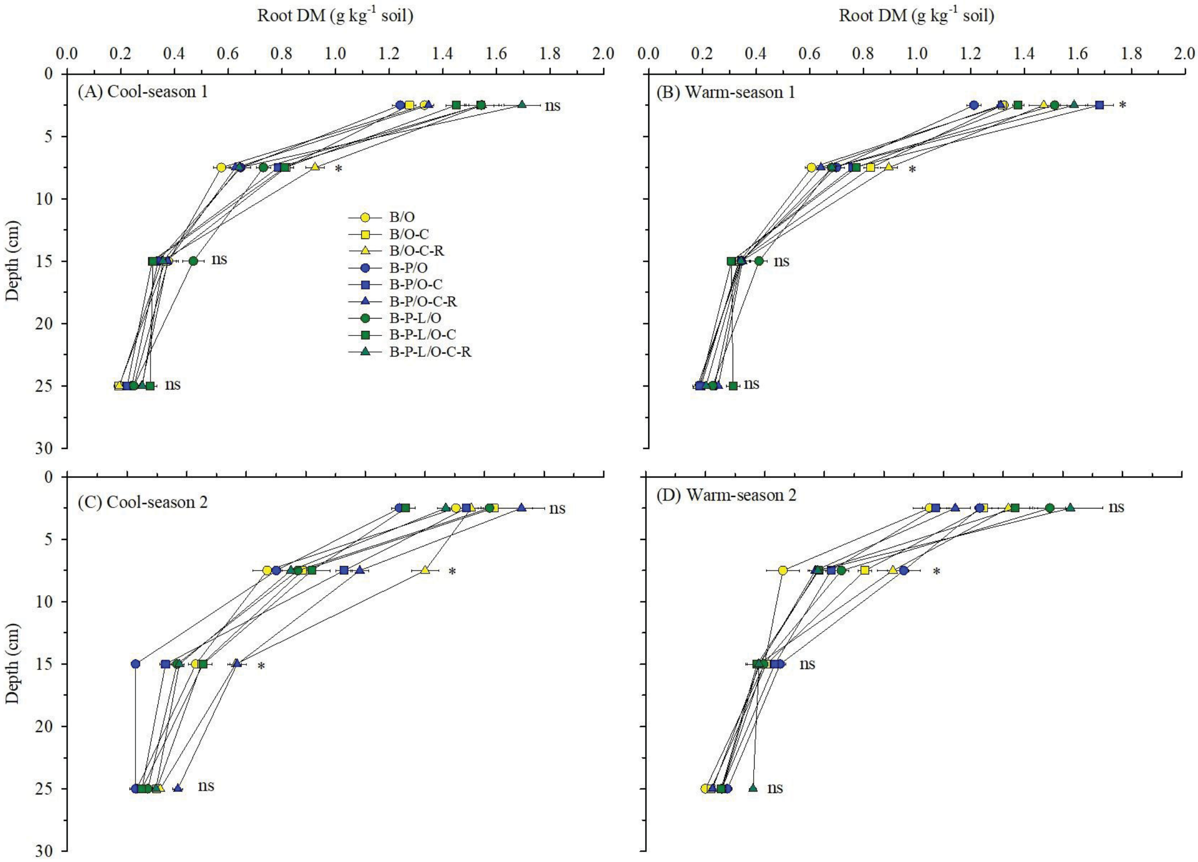

Figure 4. Root dry mass concentration (g dm^{-3} soil) of forage mixtures of increasing diversity levels in cool and warm seasons over two years in subtropical Brazil: cool-season 1 (A), warm-season 1 (B), cool-season 2 (C) and warm-season 2 (D) in subtropical Brazil. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). * for each soil layer for season/year represents statistical difference among treatments according to Scott-Knott test (*p*<0.05), ns= not significant. Horizontal lines for each symbol represent the SEM (n=4).

SOURCE: The author.

1.5.4 Total C and N concentration in aboveground and root biomass

In general, aboveground (Table 4) and root (Table 6) C concentrations were not affected by forage diversity (*p*>0.05). Aboveground C concentration ranged from 395 to 429 g kg^{-1} and from 363 to 425 g kg^{-1} in roots. Nitrogen concentration, however, was affected by treatments in two seasons, for aboveground biomass (Table 5) and roots (Table 7) (*p*<0.05). In general, N concentration in aboveground and roots was higher in treatments with presence of legumes forage peanut in warm-season 1 and clover in the cool-season 2. However, higher levels of species diversity, for example with more than three grasses, tended to reduce N concentration in aboveground and root biomass. Aboveground N concentration ranged between 24 to 41 g kg^{-1} and 7 to 20 g kg^{-1} in roots.

Table 4. Total carbon concentration (g kg^{-1}, means±SE, n=4) in the aboveground biomass of forage mixtures of increasing diversity levels in cool and warm seasons.

Treatment	Cool-season 1	Warm-season 1	Cool-season 2	Warm-season 2
B/O	395.3±2.2 ^{ns}	418.0±2.4$^{n\ s}$	406.9±2.9 ^{ns}	425.6±3.7 ^{ns}
B/O-C	398.7±1.8	415.8±2.9	410.7±2.0	429.2±4.4
B/O-C-R	400.1±2.6	416.2±2.2	405.4±2.5	414.4±1.6
B-P/O	396.5±1.7	416.8±1.6	410.8±3.3	426.0±2.3
B-P/O-C	397.4±2.5	422.6±1.0	409.0±2.0	413.5±3.0
B-P/O-C-R	396.7±2.1	419.4±1.7	412.8±1.5	427.2±2.4
B-P-L/O	399.5±2.1	427.3±2.4	412.1±1.7	424.4±2.4
B-P-L/O-C	393.6±2.7	415.5±1.9	408.6±3.6	422.4±2.4
B-P-L/O-C-R	398.6±2.1	429.0±5.1	407.1±4.0	425.8±3.2

LEGEND: Concentrations are the average of three samplings: early, mid and late season. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters in the column indicates significant statistical difference among treatments according to Scott-Knott test (*p*<0.05), ns= not significant.

SOURCE: The author.

Table 5. Total nitrogen concentration (g kg^{-1}, means±SE, n=4) in the aboveground biomass of forage mixtures of increasing diversity levels in cool and warm seasons.

Treatment	Cool-season 1	Warm-season 1	Cool-season 2	Warm-season 2
B/O	38.6±2.2 ^{ns}	33.6±2.7 a	33.0±0.9 b	25.5±1.8 ^{ns}
B/O-C	43.8±1.0	33.5±0.6 a	36.4±1.9 a	27.6±3.3
B/O-C-R	37.4±1.0	28.8±0.6 b	31.2±0.9 c	29.0±3.4
B-P/O	40.8±1.5	35.9±0.7 a	33.0±1.7 b	25.3±0.9
B-P/O-C	41.3±1.4	36.2±0.8 a	33.9±1.6 b	28.0±3.2
B-P/O-C-R	39.1±1.8	34.5±0.2 a	33.7±1.3 b	30.1±2.5
B-P-L/O	39.0±1.9	29.8±0.9 b	31.7±0.9 c	24.7±0.9
B-P-L/O-C	39.5±1.5	28.1±1.0 b	33.8±1.9 b	27.4±2.4
B-P-L/O-C-R	37.3±1.3	29.4±0.9 b	31.8±1.0 c	27.1±2.9

LEGEND: Concentrations are the average of three samplings: early, mid and late season. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters in the column indicates significant statistical difference among treatments according to Scott-Knott test (*p*<0.05), ns= not significant.

SOURCE: The author.

Table 6. Total carbon concentration (g kg^{-1}, means±SE, n=4) in the root biomass of forage mixtures of increasing diversity levels in cool and warm seasons.

Cool-season 1	Cool-season 1	Warm-season 1	Cool-season 2	Warm-season 2
B/O	371.3±8.3 ^{ns}	395.2±2.3 a	394.6±18.2 ^{ns}	395.7±8.2 ^{ns}
B/O-C	369.8±2.6	401.9±3.0 a	388.1±8.2	406.8±6.1
B/O-C-R	365.3±5.3	392.1±3.8 a	388.9±1.4	395.3±8.1
B-P/O	3681±11.4	377.7±3.1 b	389.7±6.9	407.1±13.9
B-P/O-C	363.2±8.2	384.1±8.1 b	374.4±4.2	425.5±4.0
B-P/O-C-R	366.1±4.6	378.7±3.4 b	380.3±7.6	390.9±19.5
B-P-L/O	376.0±10.1	396.9±1.5 a	384.2±7.3	412.8±2.5
B-P-L/O-C	366.7±3.1	393.4±0.9 a	371.3±9.9	399.3±4.5
B-P-L/O-C-R	372.4±6.5	398.8±3.4 a	384.7±3.6	388.7±6.9

LEGEND: Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters in the column indicates significant statistical difference among treatments according to Scott-Knott test (*p*<0.05), ns= not significant.

SOURCE: The author.

Table 7. Total nitrogen concentration (g kg^{-1}, means±SE, n=4) in the root biomass of forage mixtures of increasing diversity levels in cool and warm seasons.

Cool-season 1	Cool-season 1	Warm-season 1	Cool-season 2	Warm-season 2
B/O	13.6±0.5 ^{ns}	16.2±0.1 c	8.9±0.2 b	13.0±0.7 ^{ns}
B/O-C	12.6±0.3	17.9±0.4 b	9.9±0.6 a	14.9±1.2
B/O-C-R	13.3±0.7	16.5±0.4 c	7.5±0.4 b	14.8±1.9
B-P/O	13.9±0.4	19.1±1.0 b	8.6±0.2 b	12.4±0.3
B-P/O-C	14.3±0.6	18.4±0.3 b	9.8±1.1 a	14.8±1.9
B-P/O-C-R	15.4±1.3	20.5±0.2 a	8.0±0.6 b	15.6±1.7
B-P-L/O	14.0±0.7	17.5±0.4 c	7.7±0.1 b	12.4±0.6
B-P-L/O-C	12.7±0.4	18.2±0.2 b	9.0±0.4 a	14.7±1.7
B-P-L/O-C-R	13.0±0.6	17.0±0.4 c	9.6±0.4 a	13.9±1.1

LEGEND: Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters in the column indicates significant statistical difference among treatments according to Scott-Knott test (*p*<0.05), ns= not significant.

SOURCE: The author.

1.5.5 Carbon and nitrogen accumulation

Carbon and N accumulation in forage aboveground mass (Figure 5) followed a similar pattern to the forage mass production (Figure 1) across the seasons evaluated. A wide variation among seasons was observed, with higher C and N accumulation in aboveground mass in cool-season 1 and warm-season 2, ranging from 2,500 to 3,800 kg C ha^{-1} and from 170 to 325 kg N ha^{-1}, while in warm-season 1 and cool-season 2, it ranged from 980 to 2,200 kg C ha^{-1} and from 50 to 165 kg N ha^{-1}. During the cool-seasons, C accumulation was 10-15% higher at O-C (cool-season 1 only) and O-C-R mixtures compared to oats only, regardless of the warm-season mixture (*p*<0.05). Nitrogen accumulation, however, was not affected by treatments in both cool-seasons, ranging from 244-323 kg N ha^{-1} and 147-185 kg N ha^{-1} in the respective seasons. In the warm-seasons, treatments with B-P-L resulted in 30-88% higher C accumulation and 30-63% higher nitrogen accumulation compared to bahiagrass alone, regardless of cool-season mixture. The treatment with bahiagrass mixed with forage peanut presented intermediate values for N accumulation, still higher than bahiagrass alone.

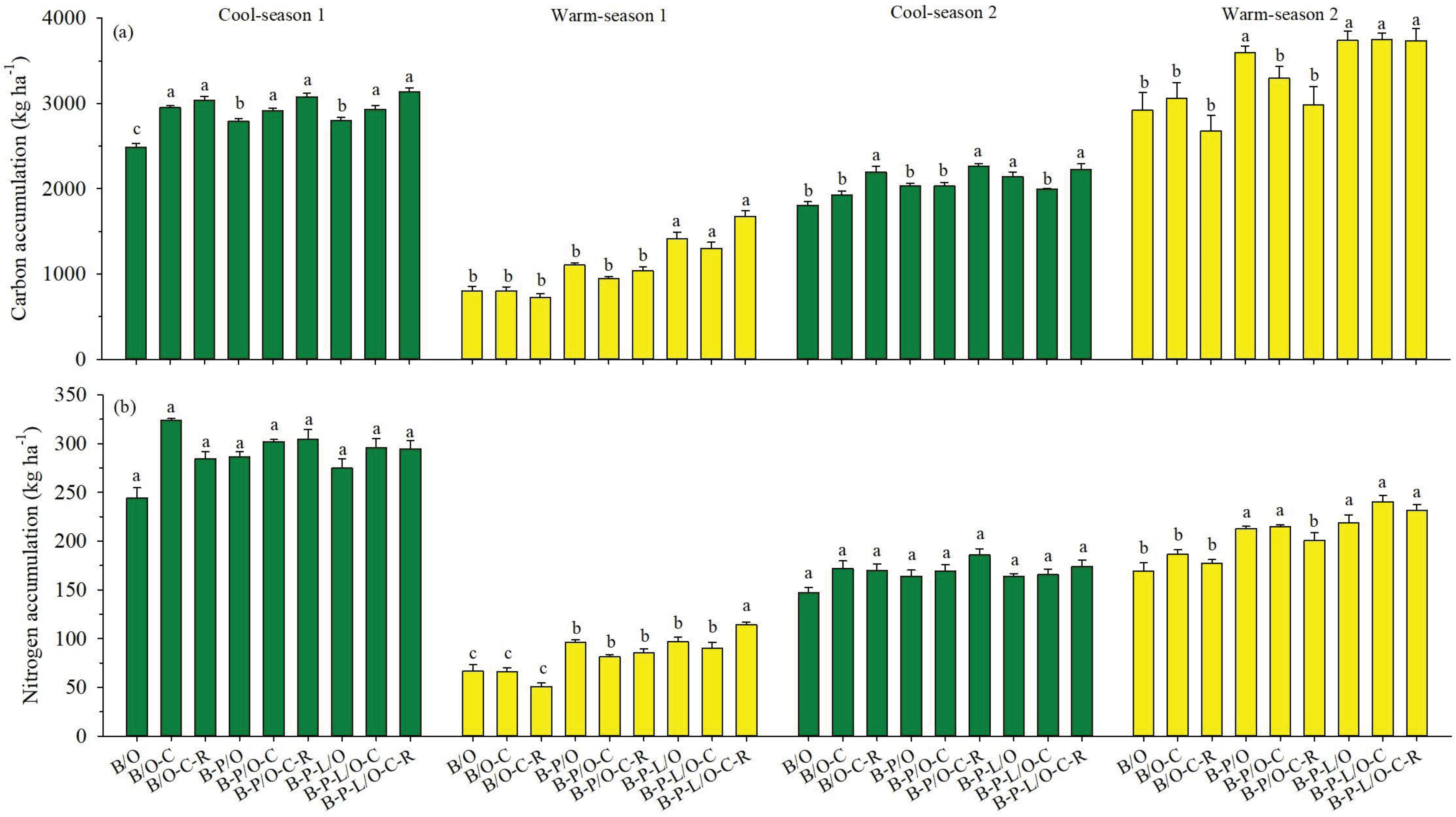

Figure 5. Aboveground carbon (a) and nitrogen (b) accumulation (kg ha^{-1}) of forage mixtures with increasing diversity levels in cool and warm seasons over two years in subtropical Brazil. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters for each season/year represents statistical difference among treatments according to Scott-Knott test ($p<0.05$). Vertical lines represent the SEM (n=4).

SOURCE: The author

Carbon and N accumulation in root biomass to 30 cm depth (Figure 6) was constant among seasons and less affected by treatments than aboveground accumulation. Carbon accumulation (Figure 6 a) in roots ranged from 500-800 kg C ha^{-1}, and N accumulation (Figure 6 b) ranged from 15-30 kg N ha^{-1}. Carbon accumulation was mainly affected on the 5-10 cm layer where treatment with higher diversity had higher root C accumulation ($p<0.05$), although not always consistent among seasons. Nitrogen accumulation in roots was affected at different layers in each season, but clearly the less diverse treatment (B/O) always presented the lowest values ($p<0.05$). Those differences resulted in higher N accumulation by roots from diverse mixtures in the whole 30 cm in the last 3 season of evaluation.

Despite the seasonal and yearly variations, C and N followed a clear pattern on average annual accumulation (Figure 7), in which the higher level of species diversity led to higher total C and N accumulation in aboveground and root mass. After 2 years of cultivation of cool and warm-season forage mixtures, the triple warm-season mixture resulted in the highest C accumulation in aboveground with 5,140±184 kg C ha^{-1}, regardless of cool-season mixtures, while in the less diverse treatment (B/O) C accumulation was 4,009±246 kg C ha^{-1} ($p<0.05$) (Figure 7 a). Other combinations with 3 to 5 species, regardless of the season, accumulated in aboveground mass an average of 4,545±148 kg C ha^{-1}, and were also higher than B/O. For the annual average N accumulation, all mixtures, except B/O-C-R, increased in relation to B/O ($p<0.05$) (Figure 7 b). In aboveground mass, the mixtures were similar (except B/O-C-R) and averaged 432±7 kg N ha^{-1}, 23% higher than B/O (351±17 kg N ha^{-1}).

The increase in species diversity level to 3 to 6 species also increased the average C (Figure 7 a) and N (Figure 7 b) accumulation in root mass at 0-30 cm depth, which were 13% higher for C (average of 1,323±37 kg C ha^{-1}) and 21% higher for N (average of 46±1.6 kg N ha^{-1}), compared to B/O ($p<0.05$). In both cases an exception occurred in B-P/O, that had a similar C and N accumulation to B/O, otherwise all mixtures were similar.

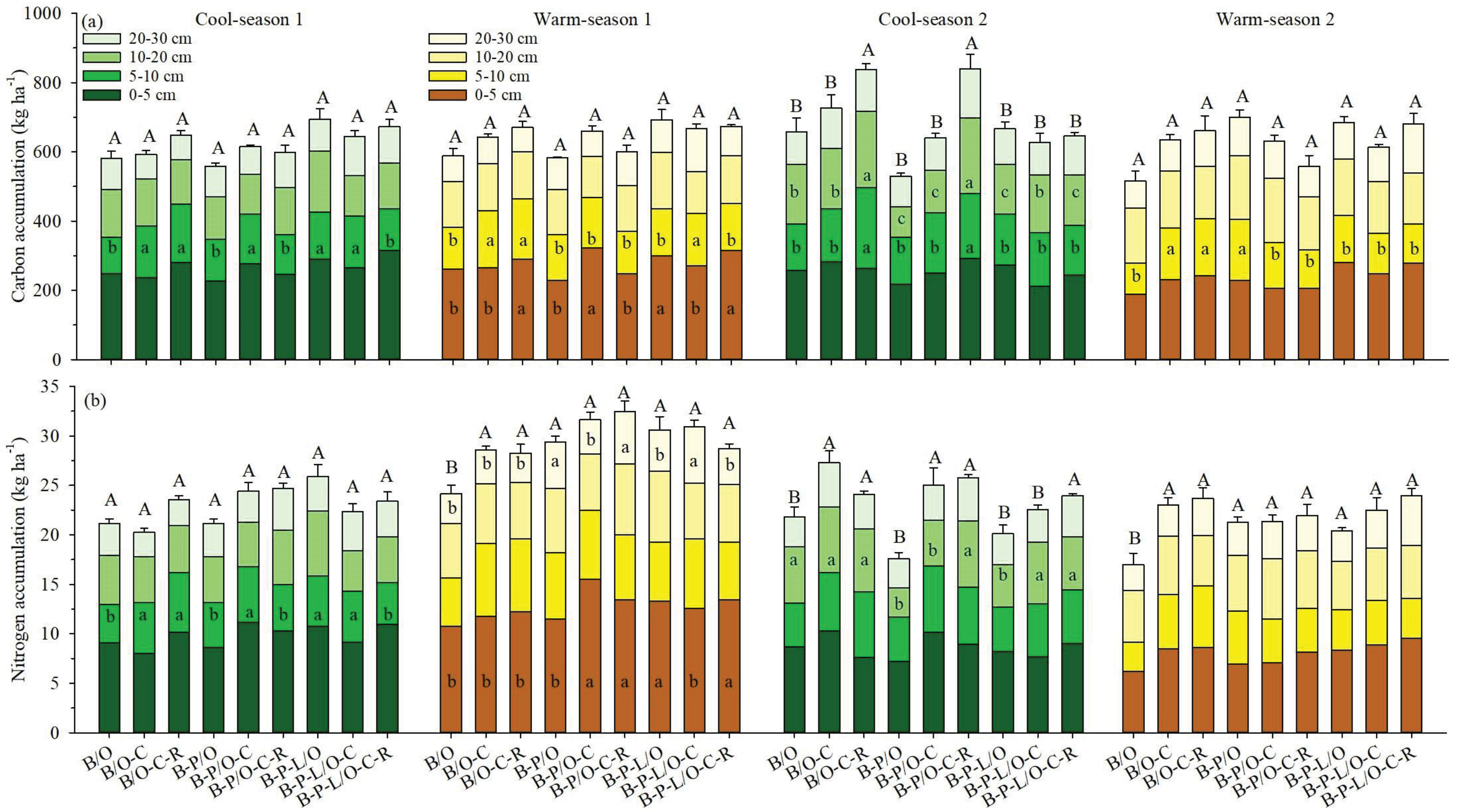

Figure 6. Carbon (a) and nitrogen (b) accumulation (kg ha^{-1}) in roots at 0-30 cm depth of forage mixtures with increasing diversity levels in cool and warm seasons over two years in subtropical Brazil. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters for each season/year represents statistical difference among treatments according to Scott-Knott test ($p<0.05$). The absence of letters for some layer/seasons represents no significant difference according to Scott-Knott test ($p<0.05$). Uppercase letters represent the statistical significance of the cumulative C and N accumulation in the whole 0-30 cm depth. Error bars represent the SEM of the cumulative 0-30 cm depth (n=4).

SOURCE: The author.

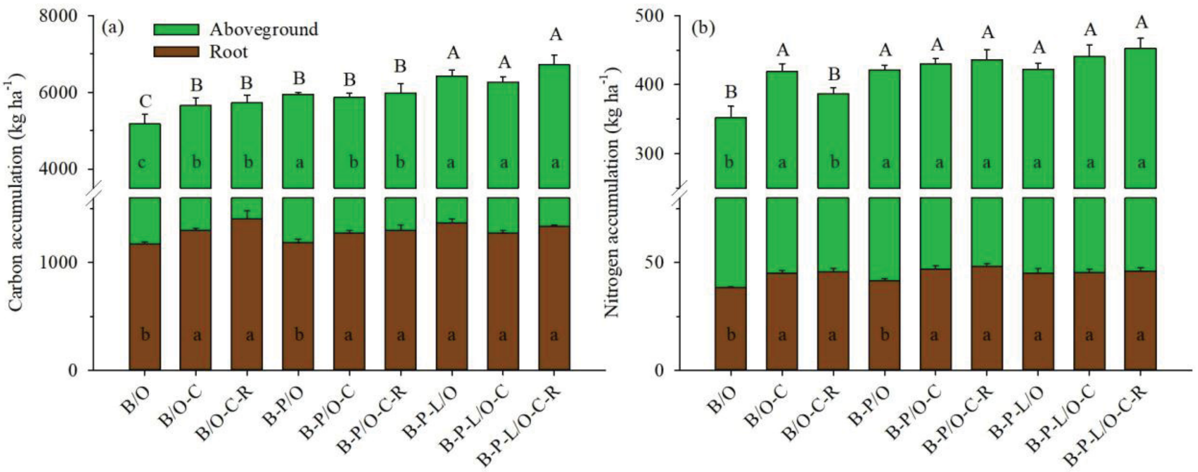

Figure 7. Carbon (a) and nitrogen (b) accumulation (kg ha^{-1} year) in aboveground and roots (0-30 cm depth) of forage mixtures with increasing diversity levels in cool and warm seasons over two years in subtropical Brazil. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters for each season/year represents statistical difference among treatments according to Scott-Knott test ($p<0.05$). Uppercase letters represent the statistical significance of the cumulative C and N accumulation (roots=aboveground). Vertical lines represent the SEM (n=4).

SOURCE: The author

1.5.6 Particulate organic matter

The mass of the soil fraction POM+sand after two years of experiment conduction was not affected ($p>0.05$) by the level of forage species diversity and was in average 16.6±0.2% of the whole soil at 0-5 cm layer and 13.8±0.3% at 5-10 cm layer (Table 8). Carbon concentration in the POM+sand fraction at 0-5 cm was higher under any forage mixture (average of 69.9 g kg^{-1}) compared to the less diverse stand (B/O) (58.4 g kg^{-1} fraction POM+sand) ($p<0.05$) but was not affected in the 5-10 cm layer (average of 50.4±1.3 g kg^{-1} fraction POM+sand). Nitrogen concentration in POM+sand was not affected by the level of forage mixtures and was 4.4±0.1 and 3.1±0.1 g kg^{-1} at 0-5 and 5-10 cm layer, respectively.

The soil POM C:N ration was similar throughout treatments and layers, ranging from 15.0 to 17.4 (Table 8). When C and N concentrations were normalized to the percentage of POM in the whole soil, there was no treatment effect. Carbon concentrations were 11.4 and 6.9 g C kg^{-1} soil, at 0-5 and 5-10 cm respectively, while N concentrations were 0.7 and 0.4 g N kg^{-1} soil, respectively.

Table 8. Total carbon and nitrogen concentration (g kg^{-1}, means±SE, n=4) in particulate organic matter (POM) of soil cultivated with forage mixtures with increasing diversity levels in cool and warm seasons after two years.

Treatment	Layer (cm)	POM+sand (% whole soil)	C (g kg^{-1} fraction)	N (g kg^{-1} fraction)	C:N ratio	C (g kg^{-1} soil)	N (g kg^{-1} soil)
B/O	0-5	17.5±0.8 ^{ns}	58.4±1.5 b	3.7±0.2 ^{ns}	15.8±0.5^{ns}	10.2±0.7 ^{ns}	0.65±0.05^{ns}
	5-10	13.8±0.4 ^{ns}	44.8±2.9 ^{ns}	2.8±0.2 ^{ns}	16.2±0.5^{ns}	6.2±0.5 ^{ns}	0.39±0.04^{ns}
B/O-C	0-5	16.3±0.3	74.3±2.2 a	4.9±0.1	15.3±0.5	12.1±0.3	0.79±0.01
	5-10	13.1±0.6	46.0±1.7	2.9±0.1	16.2±0.4	6.0±0.4	0.38±0.03
B/O-C-R	0-5	16.0±0.7	67.5±2.7 a	4.4±0.3	15.3±0.3	10.9±0.9	0.72±0.07
	5-10	13.5±0.3	51.4±3.2	3.2±0.3	16.4±0.5	6.9±0.5	0.43±0.04
B-P/O	0-5	15.9±0.5	69.8±2.2 a	4.6±0.1	15.2±0.4	11.1±0.4	0.73±0.03
	5-10	14.5±0.9	57.4±2.8	3.6±0.3	16.3±0.8	8.4±0.9	0.53±0.08
B-P/O-C	0-5	16.7±0.4	65.9±1.9 a	4.5±0.2	15.5±0.5	11.6±0.4	0.75±0.05
	5-10	13.5±0.3	53.1±5.3	3.3±0.4	16.0±0.3	7.2±0.8	0.45±0.06
B-P/O-C-R	0-5	17.0±1.0	68.1±2.3 a	4.5±0.2	15.0±0.2	11.5±0.4	0.76±0.02
	5-10	14.0±1.2	49.6±0.8	3.1±0.1	16.3±0.5	7.0±0.7	0.43±0.06
B-P-L/O	0-5	16.4±0.7	71.2±3.6 a	4.5±0.3	15.8±0.6	11.7±1.1	0.75±0.09
	5-10	13.1±0.3	46.8±2.6	2.9±0.2	16.5±0.5	6.1±0.4	0.37±0.03
B-P-L/O-C	0-5	16.1±0.4	70.6±1.6 a	4.5±0.2	15.9±0.6	11.4±0.5	0.73±0.06
	5-10	13.5±0.6	52.3±5.2	3.3±0.4	16.1±0.6	7.1±1.0	0.45±0.08
B-P-L/O-C-R	0-5	17.5±0.9	67.9±1.6 a	4.2±0.2	16.5±0.6	11.9±0.7	0.73±0.07
	5-10	15.1±1.1	52.2±1.9	3.0±0.1	17.4±0.3	7.9±0.6	0.46±0.04

LEGEND: Particulate organic matter was determined by the physical fractioning described by CAMBARDELLA and ELLIOTT (1993). Soil samples were taken at the end of warm-season 2, corresponding to two years of management. Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Different letters in the column indicates significant statistical difference among treatments according to Scott-Knott test (p<0.05), ns= not significant.

SOURCE: The author.

1.5.7 Soil resistance to penetration

In general, soil resistance to penetration (RP) after two cultivation years of cool and of warm-season forage species was not affected by the treatments (Figure 8). However, in the warm-season (Figure 8 B), at 10-20 cm layer, treatments with more diverse mixtures (B-P-L) presented lower soil RP compared to the single use of bahiagrass, regardless of cool-season mixture (p<0.05). In both seasons, RP was lower in the top 10 cm depth, and increased to values above 600 kPa at 10-40 cm depth reaching a maximum of 1700 kPa in the warm-season at 30-40 cm soil layer.

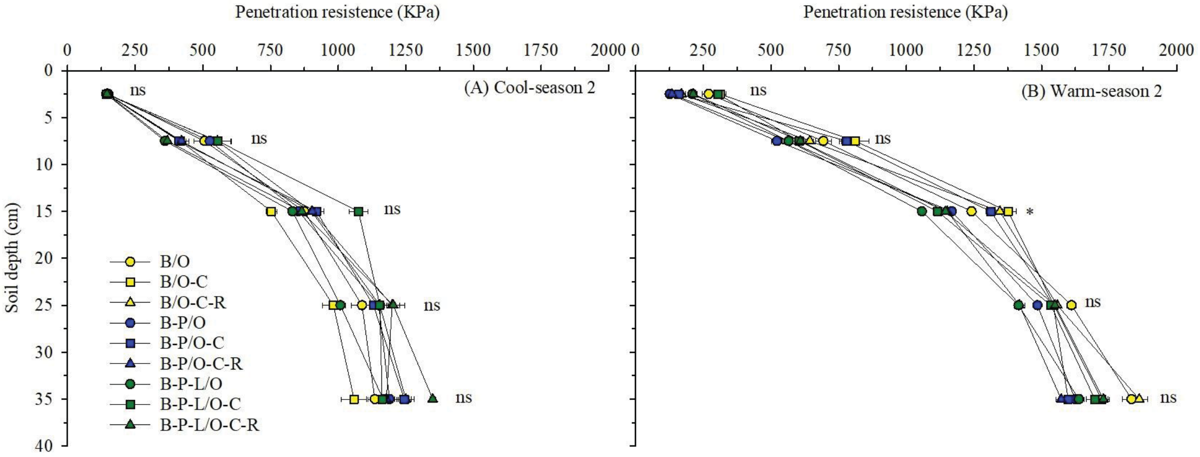

Figure 8. Soil resistance to penetration at the end of cool-season 2021 (a) and at the end of the warm-season 2021/22 (b) under increasing levels of forage species diversity Warm-season species: bahiagrass (B), forage peanut (P), limpograss (L). Cool-season species: black oats (O), white clover (C) annual ryegrass (R). Horizontal lines for each symbol represent the SEM (n=4). *Represents statistical difference among treatments for each soil layer, according to Scott-Knott test ($p<0.05$), ns= not significant.

SOURCE: The author.

1.6 DISCUSSION

1.6.1 Species diversity to increase forage yield

In this study, mixtures of cool and warm-season forage species were evaluated over two years and during their respective growing seasons. The least diverse system tested was a temporal mixture of two grasses cultivated in the same area, but with a different seasonal growth pattern: bahiagrass a perennial species that grows in the warm-season and annual black oats, that grows during the cool-season. The other treatments were composed by the addition of cool- and war-season species into the bahiagrass/oat mixture, one by one, up to a total of six species. Although all treatments are a combination of species from both seasons, the results showed that the diversity of species within each season was more relevant to increase seasonal forage yield and C and N accumulation in aboveground.

The addition of either legume or a legume + grass species increased forage accumulation rates and yield in both cool and warm-seasons, confirming our initial hypothesis. In these mixtures, legumes (white clover and forage peanut) probably increased the supply of N favoring the growth and production of the neighboring grass species (BURCHILL et al., 2014; ENRIQUEZ-HIDALGO et al., 2016), a well-documented effect in literature (HUSSE et al., 2017; LÜSCHER et al., 2014;

PIRHOFER-WALZL et al., 2012). In addition, the mixtures of grass+legume species in our study may have contributed with extra biomass by the complementary of its production in relation to the grass only system (HERNANDEZ and PICON-COCHARD, 2016; HOMEM et al., 2021). The positive effect of including clover or forage peanut into grass stands was also reported in other studies in the subtropical region. OLIVO et al. (2019) found that the addition of forage peanut to annual ryegrass and *Cynodon dactylon* mix increased the yield of the stands mainly in the warm-season. AZEVEDO et al. (2012) also found higher forage yield and better balance on forage allowance throughout the season using either red clover or forage peanut mixed with annual ryegrass and elephant grass.

When ryegrass or limpograss were added in the respective seasons to oats-clover and bahiagrass-forage peanut stands, the responses of forage yield and C and N accumulation were even higher than the addition of legumes only. The more diverse mixtures reflect the effects of complementarity on forage yield, characterized by higher uptake efficiency of available resources due to the asynchrony of growth among species during the growing season (HUSSE et al., 2017; PAREDES et al., 2018). This effect is clear in the seasonal accumulation rates of triple mixtures which were higher than less diverse treatments.

The specific characteristics of forage production and growing patterns of the different grasses are possible factors related to increasing forage yield in our experiment. One of the ryegrass traits is an extended growth cycle compared to oats (PIAZZETTA et al., 2014; ROCHA et al., 2007; SOARES et al., 2019), which provided additional forage late in the cool-season, when oats production started to decline. Ryegrass also produces leaves closer to soil surface than oats, therefore growing faster after grazing/cutting (SOARES et al., 2019). Similarly, limpograss also has temporal and spatial complementarity to bahiagrass, with superior production in mid to late-summer and taller and upright canopy (SOLLENBERGER et al., 1988), contributing to higher proportion in total forage production in the mixture with bahiagrass.

1.6.2 Species diversity increase root inputs but does not affect POM

Increasing the number of forage species from 3 to 6 also increased root mass density and its C and N accumulation throughout the cool and warm seasons, mainly by increased root mass density in the 5-10 cm layer, regardless of the seasonal species combination, compared to the less diverse system of bahiagrass/oats. The increased root mass and C and N accumulation might be related to different root traits between grasses and legumes cultivated in each season. Grasses, particularly oats, ryegrass and limpograss have a greater root length and smaller root diameter, with a deeper fibrous and branched rooting system when compared to legumes, such as clover and forage peanut (GOULD et al., 2016; QUESENBERRY et al., 2004). Differently, bahiagrass is characterized by deep-rooting and by rhizomatous thicker roots (DUBEUX JR et al., 2006). In addition, the soil in this study had no physical constraints to root growth, with regards to resistance to penetration (Figure 8), bulk density, and volume of macropores (Table 1), therefore, roots have suitable conditions for development and elongation (BENGOUGH et al., 2011; WENDEL et al., 2022).

The shallower rooting system of rhizomes of forage peanut and root-like stolons from clover, might lead to a higher density of roots in the topsoil layers, when compared to the single grass mix with bahiagrass rhizomes and oats fibrous roots. Therefore, increasing C and N input in the mixtures containing grasses and legumes. This result is different from a study with binary mixtures of bahiagrass and forage peanut (SANTOS et al., 2018a), in which no differences in root/rhizome mass from the mixtures were found compared to bahiagrass monoculture. According to the authors, the N supply through fertilization was absent in the mixture, while the monoculture received 270-360 kg N ha^{-1} year^{-1}, and this was the main factor to increase root/rhizome mass from bahiagrass, which is very responsive to N fertilization (SANTOS et al., 2018a). In our study however, the N fertilization rate was the same for all treatments (100 kg N ha^{-1}, split in half during cool and warm-season), therefore the additional increases found on root mass by the mixture reflects the complementarity between the two species.

The other mixtures with higher number of grass species also had higher complementarity in the vertical soil exploration, however, did not increase root mass when compared to seasonal binary mixtures of grass-legume. Although ryegrass and limpograss had an expressive fibrous root system, they did not surpass the root production from the bahiagrass, forage peanut, oats and clover mixtures, suggesting that these species combinations are occupying a similar vertical root niche

(HEUERMANN et al., 2019). These results might be related to a change on botanical composition of treatments containing more than 4 species, in which bahiagrass and oats, the dominant canopy species for the respective seasons, had a decreased abundance in the forage stand, giving ground to ryegrass and/or limpograss dominance, yet producing the same amount of roots. In a mixture of oats, clover, mustard and phacelia, HEUERMANN et al. (2019) found that both oats and clover had a reduced root mass in expense of the other species, showing that they can be outcompeted by species with a more aggressive and deeper rooting development. Although in HEUERMANN et al. (2019) study, the mixture also had higher root mass in the profile than the monocultures, similar to what we found in our study. One limitation of our study could be the depth of root evaluation. Although many studies found that most of grassland roots are at the top 30 cm depth (BOLINDER et al., 2002; FAN et al., 2016; SAINJU et al., 2017; WANG et al., 2019), others might suggest possible effects of mixtures bellow that layer (FORNARA and TILMAN, 2008; LI et al., 2019; SKINNER et al., 2006; YANG et al., 2019).

Despite the greater root C and N inputs in mixtures compared to bahiagrass/oats, there was no significant changes in soil POM after the two years of experimentation. The short-term conduction of this trial, mild temperatures and the initially high SOC concentration in the soil might have slowed down the treatment response on soil POM. In a similar climate, evaluating a 9- and 10-year annual mixture of black oats and ryegrass pasture under grazing intensities, ASSMANN et al. (2014) and SILVA et al. (2014) found that increased C input by plant below and aboveground led to increases in C-POM stocks up to 40 cm depth. Evaluating 11 globally distributed grasslands with 6-10 years of fertilization management ROCCI et al. (2022), also found that treatments that increased aboveground and root production, did not always increase C-POM concentration in the whole soil. According to the authors, soils with low sand proportion and lower temperatures, generally with high initial SOC pools, were less likely to respond on C-POM changes. Similar results were also found by SANTOS et al. (2019) in a subtropical sandy soil evaluating mixtures of bahiagrass and forage peanut, which did not affected C and N stocks in soil POM compared to bahiagrass monocultures (heavily N-fertilized), but increased it when compare to forage peanut monoculture after four years of establishment. According to the authors, differences in POM occurred when root mass was increased. Therefore, we can expect that over time, differences in POM will start to appear in our study, since the mixtures with 3 to 6 species are adding more C and N by roots compared to bahiagrass/oats system and already presented a higher C concentration within the POM+sand fraction at the 0-5 cm layer.

1.6.3 Future perspectives of increasing forage diversity in grassland systems

This study was a 2-year experiment with forage and root measurements throughout cool and warm-seasons and showed that mixtures can increase root production in a relatively short-term period. In the long-term it is estimated that root biomass, mainly from perennial forages are increased (MEURER et al., 2019; YANG et al., 2019), therefore providing additional benefits to soil.

The complementarity between different plant functional groups regarding rooting patterns (shallow rhizomatous roots vs deeper fibrous branched roots) can also benefit soil physical properties by increase aggregate stability, as result of finer root system from grasses, and increasing hydraulic properties and macroporosity by the thicker roots from legumes (GOULD et al., 2016; POIRIER et al., 2018). In our 2-year study improvements on soil resistance to penetration have already been seen when mixtures with grass-legumes were cultivated in relation to the grass only system (Figure 8). A better soil structure is often reflected on soil health and can bring benefits to the growing forages, as well as favor C sequestration (GOULD et al., 2016; POIRIER et al., 2018).

The higher aboveground forage accumulation can also increase soil organic matter in the top layers by the decomposition of litter (LAVALLEE et al., 2020; SANTOS et al., 2018a) and, considering animal grazing, by incorporation of residue by animal trampling and additions of manure (RIBEIRO et al., 2020). Also, mixtures can enhance the soil microbial community, improving ecosystem function and nutrient cycling (YAN et al., 2022). In addition, the nitrogen input by the legumes growing in mixture with grasses, can substitute the external demand of synthetic fertilizer-N (LÜSCHER et al., 2014), reducing environmental burdens related to nitrous oxide emissions (BRACKEN et al., 2022), or nitrate leaching to ground water (ERIKSEN et al., 2015).

1.7 CONCLUSIONS

Increasing diversity with a mixture of bahiagrass, forage peanut, limpograss, oats, clover and ryegrass increased forage, carbon and nitrogen accumulation in aboveground mass compared to the simple bahiagrass/oats mixture. Higher diversity within each season was responsible for higher forage yield gains, being 14% higher in the cool-season and 57% higher in the warm-season. The positive interaction among species and the complementary traits, with additional N supply from legumes, and differences in temporal and spatial forage growth from the grasses, are probably the main factors for the success of mixtures.

A similar pattern was observed in root mass and its carbon and nitrogen accumulation, which were increased by most mixtures relative to bahiagrass/oats. Mixtures with either 3 warm-season species or 2-3 cool-season species increased carbon and nitrogen accumulation in roots to 30 cm depth by 13% and 21%, respectively, on average across the two years. Mixtures increased root mass mainly at the 5-10 cm layer, showing that the use of diverse systems, with different root architecture and rooting depth among species can be a strategy to explore this niche.

Despite the increase in carbon and nitrogen input by roots after these two years, the soil particulate organic matter was not affected by the different forage mixtures. The short-term experiment, the subtropical climate, high initial soil organic matter concentration and finer texture of this soil might be factors delaying the response of POM to the changes in C and N input by roots. However, if those changes in root inputs are kept constant in the long-term, increasing carbon and nitrogen input to soil, we expect an increase in C and N concentration in organic matter lighter fractions, therefore presenting an opportunity to increase soil carbon sequestration over time.

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2 CHAPTER II: EFFECTS OF RECURRING INUNDATION ON DIVERSIFIED PASTURES PRODUCTIVITY, ROOT DISTRIBUTION AND SOIL CARBON AND NITROGEN

2.1 ABSTRACT

Intense spring precipitations has resulted in inundations in pasture areas in Ohio. Inundation affects the pattern of carbon inputs by plants above and belowground, leading to changes in soil carbon storage. However, higher forage diversity can be an alternative for grassland adaptation to inundation. The aim of this study was to evaluate forage and root production and particulate organic matter (POM) of diversified pastures under recurring short-term inundations. A field experiment was conducted in Southern Ohio in a prone-to-inundation pasture (soil inundations for few days after heavy rain events) with an adjacent non-prone-to-inundation pasture. Three pastures of varying degrees of species combinations were evaluated: P1) predominantly tall-fescue; P2) mixture of cool-season perennials, tall-fescue, orchardgrass, bluegrass and white and red-clover; P3) same as in P2, plus annuals oats and rye during fall. Roots were sampled in summer and fall of 2021 and 2022 to a depth of 30 cm. Cumulative aboveground biomass was determined in 5 and 4 periods of the growing season of each year, respectively. After 3 years of mixtures establishment, soil POM was evaluated (0-10 cm). Inundation reduced forage and root production and led to a reduction in C-POM stocks ($p<0.10$). The main change caused by inundation was a shift on forage botanical composition, mainly a higher occurrence of weeds and less productive species with shallow roots. A cool-season mixture of perennial species did not increase forage production, but increased root production, mainly at deeper layers and consequently C and N-POM stocks ($p<0.10$). Mixtures with annuals were not well established and reduced forage and root mass, consequently decreasing soil C and N-POM stocks ($p<0.10$). A cool-season mixture of perennial forage species can be used to increase resilience of pastures to adverse climatic events such as inundations and droughts.

Keywords: Cool-season forages. Flooding. Grasslands. Mixture. Particulate organic matter.

2.2 RESUMO

O aumento na intensidade de chuvas de primavera tem resultado em inundações em áreas de pastagens em Ohio. A inundação afeta o padrão de adições de carbono por parte aérea e raízes das plantas, levando à alterações no armazenamento de carbono no solo. Porém, uma maior diversidade de forrageiras pode ser uma alternativa para adaptação de pastagens à inundação. O objetivo deste estudo foi avaliar a produção de forragem e raiz e a matéria orgânica particulada (MOP) de pastagens diversas sobre inundação recorrente de curto prazo. Um experimento de campo foi conduzido no Sul de Ohio em uma área de pastagem sujeita à inundação (inundação do solo por alguns dias após chuvas intensas), com uma pastagem adjacente não sujeita à inundação. Três pastagens com diferentes graus de combinação entre espécies foram avaliadas: P1) predominantemente festuca; P2) consórcio de perenes de estação fria, festuca, dátilo, grama azul e trevo branco e vermelho; P3) mesmo consórcio de P2, com adição de anuais de inverno aveia e centeio no outono. As raízes foram amostradas no verão e outono de 2021 e 2022 até 30 cm de profundidade. A biomassa de parte aérea acumulada foi obtida em 5 e 4 cortes durante a estação de crescimento de cada ano, respectivamente. Após 3 anos do estabelecimento dos consórcios, a MOP foi avaliada (0-10 cm de profundidade). A inundação reduziu a produção de forragem e raízes e levou à redução nos estoques de C-MOP ($p<0.10$). O principal efeito causado pela inundação foi uma mudança na composição botânica da forragem, principalmente uma maior ocorrência de daninhas e espécies menos produtivas com raízes superficiais. O consórcio de perenes de estação fria não aumentou a produção de forragem, mas aumentou raízes, principalmente em camadas mais profundas, e consequentemente aumentou os estoques de C e N-MOP ($p<0.10$). O consórcio com as anuais de inverno não teve um estabelecimento adequado, reduzindo massa de forragem e raízes e consequentemente os estoques de C e N na MOP ($p<0.10$). O consórcio de forragens perenes de estação fria pode ser usado para aumentar a resiliência das pastagens à eventos climáticos adversos como inundações e estiagens.

Palavras-chave: Pastagens de estação fria. Alagamento. Pastagem. Consórcio. Matéria orgânica particulada.

2.3 INTRODUCTION

Climate change is a current and global issue, with severe events increasing in frequency and severity. Among the extreme weather events, the magnitude of heavy rainfalls, leading to increased flood risk, is predicted to increase (DONAT et al., 2016; HIRABAYASHI et al., 2013). Heavy rainfalls and associated inundations have been observed in the Midwest US region, especially in spring and summer (MALLAKPOUR and VILLARINI, 2015; 2016). In Ohio, reports on inundation and damage to corn, soybean and hayfields are common across years (LINDSEY et al., 2022; LINDSEY et al., 2017; ORTEZ et al., 2022; TURNER, 2017). Inundation affects soil properties, mainly by reducing oxygen availability and reducing redox potential (SÁNCHEZ-RODRÍGUEZ et al., 2019). This will affect plant growth due to reduced photosynthesis rates and nutrient uptake, which reduces C fixation and yield by plants (PLOSCHUK et al., 2017; TONG et al., 2021). In long-term, inundation can also reduce soil fertility, by releasing phosphorus and ammonium to the soil solution, which can be leached and cause eutrophication of water bodies (SÁNCHEZ-RODRÍGUEZ et al., 2019; SCALENGHE et al., 2012).

Grasslands are the most common land use in the United States, covering about 1/3 of the country, and are predominantly dominated by grass pastures and rangelands (BIGELOW, 2017; DUBEUX et al., 2022). These grasslands are responsible for a wide range of ecosystems services such as provisioning (food, fiber), regulating (climate and water regulation), supportive (soil stability and nutrient cycling) and cultural services (DUBEUX et al., 2022). Areas prone to inundation are usually cultivated with grasslands due to its higher resilience to extreme events, lower investment risk, and higher potential to uptake nutrients which reduce nutrient loss (BOHLEN and VILLAPANDO, 2011). Roots of perennial forages can develop deeper in the soil profile and capture nutrients that would leach under cash and annual crops, characterized by a shallower root system (DUBEUX et al., 2022). A better root development also improves soil structure and favor water percolation into the soil, reducing implications of high soil moisture (FISCHER et al., 2019).

Grasslands are also responsible for a larger capacity to sequester and store C in the soil, mainly due to increased C inputs belowground (BAI and COTRUFO, 2022; ZHOU et al., 2017). Belowground inputs efficiently contribute to the formation and stabilization of soil organic matter, due to the higher recalcitrance and lower decomposition of roots (RASSE et al., 2005), and by the readily available compounds from exudates, that will be energy source to soil microorganisms (BOLINDER et al., 2012; SOKOL et al., 2019). However, soils under inundation can result in decreased C inputs by plants due to limiting growth conditions and can result in loss of C bound to the Fe-oxides, due to anaerobic conditions (HUANG and HALL, 2017; HUANG et al., 2020), which can lead to depletion of soil C stocks. In irrigated grasslands in New Zealand, MUDGE et al. (2017) found that long-term excessive irrigation reduced soil C and N stocks, mainly due to lower root input by plants and larger decomposition and leaching of soil C and N after irrigation. However, there is still a lack of studies evaluating effects of natural recurring inundation events on grasslands C and N inputs and impacts on soil stocks.

Management strategies that increase plant production, such as the increase of forage species diversity, can be an option to improve water use and reduce detrimental impacts of inundation in grassland (FISCHER et al., 2019). Different forage species with complementary traits growing in a mixture, can increase aboveground mass production and stability (WAGG et al., 2022), and increase rooting depth due to different root architecture (HUSSE et al., 2017; SKINNER and DELL, 2016). In US humid regions, tall-fescue (*Schedonorus arundinaceus* (Schreb.)

Dumort., nom. cons.), a cool-season perennial grass, is the predominant species in grasslands and is characterized by high production in spring, followed by a summer-slump (KEYSER et al., 2022; TRACY et al., 2010). Some studies have shown that cool-season mixtures of perennial grasses and legumes increased forage and root mass production, more than in pastures of poor diversity (SKINNER and DELL, 2016; YANG et al., 2019). According to the authors, the higher complementarity among different species resulted in better soil exploration by roots, which were also an important factor in increasing soil C stocks.

Annual forage species can also be used to complement cool-season forages and increase the length of forage offer throughout the year, mainly in the fall/winter season when annuals can be stockpiled (DUBEUX et al., 2016; VILLALOBOS and BRUMMER, 2017). Overseeding winter annual grasses into clover stands resulted in successful establishment and high production and protein than clover monoculture in the fall and spring (CONTRERAS-GOVEA and ALBRECHT, 2005). Similarly, winter annual forages were successfully established into warm-season perennial grasslands, increasing yields on the following spring (WATCHARAANANTAPONG et al., 2020). Yet, results with winter annuals mixed to predominant cool-season grasses are still lacking. As inundation in the spring is expected to affect cool-season species development, the inclusion of annuals can possibly fill this gap on forage production and benefit belowground inputs to soil. Roots from annual species are short-lived and could contribute to a rapid input to form organic matter compared to perennials only (BOLINDER et al., 2012; HOUDE et al., 2020).

Although changes in C and N inputs by plants and soil organic matter decomposition rates can affect soil C and N stocks in the long-term, short-term and subtle changes can be observed in more sensitive organic matter fractions. Particulate organic matter (POM) is a pool that still presents organic matter that resembles plant characteristics (FRANZLUEBBERS and STUEDEMANN, 2002), and is an important point of entry to formation of stabilized organic matter in the mineral fraction (CAMBARDELLA and ELLIOTT, 1992; LAVALLEE et al., 2020). FRANZLUEBBERS and STUEDEMANN (2002), observed that increased input of organic material by plants increased POM at surface layer, as result of increased aboveground residue, and below 5 cm depth, indicating higher C and N input by plan roots.

Despite many studies with forage mixtures showing positive effects on aboveground production and soil C stocks, there is still need to understand how forage species diversity affects grasslands prone to inundation. Therefore, we hypothesize that forage mixtures will shift the botanical composition improving forage yield and quality and increasing root production, increasing soil labile organic matter, in relation to a grass monoculture. In this research we evaluated the long-term effects of natural recurring inundation events in a grassland field composed predominantly by tall-fescue and the short-term effects of introducing different forage mixtures in this area. The objectives of this study were to assess effects of natural recurring inundation events and of forage species mixtures on i) forage yield, nutritional value, and botanical composition, ii) root production and iii) soil particulate organic matter.

2.4 MATERIAL AND METHODS

2.4.1 Experiment

A field experiment was conducted at Jackson, Ohio (39°01’25” N and 82°36’39” W, 210 m altitude), in an area that had been under long-term pasture. The climate is humid continental (Dfa according to Koppen’s classification), with average (1981-2010) temperatures in the coldest month of -2.8 ^{o}C (January) and in the warmest of 23.1 ^{o}C (July), with an annual rain precipitation of 1067 mm and snowfall of 584 mm (U.S. CLIMATE DATA, 2020). The growing season lasts 7 months from April to October when the temperature is non-freezing, while from December to March the soil is usually covered by snow. Because of these climate conditions, growing two crops per year or a sequence of cool and warm forage species in a year is not common in the region. Soil is described as a Piopolis silt loam (Fine-silty, mixed, active, acid, mesic Fluvaquentic Endoaquepts), based on USDA soil classification (SOIL SURVEY STAFF, 2014) and a Fluvisols based on international classification (WRB, 2015). The average slope in the area is 0.4%.

The experiment was established on a farm, in 4.6 hectares which had been previously under grazing of perennial grasses for ~15 years. The region where the farm is located presents a long-term natural occurrence of soil inundation in some areas. Rotational grazing from spring to late summer was performed by beef cattle, generally in three grazing cycles. Occasionally, hay was harvested in late August. The predominant species found in the area was tall-fescue (*Schedonorus* *arundinaceus* (Schreb.) Dumort., nom. cons.), with some scattered occurrence of white clover (*Trifolium repens* L.) and orchardgrass (*Dactylis glomerata* L.). The historical annual fertilization rates in the area were 20 kg ha^{-1} of N, 100 kg ha^{-1} of P_2O_5 and 100 kg of K_2O ha^{-1}, broadcasted in a split application in early spring and summer.

Treatments comprised two inundation levels, namely inundated and non-inundated, and three forage-species mixtures. Inundated plots were established in the terrain part where standing water commonly occurred after rainfalls and bare-ground patches were common. Inundation events usually occur after 20 mm rain and last for no more than 5 days. The inundation is a long-term natural event that affects this area every year. Non-inundate plots were established adjacently in a slightly higher terrain part (Figure 1). In each area, three blocks were identified, in which plots with dimensions of 6.5 × 55 m were designed.

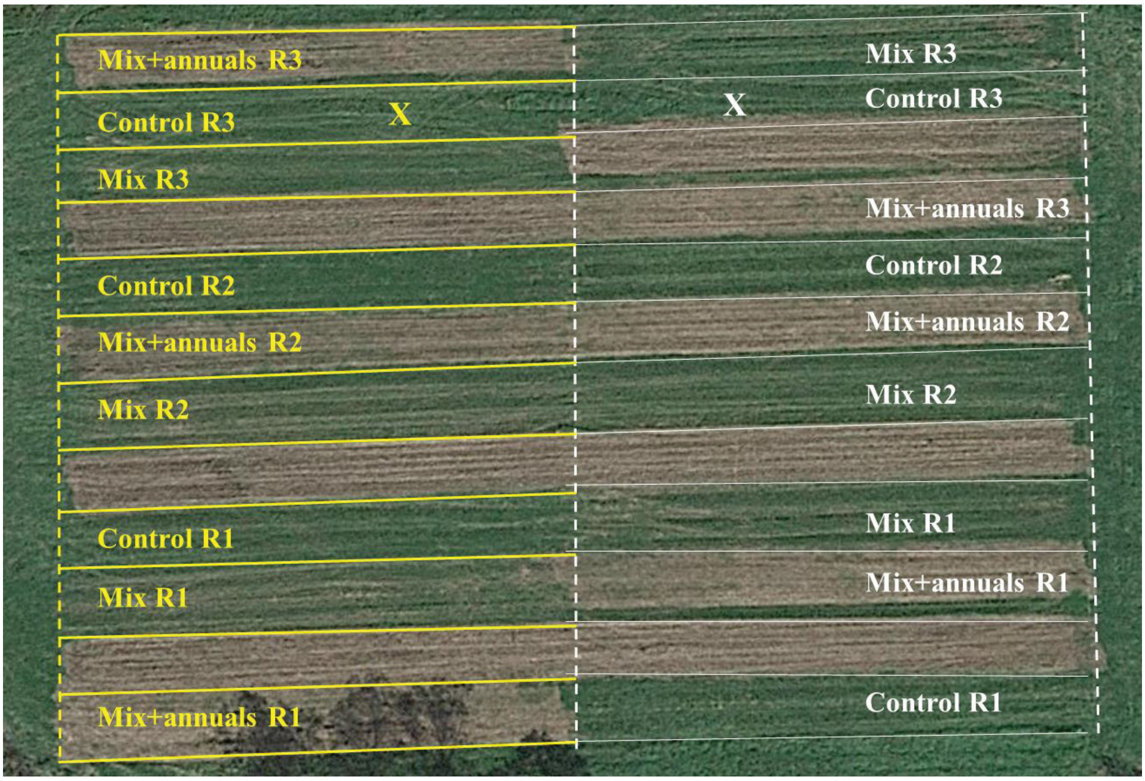

Figure 1. Experimental layout of plots under inundation (yellow) and non-inundation (white) conditions in Jackson-OH. Treatments are Control (predominantly tall fescue); Mix (mixture of cool-season species tall fescue, orchardgrass, Kentucky bluegrass and white and red clover); and Mix+annuals (same cool-season Mix, with addition of winter annuals rye and black oat). The R# represents the repetition.

SOURCE: The author.

The three forage mixtures were (1) a control predominantly of tall fescue (current long-term farm management); (2) a cool-season mixture of tall fescue (cv Kentucky 31), orchardgrass (cv Potomac), Kentucky bluegrass (*Poa pratensis* L. cv Ginger), white clover (*Trifolium repens* L. [Ladino type] variety not stated) and red clover (*Trifolium pratense* L. variety not stated), hereafter named as mix; and (3) the same previous mixture plus winter annuals rye (*Secale cereale* L. cv Elbon) and black oats (*Avena strigosa* Schreb. variety not stated), hereafter named as mix+annuals. Treatments were set up in a 2×3 split-plot design with three replicates. The main plot was inundation level, while subplots the forage-species mixtures. Figure 1 shows the plots layout in the field. Prior to experiment establishment, soil samples were taken for chemical and particle size distribution (Table 1).

Table 1. Chemical characteristics and particle size of a Piopolis silt loam soil prone and not prone to inundation prior to the experiment establishment. Jackson-OH, USA.

Variable	Unit	Layer (cm)	Inundated	Non-inundated
pH	-	0-15	5.8±0.1	5.8±0.0
K^+	$cmol_c\ dm^{-3}$	0-15	0.13±0.01	0.16±0.01
Ca^{2+}	$cmol_c\ dm^{-3}$	0-15	3.4±0.1	3.0±0.1
Mg^{2+}	$cmol_c\ dm^{-3}$	0-15	1.4±0.0	1.2±0.0
P	$mg\ dm^{-3}$	0-15	7.5±1.0	10.8±0.4
SOC	$g\ dm^{-3}$	0-15	20.3±0.1	19.5±0.1
Clay	%	0-10	24.8	21.9
	%	10-30	30.6	23.5
	%	30-50	32.5	21.2
Silt	%	0-10	50.1	44.5
	%	10-30	48.4	40.7
	%	30-50	39.4	35.0
Sand	%	0-10	25.1	33.6
	%	10-30	21.0	35.8
	%	30-50	28.1	43.8

LEGEND: pH: determined in water at a rate 1:1 (soil:solution); Ca^{+2}, Mg^{2+}, K and P extracted with Mehlich-III and determined in ICP (Thermo 6000 series); SOC determined by dry combustion (LECO FP828); particle size determined by Hydrometer method (Soil Survey Staff, 2009). For chemical characteristics results are average of 4 points ± SE; for particle size four subsamples were composite to form a sample for each layer.

SOURCE: The author.

The control tall-fescue represented the usual pasture management in Ohio, without further improvements. For mix and mix plus annuals treatments, plots were sprayed with glyphosate and 2,4 D on May 12th, 2020, at rates of 840 and 750 g a.i. ha^{-1}, respectively. On May 15th, 2020, the cool-season species were drilled in both treatments at a depth of 2 cm and row spacing of 15 cm. Seeding rates are presented on Table 2. Due to low establishment success, the entire area was grazed by beef cattle during summer and the treatments were sprayed (same as described above) and drilled again in August 2020. On mid-October 2020, rye and oats were drilled in the plots of cool-season + winter annuals mix at a rate 26.9 and 97.5 kg seeds ha^{-1}, respectively, after mowing the cool-season to 10 cm heigh and applying half of glyphosate dose (420 g a.i. ha^{-1}). The winter annuals were reseeded at the same rate in the following year in late October 2021, with the same management as described above.

Fertilization rates in 2021 were 17 kg ha^{-1} of N, 67 kg ha^{-1} of P_2O_5 and 67 kg ha^{-1} of K_2O broadcasted on April 24th and 67 kg ha^{-1} of P_2O_5 and 67 kg ha^{-1} of K_2O broadcasted on September 15th. In 2022, 6 kg ha^{-1} of N, 23 kg ha^{-1} of P_2O_5 and 74 kg ha^{-1} of K_2O were broadcasted on March 3rd and 67 kg ha^{-1} of P_2O_5 and 67 kg ha^{-1} of K_2O broadcasted on September 12th.

Table 2. Seeding rates of forage mixtures in a cool-season mix and in a cool-season mix with the addition of winter annuals. Jackson-OH, USA.

Species	Cool-season mix	Cool-season mix + winter annuals
	kg ha^{-1}	
Tall fescue	5.04	5.04
Orchardgrass	3.4	3.4
Kentucky bluegrass	2.8	2.8
White clover	0.6	0.6
Red clover	0.6	0.6
Rye	-	26.9
Oat	-	97.5

SOURCE: The author.

2.4.2 Inundation monitoring

The water table depth was monitored with a well in the inundated area and another well in the non-inundated area (Figure 1) from March 2021 to December

2022. Wells were made of a 1.4 m long and 8 cm diameter PVC pipe, with 1.5 cm diameter holes covered with a mesh nylon filter, located in the bottom 0.5 m of PVC pipe, which were inserted into the soil to a depth of 1.3 m. In each well, a HYDROS-21 CTD sensor (Meter®) was placed for the monitoring. Soil water content was also monitored at 30 cm depth by two extra TEROS-21 sensors (Meter®) installed nearby each well. Data was collected and recorded every 15 min in a ZL6 data-logger (Meter®).

Inundation events were monitored and characterized according to RINDERER *et al*. (2012). After high intensity rainfall events (usually above 20 mm day^{-1}), 20 random points per plot, with approximately 1 m apart, were visually assessed for: 1) soil not saturated and without surface pounding, totally dry; 2) soil saturated and without surface pounding, characterized by a squelchy noise when stepping in the ground; and 3) soil saturated and with visible surface pounding at soil surface. When classified as 3, the height of the surface pounding was recorded. The area covered with inundation was the sum between classifications 2 and 3 divided by the number of sampling points. The relation between area covered by inundation and water depth was used to determine a water table height threshold in which there is a probability of 25% or more of the area with inundation at the soil surface. These evaluations were recorded throughout the growing seasons of 2021 and 2022.

2.4.3 Forage yield, quality, and botanical composition

Forage samples were collected5 times in 2021 and 4 times in 2022, in spring, late spring (only for 2021), early-summer, late-summer, and fall, with dates described in table 3. Forage biomass was evaluated in two subsamples (0.5 m × 0.5 m) per plot by clipping plants at 20 cm height. After samples were taken, plots were mowed at a 20 cm height with a hay mower-conditioner (John Deere 630 MoCo ®) attached to a tractor. Then, after 3-5 days of mowing, the biomass was baled and removed from plots.

Fresh samples were frozen (-10 ºC) and then manually separated according to the species planted (tall-fescue, orchardgrass, bluegrass, clover, rye and oats) for botanical composition determination. Other grasses were commonly found in the area, such as timothy (*Phleum pratense* L.), foxtail (*Setaria pumila* Poir.), crabgrass (*Digitaria ischaemum* Schreb.), bentgrass (*Agrostis castellana* Boiss. & Reuter), field paspalum (*Paspalum laeve* Michx.) and barnyardgrass (*Echinochloa crus-galli* L.), which were grouped as “other grasses”. A category of weeds included broadleaves such as yellow nutsedge (*Cyperus esculentus* L.), morning-glory (*Ipomoea hederacea* Jacq.), plantain (*Plantago lanceolata* L.), dandelion (*Taraxacum officinale* F.H. Wigg.), cocklebur (*Xanthium strumarium* L.) and wild garlic (*Allium vineale* L.). Dead material was also identified as an additional category and included dead material from any species. After species/categories were identified, subsamples were dried to 60 °C and weighted separately. Forage yield was calculated by the sum of dry mass of all species and categories within a sample, extrapolated to Mg ha^{-1}. The botanical composition was then calculated as the percentage of dry mass of each species or category in relation to the total dry mass of the sample.

Table 3. Timeline of aboveground forage sampling.

Season	Year-1	Year-2
Spring	April 24th	May 12th
Late-spring	June 14th	-
Early-summer	July 22nd	July 23rd
Late-summer	September 14th	September 12th
Fall	November 22nd	November 16th

SOURCE: The author.

After weighing, the aboveground dry mass from each species/category per plot, was composite in one sample and grounded in a Thomas Model 4 Wiley® mill (Thomas Scientific ®) with a 2 mm sieve. Then, samples were further grounded in a Cyclone mill (Udy Corp) with a 1 mm screen sieve. Ground samples were analyzed for nutritional value (crude protein, lignin, NDF, NDA and digestibility) using a near-infrared reflectance spectroscopy (NIRS DS-3 FOSS®).

2.4.4 Root evaluations

Soil cores were sampled in August and November 2021 and 2022 to determine root biomass. Two sub-samples of the 0-5, 5-10, 10-20 and 20-30 cm layers were taken at three points per plot with a soil core sampler (AMS®) equipped with a slide-hammer. In the sampler, three rings of 5 cm height each and 5.3 cm diameter were fit in a stainless-steel cup of 15 cm height for extraction of undisturbed soil samples. For each layer and point, soil samples were removed from rings and frozen (–10°C). For processing, samples were thawed for 12h and gently broken and washed with water on a 1.0 mm mesh sieve to retain roots. Debris retained on the sieve were removed with tweezers, and roots washed and transferred to a 70% ethanol solution. After all samples were washed, roots were filtered from the ethanol solution, rinsed in tap-water and oven-dried at 45 °C to determine root biomass in each layer. The total root biomass to 30 cm depth was then calculated.

2.4.5 C and N concentrations in the aboveground and root tissue

Before grinding, root samples across all layers and the three points for the same replicate were all combined to determine the average concentrations of C and N in the root tissue of each mixture. For aboveground samples, only two sampling times per year (spring and late-summer) were selected for C and N analysis. After weighted and ground to <2mm (Wiley mill), aboveground and root dry samples were ground in a mortar and sieved to <250 µm. Then a subsample of ~12 mg was weighted and analyzed by dry combustion (Thermo Scientific Flash 2000, Thermo Scientific, MA, USA) to determine C and N concentrations.

2.4.6 Total C and N in soil

Soil samples were taken on April 2021 to assess the baseline C and N stocks of the inundated and non-inundated areas. Two pits of 50 × 50 cm and 100 cm depth dimensions were excavated in each control plot. Undisturbed samples of approximately 500 g each were taken from the 0-5, 5-10, 10-20, 20-30, 30-50, 50-75 and 75-100 cm in one of the walls of the pit. Samples were air dried and sieved to <2.0 mm. A subsample of ~10 g was ground and sieved to <250 µm and analyzed for total organic carbon (TOC) and total nitrogen (TN) by dry combustion (Thermo Scientific Flash 2000, Thermo Scientific, MA, USA). TOC and TN stocks were calculated taking into account the TOC and TN concentrations, bulk density and the equivalent soil mass approach (ELLERT and BETTANY, 1995; SISTI et al., 2004). An adjacent grass area that had been under hay production in the past 20 years was used as a reference soil for the equivalent mass calculation; and here three pits were excavated, and samples were collected similarly as in the control plots.

Two core samples were taken in each layer in one of the pit´s wall (control inundate, control non-inundated and hay field) to determine soil bulk density. Metal rings of 5 cm diameter × 5 cm height were used. In 5-cm-thick layers, rings were placed side by side, while in 10-cm-thick layers rings were placed one on top of the other. In the 30-50 cm layer rings were taken at 32.5-37.5 and 42.5-47.5 cm. In the 50-75 and 75-100 cm layers, rings were taken at 55-60 and 65-70 cm, and 80-85 and 90-95 cm layers, respectively. Then, soil samples were oven-dried at 105 ^{o}C until constant mass to determine the bulk density.

2.4.7 Particulate organic matter

Undisturbed soil samples were collected from the 0-5 and 5-10 cm layers of all treatments to evaluate particulate organic matter (POM). Duplicate samples in each layer (two points in the plot) were collected on July 7th, 2022 with the same core sampler used for root sampling. Samples were air-dried and sieved to <4.0 mm and a ¼ sub-sample was separated to the physical particle-size fractionation to obtain POM and mineral associated organic matter (MAOM) (CAMBARDELLA and ELLIOTT, 1993). Briefly, 20 g of dried soil were weighted and added to 70 mL hexametaphosphate solution (5 g L^{-1}). After 16 hours in orbital shaker (180 rpm), the suspension was poured through a 53-µm mesh to recover the POM+sand fraction, which was then oven-dried at 45 ^{o}C and ground to <250 µm. A subsample of ~35 mg was analyzed for TOC and TN determination (Thermo Scientific Flash 2000, Thermo Scientific, MA, USA).

2.4.8 Statistical analysis

Data of forage yield and nutritional value, root density and their C and N concentrations, from both years, as well as soil POM, TOC and TN, were submitted to analysis of normality (Shapiro-Wilk) and homogeneity of variances (Bartlett). When the assumptions were not significant ($p<0.10$) Box-Cox optimum potency transformation was applied. Then, data were submitted to ANOVA to test the effects of inundation and levels of forage species diversity, when significant ($p<0.10$) means were compared by Tukey test ($p<0.10$). When comparing only the effect of inundation, the F test from ANOVA was conclusive ($p<0.10$). The analyses were performed with R software (v. 4.0.5) and packages MASS and ExpDes (R Core Team, 2022).

2.5 RESULTS

2.5.1 Weather, water table height, inundation frequency and soil moisture

The two years showed contrasting weather conditions, as precipitation from April to November was 987 mm in 2021 (Figure 2A) and only 389 mm in 2022 (Figure 2B). In addition, precipitation in the first year was well distributed among seasons, while in the second a long period of about 110 days in mid-summer and fall had no more than 20 mm precipitation. The water depth followed a similar pattern to the rain precipitation and was close to the soil surface after heavy intensity rains. In most of the period, water depth in the inundated area was close to the soil surface, with average depth of 533 mm in 2021 and 943 mm in 2022, which were shallower than under non-inundated, with 709 and 1037 mm, respectively.

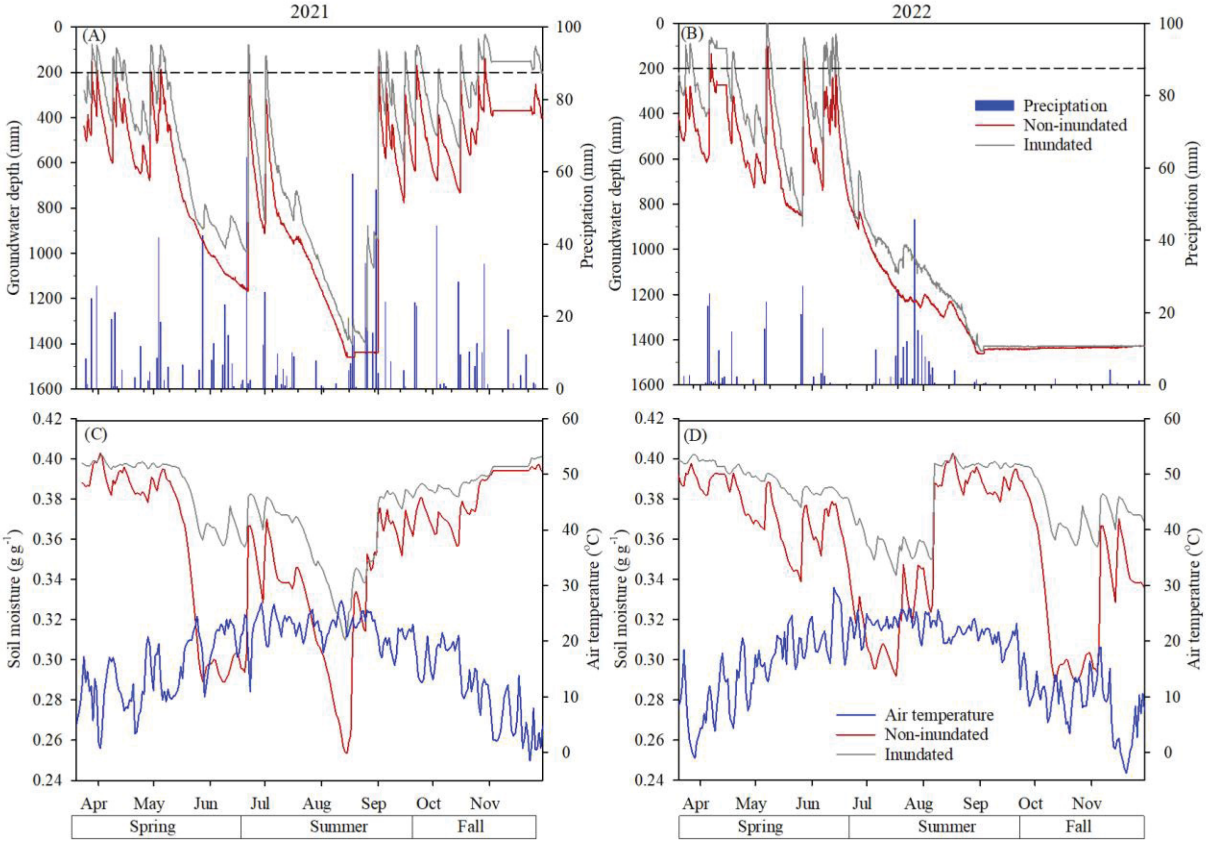

Figure 2. Water table depth (A, B) and soil moisture (C, D) in grassland inundated and non-inundated in 2021 and 2022. Rain precipitation and mean air temperature were obtained from a nearby weather station. The horizontal dashed-line represents a threshold in which inundation occurrence at the soil surface covers more than 25% of the area (See figure 3).

SOURCE: The author.

According to the threshold of 200 mm that indicates the probability of inundation occurrence in more than 25% of the soil surface area (Figure 3), in 2021, 25.3% of the time during the growing season was possibly saturated with water in the inundated plots, while in the non-inundated area that was only 0.7% of the time above that threshold. Inundation events occurred mainly in the spring, but also some in late summer and during fall. In 2022, inundation frequency was lower, covering 10.7% of the time under inundated area and 0.8% in the non-inundated area, and occurred only during spring.

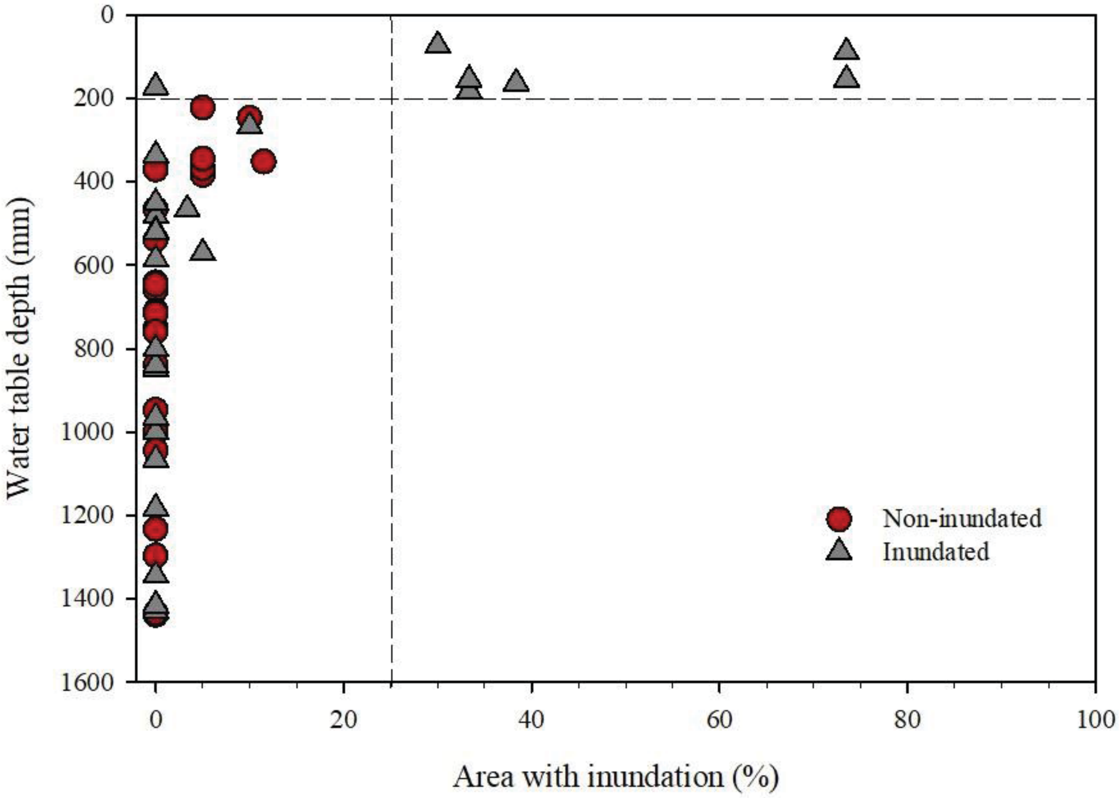

Figure 3. Relationship between groundwater table depth and area covered with inundation (based on visual evaluations during inundation events) in a grassland prone and not prone to inundation. Dashed lines indicate a threshold of 200 mm in which there is a chance of inundation occurrence covering more than 25% of the area.

SOURCE: The author.

Mean air temperature was similar between the years averaging 16.5 °C (Figure 2C and D). Temperatures lower than 5 °C occurred mainly until mid-April and after mid-November in both years and peaked around 20±5 °C from mid-June to October. Soil moisture at 30 cm depth ranged from 0.26 to 0.40 g g^{-1}, and most of the time was higher in the inundated areas. The lowest soil moisture values were observed during the summer season of both years and in fall of 2022, with sharp decreases under non-inundation, while the inundated area maintained more constant moisture.

2.5.2 Forage yield, botanical composition and nutritional value

Total forage yield ranged from 7.5 to 9.6 Mg DM ha^{-1} in 2021 (Figure 4) and from 2.5 to 8.3 Mg DM ha^{-1} in 2022 (Figure 4). Inundation affected forage yield in both years, by reducing the seasonal yields (p<0.10) mainly on control and cool-season mix plots, during spring and fall of 2021, and on control plots during the spring 2022; but had no clear effect on the mix+annuals. Overall, control and cool-season mix had similar cumulative and seasonal yields across years, regardless of inundation. The higher yields for control and cool-season mix occurred in spring, late spring and fall of 2021, and in early summer and fall of 2022.

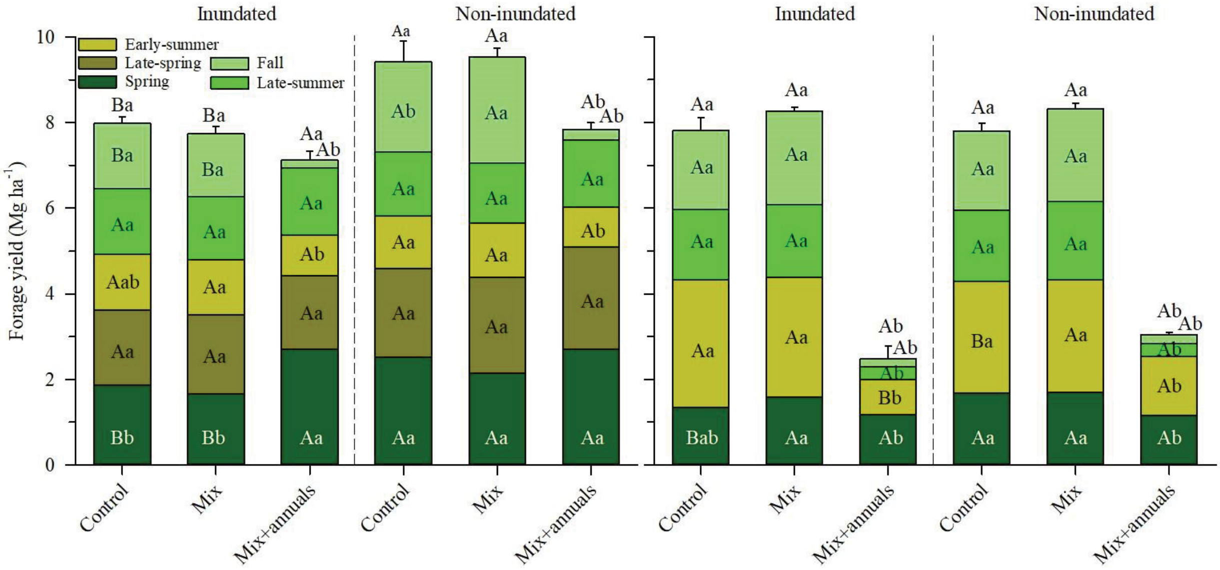

Figure 4. Forage yield (means ± SE, n=3) as affected by forage species mixtures in inundation and non-inundation conditions in 2021 and 2022. Uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare levels of species diversification within the same inundation treatment, according to Tukey`s test (p<0.10). Error bars above the bars represent the SE (n=3) for the cumulative forage yield in the year. Jackson-OH.

SOURCE: The author.

The mix+annuals had a reduced cumulative forage production (p<0.10) by 12-20% and 156-215%, in each year respectively, compared to the control, regardless of inundation (Figure 4). Although this treatment presented the highest yields in spring 2021, reduced yields occurred late in 2021 and throughout all the year of 2022.

In general, tall-fescue was the predominant species in terms of dry mass throughout the growing season of the two years, averaging 45% in inundated and 57% in non-inundated controls (Figure 5). Botanical composition in control plots was significantly affected by inundation in both years, where the proportion of tall-fescue reduced and that of clover (2021), other grass (2021 and 2022) and weeds (2021 and 2022) increased compared to non-inundated plots. Red clover was not identified in the samples throughout the experimental evaluations, therefore, clover observations refer to white clover only.

The cool-season mix significantly increased the orchardgrass proportion ($p<0.10$) in the stands to 30% in 2021 and 10% in 2022, compared to the control in both inundated and non-inundated conditions (Figure 5). The proportion of species in this mix was not significantly affected by inundation, but there was a reduction in tall-fescue (45%) and increase in other grasses (18%), compared to the non-inundated (50 and 7% respectively) in 2022.

In the cool-season mix + annuals, tall-fescue proportion decreased to 18-21% and that of winter annuals increased to 30-35% in 2021, while for 2022 tall-fescue proportion was 2-5%, followed by an increase in bluegrass (20-25%), clover (25-38%) and other grasses (15-27%) proportions (Figure 5). In the second year, the proportion of winter annuals was lower (5-7%) compared to bluegrass and clover. In general, inundation did not affect species proportion in this treatment, except for a reduction in clover and increase in other grasses in 2022.

The higher forage diversity as the cool-season mix and mix+annuals on inundated plots reduced the proportion of weeds and bluegrass, compared to the control in 2021, but with no effects in 2022 (Figure 5). In non-inundated plots the increase on the level of species diversity reduced only tall-fescue (2021 and 2022) and bluegrass (2021) proportions compared to the control. The mix+annuals presented a different pattern in 2022, with an increase in bluegrass, clover and other grasses proportions compared to the other treatments under inundated and non-inundated conditions.

In general, inundation did not affect the nutritional value of forage, but it increased lignin in control 2021 and 2022 and in mix 2022, and increased ADF and NDF in mix+annuals in 2022 ($p<0.10$) (Table 4). In general control and cool-season mix presented similar nutritional value across years, with mean CP of 13.6 and

11.0% for each year respectively. However, mix+annuals reduced ADF and NDF in both years compared to control and mix, and increased CP and lignin in 2022. Overall, better nutritional value occurred in the spring of both years, but higher CP was observed in fall of 2021 and late-summer of 2022 (Table 5).

2.5.3 Root mass

Inundation affected root mass in the 0-30 cm layer, mainly during summer of both years, in which control and mix had 16% and 13% lower root mass compared to non-inundated conditions, respectively ($p<0.10$) (Figure 6). In both summers, the reduction in root mass as effect of inundation occurred at 10-20 and 20-30 cm depth. The average root mass of control and cool-season mix (summer and fall) was 5.3 Mg ha^{-1} in inundated area and 5.8 Mg ha^{-1} in non-inundated area. During summer and fall 2021, control presented higher root mass than cool-season mix in the top 0-5 cm depth, but was lower in 5-10 and 10-20 cm depth, while in following year both treatments were generally similar across soil layers, regardless of inundation.

Root production in the mix+annuals was also reduced by inundation ($p<0.10$), but only in the first year (Figure 6). In general, this treatment presented lower root mass in all layers compared to control or mix, and followed a similar pattern as the aboveground, with a decline in root mass as the seasons passed. In the last evaluation, fall 2022, the root mass in this treatment for the entire 0-30 cm depth, was lower than root mass at 0-5 cm in control and cool-season mix treatments.

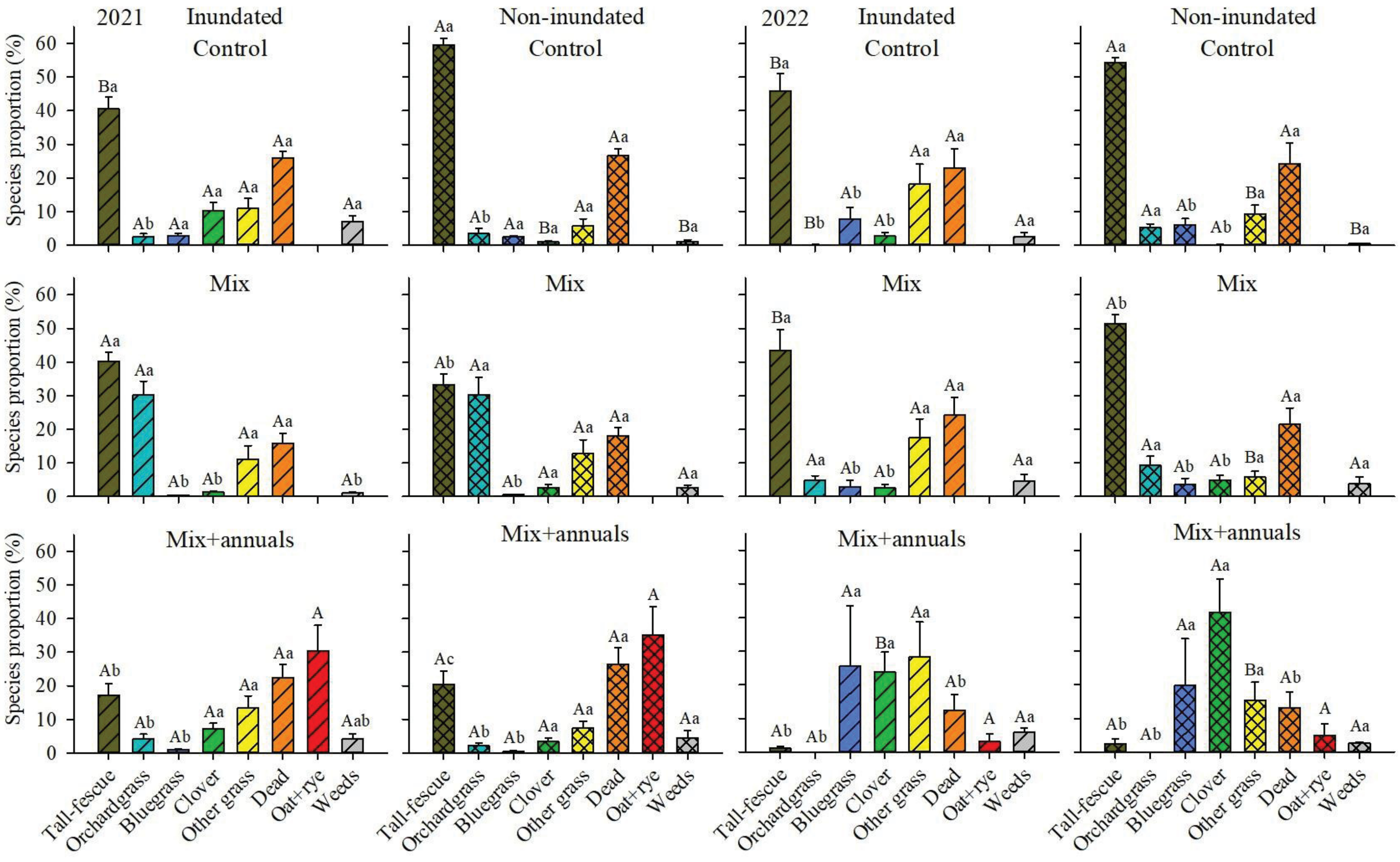

Figure 5. Forage dry mass botanical composition (means ± SE, n=12) as affected by levels of forage species in inundation and non-inundation conditions in 2021 and 2022. Data is the average across 4 cuts in each year. Uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare levels of species diversification within the same inundation treatment, according to Tukey`s test ($p<0.10$). Jackson-OH.

SOURCE: The author.

Table 4. Nutritive value of forage (means ± SE, n=12) during growing season of 2021 and 2022, as affected by levels of forage species in inundation and non-inundation conditions. Jackson-OH.

Parameters	Inundated			Non-inundated		
	Control	Mix	Mix+annuals	Control	Mix	Mix+annuals
			2021			
CP (%)	14.0±0.1 ^{ns}	12.6±0.6	12.9±0.6	14.3±0.6	13.4±0.7	11.7±0.6
ADF (%)	33.6±1.2 Aa	32.9±1.8 Aab	30.4±2.9 Ab	33.8±1.0 Aa	32.7±1.7 Aa	31.1±3.1 Aa
NDF (%)	55.0±2.3 Ba	56.3±2.8 Aa	51.0±4.4 Aa	59.6±1.8 Aa	56.4±2.6 Aab	53.0±4.7 Ab
IVDMD (%)	77.3±2.1 ^{ns}	78.5±2.4	79.6±3.6	75.9±2.0	78.3±2.3	78.5±3.7
Lignin (%)	5.1±0.4 Aa	4.1±0.4 Ab	4.2±0.9 Ab	4.4±0.3 Ba	4.2±0.5 Aa	4.0±0.9 Aa
			2022			
CP (%)	10.8±0.5 Ab	10.5±0.5 Ab	15.7±1.1 Ba	11.3±0.7 Ab	11.5±0.4 Ab	17.6±1.1 Aa
ADF (%)	34.9±0.8 Aa	34.6±0.9 Aa	30.1±0.8 Ab	34.7±0.8 Aa	34.4±1.1 Aa	28.3±0.6 Bb
NDF (%)	58.3±1.0 Aa	57.6±1.4 Aa	49.4±1.2 Ab	59.4±1.1 Aa	57.41±1.2 Aa	45.7±1.3 Bb
IVDMD (%)	73.5±1.5 Ab	74.0±1.6 Ab	80.0±1.7 Aa	73.5±1.5 Ab	75.0±1.6 Ab	81.2±0.9 Aa
Lignin (%)	4.2±0.3 Ab	4.4±0.4 Ab	5.1±0.5 Aa	3.8±0.3 Bb	3.9±0.3 Bb	5.4±0.5 Aa

LEGEND: CP: crude protein; ADF: acid-detergent fiber; NDF: neutral-detergent fiber; IVDMD: digestibility in vitro for 48 hours. Uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare levels of species diversification within the same inundation treatment, according to Tukey`s test (p<0.10); ns: not significant. Data is an average of 4 cuts in each year.

SOURCE: The author.

Table 5. Nutritive value of forage (means ± SE, n=36) during growing season of 2021 and 2022, as affected by seasonality. Jackson-OH.

Parameters	Spring	Early Summer	Late Summer	Fall
		2021		
CP (%)	14.3 B	12.1 C	11.9 C	15.3 A
ADF (%)	23.4 C	37.0 A	36.6 A	33.6 B
NDF (%)	40.5 C	61.5 AB	61.7 A	59.7 B
IVDMD (%)	90.8 A	73.0 C	71.7 C	75.3 B
Lignin (%)	1.6 D	5.3 B	5.7 A	5.0 C
		2022		
CP (%)	12.7 B	10.8 C	15.0 A	11.3 C
ADF (%)	32.0 B	34.6 A	34.5 A	30.7 B
NDF (%)	54.4 B	54.5 B	58.1 A	53.5 B
IVDMD (%)	81.4 A	72.6 C	72.1 C	77.8 B
Lignin (%)	2.9 B	5.5 A	5.6 A	3.1 B

LEGEND: CP: crude protein; ADF: acid-detergent fiber; NDF: neutral-detergent fiber; IVDMD: digestibility in vitro for 48 hours. Uppercase letters compare season for the same year, according to Tukey`s test (p<0.10); ns: not significant. Data is an average of 4 cuts in each year.

SOURCE: The author.

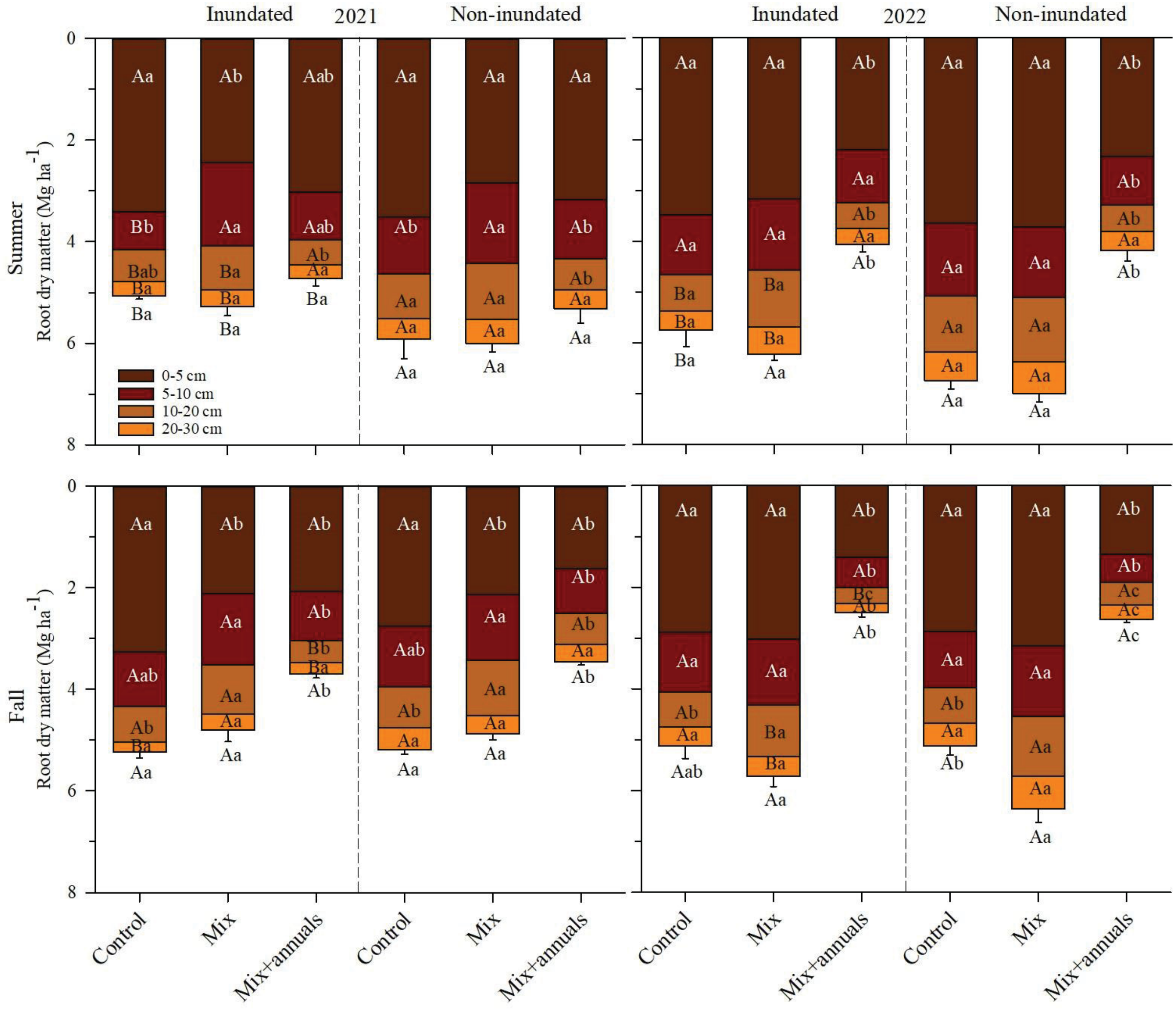

Figure 6. Root dry mass stock (Mg ha^{-1}) as affected by levels of forage species in inundation and non-inundation conditions during summer and fall of 2021 and 2022. For the same soil layer, uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare the level of species diversification within the same inundation treatment, according to Tukey`s test (p<0.10). Error bars below the bars represent the SE (n=9) for the cumulative root dry mass stock (0-30 cm). Jackson-OH.

SOURCE: The author.

2.5.4 Forage and root total C and N concentration

Inundation and forage mixtures did not affect C concentration in aboveground and roots during the 2 years of evaluation (p<0.10), except for aboveground in the first year when mix+annuals increased C concentration by 4-6% in relation to the control (Table 6). C concentration was generally higher in the aboveground, with an average of 439±3 g C kg^{-1}, than in roots, average of 366±12 g C kg^{-1}. N concentration in aboveground and roots was not affected by treatments in the first year, with averages of 26.3±1.1 and 15.8±1.1 g N kg^{-1}, respectively. But in the second year, regardless of inundation, mix+annuals increase aboveground N concentrations by 62% and root N concentrations by 28%, in relation to control and cool-season mix.

Table 6. Total carbon and nitrogen concentrations (means ± SE, n=3) on aboveground and roots (0-30 cm) during 2021 and 2022, as affected by levels of forage species in inundation and non-inundation conditions. Aboveground results are presented as the average of spring and late-summer samples. Jackson-OH.

Parameters	Inundated			Non-inundated		
	Control	Mix	Mix+annuals	Control	Mix	Mix+annuals
			2021			
Aboveground						
C (g kg^{-1})	422.6±7.9 Ab	448.6±2.3 Aa	448.5±1.0 Aa	436.2±2.5 Ab	442.9±3.6 Aab	451.4±0.8 Aa
N (g kg^{-1})	26.3±3.6 ^{ns}	24.5±0.2	27.4±1.1	27.4±0.5	26.0±0.4	25.9±0.9
Roots						
C (g kg^{-1})	360.2±12.2^{ns}	353.7±2.5	368.4±11.1	373.2±5.3	357.4±2.2	376.8±10.0
N (g kg^{-1})	16.7±1.5 ^{ns}	14.8±0.4	17.8±1.5	14.4±0.9	14.6±1.0	16.4±1.4
			2022			
Aboveground						
C (g kg^{-1})	436.5±0.4 ^{ns}	440.4±2.8	436.3±3.0	437.1±2.2	438.3±1.4	430.3±5.0
N (g kg^{-1})	23.0±2.3 Ab	23.7±1.1 Ab	38.2±1.1 Aa	21.8±0.6 Ab	21.4±0.7 Ab	34.8±3.0 Aa
Roots						
C (g kg^{-1})	364.9±15.4 ^{ns}	368.2±20.6	355.3±8.5	377.8±17.3	366.9±6.9	366.5±10.7
N (g kg^{-1})	13.3±0.2 Ab	12.7±0.1 Ab	16.9±0.8 Aa	13.7±0.2 Ab	12.8±0.3 Ab	16.8±0.5 Aa

LEGEND: Uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare levels of species diversification within the same inundation treatment, according to Tukey`s test (*p*<0.10); ns: not significant.

SOURCE: The author.

2.5.5 C and N accumulation in aboveground and roots

The average total C and N accumulation from the two years ranged from 3,479-6,124 and 223-294 kg ha^{-1}, respectively (Figure 7). The aboveground compartment contained ~63% of all C and ~72% of all N accumulated in the plant. From the root compartment, ~28% of total C and ~22% of N are accumulated in the 0-10 cm layer, while ~8 and ~6% are in the 10-30 cm layer, respectively.

The total C accumulation was 10% lower under the inundated plots, compared to non-inundated, regardless of the mix (Figure 7a). Inundation reduced C accumulation in the aboveground (-10%) and in roots at 10-30 cm layer (-24%).

Control and the mix of cool-season perennials had similar C accumulation in aboveground and roots at 0-10 cm depth, but the mix increased it by 40 and 22% in roots at 10-30 cm depth, in inundated and non-inundated, respectively. The mix + annuals reduced C accumulation by 20-40% in aboveground and root compartments in relation to control and mix.

Total N accumulation was not affected by inundation, but it reduced N accumulation in roots at 10-30 cm layer by 17-22% in all mixtures, compared to the respective treatments under non-inundation (Figure 7b). The cool-season mix of perennials increased N accumulation in roots at 10-30 cm depth, by 23-30% compared to control. The mix + annuals reduced N accumulation by 11-22% in aboveground and by 16-21% in roots, compared to control and mix.

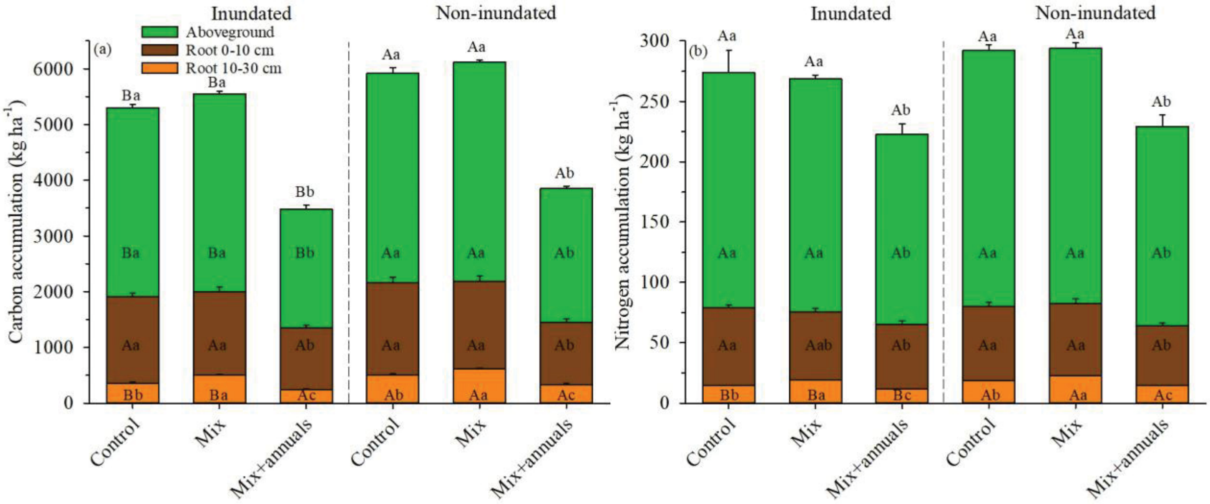

Figure 7. C and N accumulation (kg ha^{-1}) in aboveground and roots as affected by levels of forage species in inundated and non-inundated conditions (average of 2021 and 2022). Uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare the level of species diversification within the same inundation treatment, according to Tukey`s test (p<0.10). Error bars represent the SE (n=3). Jackson-OH.

SOURCE: The author.

2.5.6 Soil bulk density, C and N concentration and stocks

In general, soil bulk density at the beginning of the experiment was not affected by inundation (p<0.10) (Table 7). Soil bulk density was lower (average 0.78 g dm^{-3}) in the top 5 cm of soil and increased to 1.00-1.09 g dm^{-3} up to the 20 cm depth. Soil bulk density also increased to an average of 1.25 g dm^{-3} from 20 to 100 cm depth.

Table 7. Baseline soil bulk density (g dm^{-3}, means ± SE, n=6) affected by long-term natural inundation and non-inundation in predominantly tall fescue grasslands (control plots).

Soil layer (cm)	Inundated	Non-inundated
0-5	0.79±0.01 A	0.77±0.02 B
5-10	1.00±0.01 ^{ns}	1.04±0.02 ^{ns}
10-20	1.06±0.01 B	1.09±0.01 A
20-30	1.17±0.02 ^{ns}	1.22±0.02 ^{ns}
30-50	1.24±0.01 ^{ns}	1.29±0.03 ^{ns}
50-75	1.28±0.01 ^{ns}	1.33±0.01 ^{ns}
75-100	1.27±0.01 B	1.29±0.02 A

LEGEND: Uppercase letters compare inundation effects for the same soil layer according to F test ($p<0.10$); ns: not significant.

SOURCE: The author.

Soil TOC and TN concentrations and their C:N ratio were not affected by inundation ($p<0.10$), except at the 5-10 cm soil layer, when inundation reduced TOC concentrations by 15% compared to the non-inundated (Table 8). TOC and TN concentrations were higher in the topsoil layers, with 47.5 and 4.0 g kg^{-1} soil respectively, decreasing to 2.4 and 0.3 g kg^{-1} soil respectively, at the 75-100 cm layer. TOC and TN stocks were not affected by inundation, so that the 87.4 Mg C ha^{-1} stored to 1 m depth in inundated soil did not differ from the 93.8 Mg C ha^{-1} stored in non-inundated soil.

Table 8. Baseline soil TOC and TN concentration, stocks and C:N ratio (means ± SE, n=6) affected by long-term natural inundation and non-inundation in tall fescue grasslands (control plots).

Layer (cm)	Inundated	Non-inundated	Inundated	Non-inundated	Inundated	Non-inundated
	TOC (g kg^{-1})		*TN (g kg^{-1})*		*C:N ratio*	
0-5	46.4±2.0^{ns}	48.5±2.9	4.2±0.1 ^{ns}	3.9±0.2	11.1±0.4 ^{ns}	12.4±0.3
5-10	26.4±0.6 B	31.0±1.6 A	2.6±0.1 ^{ns}	2.8±0.1	10.1±0.2 B	11.0±0.3 A
10-20	14.3±0.4 ^{ns}	16.1±0.7	1.4±0.1 ^{ns}	1.5±0.1	9.9±0.3 ^{ns}	10.4±0.2
20-30	9.3±0.5 ^{ns}	7.7±0.2	1.0±0.1 ^{ns}	0.8±0.1	9.1±0.4 ^{ns}	9.8±0.9
30-50	4.9±0.4 ^{ns}	4.9±0.2	0.6±0.1 ^{ns}	0.6±0.0	8.8±0.3 ^{ns}	8.7±0.3
50-75	2.8±0.1 ^{ns}	3.5±0.5	0.4±0.0 ^{ns}	0.4±0.1	8.0±0.2 ^{ns}	8.5±0.2
75-100	2.4±0.0 ^{ns}	2.4±0.1	0.4±0.0 ^{ns}	0.3±0.0	6.7±0.2 ^{ns}	8.0±0.9
	TOC stock (Mg ha^{-1})		*TN stock (Mg ha^{-1})*			
0-10	31.9±0.9 ^{ns}	34.9±1.8	3.0±0.0 ^{ns}	3.0±0.1		
0-30	58.4±1.2 ^{ns}	61.7±2.6	5.8±0.0 ^{ns}	5.6±0.3		
0-100	87.4±1.8 ^{ns}	93.8±2.0	9.5±0.2 ^{ns}	9.5±0.5		

LEGEND: Uppercase letters compare inundation effects for the same soil layer according to F test ($p<0.10$); ns: not significant.

SOURCE: The author.

2.5.7 Soil particulate organic matter

Inundation reduced ($p<0.10$) the mass of POM+sand in all mixtures by 41.6 and 44.4% compared to non-inundated at 0-5 and 5-10 cm depth, respectively (Table 9). However, C and N concentration in POM+sand were higher ($p<0.10$) in the inundate areas for both layers. C concentration for each layer averaged 47.2 and 28.9 g C kg^{-1} fraction, being 45 and 62% higher than those under non-inundated, respectively. N concentration followed a similar pattern, being 50 and 71% higher than concentrations in non-inundated area at 0-5 and 5-10 cm depth respectively. However, C-POM stocks at 0-5 cm were 15% lower ($p<0.10$) under inundated conditions compared to non-inundated (3.9 Mg C ha^{-1}), regardless of the forage mixture. Under inundation, the cool-season mix treatment also resulted in lower C stock at 5-10 cm and lower N stocks at 0-5 cm depth when compared to non-inundate. The different forage mixtures did not affected POM+sand mass, or its C and N concentration ($p<0.10$). However, control and cool-season mix resulted in 28% higher C higher N stocks than the mix+annuals at 5-10 cm layer.

Table 9. Soil carbon and nitrogen concentrations and stocks (means ± SE, n=6) in the POM+sand fraction after 2 years as affected by levels of forage species in inundation and non-inundation conditions. Jackson-OH.

	Inundated			Non-inundated		
	Control	Mix	Mix+annuals	Control	Mix	Mix+annuals
Mass of POM+sand (% of soil)						
0-5 cm	18.7±1.5 Ba	17.8±1.2 Ba	19.4±1.6 Ba	29.5±2.2 Aa	32.6±3.9 Aa	33.7±1.8 Aa
5-10 cm	18.7±1.4 Ba	18.4±1.0 Ba	17.7±1.8 Ba	30.3±2.4 Aa	33.4±5.1 Aa	34.9±2.6 Aa
C concentration (g kg^{-1} fraction*) in POM +sand*						
0-5 cm	49.3±.4.3 Aa	46.1±3.1 Aa	46.1±3.5 Aa	35.3±1.3 Ba	32.1±4.0 Ba	30.2±4.0 Ba
5-10 cm	28.1±1.8 Aa	29.6±2.8 Aa	29.2±2.9 Aa	20.0±2.6 Ba	19.5±1.6 Ba	13.8±1.8 Ba
C stock (Mg ha^{-1}) in POM+sand						
0-5 cm	3.4±0.2 Ba	3.1±0.1 Ba	3.4±0.3 Ba	3.9±0.2 Aa	4.0±0.5 Aa	3.9±0.5 Aa
5-10 cm	2.7±0.1 Ba	2.8±0.2 Ba	2.6±0.1 Aa	3.0±0.2 Aab	3.4±0.4 Aa	2.5±0.3 Ab
N concentration (g kg^{-1} fraction) *in POM +sand*						
0-5 cm	3.9±0.4 Aa	3.4±0.2 Aa	3.6±0.3 Aa	2.5±0.1 Ba	2.3±0.3 Ba	2.4±0.3 Ba
5-10 cm	2.3±0.2 Aa	2.4±0.3 Aa	2.4±0.2 Aa	1.6±0.2 Ba	1.5±0.2 Ba	1.1±0.1 Ba
N stock (Mg ha^{-1}) in POM+sand						
0-5 cm	0.27±0.02 Aa	0.23±0.01 Ba	0.27±0.02 Aa	0.28±0.01 Aa	0.29±0.04 Aa	0.31±0.03 Aa
5-10 cm	0.22±0.01 Aa	0.22±0.02 Aa	0.21±0.01 Aa	0.24±0.02 Aab	0.25±0.03 Aa	0.19±0.01 Ab

LEGEND: Soil sand+POM fraction was obtained by the physical fractioning of organic matter according to Cambardella and Elliott (1993). Uppercase letters compare inundation effects for the same level of species diversification, while lowercase letters compare levels of species diversification within the same inundation treatment, according to Tukey`s test (p<0.10); ns: not significant.

SOURCE: The author.

2.6 DISCUSSION

2.6.1 Effects of inundation and mixtures on forage yield and nutritional value

Natural recurring inundation events reduced forage yield, mainly during the seasons when inundation intensity was higher, in spring and fall of 2021 and spring of 2022. Inundation reduced the proportion of tall-fescue, the predominant species in the pasture, and increased occurrence of weeds, clover and other grasses, which are characterized by occupying a lower canopy stratum and by producing less mass than tall-fescue (DEAK et al., 2007; FRANZLUEBBERS et al., 2013). Due to lack of oxygen in the soil, inundation can cause plants to close stomata, reduce transpiration and net photosynthesis rates, reducing plant C fixation and biomass (PLOSCHUK et al., 2017; SÁNCHEZ-RODRÍGUEZ et al., 2019). Therefore, under inundation, plants of tall-fescue can have a reduced tillering and leaf elongation (LIU et al., 2017), creating space for other species and weeds to grow. DEAK et al. (2007) also observed a reduction of tall-fescue proportion and increase on clover and weeds in the mixture in years with high rainfall in Northeast USA.

Surprisingly, the cool-season mixture of perennial species did not increase yield or nutritive value of forage relative to tall-fescue control, regardless of inundation. Our expectation was that the diverse mix would increase seasonal yields, mainly during spring inundation, when the use of shallow-rooted species, such as bluegrass and clover, that are better adapted to high sub-soil moisture can perform better (CASLER et al., 2020; SHEAFFER et al., 2020), but we did not observe that in our study. This can be related to the dominance of tall-fescue in the canopy, which can reduce positive effects from other species growing in the mixture (SKINNER and DELL, 2016). Other studies evaluating complex forage mixtures containing tall-fescue also showed that this species tended to dominate and outcompete other forages due to its fast and taller growing habit that shaded less competitive species (DEAK et al., 2007; SKINNER et al., 2006).

When winter annuals were introduced to the cool-season perennial mix in our study, forage yield gains were observed during spring 2021, but declined after that. This treatment had a higher forage nutritional value and N concentration in 2022, probably as result of clover dominance in the stands, which provides additional N to the system (HUSSE et al., 2017; PIRHOFER-WALZL et al., 2012). Other studies had shown that when the proportion of legume increases in a mixture, higher CP are found, but when the proportion of grasses increases, higher NDF are expected(DEAK et al., 2007), which is similar to our 2022 results.

However, despite the increase on forage quality, the inclusion of winter annuals, oats and rye, reduced forage yield by two-fold compared to the control and mix in 2022. The winter annuals had a successful establishment in the fall 2020, when the other cool-season mix were not fully developed yet, resulting in a good plant stand and higher yields in the following spring of 2021. But the successful winter annuals stand undermined the development of the cool-season species which resulted in lower yields in the following summer. In other hand, fall of 2021 was very wet, and the establishment of winter annuals was not successful due to poor germination, resulting in a higher occurrence of other grasses and a dominance of bluegrass and clover in the following spring, characterized by lower mass production compared to tall-fescue (DEAK et al., 2007). Classical research on the effects of inundation in winter cereals showed that plant tillering and aboveground and root mass were decreased, and some plants died due to rotting when inundation occurred in the first weeks after planting (WATSON et al., 1976), which possibly was the case in this study in the fall 2021. Therefore, the introduction of oats and rye to perennial cool-season mixtures in our study presented a risk for forage production, since its establishment is not always successful, and competition among annuals and perennials can decrease yields of both.

2.6.2 Effects of inundation and mixtures on root production

Natural recurring inundation events also reduced root production, in a similar way to the aboveground mass. Root mass in summer 2021 and 2022, which express the effects of inundation from spring to summer, reduced significantly with soil depth. Possibly the elevated water table during spring reduced oxygen concentration in soil and therefore root respiration and ATP synthesis, causing energy deficiency to plants and inhibiting root growth to deeper layers (BAILEY-SERRES and VOESENEK, 2010; TONG et al., 2021). In addition, inundated plants have lower photosynthetic rate and C assimilation, and consequently lower translocation of photoassimilates for root growth (PLOSCHUK et al., 2017). The higher soil moisture at shallow layers will also reduce allocation of photoassimilates to deeper roots for water uptake, since this is not a limiting factor to stimulate root growth (POORTER et al., 2012). JANSEN et al. (2005) also observed reduced root mass and length from different grass forages, including fescues, to simulated inundation, and attributed such effect to the low ability of those species to grow new adventitious roots to overcome low oxygen concentration in the soil.

The lower root mass due to inundation can also be an effect of changes in forage botanical composition. Inundation reduced tall-fescue proportion and increased clover, other grasses and weeds, possibly causing a change in rooting patterns. White clover is characterized by a shallow stoloniferous root system (FRANKOW-LINDBERG and DAHLIN, 2013), while tall-fescue presents deeper fibrous roots (NIE et al., 2008; SKINNER and COMAS, 2010). This difference in rooting depth and architecture is evidenced when we observed similar root mass at 0-5 cm, but lower mass at 5-30 cm layers under inundated compared to non-inundated conditions.

When the mixture of cool-season perennial forages was established in the area prone to inundation, there was a higher root mass than the tall-fescue monoculture, mainly deeper in the soil profile. Despite of not increasing forage yields throughout the seasons, this mixture showed a better response in root development to contrasting environmental events, with higher root mass in the summer seasons, after the high inundation occurrence in the preceding months, but also higher root mass in the fall seasons, especially in the second year, which was characterized by a water shortage. Although tall-fescue roots are expected to be predominant in the soil profile, due to higher abundance of fescue in the area and by its more extensive root system (MONCADA et al., 2022; VAN EEKEREN et al., 2010), the presence of other grasses could have contributed to increased total root mass. SKINNER and DELL (2016), also found that mixtures of 5 cool-season perennials led to higher root mass during dry seasons, when compared to a binary mixture of orchardgrass and white clover. The authors suggested that the complementary characteristics among the different species, with different rooting patterns lead to a better adaptation to access water in deeper soil profile, increasing the ability of plants to sustain forage production. Evaluating the effects of drought, SKINNER and COMAS (2010) also found that roots of bluegrass and orchardgrass increased in depth when submitted to water deficit. Similarly, SKINNER et al. (2006), found that mixtures with 2, 3 and 11 species had similar root mass on the top 5 cm, but the more diverse systems significantly increased it at 5-60 cm layers, as a strategy to increase water uptake to sustain higher forage yields.

Despite the similarities of our results with the above-mentioned studies conducted under drought conditions, our experimental conditions reflect the opposite during spring (i.e. excess of water). However, it is not clear, based on our evaluations, why the cool-season mixture had a better performance in root mass than the tall-fescue monoculture under high moisture content. Evaluating tall-fescue and orchardgrass in monoculture or growing it in a triple mix of grasses under favorable rainfall conditions, MONCADA et al. (2022) found higher root mass in the mixture at the top 5 cm, but similar to tall-fescue monoculture at deeper layers, the opposite to what we found in our study. Although we did not measured soil moisture at individual plots, we can infer that more diverse systems might have reduced soil moisture at deeper layers probably due to increased transpiration, as observed by FISCHER et al. (2019) and XI et al. (2022), therefore, promoting favorable conditions for roots to grow deeper into the soil.

However, when the degree of species diversity was increased by adding two winter annual forages to the cool-season perennial mix, there was a reduction in root mass, to a similar extent to the reduction in the aboveground mass. As discussed in the previous section, the poor establishment of the annuals, and the dominance of less productive species with shallower root system diminished root mass in the soil profile. Overall, the proportion of root mass in relation to aboveground is constant for the same species, therefore a reduction in aboveground will also proportionally reduce roots (BOLINDER et al., 2007), which corroborates with our results. In addition, the presence of the annual species and a high proportion of clover roots, might have resulted in higher decomposition of roots (ROUMET et al., 2006; SHI et al., 2012), mainly in the summer months when the cycle of annuals finishes. After plant senescence, roots from annual species will decay faster than perennials due to higher concentration of water-soluble C, which are rapidly decomposed by soil microorganisms (SHI et al., 2012). Although different patterns of root decomposition among species are an interesting feature to increase soil C, in our study this was not reliable due to strong reduction in the total root C input to the soil.

2.6.3 Effects of inundation and mixtures on soil particulate organic matter

The effects of long-term natural recurring inundation events reduced the stocks of C-POM at 0-10 cm, although no clear effect on N-POM stocks. POM reflects the most labile soil C pool, which are comprised of root fragments and aboveground litter, still reassembling to plant characteristics (LAVALLEE et al., 2020). Therefore, the reduction on the input of plant material by above and belowground parts of plants will directly affect this pool. Considering that the two years assessed in this study represent the average range of C and N inputs by this grassland ecosystem, we can assume that the low production under inundation conditions at some seasons, over time could have depleted this organic matter pool (FRANZLUEBBERS and STUEDEMANN, 2002). Although, the whole soil C and N stocks were not significantly affected by inundation, which can be explained by the slower organic matter decomposition under lower oxygen concentration on inundated soils (GARCIA-NAVARRO et al., 2018), we can observe a reduction in C concentration in the whole soil, which is an indication of potential stock depletion (FRANZLUEBBERS and STUEDEMANN, 2002). Furthermore, soils under inundation can have a lower stabilization of organic matter, because of the anaerobic conditions that can result in Fe reduction, which release C bound to Fe and increases MOS mineralization (HUANG and HALL, 2017; HUANG et al., 2020). Indeed, mottling patches with greyish and reddish color were observed in the inundated area below 5 cm depth, indicating Fe reduction and possible loss of stabilized C.

After three years since the establishment of mixtures, the stock of C-POM and N-POM was increased by the cool-season mixtures in relation to tall-fescue monoculture, although the effects were only evident in the non-inundated area. The higher input of C and N by this mixture, mainly by the improved root mass, could be the determining factor to increase C-POM and N-POM stocks. A greater plant C input is expected to increase its concentration in POM, but also in more stable mineral-associated fractions (MITCHELL et al., 2018). The fact that this treatment also increased root mass at deeper layers is an indicator of organic matter stabilization, since roots will be better protected from decay within the soil minerals and aggregates (POIRIER et al., 2018; RASSE et al., 2005). Therefore, it is expected that this treatment will increase soil C and N stocks in the long-term due to rapid turnover of roots at shallow layers, and longer residence time at deeper layers (HOUDE et al., 2020). Although under inundation conditions, positive effects of higher belowground input may take longer to occur due to lower residue decomposition rates (GARCIA-NAVARRO et al., 2018). SKINNER and DELL (2016), also found a positive relation between root mass and soil C concentrations, mainly at 10-30 cm, when a 5-species mixture was cultivated by 9 years and increased roots and soil C in relation to a binary grass-legume mixture.

In contrast, the addition of winter annuals to the cool-season perennial mix decreased C-POM and N-POM. This is probably related to the lower input and higher decay rate of annuals roots (BOLINDER et al., 2012; HOUDE et al., 2020), during the two years. In addition, the management to establish the mixture, with soil disturbance to drill the annuals could stimulate a decay on soil organic matter (BOLINDER et al., 2012). In addition, the higher proportion of clover in this stand could have increased the available N concentration in the soil, accelerating the decomposition of root and POM (BLECKEN et al., 2022). Therefore, the inclusion of annual forages into perennial mixtures under our conditions represents a risk to forage production and also a risk for the sustainability of the system, causing loss of native soil organic matter.

2.7 CONCLUSIONS

Long-term recurring inundation reduced forage yield by 2-11% and root production by 13-16% on a predominantly tall-fescue pasture, in relation to the non-inundated pasture. Inundation mainly affected the botanical composition of the pasture, by reducing the predominancy of tall-fescue and increasing other grasses and weeds, characterized by a lower yield potential and less aggressive root development. Such reductions caused by inundation in the above and belowground compartments led to reduced soil C concentration and lower C and N stocks in the particulate organic matter fraction.

The inclusion of a cool-season perennial mixture, with tall-fescue, bluegrass, orchardgrass and clover, did not affected forage yields or its nutritive value, in comparison to a predominantly tall-fescue pasture, but it increased root production in the second year by 8-20% and C stocks in the particulate organic matter fraction after three years of establishment, indicating a potential to increase soil C sequestration in pastures prone and not prone to inundation. However, the inclusion of winter annuals, rye and oats, to this perennial mix, did not promote benefits to forage and root production and even reduced its mass two-fold and reduced C and N stocks in the particulate organic matter fraction. Difficulties to establish the annuals due to above normal wetness in fall of 2021, and the competition among species in this mixture, which caused a decline in the development of the perennials after the end of the annuals cycle, increased the occurrence of shallow-rooted weed species in the plots, leading to an overall decline in total root mass and depletion on soil C and N pools.

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GENERAL CONCLUSION

Different levels of forage species diversity under two climates were evaluated in this research during two years, and results showed the potential of more diverse systems to increase forage and root production, but not always increase soil particulate organic matter. Complementary characteristics among the species used, including different seasonal growth and different root architecture and rooting depth, the use of species with potential to biologically fix N and ability to coexist without causing harmful competition were important facts for the success of mixtures.

In subtropical climate, forage mixtures with 3 to 6 warm and cool-season species, including grasses and legumes, resulted in higher forage yields and higher root production over the two years of evaluation. Although more diverse pastures resulted in improvements and higher C and N input to the soil compared to a binary mixture of one warm and one cool season grasses, no changes were observed in the soil particulate organic matter after 2 years. This suggested that more time under increased belowground input is needed to affect soil organic matter pools in more clayey soils with already higher organic matter contents.

In other hand, under temperate climate in a silt loam soil, a diverse mixture of cool-season perennials increased root production and soil particulate organic matter but did not affect forage yields compared to a perennial grass monoculture. This cool-season mixture also presented a better adaptation to the recurring natural inundation events, producing more roots and contributing with higher belowground C and N input. This provides an alternative for management of grasslands prone-to-inundations, which are characterized by lower belowground production and reduced soil organic matter. However, when annuals were included in the perennial's mixtures, establishment was not successful, and weed pressure and competition among species increased, causing reduction in forage yields, roots and soil particulate organic matter.

Overall, forage mixtures can be used to improve resilience of subtropical and temperate grasslands, providing additional forage and increasing soil organic matter. Future studies could evaluate the long-term (more than 5 years) mixtures to provide more accurate recommendations on species combinations that promote increased on productivity and on soil C. Future research could focus on establishment strategies, such as seeding rates, use of heavy grazing, tillage or herbicides before sowing,

sowing in line or broadcasting and stablishing different species in leys instead of mixing together. Finding management strategies that reduce species competition and weed pressure is the way forward to promote successful mixed forage stands.

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SUPPLEMENTARY MATERIAL

This section is comprised of additional information (Supplementary Tables 1 to 5) used in the literature survey presented in the General Introduction section.

The survey focused on publications indexed in Web of Science main collection and Scholar Google database, from 2000-2020 comparing forage yield and root production from grassland monocultures and mixtures. Search terms included: “mix or mixture or intercropping or complementary or diverse” in the first line; “forage or pasture or grass or grassland” in the second line; and “production or yield or biomass or dry matter or aboveground or belowground or root” in the third line. The search retrieved a total of 1,326 publications which were filtered based on some requirements: 1) response variable (either forage yield or root production); 2) treatment with monoculture comparing to a mixture of species; 3) identification of species in each treatment; 4) same management conditions to monoculture and mixtures; 5) field experiment; 6) clear description of experimental replication and how sampling was performed. Only 112 publications were selected for forage production and 7 for root production based on the above criteria and are listed at the end of this section.

Supplementary Table 1. Distribution of studies on forage production according to Köppen climatic classifications comparing forage mixtures to a monoculture.

Köppen climates	Number of studies	Number of observations	Forage yield increase with a mixture of species (%)
Aw	7	64	21.5
BSh	3	38	2.3
BSk	9	161	19.25
BWh	2	40	10.4
BWk	1	6	49.0
Cfa	23	304	24.0
Cfb	19	257	25.6
Csa	21	615	36.9
Csb	1	18	-40.3
Cwa	1	1	53.4
Dfa	4	158	3.7
Dfb	23	781	15.8
Dfc	2	14	-5.1
Dwa	1	2	22.3
Dwb	1	23	-0.4

LEGEND: the sum of studies is higher than 112 because some studies evaluated multiple locations.

SOURCE: The author.

Supplementary Table 2. Distribution of studies on forage production based on the number of species in the mixture.

Number of species in mixture	Number of studies	Number of observations
2	88	1438
3	29	592
4	17	247
5	9	98
6	2	35
7	1	3
9	3	12
10	2	54
11	1	4
16	1	20

LEGEND: the sum of studies is higher than 112 because some studies evaluated multiple treatments with varying number of species.

SOURCE: The author.

Supplementary Table 3. Distribution of studies on root production according to Köppen climatic classifications, comparing forage mixtures to a monoculture.

Köppen climates	Number of studies	Number of observations	Root mass increase with a mixture of species (%)
BSk	1	6	14.3
Cfa	2	11	18.9
Cfb	3	28	71.0
Dwa	1	2	4.6

SOURCE: The author.

Supplementary Table 4. Distribution of studies on root production based on the number of species in the mixture.

Number of species in mixture	Number of studies	Number of observations
2	4	22
3	3	8
4	1	6
5	1	2
8	1	5
11	1	1
16	1	3

LEGEND: the sum of studies is higher than 7 because some studies evaluated multiple treatments with varying number of species

SOURCE: The author.

Supplementary Table 5. Frequency (%) of occurrence of top 40 species in the surveyed studies.

Species	Common name	Family	Cycle	Freq. (%)
Medicago sativa	Alfalfa	Fabaceae	Perennial	12.5
Trifolium pratense	Red-clover	Fabaceae	Perennial	5.9
Lolium perenne	Perennial Ryegrass	Poaceae	Perennial	4.5
Dactylis glomerata	Orchardgrass	Poaceae	Perennial	3.9
Phleum pratense	Timothy	Poaceae	Perennial	3.3
Rapanus sativus	Radish	Brassicaceae	Annual	3.0
Panicum milaceum	Proso millet	Poaceae	Annual	2.9
Schedonorus arundinaceus	Tall-fescue	Poaceae	Perennial	2.9
Vicia sativa	Vetch	Fabaceae	Annual	2.8
Andropogon gerardii	Big blue stem	Poaceae	Perennial	2.7
Agropyron cristatum	Crested wheatgrass	Poaceae	Perennial	2.6
Agropyron intermedium	Intermediate wheatgrass	Poaceae	Perennial	2.6
Avena sativa	Oat	Poaceae	Annual	2.3
Bromus riparius × Bromus inermis	Brome (hybrid)	Poaceae	Perennial	2.1
Trifolium repens	White-clover	Fabaceae	Perennial	2.1
Panicum virgatum	Switchgrass	Poaceae	Perennial	2.0
Triticosecale	Triticale	Poaceae	Annual	1.9
Cynodon dactylon	Bermudagrass	Poaceae	Perennial	1.7
Poa pratensis	Kentucky bluegrass	Poaceae	Perennial	1.6
Cichorium intybus	Chicory	Asteraceae	Perennial	1.5
Lotus corniculatus	Bird’s-foot trefoil	Fabaceae	Perennial	1.5
Paspalum dilatatum	Dallisgrass	Poaceae	Perennial	1.4
Bromus inermis	Smooth brome	Poaceae	Perennial	1.4
Chloris gayana	Rhodes grass	Poaceae	Perennial	1.4
Phalaris arundinacea	Reed canarygrass	Poaceae	Perennial	1.3
Hordeum vulgare	Barley	Poaceae	Annual	1.2
Festuca pratensis	Meadow-fescue	Poaceae	Perennial	1.0
Pennisetum glaucum	Pearl millet	Poaceae	Annual	1.0
Pisum sativum	Pea	Fabaceae	Annual	1.0
Bromus riparius	Meadow brome	Poaceae	Perennial	0.9
Elymus trachycaulus	Slender wheatgrass	Poaceae	Perennial	0.9
Crotalaria juncea	Sunn hemp	Fabaceae	Annual	0.9
Sorghastrum nutans	Indiangrass	Poaceae	Perennial	0.9
Festulolium	Festulolium	Poaceae	Perennial	0.8
Lolium multiflorum	Ryegrass	Poaceae	Annual	0.8
Panicum maximum	Guineagrass	Poaceae	Perennial	0.8
Secale cereale	Rye	Poaceae	Annual	0.7
Trifolium alexandrinum	Egyptian clover	Fabaceae	Annual	0.7
Onobrychis sativa	Sainfoin	Fabaceae	Perennial	0.7
Vicia villosa	Winter vetch	Fabaceae	Annual	0.6
Others (72 species)				15.6

LEGEND: frequency calculated based on the number of observations containing each species in relation to all observations.

SOURCE: The author.

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