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RESEARCH ARTICLE

Yield benefit of tropical grass-legume compared to grass-alone pastures enhanced by biological nitrification inhibition

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Abstract

Background and aims In Amazonia pastures are usually established after deforestation by sowing *Urochloa* grasses. Productivity declines due to nitrogen (N) limitation. Integrating N_2-fixing legumes and grasses with Biological Nitrification Inhibition (BNI) capacity are considered as options for improving pasture sustainability.

Methods We studied grass-alone (GA) and grass–legume (GL) pastures on seven farms in Colombian Amazonia. Each treatment included grass with either low (*Urochloa brizantha*), intermediate (*U. decumbens*), or high (*U. humidicola*) BNI capacity. Biomass yields were measured over six harvests. Total N and $\delta^{15}N$ of grasses and legumes and mineral N in topsoil (0–0.1 m) were analyzed in the first harvest.

Results GL pastures with high BNI *U. humidicola* showed a biomass yield benefit of 1.6 t ha^{-1} over six harvests and 9.3 kg N ha^{-1} higher N yield for the first harvest compared to GA pastures. More than 70% of the legume N was derived from atmosphere, irrespective of *Urochloa* grass present. Plant $\delta^{15}N$ indicated that *Urochloa* grasses obtained 20% less atmospheric N_2 in GL than GA pastures. In contrast to *U. brizantha*, soil mineral N under *U. humidicola* did not increase significantly in GL pastures, indicating that BNI reduced nitrification and enhanced N use efficiency.

Conclusion Under conditions in Amazonia, legumes enhance the yield and N supply when associated with high BNI grass. The importance of different N sources and isotopic fractionation during N uptake

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of *Urochloa* grasses requires further investigation. These results highlight the potential of GL–high BNI systems to support pasture sustainability.

Keywords Tropical pastures · Amazonia · *Urochloa* grasses · Legume · Biological nitrification inhibition · Nitrogen fixation

Abbreviations

GA	Grass-alone
GL	Grass-legume
BNI	Biological nitrification inhibition
Ndfa(%)	Proportion of nitrogen derived from atmosphere

Introduction

Conversion of tropical forest for agriculture persists as a critical ecological threat of our time, with severe implications for global biodiversity and climate change (Armenteras et al. 2013; Carriazo et al. 2020; Hänggli et al. 2023). Colombia has one of the highest deforestation rates in South America (Asner et al. 2004; Wassenaar et al. 2007), with approximately 2.4 million ha of forests converted to other land covers between 1967 and 2012 (Clerici et al. 2019). In the Amazon basin, most of the cleared land has been transformed to pastures for cattle ranching (Asner et al. 2004; Jank et al. 2014; Murillo-Sandoval et al. 2023). The vast majority of pastures have been sown with grass monocultures of the genus *Urochloa* (Jank et al. 2014), formerly known as *Brachiaria* (Cook and Schultze-Kraft 2015), hereafter referred to as *Urochloa* grasses.

Vast areas of tropical pastures are in some stage of degradation (Asner et al. 2004; de Oliveira et al. 2004; de Souza et al. 2020; Mosquera et al. 2012), and in Colombian Amazonia, it is one of the drivers of continued deforestation (Del Río Duque et al. 2022). Pasture degradation is characterized by declining forage biomass yield and nutritional quality, going along with degradation of chemical, physical and biological soil properties (Asner et al. 2004; Boddey et al. 2004; de Oliveira et al. 2004; Fonte et al. 2014; Mosquera et al. 2012; Nesper et al. 2015). Overgrazing and the lack of maintenance fertilization lead to reduced pools of available nitrogen (N) and phosphorus (P) in soil and are the principal causes of pasture degradation (Boddey et al. 2004; Dias-Filho et al. 2001; Neill et al. 1999; Nesper et al. 2015). Farmers in the Amazon region seldom apply organic or/and mineral N fertilizers to pastures, if at all (Boddey et al. 2020; Villegas et al. 2020). Thus, the soil organic matter is the main source of this N, resulting in depletion of available soil N with time. Two complementary strategies have been proposed to mitigate pasture degradation via enhanced plant N supply, which are the introduction of N_2-fixing legumes (Miles et al. 2004), and of grasses with the capacity of Biological Nitrification Inhibition (BNI) (Rao et al. 2014).

Grass-legume (GL) associations are an important alternative to grass-alone (GA) pastures because of the N input from the legume through symbiotic N_2 fixation (Homem et al. 2021; Schultze-Kraft et al. 2018). Under conditions of highly weathered, acidic soils, legumes, e.g., *Arachis pintoi* obtained between 50 and 80% of their N from the atmosphere in researcher-managed (Homem et al. 2021) and farmer-managed (Villegas et al. 2020) pastures. Despite their beneficial effects on N supply and forage quality, which have mostly been demonstrated on research stations or experimental farms, legume adoption by farmers has remained low (Schultze-Kraft et al. 2018). To sustain stable *Urochloa*-legume mixtures, appropriate grazing management is required (Fisher and Kerridge 1996; Schultze-Kraft et al. 2018). Hence, research in farmer managed pastures is needed to evaluate legume integration under low N input conditions that constrain productivity and risk long-term degradation.

Urochloa genotypes have the capacity to inhibit nitrification in soil, which is termed as biological nitrification inhibition, BNI (Subbarao et al. 2009). This BNI capacity is being intensively researched as it holds great promise to reduce N losses in the form of nitrous oxide (N_2O), a potent greenhouse gas, and nitrate (NO_3^-), a water pollutant (Coskun et al. 2017; Rao et al. 2014). For *Urochloa humidicola*, the main responsible substance for BNI capacity has been identified as the root-exuded fusicoccane-type diterpene brachialactone (Subbarao et al. 2009), isomers and derivatives of it (Egenolf et al. 2020). It blocks the enzymatic ammonia monooxygenase and hydroxylamine oxidoreductase pathways of nitrifiers as *Nitrosomonas europaea* (Subbarao et al. 2009). More recently, Egenolf et al. (2023) have identified vanillin from *Urochloa* root exudates as the BNI active compound. The BNI potential has

usually been demonstrated using a soil incubation assay, where soil is amended with a high dose of ammonium sulfate (Karwat et al. 2017; Nuñez et al. 2018). Similarly, reduced abundance of nitrifiers in soils (Nuñez et al. 2018) and reduced nitrate leaching potential (Karwat et al. 2018) from soil under high BNI compared to low BNI capacity grasses have been shown under large amounts of ammonium fertilizer inputs, which are not typical for the growth conditions of *Urochloa* grasses in farmers' pastures (Boddey et al. 2020; Villegas et al. 2020). Further, in soils with low mineral N contents, BNI may reduce N losses by promoting the immobilization of mineralized N by microorganisms (Vázquez et al. 2020). Despite this evidence, the role of BNI under the low-input conditions, typical of smallholder farming systems, and particularly in grass–legume mixtures, remains poorly understood.

Another mechanism of *Urochloa* grasses to improve N nutrition is N_2 fixation. They can obtain 20% to 40% of their total biomass N from the atmosphere through the association with N_2-fixing bacteria (associative and endophytic N_2 fixation by diazotrophic bacteria in the rhizosphere and roots of non-legumes, termed associative N_2 fixation hereafter) (Boddey and Victoria 1986; Reis et al. 2001). A high genetic diversity of diazotrophic bacteria has recently been isolated from the rhizosphere and the roots of twenty *Urochloa* genotypes (Ribeiro et al. 2020). However, the contribution to N supply in low input farmer managed pastures and grass-legume mixtures has not been quantified.

Urochloa humidicola (cultivar Humidicola) is the most widely used grass in Colombian Amazonia, because of its strong stoloniferous growth habit contributing to cover ground rapidly and compete with weeds, of its good adaption to poorly drained soils, and its adaption to low soil N supply and low P and calcium (Ca) requirements (Rao et al. 1996). Other *Urochloa* grasses such as *U. brizantha* and *U. decumbens* are also used, and some pioneer farmers have integrated forage legumes to enhance animal productivity (Mosquera et al. 2012; Villegas et al. 2020). Whilst the high BNI capacity of *U. humidicola* has not been a selection criterion for farmers, its impact on biomass yield, N sources and soil N compared with low BNI grasses, and when grown alone or combined with legumes under low input farmers' conditions is unknown.

To this end, we studied a) the yield of *Urochloa* grasses and of *Urochloa*-legume mixtures during six consecutive regrowth cycles, b) the N yield and N sources of the pasture plants during one regrowth using ^{15}N natural abundance methods (Reis et al. 2001; Unkovich et al. 2008) and c) the mineral N status of the soil in seven cattle farms located in Caquetá Department, Colombia. Our hypotheses were that i) dry matter (DM) yield is higher in high BNI capacity grass-legume mixtures compared to grass-alone and low BNI pastures; ii) symbiotic and associative N_2 fixation is a more important N source in pastures with low BNI grasses (because of higher N losses reducing plant available N), and iii) soil N availability is higher in high BNI grass-legume pastures, because less ammonium is converted to nitrate, and ammonium is retained in the topsoil.

Material and methods

Study region and experimental design

The study was conducted on seven farms within a 70 km radius around Florencia (N 1.615351°, W 75.608735°), the capital of the Caquetá department of Colombia. The region is situated in the Amazonia Piedmont of the eastern Andean mountain range. Cattle ranching (dairy and meat) is a major economic sector of the region and characterized by extensive grazing systems (Torrijos-Rivera 2019). Seventy percent of the farms have between < 10 and 100 animals, and less than 0.2% are considered as large farms (Torrijos-Rivera 2019). The landscape is characterized by pastures and small patches of native forest embedded in an undulating topography. The climate is tropical humid (IDEAM 2023). Annual rainfall is 3,700 mm and mean temperature 26.1 °C (max 31 °C, min 21 °C) (IDEAM 2023). During rainy months (i.e., March-November) daily solar radiation is about 4–5 h and during drier months > 5 h (IDEAM 2023). Precipitation during the six weeks of regrowth cycles of pastures (see below) ranged from 200–800 mm (Fig. S1). The experimental design comprised three different grass treatments with the principal grasses being either *Urochloa brizantha*, or *U. decumbens,* or *U. humidicola*. Each grass treatment was present as monoculture (grass-alone, GA) or combined with a legume (grass-legume, GL), with GA and GL being

denoted as pasture type resulting in six pastures combinations. With participation of the farmers, a total of 18 paired GA and GL plots were identified across seven farms (Fig. 1). A pair denotes two plots on the same farm with the same grass treatment, once present as GA and once as GL. In every plot, always only one principal grass species was present. In total the design included five pairs with *U. brizantha,* four pairs with *U. decumbens* and nine pairs with *U. humidicola*. Each farm contributed at least one pair, with some farms having up to four pairs of different *Urochloa* grasses (Fig. 1).

Based on published reports, the three *Urochloa* grasses have been classified as low (*U. brizantha* cv. Marandú CIAT 6780), intermediate (*U. decumbens* cv. Basilisk, CIAT 606) and high (*U. humidicola*, cv. Humidicola, CIAT 679) in their BNI capacity (Odokonyero et al. 2017; Subbarao et al. 2007, 2006). The term BNI capacity refers to the ability to release BNI compounds from the roots, as well as the amount of these compounds released (Nuñez et al. 2018; Subbarao et al. 2006). Legume species in GL pastures were *Arachis pintoi* cv. Mani Forrajero, CIAT 17434*; Desmodium heterocarpon* subsp. *ovalifolium* cv. Maquenque, CIAT 13651; or *Pueraria phaseoloides* cv. Kudzu, CIAT 9900, with *Arachis pintoi* being the most often present on 10 out of 18 GL plots (Table S3).

According to farmer reports, the selected pastures ranged from 5 to 30 years in age, defined as the time since establishment or renovation by sowing or planting (Table 1). Most plots have received occasional applications of soil amendments such as lime (1 t ha^{-1} per application) or organic fertilizers like compost (1 t ha^{-1} per application) and supermagro, a biofertilizer made from fermented manure, milk, molasses, and rock powder, diluted at a 1:20 ratio with water (Hermann-Bandera et al. 2023) and applied at rates of 800–1000 L ha^{-1}. However, these inputs have been used infrequently. The last application of soil amendments/fertilizers took place at a minimum of half a year before our study started, and 3 to 6.5 years ago on average across treatments. Pasture regrowth time before the experiment started,

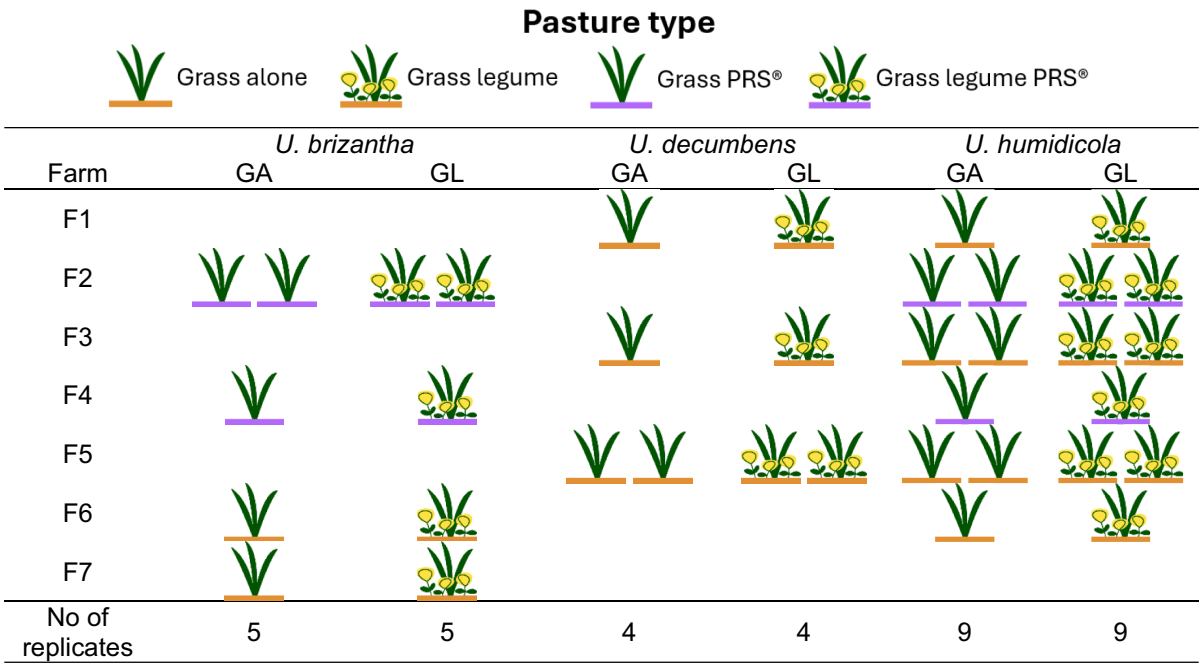


Fig. 1 Distribution of grass treatments (*U. brizantha, U. decumbens, U. humidicola*) and pasture types (grass-alone GA; grass-legume GL) across the seven farms, with the total number of replicates per treatment. Each icon represents one fenced plot located on existing pasture at the farm. Empty cells indicate that no plot of that grass treatment and pasture type combination was present on the corresponding farm. Cells with two icons denote that two plots of the same combination occurred on that farm, installed in different pastures. Purple ground marks plots where PRS® probes were applied

Table 1 Age and management of pastures; numbers in brackets indicate the standard error

Principal grass	Pasture type	System	Pasture age	Fertilized pastures[a]	Amendment/Fertilizer type[b]			Last fertilizer application	Legume management	Regrowth time[c]	Stocking rate[d]
			years	with/without	Supermagro	Organic	Lime	years		days	animals ha^{-1}
U. brizantha	GA	Dairy	19.0 (±2.8)	4/1	1	3	2	3.0 (±1.3)	sown/native	28.5 (±0.4)	0.9 (±0.0)
	GL	Dairy	19.5 (±1.7)	4/1	1	2	3	3.0 (±1.3)	sown/native	28.5 (±0.4)	0.9 (±0.0)
U. decumbens	GA	Dairy + Meat	16.3 (±6.6)	4/0	2	0	2	6.5 (±4.6)	sown	24.8 (±1.9)	0.8 (±0.2)
	GL	Dairy + Meat	11.7 (±5.8)	4/0	2	0	2	3.3 (±1.6)	sown	24.3 (±1.4)	0.8 (±0.2)
U. humidicola	GA	Dairy + Meat	18.8 (±2.6)	7/2	2	0	5	4.9 (±1.9)	sown/native	22.5 (±3.2)	0.8 (±0.1)
	GL	Dairy + Meat	17.0 (±2.3)	7/2	2	0	5	5.4 (±1.8)	sown/native	26.0 (±1.0)	0.8 (±0.1)

a: Number of pastures, i.e., plots with and without fertilization.

b: Number of pastures, i.e., plots with fertilizer type applied. Supermagro is a biofertilizer made from fermented manure, milk, molasses, and rock powder, diluted at a 1:20 ratio with water (Hermann-Bandera et al. 2023) and applied at rates of 800–1000 L per hectare. Organic fertilizer applied was compost.

c: Farm no 4 no data.

d: Animals per ha of total pasture area per farm.

ranged from 20 to 29 days. The stocking rate, i.e., animals per total pasture area per farm ranged from 0.45 to 1.2 animals ha^{-1} across farms, with little difference across treatment means. Pasture regrowth time and stocking rate reflects typical regional practices (Landholm et al. 2019; Motta-Delgado and Ocaña-Martínez 2018; Rivas and Holmann 1999).

Soils in the region have been classified as Acrisols and Cambisols (IGAC 2012). Acricols are characterized by a high level of weathering, high acidity, low cation exchange capacity, low base saturation and high exchangeable aluminum saturation, whereas Cambisols are more fertile because they are less developed and therefore less weathered (IUSS Working Group WRB 2022). The characteristics of the topsoil (0–0.1 m) by pasture type are presented in Table 2. Soil characteristics by grass treatment and pasture type can be found in supplementary Table S2.

Experimental plots, sampling and sample preparation

To investigate pasture productivity and N sources of the plants, a surface of 4 m^2 was fenced within each plot in September 2022, to exclude grazing during the entire study (Fig. S4). For plant height standardization, all plants on the 4 m^2 plots were cut. For yield determination, only the harvest from a central 1 m^2 subplot was considered, to avoid border effects. For the first regrowth, plots with *U. brizantha* and *U. decumbens* were standardized and harvested at 0.2 m above ground, and plots with *U. humidicola* at 0.1 m above ground due to different phenology. After the first harvest *U. brizantha* and *U. decumbens* were also standardized at 0.1 m and for harvests 2 to 6 all plots were harvested at 0.1 m for consistency. Duration of regrowth between height standardization and the following harvest, and between harvests, was 34 to 45 days. Harvested biomass was separated in the botanical fractions of a) principal grass *(U. brizantha, or U. decumbens, or U. humidicola),* b) legume (*Arachis pintoi, or Desmodium ovalifolium, or Pueraria phaseoloides*), c) secondary grass (species such as *Homolepis aturensis, Paspalum* spp.), d) *Cyperaceae,* and e) forbs. For harvests 2 to 6 only the principal grass, and in GL pastures additionally the legume fraction, were separated from the remaining biomass (with the principal grasses und legumes usually being > 90% of total pasture biomass; Table S3). After separation, plant material was air-dried for seven days

Table 2 Average physical and chemical soil characteristics (0–0.1 m) of the fenced plots per pasture type of grass-alone (GA) and grass-legume (GL) association ± standard error. Samples were taken in May 2022

Pasture type	Sand %	Silt %	Clay %	pH[a]	CEC[b] cmol kg^{-1} soil	Al[c] cmol kg^{-1} soil	Total N[d] g N kg^{-1} dry soil	Total C[d] g C kg^{-1} dry soil	C:N ratio[e]	P[f] mg kg^{-1} soil
GA	31.5 (±4.4)	25.9 (±3.4)	42.7 (±2.3)	4.7 (±0.0)	14.7 (±0.9)	2.5 (±0.4)	2.9 (±0.2)	33.5 (±1.7)	11.4 (±0.2)	7.1 (±1.1)
GL	27.0 (±3.9)	28.3 (±3.9)	44.7 (±1.6)	4.6 (±0.1)	13.7 (±0.5)	2.5 (±0.3)	3.0 (±0.1)	32.5 (±0.9)	11.0 (±0.2)	6.2 (±0.8)

a: measured in deionized water 1:1; determined potentiometrically.

b: Cation exchange capacity (CEC) determined by extraction with 1 M ammonium acetate (pH 7.0) and quantified volumetrically by titration with 0.1 M NaOH (ICONTEC 2014; McKean 1993).

c: Exchangeable aluminum extracted with 1 M unbuffered KCl; determined by titration with NaOH after extraction (ICONTEC 2023; McKean 1993).

d: Dry combustion using a NCS elemental analyzer (Vario PYRO cube, Elementar, Germany).

e: Ratio of total C to total N.

f: Bray II extractable phosphorus; analyzed colorimetrically by UV–VIS spectrophotometry (Borrero Tamayo et al. 2017).

prior to DM weight determination. A maximum of 20 g per each botanical fraction of the first harvest was packed and transported to Switzerland. There, a subsample of each fraction was ball-milled for further chemical and isotopic analysis.

To determine soil mineral and total N contents, four soil samples per plot were taken at 21 days after the initial height standardization during the first regrowth from the 0–0.1 m topsoil layer with a soil auger on the experimental plot around the 1 m^2 central area and stored at 4 °C. One to four days after sampling, moist samples were homogenized and sieved at 4 mm for mineral N extraction from field moist soil.

Soil and plant analyses

Mineral N was extracted from 25 g of field moist soil using 125 ml of 2 M KCl according to Davidson et al. (1991) (thereafter termed mineral N batch extraction). The suspension was filtered through Whatman No. 1 filters prior to freezing for storage and transport to Switzerland. Another 20 g of field moist soil was dried at 60 °C to determine the gravimetric water content for dry soil correction of mineral N content and transport to Switzerland for the determination of total bulk soil N and C concentrations and bulk soil $\delta^{15}N$.

To quantify the supply of ammonium (NH_4^+) and nitrate (NO_3^-) in the topsoil during the first regrowth, three pairs of anion and cation exchange resins (PRS® probes; Qian and Schoenau 2005) were installed after plant height standardization in *U. brizantha* and *U. humidicola* GA and GL plots on two farms, resulting in three replicates per combination of each GA and GL pasture treatment (12 plots in total; Fig. 1). The PRS® probes consist of anion or cation exchange resin membranes that are inserted at 0.03–0.09 m soil depths. After 21 days, probes were removed, at the same time as soil sampling for the mineral N batch extraction took place. Probes were washed directly in the field with deionized water, and probes were stored at 4 °C until elution. Three anion and cation probes each were separately pooled per plot by eluting with 17.5 ml of 2 M KCl per probe resulting in 52.5 ml eluent per batch. Elution extracts were frozen prior to analysis.

The NH_4^+ and NO_3^- concentrations in soil extracts and eluents of the PRS® probes were analyzed colorimetrically at 690 nm and 540 nm, respectively (Kandeler

and Gerber 1988; Krom 1980). Soil mineral N contents are reported as NH_4^+-N and NO_3^--N, and total mineral N as sum of NH_4^+-N and NO_3^--N. Milled plant and soil samples were analyzed for total C and N concentrations as well as $^{15}N/^{14}N$ ratio by dry combustion using a NCS elemental analyzer coupled to an Isotope Ratio mass spectrometer (Vario PYRO cube, Elementar, Germany and IsoPrime100 IRMS, Isoprime, United Kingdom) at Eschikon research station of ETH Zurich.

Calculations

Biomass yield has been calculated as sum of dry weight of botanical fractions and reported as g dry matter (DM) m^{-2}.

The N yield per botanical fraction was calculated by multiplying the respective N concentration by harvested biomass per m^2. Total N yield was calculated as sum of the N yield in botanical fractions. Total soil N and soil mineral N contents are consistently indicated per kg dry soil.

Natural abundance values are expressed as $\delta^{15}N$ per mil (‰) ^{15}N excess or depletion over the $^{15}N/^{14}N$ of the air ($R_{air} = 367.6 \times 10^{-5}$) (Shearer and Kohl 1986):

$$\delta^{15}\mathrm{N}(‰) = \frac{\left(^{15}\mathrm{N}/^{14}\mathrm{N}\right)_{\mathrm{sample}} - \left(^{15}\mathrm{N}/^{14}\mathrm{N}\right)_{\mathrm{air}}}{\left(^{15}\mathrm{N}/^{14}\mathrm{N}\right)_{\mathrm{air}}} \times 1000 \quad (1)$$

For calibration, IAEA-N-1 ($\delta^{15}N = 0.4$ ‰), peptone ($\delta^{15}N = +6.7$ ‰), glycine ($\delta^{15}N = +12.2$ ‰) for N was used. Repeated measurement of sulphanilamide internal standard ($\delta^{15}N = -0.8$ ‰) was carried out to correct for instrumental drift. Wheat flour ($\delta^{15}N = +2.2$ ‰) and standard soil ($\delta^{15}N = +5.5$ ‰,) was repeatedly measured to check for matrix effects for plant and soil samples, respectively.

Nitrogen proportion derived from atmosphere Ndfa(%) in legumes and *Urochloa* grasses was calculated as:

$$\mathrm{Ndfa}(\%) = \frac{\delta^{15}N_{ref} - \delta^{15}N_{fix}}{\delta^{15}N_{ref} - B} \times 100 \quad (2)$$

where $\delta^{15}N_{ref}$ refers to the $\delta^{15}N$ of a non-fixing reference plant depending exclusively on soil mineral N, $\delta^{15}N_{fix}$ is the $\delta^{15}N$ value of the N-fixing legume or *Urochloa* grass shoot, and B refers to isotopic fractionation taking place during N_2 fixation and transport of fixed N from nodules/roots to N-fixing plant shoots (Shearer and Kohl 1986). The B-value for *A. pintoi* of −0.88 ‰ resulted from the mean of three B values from *A. hypogaea*; for *P. phaseoloides* −1.22 ‰ according to Villegas et al. (2020) and −1.3 ‰, an average of values reported for *D. ovalifolium* and *D. intortum* (Unkovich et al. 2008; Yoneyama et al. 1986). For *Urochloa* grasses the lowest measured $\delta^{15}N$ in this dataset was used following the approach adopted in previous studies (Oberson et al. 2013; Reis et al. 2001; Unkovich et al. 2008). The B values were −0.09 ‰ for *U. brizantha*, −1.45 ‰ for *U. decumbens* and −2.01 ‰ for *U. humidicola*.

Cyperaceae plants growing in the same plot (of 1 m^2) were used as reference plants, because their $\delta^{15}N$ was closest to that of total soil N (Fig. 4). If no *Cyperaceae* plant was present a forb growing in the same plot was used as a reference plant.

Statistical analysis

Statistical calculations were performed using R software version 4.0.2 (R Core Team 2023). For the six consecutive harvests, a linear mixed model (LMM) was applied with principal grass (*U. brizantha, U. decumbens, U. humidicola*), pasture type (GA, GL) and harvest as fixed factor and farm and plot ID as random factor. The response variable was total biomass yield. For cumulative yields the same model structure was used, excluding the harvest factor. Prior to plot-wise yield cumulation for statistical analysis, missing values (plot ID 19, 20, 33 and 34 at harvests 5 and 6) were replaced using predictions from the LMM of total biomass yield. These plots were excluded from the biomass analysis because they experienced external disturbances unrelated to the experimental treatments such as animals grazing and termites damaging the sward inside the plot. For visualization only observed data was used: cumulative yield was calculated from treatment-level means by first averaging biomass across plots within each treatment and harvest, and then summing these treatment means across the six harvests. For the first harvest the fixed factors included principal grass, pasture type and botanical fraction, with N yield of different plant fractions as response variables. When $\delta^{15}N$ was the response variable, soil was included among botanical fractions factors. For models with total

N yield, N derived from the atmosphere (Ndfa) via associative N_2 fixation by *Urochloa grasses* and soil N mineral N forms (NO_3^--N, NH_4^+-N, total mineral N) as response variables, the fixed factors were principal grass and pasture type. The significance of fixed effects was evaluated using a Type III analysis of variance (ANOVA). Multiple comparisons between pasture types across principal grass treatments, botanical fractions, mineral N forms and harvests were conducted using estimated marginal means (emmeans), with p-values adjusted using the Sidak method. Significance level was set at $p < 0.05$ and confidence interval at 95%. Significant levels are denoted as *** $p < 0.001$, ** $0.001 < p < 0.01$ and * $0.01 < p < 0.05$. Pearson correlation coefficients were calculated and visualized by corrplots (package Hmisc) with significance levels indicated as above. All figures were generated using the ggplot2 package.

Results

Biomass yields

Biomass yields across the six harvests were often higher in GL than GA pastures with intermediate (*U. decumbens*) and high (*U. humidicola*) BNI grasses (Fig. 2b-c). GL and GA pastures with *U. brizantha*

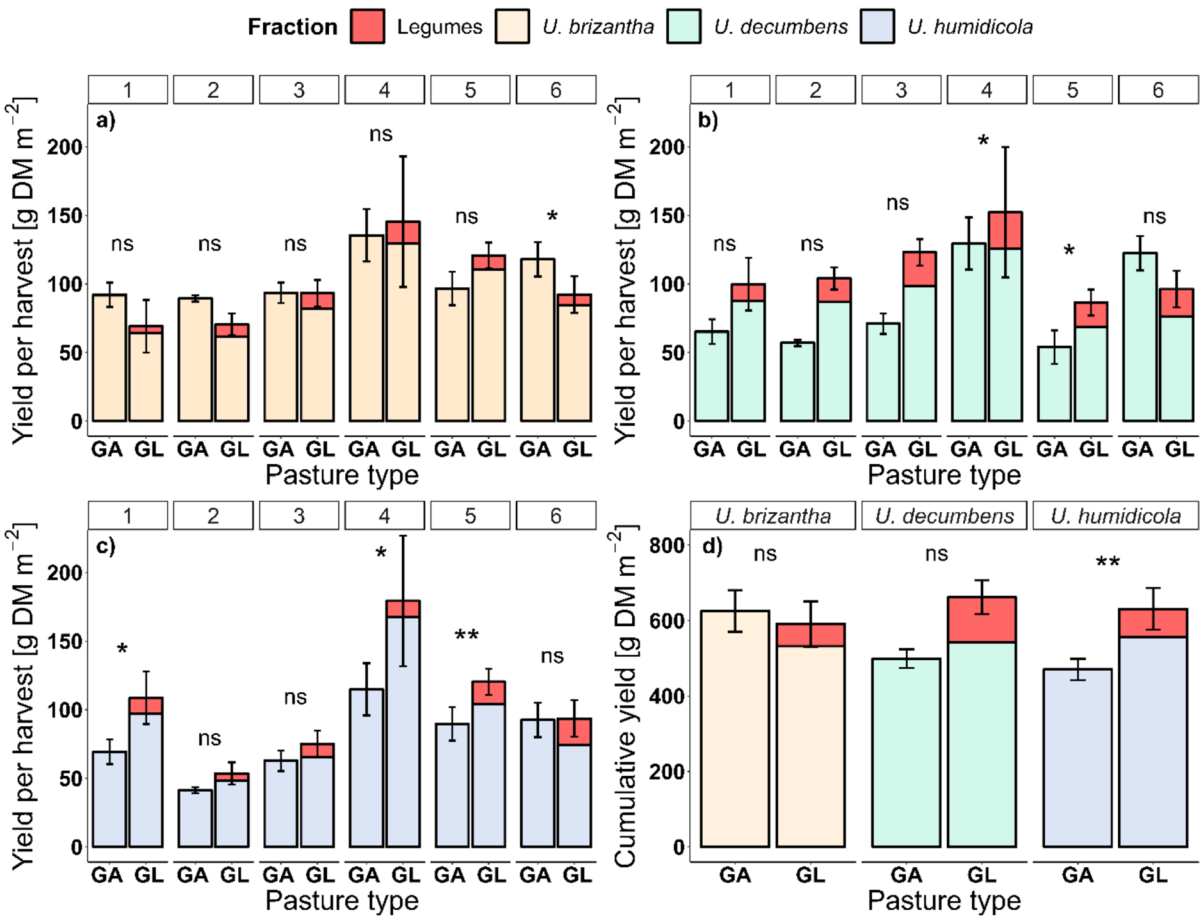


Fig. 2 Mean yield in g dry matter (DM) $m^{-2} \pm$ standard error of grass-alone (GA) and grass-legume (GL) pastures of harvests 1–6 with the same principal grass **a)** *U. brizantha*/low BNI **b)** *U. decumbens*/intermediate BNI **c)** *U. humidicola*/high BNI. Regrowth duration was 34, 43, 41, 45, 41 and 42 days in the order of harvests. **d)** Mean cumulative yield of the six harvests per pasture type. Colors indicate yield per each botanical fraction. Significant differences between pasture types per grass treatment correspond to significant levels $p < 0.001$, $0.001 < p < 0.01$ and $0.01 < p < 0.05$ and are denoted as ***, ** and * respectively. ns = not significant

(low BNI) had similar yields (Fig. 2a). Yields differed significantly across the six harvests ($p < 0.001$, Table S6) but did not align with cumulative precipitation patterns (Fig. S1) indicating other environmental factors influenced seasonal yield variation. A significant interaction between principal grass and pasture type was found ($p < 0.05$). Specifically, mean yield was significantly ($p < 0.05$) higher in GL than GA pastures of intermediate BNI *U. decumbens* for harvest 4 and 5, increasing from 54 to 86 g DM m^{-2} and from 129 to 152 g DM m^{-2}, respectively. For high BNI *U. humidicola* the total yield of GL pastures was also significantly higher in harvests 1, 4 and 5 compared to GA ($p < 0.05$ to $p < 0.01$) ranging from 69 (GA) vs. 109 (GL) at the lower end to 114 (GA) vs. 179 (GL) g DM m^{-2} at the higher end. The mean proportion of legume biomass on yield DM in the six harvests was 7–25% for *U. brizantha*, 10–19% for *U. decumbens* and 9–23%, for *U. humidicola* GL pastures (Fig. S7). Legume biomass had a synergistic effect for the significant increases of GL yields with *U. decumbens* and *U. humidicola* mentioned above, except for harvest 4 with *U. decumbens.*

Higher cumulative forage yields in GL than GA pastures with *U. decumbens* and *U. humidicola* can be attributed to a combination of increased grass and legume biomass (Fig. 2d). Only for *U. humidicola*, cumulative yield was significantly higher in GL than GA ($p < 0.01$), with 470 g DM m^{-2} in GA vs. 630 g DM m^{-2} in GL. With *U. brizantha* GA and GL pastures had similar cumulative yields (GA 625 g DM m^{-2} vs. GL 591 g DM m^{-2}) and the legume substituted the grass biomass. Interestingly, these yields were higher compared to GA pastures with *U. decumbens* and *U. humidicola* (499 g DM m^{-2} and 470 g DM m^{-2}), although not statistically significant. Moreover, they were at a comparable level to the respective GL pastures (662 g DM m^{-2} and 630 g DM m^{-2}).

Plant nitrogen yield

The total mean N yield during the first regrowth cycle was higher for GL than GA pastures, with an increase from 0.8 to 1.7 g N m^{-2} ($p < 0.01$) for high BNI *U. humidicola* and from 0.8 to 1.5 g N m^{-2} for intermediate BNI *U. decumbens* (not significant) (Fig. 3). The additional N yield in GL pastures for *U. decumbens* and *U. humidicola* was synergistic, what led to higher N yield for the principal grass and additionally from the legume. In GL pastures with *U. brizantha* the grass N yield did not increase. As already observed for DM yield among GA pastures, the N yield of *U. brizantha* in GA pastures (1.1 g N m^{-2}) was higher compared to *U. decumbens* and *U. humidicola* as well. However, mean N yield did not increase in GL pastures with *U. brizantha* (1.2 g N m^{-2}).

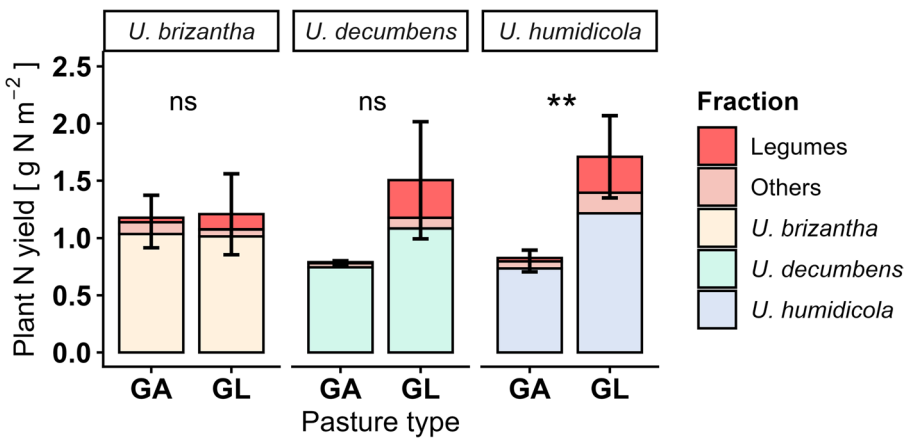


Fig. 3 Mean nitrogen yield in g N $m^{-2} \pm$ standard error by botanical fractions in grass-alone (GA) and grass-legume (GL) pastures with *U. brizantha*/low BNI, *U. decumbens*/intermediate BNI and *U.* humidicola/high BNI as principal grass. Colors indicate N yield of different botanical fractions. Significant differences of total N yield between pasture types per grass treatment correspond to significant levels $p < 0.001$, $0.001 < p < 0.01$ and $0.01 < p < 0.05$ and are denoted as ***, ** and * respectively. ns = not significant

Plant nitrogen sources

δ15N of soil and plant fractions

The mean $\delta^{15}N$ of soil total N varied between + 6.3 and + 8.1 ‰ (Fig. 4). It was significantly higher in soils of GA pastures with *U. humidicola* compared to GL pastures with *U. decumbens* as principal grass. Nitrogen of all plant fractions had lower $\delta^{15}N$ than the soil with lowest values in legumes (overall mean of −0.8 ‰), followed by *Urochloa* grasses (+ 1.2 ‰) and highest in the *Cyperaceae* (overall average of + 5.1 ‰). Pasture type and principal grass hardly affected the $\delta^{15}N$ of plant fractions (Table S6). Only the $\delta^{15}N$ of the plant tissue of secondary grasses in GA pastures with *U. humidicola* (0.0‰) was significantly lower than in pastures with *U. brizantha* as the main grass (GA + 1.7 ‰, GL + 2.4 ‰), and $\delta^{15}N$ in GL *U. humidicola* (+ 1.0 ‰) was also lower than in GL *U. brizantha* (+ 2.4 ‰).

Nitrogen derived from the atmosphere

The proportion of N in legumes derived from the atmosphere (Ndfa) was above 70% regardless of the associated principal grass (Fig. 5a). The estimated proportion of Ndfa of *Urochloa* grasses assuming that their low $\delta^{15}N$ values were due to isotopic dilution by fixed atmospheric N_2 was significantly lower in GL compared to GA pastures ($p < 0.05$, Table S6), with average Ndfa values across principal grasses of 67% (± 11) in GA pastures and 47% (± 15) in GL pastures (Fig. 5b). The Ndfa(%) was highest in GA pastures of *U. brizantha* (79% ± 7) and lowest in GL of *U. decumbens* (40% ± 16).

Nitrogen status of the topsoil

Mineral and total nitrogen

The pasture type had a significant effect on NH_4^+-N, NO_3^--N and total mineral N contents in the topsoil ($p < 0.01$, $p < 0.01$ and $p < 0.001$ respectively, Fig. 6).

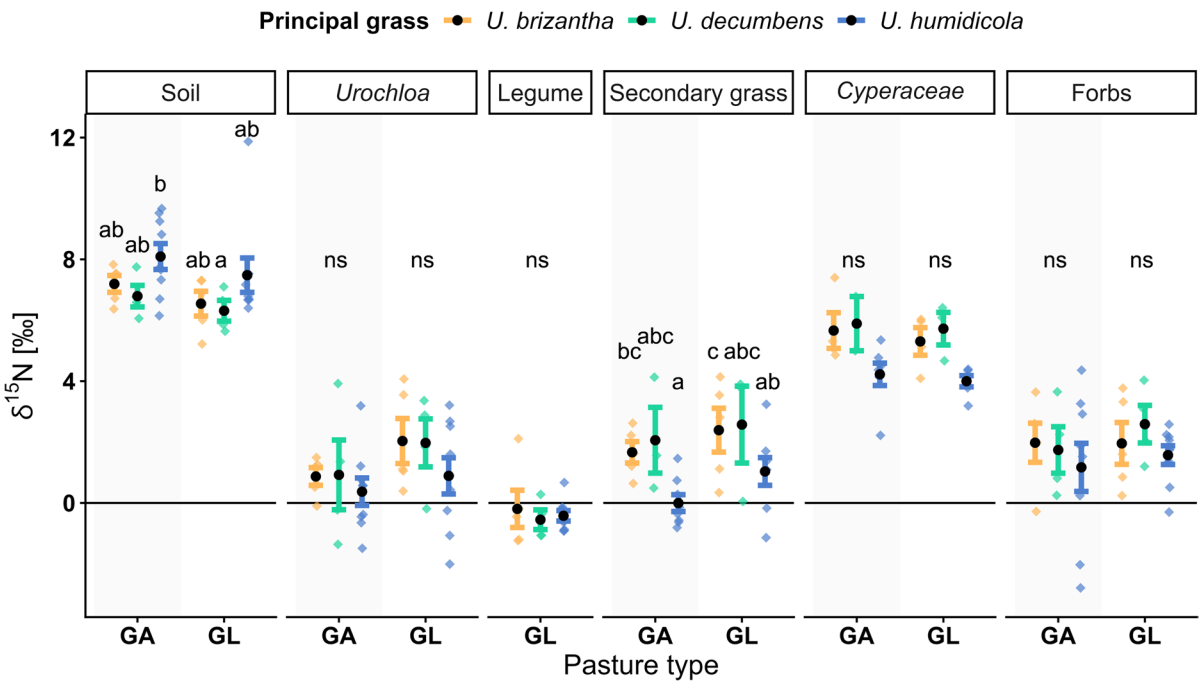


Fig. 4 $\delta^{15}N$ (‰) of total nitrogen of the 0–0.1 m soil layer and of plant N in different botanical fractions in grass-alone (GA) and grass-legume (GL) pastures. Black dot indicates mean ± standard error as colored bar. Colors indicate grass treatment with principal grass species *U. brizantha*/low BNI, *U. decumbens*/intermediate BNI and *U. humidicola*/high BNI. Letters indicate differences within soil or botanical fractions across all six pasture treatments at $p < 0.05$. Plant and soil samples from first harvest (November 2022)

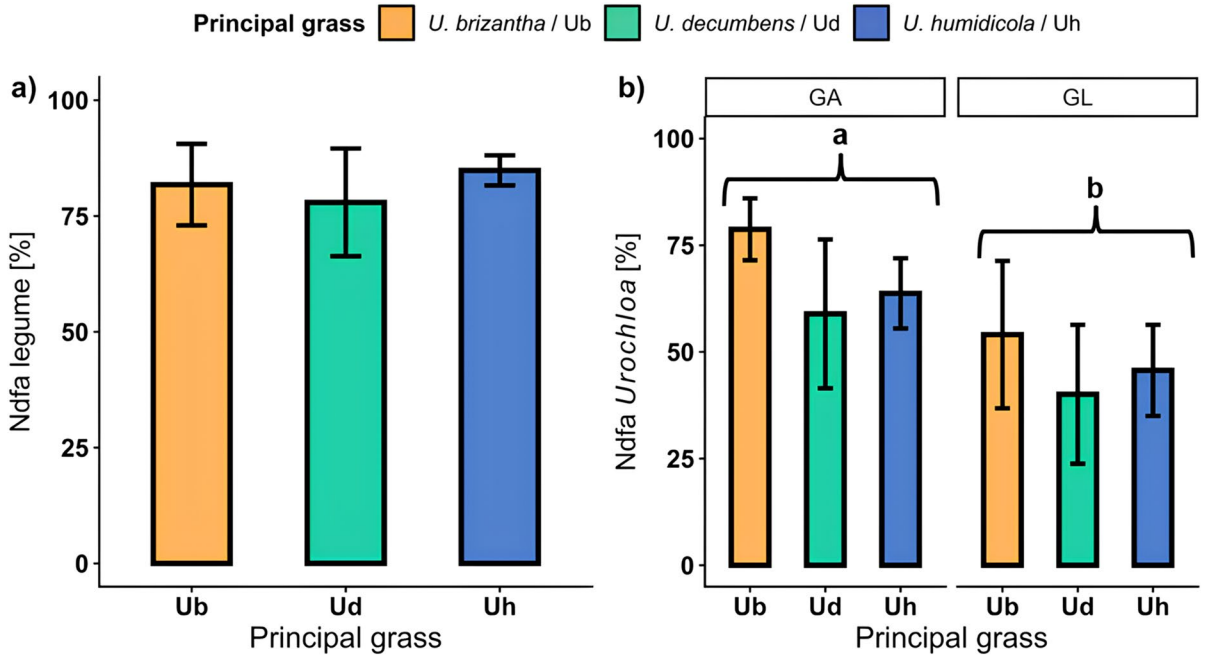


Fig. 5 **a**) Nitrogen derived from the atmosphere Ndfa(%) by the legume associated with each principal grass **b**) Ndfa(%) of the principal grass in grass-alone (GA) and grass-legume (GL) pastures. Colors indicate presence of same principal grass species *U. brizantha*/low BNI, *U. decumbens*/intermediate BNI and *U. humidicola*/high BNI. Letters indicate differences between pasture types at $p < 0.05$

Soil mean value of NH_4^+-N was higher in GL (6.2 mg N kg^{-1} dry soil) compared to GA pastures (4.1 mg N kg^{-1} dry soil) as well as NO_3^--N (2.1 mg N kg^{-1} vs. 0.6 mg N kg^{-1} dry soil) and total mineral N (8.3 mg N kg^{-1} vs. 4.7 mg N kg^{-1} dry soil) (Fig. 6). The mean soil NH_4^+-N content was higher than the NO_3^--N content: 5.2 mg NH_4^+-N kg^{-1} (±0.9) vs. 1.3 mg of NO_3^--N kg^{-1} (±0.5).

When comparing principal grasses, *U. brizantha* (low BNI) showed significantly higher mean NH_4^+-N in the topsoil of GL pastures (6.8 mg N kg^{-1}) compared to *U. humidicola* (high BNI) in GA pastures (3.4 mg N kg^{-1}). Moreover, NO_3^--N and total mineral N contents in topsoil of GL pastures with low and intermediate BNI grasses, *U. brizantha* (2.0 and 8.8 mg N kg^{-1}) and *U. decumbens* (3.4 and 9.6 mg N kg^{-1}), were significantly higher compared to high BNI grass *U. humidicola* (0.4 and 3.7 mg N kg^{-1}). In contrast, for *U. humidicola*, the high-BNI grass, total mineral N content was not significantly greater in GL than in GA pasture soils. Total bulk soil N content in the topsoil was on average around 3 g N kg^{-1} dry soil and did not differ between pasture types and principal grasses.

Soil ammonium and nitrate supply determined by anion and cation exchange membranes

Ammonium was the mineral N form dominating N supply determined by PRS® probes, except in GL pastures with *U. brizantha* (Table 3). Furthermore, the supply of NO_3-N in GL pastures tended to be higher in *U. brizantha* (74.9 µg N 10 cm^{-2} 21 $days^{-1}$) compared to *U. humidicola* (3.0 µg N 10 cm^{-2} 21 $days^{-1}$) due to an outlier (205.3 µg N 10 cm^{-2} 21 $days^{-1}$), resulting in extremely high variability among the three values of that pasture treatment. For GA pastures, almost no differences were evident between the two principal grasses. The N supply increased for all three GL compared to GA pastures except for NH_4^+-N in *U. brizantha* (10.0 in GA to 9.5 µg N 10 cm^{-2} 21 $days^{-1}$ in GL). No statistically significant differences were detected due to high spatial variability and small sample size.

Discussion

Our study addressed the impact of the integration of legumes and grasses with different BNI capacity

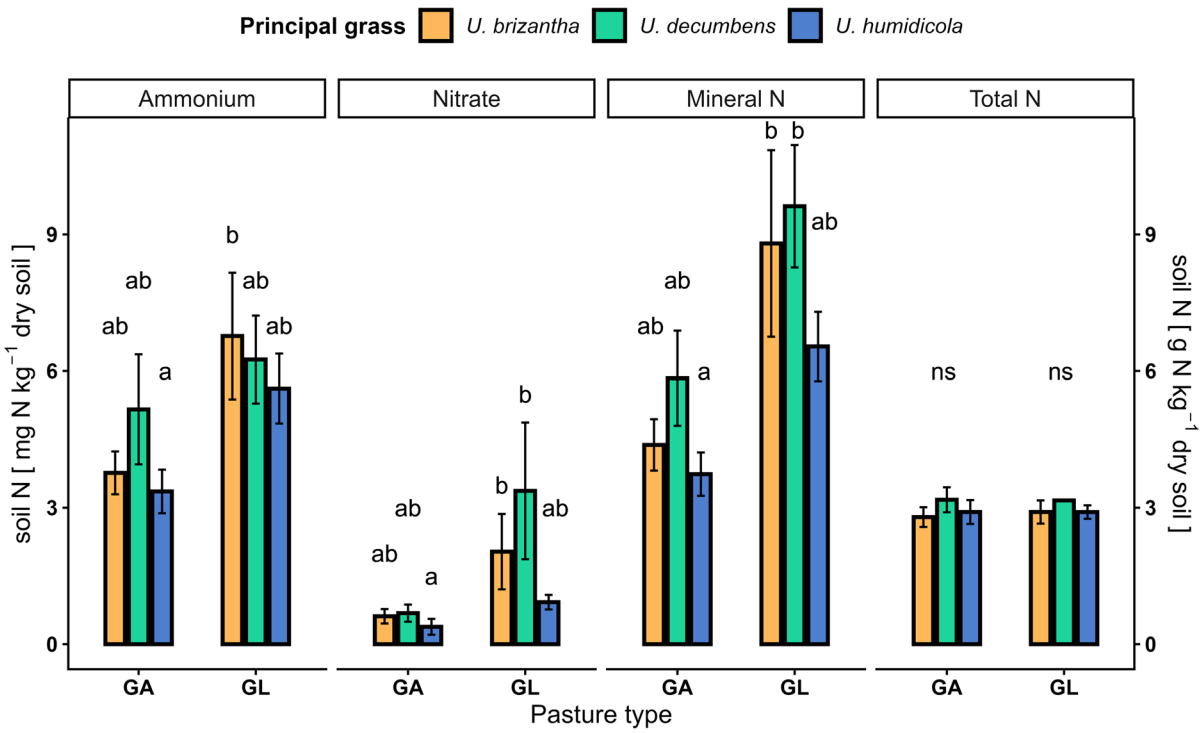


Fig. 6 Mean ammonium (NH_4^+-N), nitrate (NO_3^--N), mineral N (NH_4^+-N + NO_3^--N) in mg N kg^{-1} dry soil, and total N (total soil N) content in the 0–0.1 m topsoil ± standard error in grass-alone (GA) and grass-legume (GL) pastures. Y axis on the right (g N kg^{-1} dry soil) corresponds to total soil N. Colors indicate grass treatments with principal grass species *U. brizantha*/low BNI, *U. decumbens*/intermediate BNI and *U. humidicola*/high BNI. Letters indicate differences within soil mineral N fractions and total N across the six pasture treatments at $p < 0.05$. Three distinct statistical models were applied, with NH_4^+-N, NO_3^--N, and mineral N as response variable, respectively (Table S6)

on biomass yield, N yield and N sources of the pasture plants, and soil N under conditions of pioneer farmers management in Colombian Amazonia. This is a remote region characterized by low external inputs and extensive grazing. Our results confirm a yield benefit by the legume, but only in the presence of the high BNI grass. However, that yield benefit is relatively small, probably because legume proportions in the pasture were less than 20% of plant DM.

Yield benefit from integration of legume with high BNI grass

The biomass yield response of GL compared to GA pastures was affected by the grass species (Fig. 2). Higher biomass yield in GL mixtures than GA with *U. humidicola* resulted from the additive biomass of the legume, but also from an increase of the grass biomass. Greater yield due to the additive legume biomass has also been reported by previous studies in this region (Villegas et al. 2020).

Mean yields of our study were on average 960 kg DM ha^{-1} per harvest (Fig. 2), and are in the range of yields reported by Villegas et al. (2020) and by Fonte et al. (2014) for productive pastures in the region. However, the yield potential of grass legume-mixtures is higher. For instance, the yields of GL pastures with *U. brizantha* we observed (980 kg DM ha^{-1}) are lower compared to the 1,200–2,000 kg DM ha^{-1} per harvest reported by Pereira et al. (2020) in unfertilized *U. brizantha* grass-legume mixtures and could be the effect of a higher legume share (15–40%) in their GL pastures. Moreover, Homem et al. (2021) reported mean yields of 2,500–3,000 kg DM ha^{-1} in *U. brizantha*–legume mixtures with a legume share of 24–35% and annual fertilization of 22 kg P ha^{-1} and 41 kg potassium (K) ha^{-1}, while Dos Santos

Table 3 Supply of NH_4^+-N and NO_3^--N during regrowth of grass-alone and grass-legume pasture with *U. brizantha* or *U. humidicola* as principal grass

Principal grass	Pasture type	NH_4^+ μg N 10 cm^{-2} 21 $days^{-1}$	NO_3^- μg N 10 cm^{-2} 21 $days^{-1}$
U. brizantha	GA	8.60	0.12
		9.27	2.46
		12.24	0.68
		Ø 10.0 (± 0.9)	**Ø 1.1 (± 0.5)**
	GL	8.79	19
		10.99	0.4
		8.79	205.33
		Ø 9.5 (± 0.6)	**Ø 74.9 (± 50.7)**
U. humidicola	GA	9.94	0.49
		8.02	0.49
		11.28	1.11
		Ø 9.7 (± 0.5)	**Ø 0.7 (± 0.1)**
	GL	13.86	0.92
		8.12	2.77
		19.80	5.42
		Ø 13.9 (± 1.9)	**Ø 3.0 (± 0.8)**

Measurements during 21 days of the first regrowth in November 2022 with 3 replicates per principal grass and pasture type with PRS® probes at 0.03–0.09 m soil depths. Values in bold are mean ± standard error

et al. (2023) also observed higher biomass and N yield in such mixtures with 35% legumes across seasons. In contrast, our study found no increase in N yield for *U. brizantha* under unfertilized conditions what could be explained by the low legume share of 12%. Remarkably, despite the low legume proportions of 14% of DM in pastures with high BNI *U. humidicola*, we observed higher biomass production and N yield in these grass-legume compared to grass-alone pastures. A similar trend was observed for pastures with intermediate BNI *U. decumbens* although not significant, probably due to the smaller sample size of *U. decumbens* (4) compared to *U. humidicola* (9). Comparing the difference in yield between paired GA and GL plots across farms, yield benefits in *U. decumbens* and *U. humidicola* were more consistent (Fig. S9a-c). Annual extrapolation suggested that these GL pastures produced approximately 2.1 t DM ha^{-1} (Fig S9d) more than GA pastures. This contrasts with *U. brizantha* and may be related to the BNI activity of *U. decumbens* (intermediate BNI) and *U. humidicola* (high BNI), which could have enhanced soil N use efficiency in these pastures. Notably, in intermediate and high BNI GL pastures not only the biomass yield increased but also the N yield (Fig. 3) and the N concentrations of plant biomass of the first harvest (Table S10), what are indicators for improved forage quality.

Beyond the effects of legume integration and BNI, overall biomass production in our study was modest, likely constrained by nutrient limitations. With six harvests over 36 weeks, we harvested around 590–660 g m^{-2} dry matter, i.e., around 6 t ha^{-1} across all pasture types. Extrapolated to one year, this would be around 9 t ha^{-1}. This is at the lower end of yields compared to other studies in Colombia where no N was fertilized but lime and macro- and microelement containing mineral fertilizers have been applied (Bastidas et al. 2024; Villegas et al. 2023). In contrast, no mineral fertilization and little amounts of soil amendments/fertilizers were applied by the farmers in the present study (Table 1). Hence, other factors that N might have limited biomass production under current management and soil conditions. Phosphorus availability could also be a growth-limiting factor. P concentrations in *Urochloa* grasses sampled by Villegas et al. (2020) in the same region were on average 1.1–1.3 g P kg^{-1} DM and close to the critical threshold of 0.8–1 g P kg^{-1} DM (Rao et al. 1996).

In addition to nutrient availability meteorological conditions can constrain plant growth. Villegas et al. (2020) assume, that frequent waterlogging of soil due to heavy rains and cloudiness can limit yields in the

humid climate of the forest margins, as compared to areas with drier climate. In contrast, the temporal yield pattern of our study does not follow precipitation as it has been reported by Montenegro et al. (1999). At the same time, the Amazon region faces increasing climate stress through rising temperatures and prolonged droughts (IPCC, 2021). Hofer et al. (2017) demonstrated increased resistance and resilience of grass–legume mixtures compared to monocultures in a temperate climate through complementary drought responses of legumes and grasses. These mechanisms may also apply in tropical pastures, offering a practical way to enhance sustainability and adaptation to changing climatic conditions. Identifying the factors that limit pasture yield, and that can be overcome by management, is essential for optimizing sustainable use and maintaining productivity of GL–high BNI systems.

Symbiotic nitrogen fixation is not affected by BNI activity due to nitrogen scarcity

Contrary to our hypothesis, the symbiotic N_2 fixation of the legume was not affected by the BNI capacity of the associated grass. Overall, the Ndfa(%) was high, with more than 70% of legume N derived from the atmosphere. Earlier studies found similarly high Ndfa(%) values for tropical herbaceous legumes growing in association with grasses (Cadisch et al. 1989; Dos Santos et al. 2023; Villegas et al. 2020). This indicates a high level of competition for mineral N and/or low soil N availability (Nyfeler et al. 2011; Trannin et al. 2000). The high Ndfa(%) also underlines the good adaptation of the legumes used by the farmers, which mostly was *A. pintoi*, to the acidic soils (Rao et al. 1995). Nyfeler et al. (2011) reported that in temperate systems, Ndfa(%) in legumes significantly decreased when the legume dry matter yield exceeded 60% of total yield. Hence, increasing legume share up to 26% at plot level reported in this study did not affect Ndfa(%) significantly (correlation not shown). At the same time, the proportion of legumes was relatively low, with an average of 14% across the six harvests (Fig. S7).

The extrapolated annual net N input by symbiotic N_2 fixation was 18, 31 and 24 kg N ha^{-1} y^{-1} in pastures with *U. brizantha*, *U. decumbens* and *U. humidicola,* respectively and below the 110 kg N ha^{-1} reported by Homem et al. (2021) at a legume proportion of 24–35%. A legume proportion with a range from 20 to 45% of total pasture DM has been estimated to cover the N requirement of pasture growth in the tropics (Thomas 1992) and a more recent study suggested a legume proportion of 26–30% considering optimum radiation use efficiency (Caram et al. 2025). Despite the low legume proportion, N yield in *U. decumbens* and *U. humidicola* biomass of the first harvest was higher in GL than GA pastures by 3.5 and 5 kg N ha^{-1}, respectively, which corresponds to an estimated annual increase of 16 and 32 kg N ha^{-1} y^{-1}. This points to a greater N offer for the grasses, via transfer of legume N resulting from the mineralization of below and above ground legume residues (Trannin et al. 2000), and not to a reduced competition for soil N, since N yields of the grass fraction was larger in the mixtures than in the pure stands. This is also reflected in the high N fixation by legumes observed in this study (Fig. 5a), indicating minimal N competition for soil N with *Urochloa* grasses. However, it was challenging to find pastures with high legume cover in this study, suggesting it may be difficult to sustain legume proportions above 20% in practice. Nevertheless, evidence from temperate grasslands indicates that substantial benefits can occur at legume proportions below the theoretical optimum (O'Malley et al. 2026). This is consistent with our observations in *U. humidicola* GL pastures and highlights the capacity of grass–legume mixtures to enhance N supply in unfertilized systems.

High BNI grass *U. humidicola* suppresses an increase in soil mineral nitrogen pool

The higher soil NH_4^+-N and mineral N contents we observed in GL compared to GA pastures suggest increased net mineralization in GL soil (Fig. 6). This can be attributed to mineralization of legume litter, which has a higher N concentration than grass litter (Gijsman et al. 1997; Kohmann et al. 2019; Trannin et al. 2000). Remarkably, a significant increase in mineral N forms was observed only when comparing *U. humidicola* GA with *U. brizantha* and *U. decumbens* GL pastures. This suggests that the BNI activity of *U. humidicola* reduced nitrification (Karwat et al. 2017; Nuñez et al. 2018; Subbarao et al. 2009) and/or stimulated microbial immobilization of NH_4^+, a mechanism reported under low N availability by Vázquez et al. (2020). Whether microbial

immobilization acts as a short-term N sink remains uncertain and requires further research on microbial N turnover in soils under grasses with BNI capacity. Reduced nitrification could potentially reduce N losses via denitrification and NO_3^- leaching (Karwat et al. 2018). However, PRS® probes measurements and extracted mineral N were dominated by NH_4^+ in all pastures (Fig. 6, Table 3), suggesting that ammonification exceeded nitrification regardless of the grass's BNI capacity. Nevertheless, the higher N yield observed in pastures with *U. humidicola* indicates an improved nitrogen use efficiency enhanced by BNI capacity.

In GL pastures with low-BNI *U. brizantha*, soil mineral N increased by + 3 mg NH_4^+-N and + 1.4 mg NO_3^--N relative to *U. brizantha* GA pastures without a corresponding significant increase in plant N uptake. In contrast, with high BNI *U. humidicola* soil mineral N also increased (+ 2.3 mg NH_4^+-N and + 0.5 mg NO_3^--N) with corresponding increase in plant N uptake (+ 0.9 g N m^{-2}). Although higher soil mineral N could increase potential N losses, the absolute concentrations remain within the typical range of unfertilized tropical pastures (Vázquez et al. 2020). Nevertheless, loss pathways (e.g., nitrification, denitrification, leaching) and their magnitude in GL pastures with grasses differing in BNI capacity should be quantified in future research.

Compared to temperate grasslands and degraded pastures, the topsoil mineral N content with average values from 3 to 9 mg N kg^{-1} are low and similar to contents under mixed grass-legume swards receiving little mineral N fertilizer under the pedo-climatic conditions of an experiment at a Swiss mid-land research station (Nyfeler et al., 2024). At the same time, the total N content of the topsoil was with 3 g N kg^{-1} similar to contents reported for the 0–0.1 m topsoil of a range of grasslands in the temperate zone, though at the lower end, and similar to extensive grassland site investigated by Liebisch et al. (2013). Degraded pastures in Brazil showed N concentrations between 0.9–3.0 g N kg^{-1} soil (Neill et al. 1997; Verchot et al. 1999), and around 2 g N kg^{-1} in degraded pastures in our study region (Fonte et al. 2014). Soil organic matter, and hence organic N is largely limited to the top 0.1 m of the soil, as revealed by soil profile descriptions (not shown) and typical for the Acrisols at the research sites. Thus, whilst overall at similar levels to fertile temperate grassland soils, the limited soil volume containing total and available N may limit N supply to plants. Thus, biological N_2 fixation, together with the inhibitory effects of BNI-active grasses on soil N losses, plays a key role in maintaining and enhancing soil N fertility under these conditions. However, despite the greater N yield (Fig. 3), more available soil N under GL (Fig. 6) implies increased N availability and an extra N offer not used by the plants, suggesting other factors that N might limit plant N yield.

Associative nitrogen fixation of *Urochloa* decreases with increasing soil nitrogen availability

Although previous reports showed that *Urochloa* grasses can obtain atmospheric N via associative N_2 fixation (Boddey and Victoria 1986; Reis et al. 2001; Thiengo et al. 2024), we were surprised by finding $\delta^{15}N$ in their shoot biomass (overall average of + 1.04 ± 0.26 ‰, Fig. 4) by around 6 ‰ lower than those of total soil N. The association of *Urochloa* grasses with diazotrophic bacteria has been shown by Ribeiro et al. (2020) for all three *Urochloa* grasses included in our study. In *Urochloa* grasses sampled by Villegas et al. (2020) in pastures on farms in the same region, the $\delta^{15}N$ was + 4.5 ± 3.1 ‰ in GA and + 3.8 ± 2.9 ‰ in GL (mean ± standard deviation). Preliminary analyses of the $\delta^{15}N$ of NH_4^+ from the 2M KCl mineral N batch extraction using the diffusion method from Goerges and Dittert (1998) suggested that NH_4^+, which is the dominating mineral N form in the soil (Fig. 6), has $\delta^{15}N$ values from + 6.0 to + 8.3 ‰, with little difference to the $\delta^{15}N$ of total soil N (data not shown). Therefore, we interpret the low $\delta^{15}N$ of the grass biomass to result from associative N_2 fixation. This contrasts with Karwat et al. (2018), who suggested that low $\delta^{15}N$ in *Urochloa* reflects reduced N losses. However, in their study, plant $\delta^{15}N$ ranged from + 3.8 to + 6.7 ‰ and the pastures were fertilized with 70 kg N ha^{-1} whilst pastures of our study received no N fertilizers recently. Thus, we can assume that soil N would have been a more important source in their study, leading to higher plant tissue $\delta^{15}N$. Low $\delta^{15}N$ values have also been interpreted as indicator of low N availability to plants (Craine et al. 2009; McLauchlan et al. 2007). Therefore, considering these differences in soil N status between Karwat et al. (2018) and our work, we

interpret the low $\delta^{15}N$ in the grass biomass of our study as an indicator of high associative N_2 fixation rather than reduced N losses.

Compared to earlier studies (compilation in Chalk (1991)), we obtained relatively high proportions of Ndfa(%) in the grasses (Fig. 5b). Because we used the lowest grass species specific $\delta^{15}N$ as B value (see Material and Method section), the Ndfa(%) values are likely somewhat overestimated because with that approach, the Ndfa(%) of one replicate per grass species is set to 100%. On the other hand, two replicates were set to 0% Ndfa due to values below realistic limits. Earlier studies reported Ndfa(%) values of 30–40% for *U. humidicola* and *U. decumbens* (Boddey and Victoria 1986), 2.7–26.2% (Reis et al. 2001) and 21% in *U. brizantha* in the low N treatment by Thiengo et al. (2024). Inoculated *U. brizantha* grown in N-free medium in the greenhouse has grown well and accumulated a significant amount of N compared to the seed N reserve, strongly supporting the significant contribution of associative N_2 fixation to plant N (D. Villegas, unpublished). Nevertheless, we recommend not interpreting absolute values and limiting the focus to the relative effects of pasture type on Ndfa(%).

Our results suggest that the Ndfa(%) of *Urochloa* grasses was significantly affected by pasture type and was lower in GL than GA pastures and this is likely due to a greater N availability in soils under GL pastures. A lower C:N ratio of the grass tissue (Table S10) has been interpreted as an indicator of higher soil N availability (Heyburn et al. 2017). We observed a positive relationship between Ndfa(%) in grasses and their tissue C:N (Fig. 7). However, in our study the $\delta^{15}N$ of *Cyperaceae*, which we used as a reference plant under the assumption that it relies solely on soil N did not differ between GA and GL pastures. Moreover, we analyzed only the first harvest for isotopic composition, which is known to vary across regrowth cycles (Dos Santos et al. 2023). We agree with Giller et al. (2024) that isotopic composition alone is not sufficient to conclude on the contribution of associative N_2 fixation. Other factors such as colonization by arbuscular mycorrhiza, which has been shown for different *Urochloa* grasses (Teutscherova et al. 2019), could also influence $\delta^{15}N$ (Chen et al. 2025; Craine et al. 2009; Hobbie and Högberg 2012). Complementary techniques, such as ^{15}N tracer studies and molecular analyses of diazotroph communities, could provide more robust quantification of different N sources and clarify N cycling under BNI-legume systems in future research.

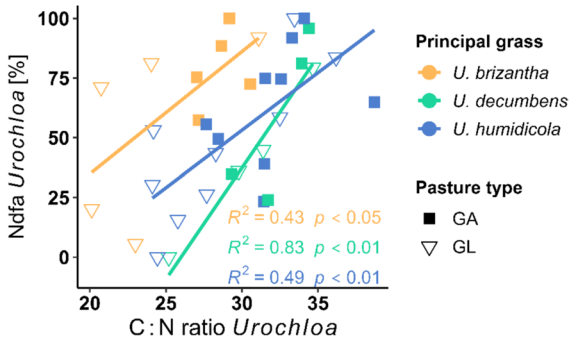


Fig. 7 Nitrogen derived from the atmosphere Ndfa(%) of *Urochloa* grasses and their plant tissue C:N ratio values

Conclusion

We showed a yield benefit of grass-legume mixtures with the high BNI grass *U. humidicola* compared to grass-alone in farmer-managed pastures. Contrary to our expectation, symbiotic N_2 fixation of legumes was not affected by BNI capacity of the grass. Low $\delta^{15}N$ values of *Urochloa* grasses suggested significant associative N_2 fixation, which decreased in GL pastures compared to GA due to increased soil N availability. Although a substantial amount of N entered the system through both associative and symbiotic N_2 fixation, overall productivity remained limited. Greater N yield and reduced soil mineral N in high BNI GL pastures suggest that BNI both suppressed nitrification and enhanced N use efficiency.

Given the low legume proportions and the lack of regular fertilizer applications observed in this study, further research should examine the effects of increasing legume abundance (20–40%) in combination with intermediate- and high-BNI grasses on biomass productivity and N yield. Such studies should also identify potential co-limitations, particularly P availability, as well as potential risks of increased losses such as N_2O emissions and NO_3^- leaching. Addressing these uncertainties is critical for evaluating the true sustainability of BNI-based grass–legume systems.

Beyond biophysical constraints, our results underscore the importance of addressing farmer

adoption barriers and the lack of maintenance fertilization. Participatory approaches that combine technical innovation with local knowledge will be essential to promote the uptake of these systems. Integrating legumes with high-BNI grasses offers a promising strategy to improve nutrient cycling and resilience of tropical pastures. Our findings provide a practical approach to increase pasture sustainability, helping to mitigate deforestation, while enhancing resilience to enable adaptation to changing climatic conditions in the Amazon region.

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Jacobo Arango: Writing – review & editing, Funding acquisition.

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Idupulapati Rao: Writing – review & editing.

Eduardo Vázquez: Writing – review & editing.

Jaime-Enrique Velásquez: Writing – review & editing, Supervision, Funding acquisition, Resources.

Daniel M. Villegas: Writing – review & editing.

Astrid Oberson: Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Resources.

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Data availability The datasets generated and analyzed during this study are openly available from the ETH Zurich Research Collection: https://doi.org/10.3929/ethz-c-000784914.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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