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Urochloa Grasses Swap Nitrogen Source When Grown in Association with Legumes in Tropical Pastures

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Abstract: The degradation of tropical pastures sown with introduced grasses (e.g., *Urochloa* spp.) has dramatic environmental and economic consequences in Latin America. Nitrogen (N) limitation to plant growth contributes to pasture degradation. The introduction of legumes in association with grasses has been proposed as a strategy to improve N supply via symbiotic N_2 fixation, but the fixed N input and N benefits for associated grasses have hardly been determined in farmers' pastures. We have carried out on-farm research in ten paired plots of grass-alone (GA) vs. grass-legume (GL) pastures. Measurements included soil properties, pasture productivity, and sources of plant N uptake using ^{15}N isotope natural abundance methods. The integration of legumes increased pasture biomass production by about 74%, while N uptake was improved by two-fold. The legumes derived about 80% of their N via symbiotic N_2 fixation. The isotopic signature of N of grasses in GA vs. GL pastures suggested that sources of grass N are affected by sward composition. Low values of $\delta^{15}N$ found in some grasses in GA pastures indicate that they depend, to some extent, on N from non-symbiotic N_2 fixation, while $\delta^{15}N$ signatures of grasses in GL pastures pointed to N transfer to grass from the associated legume. The role of different soil–plant processes such as biological nitrification inhibition (BNI), non-symbiotic N_2 fixation by GA pastures and legume–N transfer to grasses in GL pastures need to be further studied to provide a more comprehensive understanding of N sources supporting the growth of grasses in tropical pastures.

Keywords: biological nitrogen fixation; nitrogen concentration; nitrogen transfer; ^{15}N natural abundance

1. Introduction

Deforestation in the tropics has been estimated at about 0.74 million km^2 from 2000 to 2012 [1]. Nearly half of it occurred in the South American rainforest [1]. In the Amazon basin, most of the cleared land has been converted to pastures sown with introduced grasses (mostly *Urochloa* spp., formerly known as *Brachiaria*) for livestock production [2,3]. In Colombia alone, more than 8% of the remaining forest area has been lost since 2000, yielding one of the highest deforestation rates in South America [2,4].

The majority of tropical pastures exist in some stage of degradation [5]. Pasture degradation is understood as a marked reduction in livestock production due to a significant decrease in forage

yield and nutritional quality, the invasion of non-palatable plant species leading to bare soil patches (thus increasing susceptibility to erosion), soil compaction, acidification and reduced soil microbial biomass [6–8]. This phenomenon has tremendous economic and ecological implications, as it leaves large areas of degraded land and promotes a trend of continuing deforestation [9]. As an example of economic implications, in Brazil, every year, about 8 million hectares of degraded pastures require considerable investment for renewal and/or recovery [3], with an estimated annual cost of USD 100 to 200 ha^{-1}, i.e., around USD 1 billion per year in total at country level [10].

The predominant soils of the deforested area in the Colombian Amazon are highly weathered Haplic Ferralsols and Haplic Acrisols [11]. These acid soils typically have low total and available phosphorus (P) contents. Overgrazing and reduced pools of available nitrogen (N) and P in soil are seen as the principal causes of pasture degradation [6,8,12–14]. Thus, grass-legume (GL) associations are an important alternative to grass-alone (GA) pastures because of N input from the legume through symbiotic N_2 fixation [15]. Examples of legume species sown in association with *Urochloa* grasses are *Pueraria phaseoloides*, *Arachis pintoi*, *Desmodium ovalifolium*, *Centrosema* spp. and *Stylosanthes* spp. Despite the aggressive growth of *Urochloa* grasses [15,16], stable *Urochloa*/legume associations are possible where grazing is appropriately managed [17,18]. Provided sufficient P supply, tropical forage legumes obtain at least 70% of their N through symbiotic N_2 fixation [19]. A legume proportion range from 20% to 45% of total pasture dry matter has been estimated to cover the N requirement of pasture growth in the tropics [20], similar to grass-clover swards in temperate climates [21]. GL associations also significantly improve animal (meat and milk) productivity [18,22]. Such improvements have been largely related to the production of more forage biomass (mainly in dry season) and of better quality. Mixed grass-legume diets provide highly digestible protein, less structural carbohydrates and therefore increase animal forage voluntary intake, efficiency of nutrient conversion and live weight gain more than diets based on N fertilized grass monocultures [23–25]. Whilst the agricultural and environmental benefits of integration of legumes in tropical pastures have repeatedly been shown in researcher-managed fields [18], the adoption of legumes by farmers has remained rather small.

In pastoral systems, the quantity and quality of plant litter inputs are crucial for nutrient cycling [26]. In addition to aboveground litter, belowground inputs composed by root and rhizodeposition may enhance nutrient cycling and availability [27]. The belowground N input of clover growing in GL mixtures under temperate climate was around 40% of aboveground N [28] and about 50% of grass N was legume-derived [29]. Grasses growing in association with legumes thus benefit from symbiotically fixed N, with decomposing legume roots most likely being the main transfer pathway [29,30]. Preliminary research work on the determination of $\delta^{15}N$ values of shoot tissue as part of the study on pasture degradation [8] suggests that *Urochloa* spp. have different N acquisition strategies, resulting in N uptake from different sources, when growing alone than when growing with legumes. Indeed, *Urochloa* spp. have a variety of strategies, which could affect the $\delta^{15}N$ signature of their biomass [31]. First, grasses of the genus *Urochloa* can obtain 20% to 40% of their total N in the plant from the atmosphere through the association with N fixing bacteria (i.e., non-symbiotic N_2 fixation) [32,33]. Secondly, *U. humidicola* can suppress soil nitrification by releasing inhibitors from roots [34,35], which affects the $\delta^{15}N$ in plants [31]. Thus, the integration of legumes could advance toward improved N supply to the associated grass, either via the provision of fixed N_2, or via N sparing due to the reduced demand of mineralized soil N to support legume growth.

In temperate GL pastures, grasses and legumes mutually benefit to acquire N from diverse symbiotic and non-symbiotic sources, and transform N into biomass more efficiently than pure grass or legume fields [36]. Nevertheless, in spite of the fact that in tropical grasslands the beneficial effects of GL pastures on livestock productivity and soil fertility have been well studied, the underlying processes such as symbiotic N_2 fixation and N sources exploited by mixed pasture components have rarely been determined under farmers' pasture management conditions.

The objectives of this work carried out under conditions of farmers' practice, at the plot scale were to: (i) estimate the productivity and N uptake of GA vs. GL pastures; (ii) determine the symbiotic

N_2 fixation by legumes; and (iii) evaluate the N sources of grasses growing alone vs. associated with legumes. We hypothesized that legumes would fix significant amounts of N, and that the associated grasses in GL pastures would benefit from the symbiotic N_2 fixation via legume N transfer, and that this legume N transfer would result in lower $\delta^{15}N$ value of grasses in GL than in GA pastures.

2. Materials and Methods

2.1. Study Sites

The study was carried out in farms located in the Caquetá Department of Colombia, within a range of 1°19′13.2″ N to 1°44′37.51″ N, and 75°15′40.69″ W to 75°46′10.4″ W. The area is located in the Amazonia Piedmont of the eastern Andean mountain range in a landscape mostly dominated by degraded pastures. Average annual rainfall is 3758 mm and mean temperature 25.8 °C, with 1570 h of sunshine per year (adapted from IDEAM [37]).

In the study region, the landscape predominates with rolling hills of slopes lower than 25%. The soils originated from sedimentary parent material native of the Amazonic mega-basin [38]. The mineralogical fraction is constituted by gibbsite ($Al(OH)_3$), kaolinite ($Al_2Si_2O_5(OH)_4$), mica and goethite [α-$Fe_3{}^+O(OH)$] [39]. The soils present low natural fertility, with textures ranging from silty clay to sandy clay loam, low base saturation, extreme acidity, and exchangeable aluminum saturation at toxic levels for most field crops [38]. Some soil characteristics of studied plots are provided in Table 1.

Ten paired areas (from 0.22 to 3.5 ha) with grass-alone (GA) and grass-legume (GL) pastures in adjacent plots were identified in six farms. Informal interviews with the farmers indicated that pastures were aged between 16 and 32 years, and were sown using tillage with a disc harrow and applying between 0.2 to 1.0 Mg ha^{-1} of $CaMg(CO_3)_2$ in five of the farms, but no-tillage or liming was used for establishing pastures in the sixth one (E1-2, Supplementary Table S1). Five of the six farmers have repeated liming since the establishment of the pastures, with intervals of several years (e.g., the plots F1-2 received lime six years before sampling). None of the farms received maintenance fertilizers or renovated pastures by re-sowing them. The grazing management of the pastures is under rotation, usually between one to three days of grazing and between 27 to 45 days of rest to permit recovery and growth of the pasture, with a dual-purpose cattle system for milk production in five farms and beef production in one farm. The establishment and management of pastures differ between farms rather than pasture types. However, sometimes more productive animals graze on GL than on GA (e.g., non-lactating cows grazing in GA), and the grazing duration gets adjusted according to forage availability. Introduced grasses evaluated in the farms were *Urochloa humidicola* cv. Tully (CIAT 679), *U. brizantha* cv. Toledo (CIAT 26110), and *U. decumbens* cv. Basilisk (CIAT 606). The associated forage legumes were either *Arachis pintoi* cv. Mani Forrajero (CIAT 17434) or *Pueraria phaseoloides* cv. Kudzu (CIAT 9900). Detailed information about farms management and establishment of pastures, and species found per farm is provided in Supplementary Tables S1 and S2.

Table 1. Soil chemical characteristics (0–10 cm soil depth) of grass-alone and grass-legume pastures sampled on farms in the Caquetá Department of Colombia. Each value represents the mean of ten plots for grass-alone, and of eight plots for grass-legume pastures.

Pasture Type	pH [a]	Total N (mg g Dry Soil^{-1}) [b]	NH_4^+ (mg kg Dry Soil^{-1}) [c]	NO_3^- (mg kg Dry Soil^{-1}) [c]	Total C (mg g Dry Soil^{-1}) [b]	Bray-II P (mg kg Dry Soil^{-1}) [d]	C:N	$\delta^{15}N$ (‰) [b]	$\delta^{13}C$ (‰) [b]
Grass-alone	4.8 ± 0.3	2.9 ± 0.5	4.1 ± 1.0	3.1 ± 2.1	32.4 ± 5.6	1.27 ± 0.5	11.2 ± 0.7	5.9 ± 0.7	−20.6 ± 1.6
Grass-legume	4.8 ± 0.1	2.6 ± 0.7	3.9 ± 2.3	3.4 ± 4.5	28.2 ± 7.4	1.09 ± 0.6	10.6 ± 0.8	6.1 ± 1.2	−21.0 ± 1.6

[a] Measured in deionized water. [b] Total N, C, $\delta^{15}N$, and $\delta^{13}C$ determined by dry combustion using an NCS elemental analyzer coupled to an Isotope Ratio Mass spectrometer (Vario PYRO cube, Elementar, Germany and IsoPrime100 IRMS, Isoprime, United Kingdom) (precision ± 0.2‰), further details are provided in Section 2.3. [c] Mineral N extraction in 1M KCl and quantification of NH_4^+ and NO_3^- following Borrero et al. [40]. [d] Bray-II extractable P [40,41].

2.2. Plant and Soil Sampling

In each pasture, at the end of May 2019, a 25 m^2 plot was fenced to impede animal grazing and deposition of excreta for 45 days before sampling. In mid-July 2019, one sampling circle of 5 m radius was delimited per plot. Topsoil samples (0–10 cm soil depth) were collected using an Eijkelkamp Edelman soil auger in the center of the circle and in other six points that were equally distant to each other in the periphery. Soil subsamples were then mixed and a composite sample per plot was air-dried for 48 h and passed through a 2 mm sieve. A PVC frame of 1 m^2 was placed randomly inside each sampling circle. Shoot biomass in the frames was cut to ground level, and harvested after 45 days of regrowth. This relatively long regrowth period was required, because the period fell into the rainy season, with 356, 473 and 611 mm of precipitation during May, June, and July 2019, respectively [42]. Slow rates of regrowth resulted from both, high levels of precipitation and cloudiness during the day, which may have resulted in lower level of photosynthetically active radiation [43], and it may have been partially influenced by the low cutting level (<5.0 cm) used to homogenize the pasture height before harvest. The harvested biomass was split into four botanical fractions: principal grasses (*Urochloa* spp.), secondary grasses (native/naturalized e.g., *Homolepis aturensis* and/or *Paspalum* spp.), legumes, and forbs. A plant litter composite sample was also collected. Plant litter was defined as dead plant parts lying on the ground including dead and completely dry grass leaves still attached to the shoot and senescent legume leaves. Plant samples were oven-dried at 60 °C for 72 h and their dry matter (DM) weight was determined. A subsample of each fraction was ground using a cutting mill (RETSCH, model SM 100) and pulverized using a home-made ball mill with a SIEMENS engine.

At the beginning of the study, we identified 10 paired GA and GL pasture plots. Nevertheless, possibly due to seasonal changes, at the time of the sampling two GL plots showed legume proportions that were lower than 3% and these two plots were not included to the total number of GL plots. Thus, the final results reported were based on 10 GA and 8 GL plots with an average legume proportion of 35% (10–60%) with respect to the total green biomass DM of the plot.

2.3. Chemical and Isotopic Analysis of Plants and Soil

Plant and soil samples were analyzed for total N and C concentration, $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotopic ratios by dry combustion using an NCS elemental analyzer coupled to an Isotope Ratio Mass spectrometer (Vario PYRO cube, Elementar, Germany and IsoPrime100 IRMS, Isoprime, United Kingdom) at ETH Zurich, Eschikon, Switzerland. The natural ^{15}N abundance values are expressed as $\delta^{15}N$, i.e., per mil (‰) ^{15}N excess or depletion over the $^{15}N/^{14}N$ ratio of the air ($R_{air} = 367.6 \times 10^{-5}$) [44]:

$$\delta^{15}N\ (‰) = \frac{{}^{15}N/{}^{14}N \text{ ratio}_{\text{ sample}} - {}^{15}N/{}^{14}N \text{ ratio}_{\text{ air}}}{{}^{15}N/{}^{14}N \text{ ratio}_{\text{ air}}} \times 1000, \quad (1)$$

The $\delta^{13}C$ is accordingly expressed as ^{13}C excess or depletion over the $^{13}C/^{12}C$ ratio of the international Vienna Pee Dee Belemnite (VPDB) standard ($R_{VPDB} = 1123.75 \times 10^{-5}$). For calibration, we used two international standards, IAEA-N-1 ($\delta^{15}N$ = +0.4‰) and IAEA-N-2 ($\delta^{15}N$ = +20.3‰), peptone ($\delta^{15}N$ = +6.7‰) and glycine ($\delta^{15}N$ = +12.2‰) for nitrogen, and peptone ($\delta^{13}C$ = −15.7‰) and glycine ($\delta^{13}C$ = −33.25‰) for carbon. Correction for instrumental drift was done by repeated measurement of a sulfanilamide internal standard ($\delta^{13}C$ = −28‰, $\delta^{15}N$ = −0.8‰). Repeated measurement of the sulfanilamide standard gave an analytical precision of 0.3‰ for $\delta^{15}N$, and 0.2‰ for $\delta^{13}C$. Calibrated pea grain was repeatedly measured as an internal quality check ($\delta^{13}C$ = −24.7‰, $\delta^{15}N$ = +2.4‰).

The P concentration in the plant tissue was determined after digestion of 0.2 g of pulverized leaf tissue with 2 mL of distilled water and 2 mL of concentrated HNO_3 using a high-pressure single reaction chamber microwave system (turboWave, MWS microwave systems) [45]. The P concentration in the extracts was determined colorimetrically at 610 nm using the malachite green method [46].

Nutrient (N and P) uptake per botanical fraction was calculated by multiplying their nutrient concentration by the biomass production per m^2. Total nutrient uptake was determined by summing the nutrient uptake of each botanical fraction except plant litter in 1 m^2.

The weighted $\delta^{15}N$ of the swards (on a m^2 basis) was determined by applying the formula:

$$\text{Weighted } \delta^{15}\text{N } (‰) = \frac{\left[\left(\delta^{15}\text{N}_{\text{ Pg}} \times \text{N uptake}_{\text{ Pg}}\right)+\left(\delta^{15}\text{N}_{\text{ Sg}} \times \text{N uptake}_{\text{ Sg}}\right)+\left(\delta^{15}\text{N}_{\text{ Leg}} \times \text{N uptake}_{\text{ Leg}}\right)+\left(\delta^{15}\text{N}_{\text{ Forbs}} \times \text{N uptake}_{\text{ Forbs}}\right)\right]}{\text{Total N uptake of the plot}}, \quad (2)$$

where Pg: principal grass, Sg: secondary grass, Leg: legumes, N uptake: N uptake in respective botanical fraction, in g m^{-2}. The weighed $\delta^{13}C$ (‰) was calculated accordingly.

The weighted nutrient concentration of N and P of the total biomass per plot:

$$\text{Weighted nutrient concentration } \left(\text{mg g DM}^{-1}\right) = \frac{\left[\left(\text{NC}_{Pg} \times \text{biomass}_{\text{Pg}}\right)+\left(\text{NC}_{\text{Sg}} \times \text{biomass}_{\text{Sg}}\right)+\left(\text{NC}_{\text{Leg}} \times \text{biomass}_{\text{Leg}}\right)+\left(\text{NC}_{\text{Forbs}} \times \text{biomass}_{\text{Forbs}}\right)\right]}{\text{Total biomass of the plot}} \quad (3)$$

where NC is the nutrient concentrations (N or P, in mg g^{-1}).

2.4. Legume N Derived From the Atmosphere

The proportion of N derived from the atmosphere (%Ndfa, i.e., fixed N) was determined by the ^{15}N natural abundance method [44] and applying the following formula:

$$\text{Ndfa } (\%) = \frac{\delta^{15}\text{N}_{\text{ ref}} - \delta^{15}\text{N}_{\text{ leg}}}{\delta^{15}\text{N}_{\text{ ref}} - \text{B}} \times 100, \quad (4)$$

where δ^{15}Nref: $\delta^{15}N$ signature of the non-fixing reference plant shoots, δ^{15}Nleg: $\delta^{15}N$ signature of the legumes shoots, B: is the $\delta^{15}N$ of *Arachis pintoi* or *Pueraria phaseoloides* shoots relying on atmospheric N_2 as a sole source of N and it accounts for any internal isotopic fractionation of legume plants [47].

For reference plant, we used the forbs growing in the sampling area, i.e., in association with the legume. The $\delta^{15}N$ of non-N_2-fixing reference plant was assumed to be identical to the $\delta^{15}N$ of soil N taken up by the legume [48]. B values used in our study were obtained from previous reports for the legume species studied or the most closely related species of the same genus. For *Pueraria phaseoloides* the B value used was −1.22 [49]. For *Arachis pintoi* we used −0.88, the mean of three values reported for *Arachis hypogea* [49–51].

The amount of N fixed was calculated for each GL plot by multiplying the N uptake in shoot DM of legumes with the respective %Ndfa.

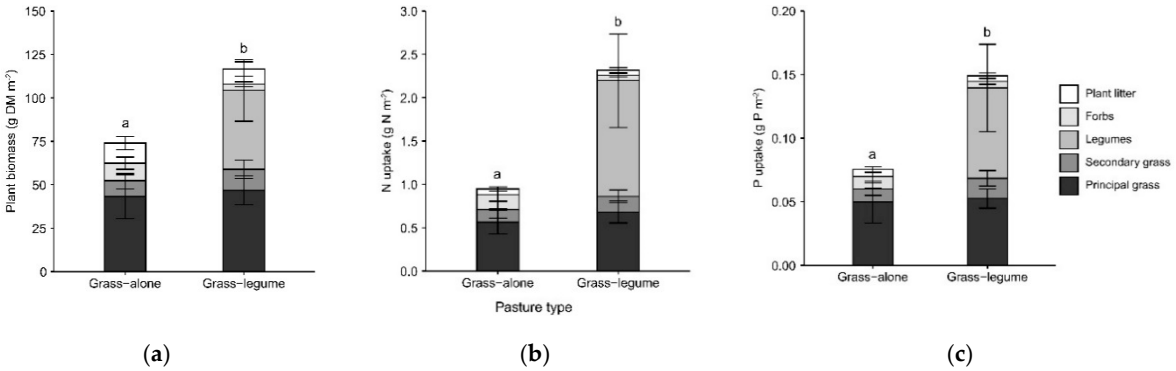
2.5. Data Analysis

Statistical analyses and figures were performed using R v3.4.4. Significant differences between botanical fractions were assessed through a linear mixed-effects model treating pasture type and botanical fraction as fixed factors, and farm as random effect using the packages 'lme4' v1.1-23 and 'nlme' v3.1-147 in R. Multiple comparisons between botanical fractions were evaluated with TukeyHSD tests using 'emmeans' v1.4.8. To evaluate total differences between pasture types, weighted nutrient concentrations and isotopic signatures were calculated per farm, and a second model was built treating pasture type as fixed factor and farm as random effect. Analysis of $\delta^{15}N$ of grass and legume species followed the same model structure as for botanical fractions, considering species and pasture type as fixed factors, and farm as random effect. Differences in %Ndfa between legume species were not statistically tested due to very high differences in sample size (n = 6 for *A. pintoi*, and n = 2 for *P. phaseoloides*). The correlation between $\delta^{15}N$ of grasses and forbs, and the $\delta^{15}N$ of grasses with grass biomass and N concentration were tested with the Pearson's correlation coefficient (r). Figures were constructed using 'ggplot2' v2.2.1.

3. Results

3.1. Dry Matter Productivity and Nutrient Uptake

Grass-legume pastures produced more plant biomass and had greater nutrient (N and P) uptake than GA pastures. Excluding the plant litter fraction, the extent of increase in GL compared to GA swards was up to 74% for shoot DM production (g DM m^{-2}: 62 in GA vs 108 in GL), while it was more than two-fold higher for N uptake (g N m^{-2}: 0.8 in GA vs 2.2 in GL) and P uptake (g P m^{-2}: 0.07 in GA vs. 0.14 in GL) (Figure 1). The proportion of biomass of forbs in the total plant biomass of the pastures was lower in the GL (3% of total DM) than in the GA pastures (16% of total DM).



(**a**) (**b**) (**c**)

Figure 1. (**a**) Shoot plant biomass production (**b**) N uptake and (**c**) P uptake in grass alone and grass-legume pastures for each plant fraction; n = 10 for grass alone and 8 for grass-legume pastures. DM = dry matter. Different letters denote statistical differences for the total plant biomass production, N and P uptake of the sward per pasture type (α = 0.05).

As expected, N concentrations were significantly higher in legume than grass shoots (Table 2). In contrast, P concentrations did not differ significantly between grasses and legumes. Weighted N concentration of GL plant biomass was 18% higher than that of the biomass in GA plots. No difference was observed in weighted plant biomass P concentration between the two pasture types. Therefore, the higher P uptake observed in GL than GA pastures resulted from greater DM production.

Table 2. Nutrient concentrations (N, P, C) and isotopic signatures of N and C in two different pasture types for each botanical fraction. Values are mean ± standard deviation, with $n = 10$ for grass alone, and $n = 8$ for grass-legume pastures. DM = dry matter, ns = not significant.

Pasture Type	Botanical Fraction	N Concentration (g N kg DM^{-1})	P Concentration (g P kg DM^{-1})	C Concentration (g C kg DM^{-1})	C:N	δ^{15}N (‰)	δ^{13}C (‰)
Grass alone	Forbs	18.8 ± 4.1 c	1.3 ± 0.4 b	411.1 ± 5.4 a	22.7 ± 4.7 a	5.3 ± 1.1 d	−25.0 ± 5.1 b
	Principal grass	14.9 ± 3.5 b	1.1 ± 0.3 b	424.0 ± 5.4 b	30.0 ± 7.6 a	4.5 ± 3.1 c	−13.3 ± 0.5 d
	Secondary grass	15.3 ± 5.3 bc	1.4 ± 0.3 b	420.5 ± 8.1 ab	30.5 ± 11.4 a	2.5 ± 3.0 c	−20.9 ± 6.2 c
	Legumes	22.1 ± 4.8 d	1.2 ± 0.1 b	447.9 ± 9.0 c	20.7 ± 4.9 a	−0.6 ± 0.6 a	−31.0 ± 0.0 a
	Plant litter	6.8 ± 1.8 a	0.4 ± 0.1 a	424.8 ± 11.7 b	67.1 ± 20.1 b	1.9 ± 2.3 b	−16.9 ± 3.8 c
	Total *	17.4 ± 3.9 A	1.2 ± 0.3 A	426.2 ± 11.5 A	25.8 ± 7.1 A	4.6 ± 2.9 B	−17.1 ± 3.8 A
Grass legume	Forbs	18.4 ± 3.1 c	1.4 ± 0.4 b	417.4 ± 7.5 ab	23.1 ± 3.7 a	5.7 ± 2.5 d	−25.4 ± 5.3 b
	Principal grass	14.8 ± 3.5 b	1.3 ± 0.5 b	426.9 ± 5.0 b	30.1 ± 6.8 a	3.8 ± 2.9 c	−12.7 ± 0.4 d
	Secondary grass	15.4 ± 2.1 bc	1.4 ± 0.3 b	419.8 ± 5.0 ab	27.6 ± 3.7 a	3.6 ± 3.2 c	−18.0 ± 3.8 c
	Legumes	27.8 ± 3.3 d	1.3 ± 0.3 b	422.1 ± 9.2 ab	15.3 ± 1.8 a	0.4 ± 1.0 a	−29.6 ± 0.3 a
	Plant litter	7.9 ± 1.3 a	0.5 ± 0.2 a	417.1 ± 8.6 ab	53.6 ± 7.3 b	1.1 ± 2.1 b	−16.0 ± 0.9 c
	Total *	20.5 ± 3.2 B	1.2 ± 0.2 A	404.9 ± 46.2 A	20.0 ± 3.3 A	2.1 ± 1.7 A	−19.9 ± 3.5 A
Source of variation **	Pasture type	ns	ns	$p < 0.05$	ns	ns	ns
	Botanical fraction	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	Pasture type x botanical fraction	ns	ns	$p < 0.001$	ns	ns	ns
	Farm (random)	$p < 0.001$	$p < 0.001$	$p < 0.05$	ns	$p < 0.001$	$p < 0.001$

* Calculated values of weighted nutrient concentration or isotopic signature of each botanical fraction (forbs, grasses and legumes only) by their nutrient uptake as in Equations (2) and (3).
** Sources of variation apply only for comparison of botanical fractions. For each variable, different lowercase letters indicate statistical differences of botanical fractions within and between pasture types. Uppercase letters indicate statistical differences for the total (weighted) concentrations between pasture types according to the Tukey HSD test ($\alpha = 0.05$).

3.2. Legume-N derived from the atmosphere

The weighted $\delta^{15}N$ signature of the combined plant biomass of GA pastures was 4.6‰, while it was 2.1‰ for GL pastures (Table 2). The $\delta^{15}N$ signature of forbs was similar to that of soil N, whereas that of principal grasses was on average by 1.4‰ (GA) and 2.3‰ (GL) less enriched than soil N (Tables 1 and 2, Figure 2). Therefore, forbs were considered as more appropriate reference plants to determine Ndfa than grasses.

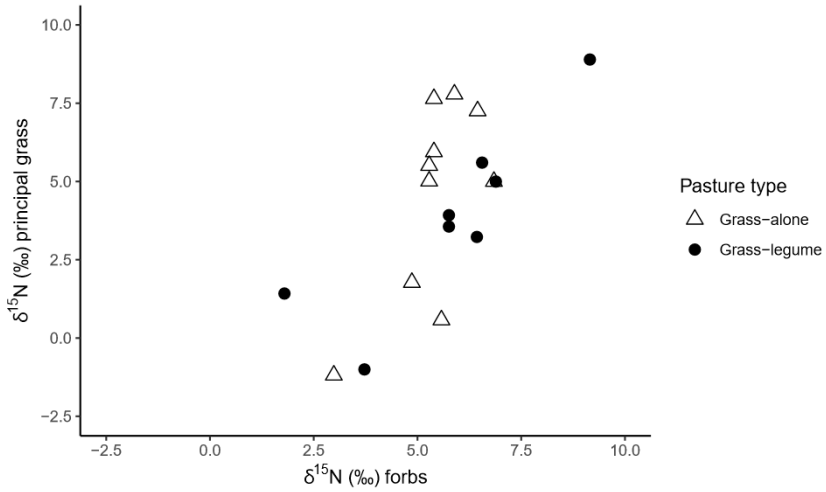


Figure 2. Relationship between shoot $\delta^{15}N$ signature of forbs and principal grasses sampled in grass-alone (open triangles, $n = 10$) and grass-legume (full circles, $n = 8$) pastures. Pearson's correlation coefficient (r) = 0.76, $p < 0.01$.

Arachis pintoi was the legume species found in six out of eight farms, and *P. phaseoloides* occurred in two farms. Average Ndfa derived for GL pastures using Equation (4) ranged from 60% to 99% (average value of 80%, Table 3). *A. pintoi* showed on average 16% higher %Ndfa than *P. phaseoloides*. The amount of N fixed in the biomass of legumes ranged from 0.15 to 3.7 g N m^{-2}.

Table 3. Grass and legume species, average $\delta^{15}N$ signature of shoots and %Ndfa ± standard deviation of the mean observed per species. Ndfa = Nitrogen derived from the atmosphere, ns = not significant.

Pasture Type	Species	$\delta^{15}N$ (‰)	Ndfa (%)
Grass alone	*U. brizantha*	4.7 ± 4.2 ab	-
	U. decumbens	5.8 ± 1.1 b	-
	U. humidicola	3.2 ± 4.2 ab	-
Grass legume	*U. brizantha*	4.9 ± 2.7 ab	-
	U. decumbens	5.0 ± 0.0 ab	-
	U. humidicola	2.0 ± 3.3 ab	-
	A. pintoi	0.4 ± 0.9 a	83.2 ± 14.0
	P. phaseoloides	1.3 ± 0.6 ab	67.5 ± 9.2
Source of variation	Pasture type	ns	-
	Species	$p < 0.05$	-
	Pasture type x species	ns	-
	Farm (random)	ns	-

Different letters indicate statistical differences according to the TukeyHSD test ($\alpha = 0.05$).

3.3. $\delta^{15}N$ and $\delta^{13}C$ Isotopic Signature of Pasture Components

Urochloa humidicola was the principal grass in four out of ten GA plots and three out of eight GL plots, while it was *U. decumbens* in four GA plots and one GL, and *U. brizantha* in two GA and four GL plots (Supplementary Table S2). The $\delta^{15}N$ signature of principal grasses varied widely among

plots of the same type of pasture. In three out of ten plots, the GA-principal grass showed δ^{15}N lower than 2‰, and in two of them (A2 and F1), even lower than the corresponding GL principal grass signature (Supplementary Table S2). The average δ^{15}N signature of shoot tissue of *U. humidicola* tended to be lower than that of *U. decumbens* and *U. brizantha*, however, this difference was not statistically significant (Table 3).

The δ^{15}N of the principal grass was negatively related to the DM production of the principal grass ($r = -0.5$, $p < 0.05$. Figure 3a), and positively related to the N concentration of the principal grass shoot tissue ($r = 0.5$, $p < 0.05$. Figure 3b). However, this latter correlation was stronger in the GA pastures ($r = 0.68$, $p < 0.05$) than in GL ($r = 0.26$, non-significant). At N concentrations higher than 14 mg g^{-1} in the shoot tissue, the δ^{15}N of grasses growing in GL pastures was lower than that of GA pastures by at least 2‰ ($p < 0.01$).

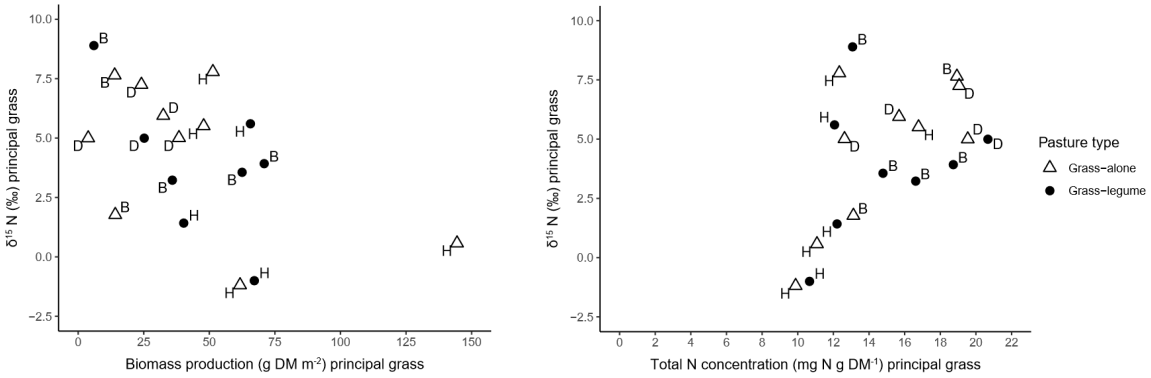


Figure 3. (**a**) Relationship between biomass production and δ^{15}N signature of principal grass shoot, $r = -0.5$, $p < 0.05$. (**b**) Relationship between total N concentration and δ^{15}N signature of principal grass, $r = 0.5$, $p < 0.05$. Open triangles represent grass-alone pastures, $n = 10$. Full circles represent grass-legume pastures, $n = 8$. H = *Urochloa humidicola*, D = *U. decumbens*, B = *U. brizantha*.

The δ^{15}N values of the principal grass and the forbs were closely related ($r = 0.76$, $p < 0.01$. Figure 2). The δ^{15}N value of the principal grass was lower than that of the forbs, on average by 0.8‰ for GA pastures, and by 1.9‰ in GL pastures (Table 2, Figure 2).

The δ^{13}C signature of grasses was significantly higher (less negative) than that of forbs and legumes (Table 2). The type of pasture had no significant effect on δ^{13}C of the botanical fractions and soil organic matter (Tables 1 and 2, respectively).

4. Discussion

4.1. Legumes Improve Pasture Productivity and Nutrient Uptake

The average biomass production of 62 g m^{-2} in GA pastures was in the range obtained by Fonte et al. [26] on farms in the same study region (average values of 101 g m^{-2} for *Urochloa* spp. GA pastures which farmers had characterized as productive, and 47 g m^{-2} for degraded pastures). Moreover, during the rainy season, as in our study and Fonte et al. [26], Gomez et al. [43] harvested from fertilized *Urochloa* spp. GA pastures on average 250 g m^{-2} after 42 days of regrowth of the pasture. Pasture biomass production was reduced by a factor of two to three during the wettest months compared to drier months [43], and June 2019 had a very high amount of precipitation. Lower biomass production during the wettest months was probably due to low soil oxygen levels caused by waterlogging and/or lower rates of net photosynthesis associated with a lower level of photosynthetically active radiation, which may have been driven by higher cloudiness [52].

The pastures containing legumes (i.e., GL) had a significantly higher biomass production (Figure 1a). This is in agreement with earlier results obtained from on-station experiments. For instance, the DM

production of GL pastures composed by *U. decumbens* and *Calopogonium mucunoides* was 1.25 times greater than that of GA pastures containing *U. decumbens* alone in an experiment near Campo Grande, Brazil [53], and DM production was doubled in experiments with *U. decumbens* and *A. pintoi* association, as compared to *U. decumbens* alone in Carimagua, Colombia [17].

Because *Urochloa* grasses obtain atmospheric N_2 via non-symbiotic N_2 fixation [32,33,54], we used the $\delta^{15}N$ of the forbs as an indicator of the $\delta^{15}N$ of available soil N. The strong depletion of $\delta^{15}N$ in legumes compared to the forbs and grasses indicated that legumes largely relied on atmospheric N (Tables 2 and 3) [44]. The average value of 35% legume DM in the total pasture biomass is within the range of 35–45% proposed by Thomas [20] required to maintain a balanced N cycle of tropical GL pastures with a herbage utilization of 50–70% by grazing animals. The proportion of N derived from the atmosphere (%Ndfa) observed with the legumes in this study was about 80% and this value is at the high end of the range reported for tropical legumes [19,55]. This is remarkable because available P concentrations of less than 2 mg kg soil^{-1} are considered as plant growth-limiting for both grasses and legumes [56,57] and could, therefore, limit N_2 fixation of legumes [19].

The amount of N fixed in the shoots of legumes observed in this study was 0.15 to 3.7 g N m^{-2} for 45 days of regrowth. Thus, the average increase in N uptake of 1.4 g m^{-2} in GL pastures than GA pastures was largely due to N_2 fixing ability by the legume (Figure 1b). Because on average around 80% was derived from N_2 fixation, some legume N transfer to grasses might still explain the overall greater N uptake in GL than GA pastures. In temperate GL meadows, about 50% of the grass N was derived from the associated legumes [29]. Trannin et al. [30] suggested that the mineralization of root residues from *Stylosanthes guianensis* was the major source of legume-N transferred to the associated *Urochloa decumbens*. Moreover, greater N deposition through litter has been reported for GL pastures than GA pastures [58].

The P uptake was doubled in GL compared to the GA pasture (Figure 1c), at similarly low available P status of the soil (Table 1). It was reported that legumes such as *A. pintoi* acquire more P from less available P pools from acid soil with its smaller root system than *Urochloa* grass [59]. Thus, the increase of 72 mg P m^{-2} acquired by GL pasture can be attributed to the superior performance of legume towards improved P cycling in the system through soil P pools [60]. According to the farmers' information, the GL pastures are grazed by more productive animals than the GA pastures, and more P may hence be exported via animal products from plots containing legumes, as suggested by Oberson et al. [61] for *Urochloa*-Kudzu pastures. Thus, legumes may take up more soil P and stimulate biological P cycling (through plant litter, animal excreta and microbial turnover) to keep it in available P forms. Although this could increase the risk of soil P mining [62], the strategic application of small amounts of P fertilizer (10 kg ha^{-1} every two years as maintenance fertilizer) may overcome the risk for soil P mining in grazed pastures [63].

4.2. What N Sources are Exploited by Grasses in Each Pasture Type

The $\delta^{15}N$ of the principal grasses, all of which were *Urochloa* spp. grasses was higher than that of the legumes except in two cases (Table 3, Supplementary Table S2). At the same time, their $\delta^{15}N$ was lower than that of the associated forbs (Table 2, Figure 2). This observation indicates that the grasses, on average, were benefiting less from atmospheric N_2 than the legumes, irrespective of the underlying process.

The N concentrations of *Urochloa* spp. grasses observed in our study were higher than previous reports [64], although it was not sufficient to sustain higher plant growth, as indicated by the lower value of biomass production. At N concentrations higher than 14 mg g^{-1}, the $\delta^{15}N$ of the principal grasses of GL was lower than that of GA pastures by at least 2‰. This suggests that legume N transfer was a process involved in the provision of atmospheric N to grasses. Atmospheric N_2 fixation seems to make a significant contribution resulting in low $\delta^{15}N$ values of the *Urochloa* grasses in both GA and GL pastures (Table 3). Still, the contribution from non-symbiotic N_2 fixation in GA may not be adequate to sustain grass growth without the supply of N from the soil through mineralization. This finding

is consistent with earlier reports that suggested no more than 20–40% of N in *Urochloa* grasses was derived from the atmosphere via non-symbiotic N_2 fixation [32,33].

C_4 grasses typically have $\delta^{13}C$ higher than −20‰, whereas C_3 legumes usually have $\delta^{13}C$ lower than −20‰ due to differences in C isotopic fractionation during CO_2 assimilation [65]. In our study, the $\delta^{13}C$ and $\delta^{15}N$ of soil was not statistically different between pasture types, around −20‰ and 6‰, respectively (Tables 1 and 2). While the former forest C_3 vegetation still affects the isotopic composition of soil organic matter C [66], the contribution of legume residues seems to not have been enough to enrich the total soil N pool.

Although the grasses with the lowest $\delta^{15}N$ and N concentration in shoot tissue were mostly *U. humidicola* (Figure 3b), no clear pattern of distribution was observed among *Urochloa* species, either for plant biomass production or N concentration (Figure 3a, b). We consider that the distribution of grass species observed in our study is representative of the pastures in the region. However, to draw valid conclusions on N uptake and utilization at the species level, a more balanced design with an equal number of observations per species will be needed in future research.

Low ^{15}N natural abundance in the shoot tissue of *U. humidicola* has been interpreted as an indicator of high capacity of biological nitrification inhibition (BNI) in that grass [31]. Indeed, in our study, *U. humidicola* showed the lowest $\delta^{15}N$ values, both in GA and GL pastures, but this grass was found to obtain a relatively significant proportion of N through non-symbiotic N_2 fixation [33]. Our results rather suggest that low $\delta^{15}N$ of the grasses is an indicator of low N availability in soil [67], and grasses adapted to N depleted soils can cope with either through BNI ability [31] and/or with non-symbiotic N_2 fixation [33].

5. Conclusions

In farmers' long-term tropical pastures established in the forest margins of Colombia, legumes associated with grasses (GL pastures) resulted in greater pasture biomass production than grass-alone (GA) pastures. Legumes derived on average 80% of their N from symbiotic N_2 fixation, despite low fertility acid soils with low plant-available P content. Legumes significantly increased both N and P uptake by the pasture biomass. The greater N uptake by legumes could be assigned mostly to N fixed from the atmosphere. The $\delta^{15}N$ signatures of grasses in GA vs. GL pastures suggested that sources of grass N are affected by legumes integrated in the pasture. While lower $\delta^{15}N$ values of grasses growing in GL than GA pastures suggest that grasses could obtain fixed N via legume N transfer, exceptionally low$\delta^{15}N$ values of grasses in GA pastures indicate significant potential for N input via non-symbiotic N_2 fixation from the atmosphere. Overall, this study indicates that *Urochloa* grasses are capable to swap N sources when these grasses are grown in association with legumes. The role of different soil-plant processes such as BNI or N_2 fixation from the atmosphere need to be further studied under field and also controlled conditions. This missing knowledge is critical to define the sources of N for grass growth, either in GA or GL pastures in the tropics.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/11/419/s1, Table S1: Establishment and management parameters of ten grass-alone (GA) and eight grass-legume (GL) paired pastures in six farms in the Caquetá Department of Colombia, Table S2: Grass and legume species, $\delta^{15}N$ signature of shoots, and %Ndfa observed per species per farm.

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