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Corresponding author:

Janerson José Coelho;
Email: janersoncoelhozoo@gmail.com

The distance from tree legumes in silvopastoral systems modifies the litter in grass-composed pastures

Amanda Maria Gallindo dos Santos¹, Jose Carlos Batista Dubeux Jr²,
Mércia Virginia Ferreira dos Santos¹, Suellen Brandão de Miranda Costa¹,
Diego de Lima Côelho¹ , Erick Rodrigo da Silva Santos³,
Nalgia Gomes de Miranda e Silva¹, Bárbara Marcélia Martins de Oliveira¹,
Valéria Xavier de Oliveira Apolinário⁴ and Janerson José Coelho⁴ 

¹Universidade Federal Rural de Pernambuco, Recife, PE 55.810-700, Brazil; ²University of Florida, North Florida Research and Education Center, Marianna, FL 32351, USA; ³University of Alberta, Edmonton, AB, CA T6G 2R3, Canada and ⁴Universidade Estadual do Maranhão, São Luís, MA 65.235-000, Brazil

Abstract

The use of silvopastoral systems with tree legumes is a viable alternative to recover and develop pastures, as they add N to the system influencing pasture growth. This study hypothesized that the herbage and litter of signalgrass (*Urochloa decumbens* Stapf) is affected by legume trees in the pasture. Treatments were composed of (1) signalgrass + *Mimosa caesalpiniiifolia* Benth.; (2) signalgrass + *Gliricidia sepium* Jacq.; and (3) signalgrass monoculture. The 3-year experiment followed a randomized complete block design with three replications. Tree legumes were planted in double rows (15 × 1 × 0.5 m), in 1 ha paddocks. Litter samples were taken in five distance points (0, 1.8, 3.7, 5.6 and 7.5 m) perpendicular to tree legume rows. Signalgrass was taller at longer distances from the trees ($P < 0.05$). Signalgrass height differed between treatments, with taller signalgrass found in pastures mixed with *G. sepium* (15.6 cm) compared to *M. caesalpiniiifolia* (9 cm) ($P < 0.05$). Herbage N content decreased with increasing distance from tree rows ($P < 0.05$). Litter N content followed a similar pattern, ranging from 23 g/kg under the trees to 12 g/kg at 7.5 m away from tree rows. Signalgrass did not grow under the tree crown (0–1.8 m), especially when intercropped with *M. caesalpiniiifolia*. The findings of this study suggest that the type of legume trees used in the silvopastoral system has the potential to modify the pattern of grass growth and content of N in pasture litter.

Introduction

Livestock production is mostly based on grasslands, which occupy around 40% of the global terrestrial surface, not including ice-covered lands (Hewins *et al.*, 2018). However, some of these grasslands are in different stages of degradation (Nesper *et al.*, 2015). Among the factors that can cause a decline in grassland productivity, low soil fertility and inefficient pasture management can lead to a series of other factors contributing to pasture and soil degradation (Sattler *et al.*, 2018; Vasques *et al.*, 2019). The extractive form of livestock exploitation has also been causing a series of deleterious effects in grassland areas, impacting its overall productivity and sustainability in many regions of the globe (Fabrice *et al.*, 2015). In Brazil, about 60% of the grasslands are under some stage of degradation (Feltran-Barbieri and Féres, 2021), leading to an urgency in finding different pasture management systems to reduce degradation and improve productivity.

Agricultural systems must maintain their production level over time and avoid the depletion of resources (e.g. soil nutrients, microbial diversity) (Bernardino and Garcia, 2010; Chourasiya *et al.*, 2017). The integration of pastures with trees, especially when the tree species are N₂-fixing legumes, has been considered a viable option to recover degraded pastures (Chaer *et al.*, 2011; Apolinário *et al.*, 2015; Dubeux *et al.*, 2017). In the long term, tree legumes can provide an increase in the production of N-rich litter, contributing to the sustainability of grass pastures (Xavier *et al.*, 2011). The deposition of the litter might influence the grass surrounding the trees due to the dynamics of litter decomposition and nutrient release.

The presence of trees and shrubs on pasture can affect grass development, primarily due to excessive shading and, in some cases, due to the competition for water and nutrients caused by arboreal-shrub species to the herbaceous stratum (Costa *et al.*, 2016). Regarding deciduous tree species, litter deposition acts as an important mechanism for nutrient return in many ecosystems. Litter is an effective pathway of nutrient return, affecting the physical-chemical-biological conditions of the soil (Machado *et al.*, 2012). In addition, the introduction of tree legumes might provide other beneficial effects, such as improving animal diet quality,

providing shade to the animals, and adding N to the system, contributing to improved production (Caldas *et al.*, 2009; Apolinário *et al.*, 2016; Dubeux *et al.*, 2017; Santos *et al.*, 2020; Gomes da Silva *et al.*, 2021; Lebrazi and Fikri-Benbrahim, 2022). Understanding how different types of legume trees, their disposition and distance in silvopasture system affect grass growth and nutrient cycling (e.g. N) can help to develop better management strategies for fertilization, tree pruning and grazing.

We hypothesized that the herbage and litter of signalgrass (*Urochloa decumbens* Stapf) are affected by the integration of the pasture with legume trees. The objective of this study was to assess the impact of integrating signalgrass with different types of legume trees, namely sabiá (*Mimosa caesalpinifolia* Benth.) and gliricidia (*Gliricidia sepium* Jacq.) on herbage mass, litter mass and N content, and soil organic matter (SOM) content in different silvopasture systems in the sub-humid tropics. Also, the effects of distance from trees on grass litter production, herbage mass and quality were examined.

Material and methods

Site description

The experiment was carried out at IPA – Itambé Research Station located in the coastal region of Pernambuco State, Brazil. Itambé is located in the Forest Zone of Pernambuco, approximately 190 m asl, with an average annual rainfall of 1200 mm, and an average annual temperature of 25°C. The average annual relative humidity of the air is 80%, and the local climate is defined as warm-humid rainy tropical with a dry summer. Rainfall has a unimodal distribution with most rains occurring from May to September (Fig. 1). The predominant soil in Itambé-PE is classified as Red-Yellow Argisol, according to FAO-WRB, or Oxisol according to Soil Taxonomy EMBRAPA (Santos *et al.*, 2018).

Experimental design, planting, management

The experiment was carried out from September 2013 to July 2015. Treatments were: (1) *U. decumbens* (signalgrass) + *M. caesalpinifolia* (sabiá); (2) *U. decumbens* + *G. sepium* (gliricidia). Experimental units were 1 ha paddocks, arranged in a randomized complete block design with three replications. In 2011, tree legumes were planted in double rows (15.0 × 1.0 × 0.5 m) and the tree population was 2500 trees/ha (Fig. 2). Signalgrass was planted between tree double rows. Crossbred Holstein × Zebu

steers with an average initial weight of 175 ± 12.5 kg were used as experimental animals, and each paddock had two tester animals. The steers were weighed every 28 days after a 16 h fast. Cattle were managed under continuous stocking with variable stocking rates using the put-and-take technique. Forage allowance was maintained at 3 kg DM of green forage mass per kg of cattle live weight, and the stocking rate was adjusted following the methods described in Sollenberger *et al.* (2005).

Signalgrass had been previously established in one block in 1969 (Lira *et al.*, 1995), while in the other two blocks, signalgrass (cultivar Basilisk) was established concurrently with tree legumes between the double rows. Briefly, the establishment of signalgrass occurred in shallow openings (approx. 5 cm deep), spaced at 1.0 × 0.5 m, with seeds placed manually (10 kg of commercial seed/ha and 40% pure live seeds).

Tree legumes were seeded in a greenhouse, and inoculated with specific *Bradyrhizobium* strains obtained from the Soil Microbiology Laboratory at Universidade Federal Rural de Pernambuco (UFRPE). All paddocks were fertilized in July 2011 using approximately 44 kg P/ha (100 kg P₂O₅ single superphosphate), and 100 kg K/ha (120 kg K₂O potassium chloride), following recommendations for the establishment of pastures of *U. decumbens* and soil chemical status (Supplemental Table 1). Legume seedlings, approximately 30 cm in height, were transplanted to the field in June 2011, and placed in furrows of 20 cm in depth.

Sampling

The sampling of herbage and litter occurred in each paddock along six transects perpendicular to the legume tree double rows (Fig. 2). Each transect had five sampling points (0.25 m²) distributed from the centre of the double rows to the middle of the pasture (0, 1.8, 3.7, 5.6 and 7.5 m) (Fig. 2). There was a total of 30 points sampled per experimental unit. A composite sample was obtained for each distance that was evaluated. The evaluation of existing litter, consisting of leaves, fruits and branches on the soil, was performed every 56 days, at the same distances, after harvesting the herbage in the same quadrat. All dead and loose material from the aerial fractions of the plants (grass and legume) found within the quadrants placed on the soil surface were considered litter. The litter was dried in a forced air oven at 55°C for 72 h to obtain a constant weight. In January 2015, it was not possible to perform measurements in the areas that contained the mixture with *M. caesalpinifolia*, due to the

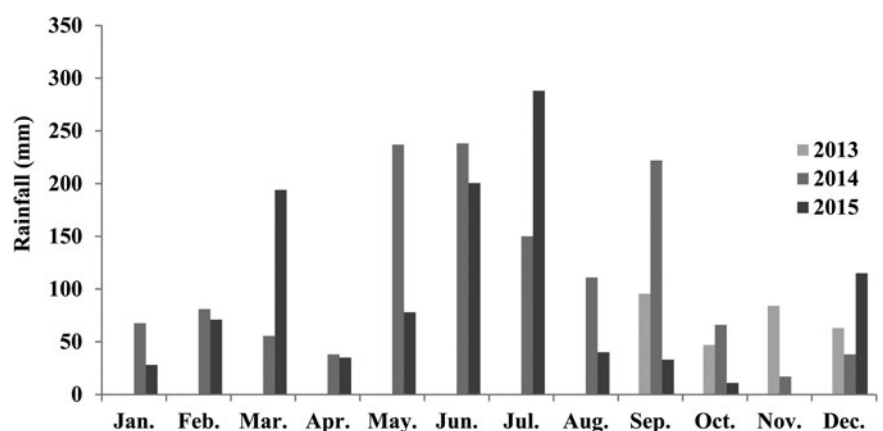


Figure 1. Monthly rainfall at the research site during the experimental period.

Source: Agência Pernambucana de Águas e Clima (APAC).

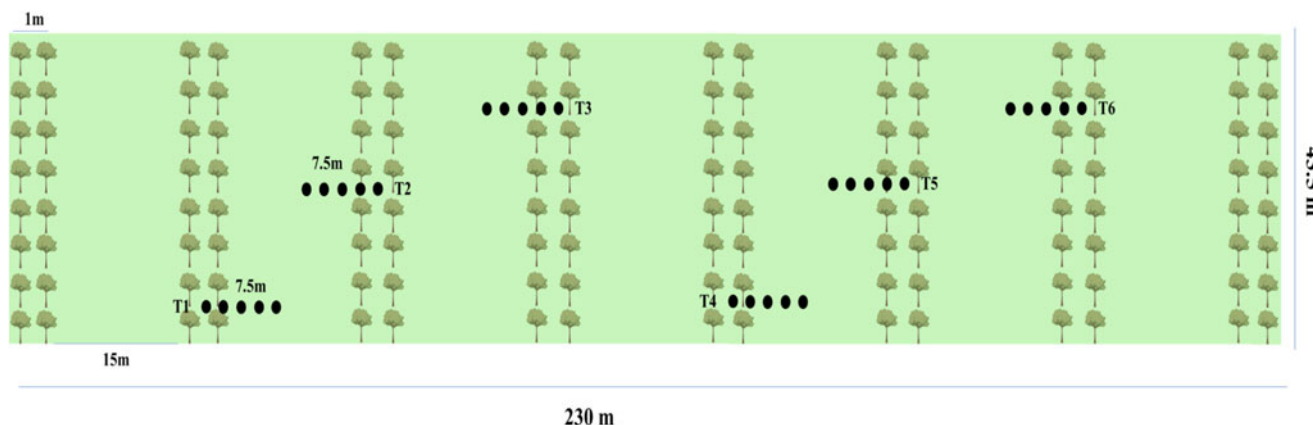


Figure 2. The layout of one experimental unit with tree legumes. Representation of legume tree double rows, and black dots representing sampling points within each transect. T1 = transect 1; T2 = transect 2; T3 = transect 3; T4 = transect 4; T5 = transect 5; T6 = transect 6.

low herbage mass resulting from irregular rainfall (Fig. 1). Consequently, animals were removed from the experimental area in February 2015 due to reduced herbage mass.

Soil samples were collected in September 2013 using the JMC Environmentalist’s Sub-Soil Probe Sampling System (Newton, Iowa, USA) at (0–10, 10–20 and 20–40 cm depth). For each distance from the trees evaluated (0, 1.8, 3.7, 5.6, 7.5 m) in the experimental units, six points were collected, with three subsamples per point, forming a composite sample for each distance and depth evaluated. Before the analysis, the soil was air-dried and sieved (2 mm) to remove debris, stones, litter and roots.

Measurements of the grass

Average compressed grass height (cm) was estimated using a Metal Disc Meter for sward measurements (own manufacture). The compressed height was measured exclusively in one distance (3.7 m) perpendicular to the double row of trees. This measurement was repeated in the six marked transects of each

experimental unit and measured every 28 days. Using the same quadrat area, herbage was harvested at the ground level.

Chemical analysis

The chemical analyses of the forage were performed considering each six subsamples from each transect in each experimental unit, totalling 18 samples per transect in each silvopasture. It is worth mentioning that the forage mass was only collected from the distance of 3.7 to 7.5, as there was no forage growth from 0 to 1.8 m from the double rows of legume trees. The dry matter content of herbage and litter samples was determined after drying the material in the oven at 105°C for 24 h, and the organic matter content was determined after the combustion at 600°C for 3 h, following Bezerra Neto and Barreto (2004). Total N content was estimated in the plant material after digestion of the samples in sulfuric acid (H₂SO₄) and hydrogen peroxide (H₂O₂), according to Thomas *et al.* (1967), followed by distillation with NaOH (13N) and titration (HCl 0.07143N). The SOM was assessed via

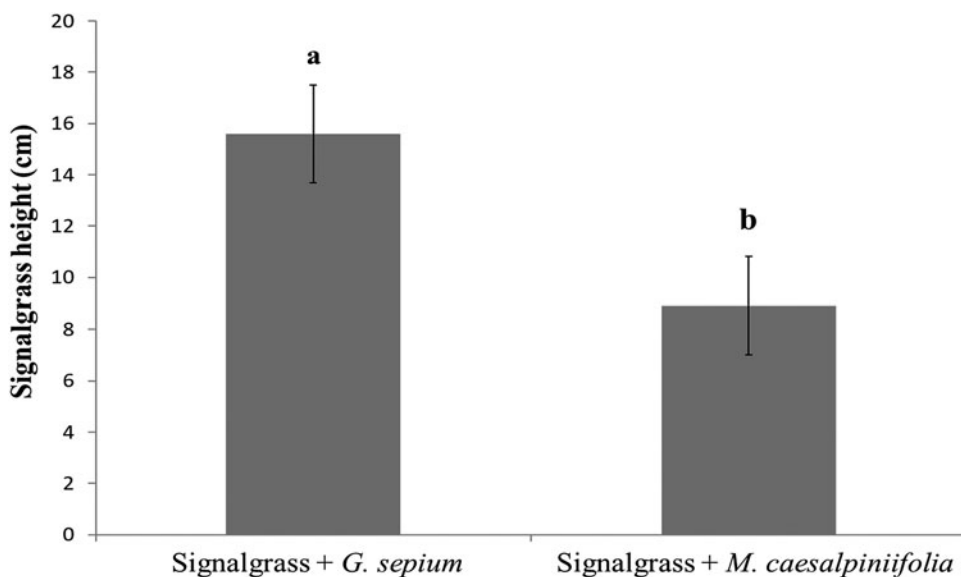


Figure 3. Average compressed canopy height (cm) of the signalgrass (*Urochloa decumbens*) at 3.5 m from the legume trees double rows in silvopastures systems with *Gliricidia sepium* Jacq. and *Mimosa caesalpinifolia* Benth. Standard error = 0.33 cm. Plant height was only measured at one distance (3.7 m) perpendicular to the double row of trees.

combustion in a muffle at 600°C for 4 h. For SOM, the results for each distance from the trees (0, 1.8, 3.7, 5.6 and 7.5 m) were based on the average SOM results of all soil depths collected (0–10, 10–20 and 20–40 cm).

Statistical analysis

Data were subjected to normality and homoscedasticity tests before the statistical analysis. The data were analysed by the Mixed procedure of the statistical package SAS (SAS Inst. Inc., 1996). In all analyses, blocks were considered a random effect. Systems and sampling points were fixed effects. Significance was declared at 5% probability. LSMEANS were compared using the PDIF procedure, and adjusted Tukey test.

Results

Signalgrass height and herbage mass

The compressed height of the signal grass was affected by treatment ($P < 0.05$). Signalgrass was, on average, taller in the system with *G. sepium* (16 cm) compared to the system with *M. caesalpinifolia* (9 cm) (Fig. 3). Herbage mass increased with the distance from the tree legume rows ($P < 0.05$) for both systems mixed with legume species (Fig. 4). At 7.5 m, the herbage mass was 1869 kg DM/ha whereas, at 3.7 m, the herbage mass averaged 1419 kg DM/ha.

Herbage N content

Signalgrass N content varied significantly ($P < 0.05$) among distances. The greatest herbage N (15.6 g/kg) was observed at a 3.7 m distance from the tree rows, whereas the lowest value (14 g/kg) was observed at a 7.5 m distance (Fig. 5). It is important to mention that sampling points near the tree row were not included due to non-existent herbage mass in most evaluations.

Litter N content

Litter N content varied with distance from the tree rows and tree legume species ($P < 0.05$). Litter N content declined with increasing distance from tree rows, ranging from 12.4 g/kg (point 0) to 23.2 g/kg at 7.5 m away from the trees (Fig. 6).

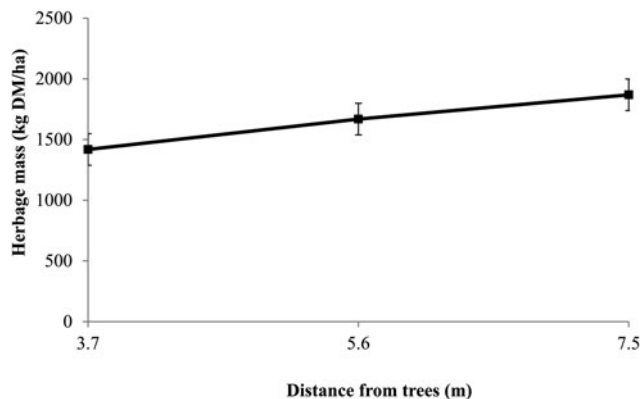


Figure 4. Herbage mass of *Urochloa decumbens* (signalgrass), according to the distances from the tree legumes. Data are averaged across tree species, evaluation dates and blocks. Standard error = 39.7 kg DM/ha. Note: Signalgrass did not grow under the tree crown (0–1.8 m), and herbage mass and composition were not measured at these specific points.

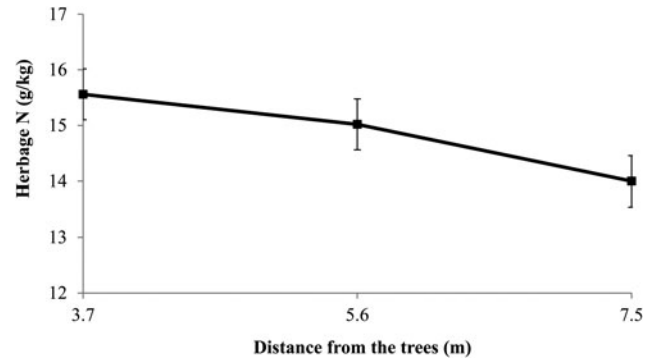


Figure 5. Nitrogen concentration in *Urochloa decumbens* (signalgrass) according to the distances from the tree legumes. Data are averaged across tree species, evaluation dates and blocks. Standard error = 0.02 N g/kg. Signalgrass did not grow under the tree crown (0–1.8 m), and herbage mass and composition were not measured at these specific points.

Litter mass

Litter mass varied differently between tree legume species. Litter mass varied with the evaluation date and sampling points for the signalgrass-*M. caesalpinifolia* system (Fig. 7). Litter mass was greater within legume rows (point 0), with an average of 1021 kg OM/ha; the lowest mean was observed at 7.5 m. Within legume rows, the litter was composed almost exclusively of material from *M. caesalpinifolia*, as there was no presence of signalgrass within the rows. *M. caesalpinifolia* is a deciduous plant that loses its leaves during the dry period to save water (Holanda *et al.*, 2019). In the months considered dry, litter deposition was greater than in the other months. Total deposition in November and January was greater, with the greatest values observed in November 2014 (1704 kg OM/ha).

Litter mass in the signalgrass-*G. sepium* pasture was quite uniform along the transect points throughout the experimental period, varying among evaluations ($P < 0.05$) (Fig. 8). Litter mass peaked in September, November and January, with means varying from 654 kg OM/ha in March to 1000 kg OM/ha in November. The months of March and May presented lower litter mass when compared to other months.

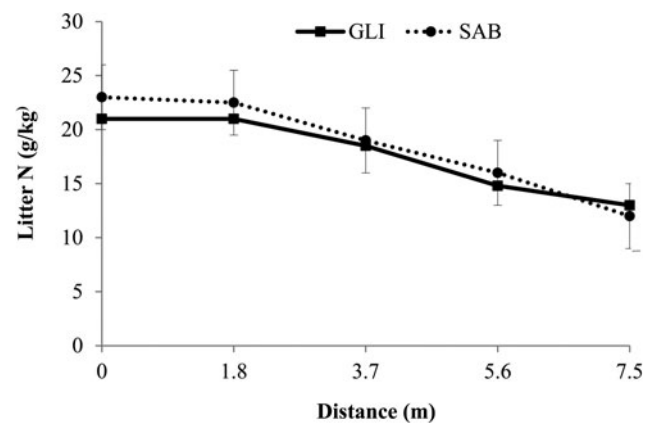


Figure 6. Litter N in mixed pastures of *Urochloa decumbens* (signalgrass) with *G. sepium* or *M. caesalpinifolia* according to the distances from tree legumes. Data are averaged across evaluation dates and blocks. Standard error = 3.8 N g/kg.

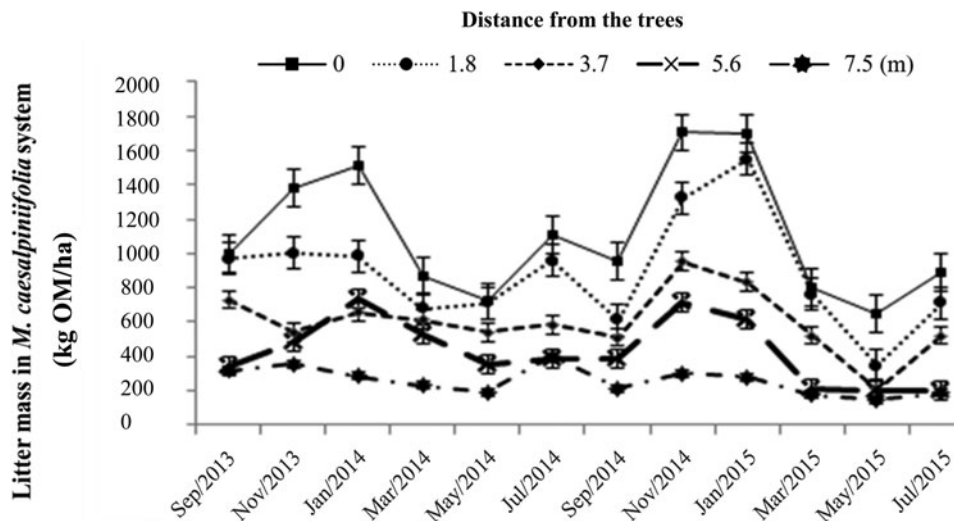


Figure 7. Litter mass in signalgrass-*M. caesalpinifolia* in silvopastoral system.

Soil organic matter

There was an interaction between the distances and the treatments for SOM content ($P < 0.05$). SOM in the *M. caesalpinifolia* treatment was greater when compared to the *G. sepium* treatment, except at 7.5 m, where *G. sepium* had a value of 38 g/kg and *M. caesalpinifolia* presented 37 g/kg. At point 0, there was lower SOM in the treatment of *M. caesalpinifolia*-signalgrass. The greatest SOM occurred at a 1.8 m distance in the mixture with *M. caesalpinifolia* and at a 5.6 m distance in the mixture with *G. sepium* (Table 1).

Discussion

Signalgrass height and herbage mass

The grass that was growing near legume trees typically exhibited less development than at longer distances in the two agrosilvopastoral systems tested, as there was no growth at the distances 0 and 1.8 m. A previous study indicated a quadratic increase in signalgrass height concerning the distance from the trees, with greater

canopy height at distant points from the trees (Cabral, 2013). This author highlighted the position and direction of the tree rows (north/south direction) in this specific agrosilvopastoral system, possibly reducing the amount of light reaching the grass canopy, especially near the legumes.

Shaded areas under the tree canopy may limit forage growth by inducing changes in light quantity and quality, along with competition for water and nutrients in the soil (Paciullo *et al.*, 2011a). This phenomenon was also observed in the current experiment at the distance interval from 0 to 1.8 m, where there was no presence of signalgrass, likely due to intense competition between legumes and grasses for nutrients, light and water, or by the occurrence of allelopathic effects. Coêlho (2016) studied the structural characteristics of *U. decumbens* Stapf. under monocrop and silvopastoral systems in the same area of the present study, and reported that during the dry period of the year, greater plant height and compressed heights of signalgrass canopy were observed in the monoculture treatment and silvopastures with *G. sepium*, suggesting a stronger competition from *M. caesalpinifolia*

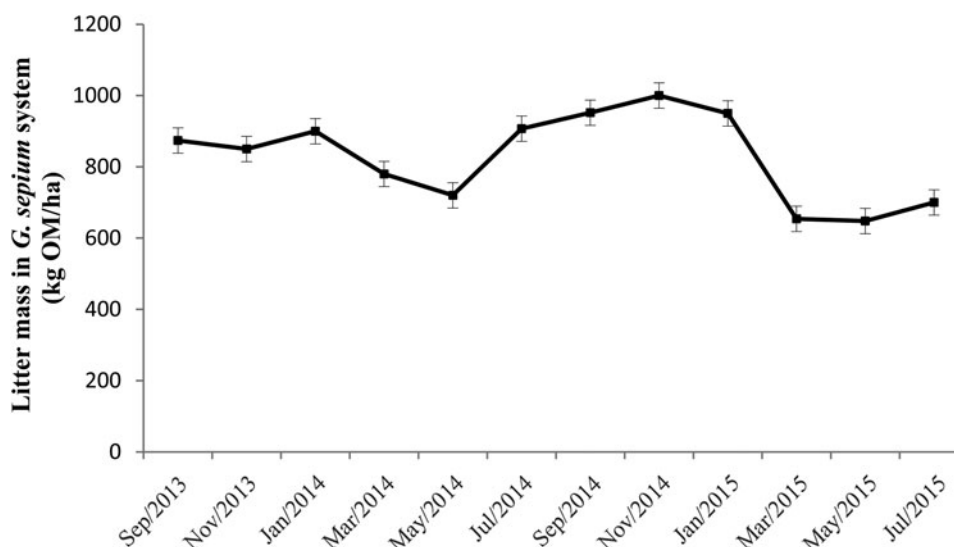


Figure 8. Litter mass in mixed pastures of signalgrass-*G. sepium* in silvopastoral system.

Table 1. Soil organic matter (SOM) content (g/kg) at different distances from trees in two silvopastoral systems

Distance (m)	System			
	<i>M. caesalpinifolia</i>		<i>G. sepium</i>	
	g/kg			
0	34.4 ± 0.2	cA	33.2 ± 0.2	abB
1.8	38.8 ± 0.6	aA	31.8 ± 0.3	bB
3.7	36.5 ± 0.5	bA	35.7 ± 0.3	abB
5.6	37.4 ± 0.4	abA	34.5 ± 0.5	abB
7.5	37.3 ± 0.2	abB	38.2 ± 0.4	aA
Standard error = 0.14				

Lowercase letters compare distances (columns) and uppercase compare treatments (lines). Means followed by the same letter do not differ statistically according to Tukey's test at 5% probability. Note: results for each distance were based on the average SOM content of all soil depths collected (0–10, 10–20 and 20–40 cm).

trees. However, both legume tree species demonstrated the potential to reduce the soil fertility of nearby areas of the signalgrass pasture over time (Herrera *et al.*, 2023).

Shade is a determining factor affecting the growth of forage plants, acting either beneficially or harmfully depending on the plant species and their tolerance to shading. Lima *et al.* (2020) reported that *U. decumbens* displayed greater herbage mass under full sun compared to shaded areas in silvopastoral systems with *M. caesalpinifolia* and *G. sepium*. According to Abraham *et al.* (2014), a greater proportion of leaves in canopies kept under the shade could be an indicator that the plants are prioritizing the allocation of photoassimilates for leaf formation. Phenotypic plasticity can be observed in tropical grasses under shaded conditions, and several morphological responses have been reported including the increase in leaf elongation rates, greater leaf surface area, reduction of leaf thickness, decrease in tillering rate, presence of longer internodes and stems, increase in the chlorophyll content (Paciullo *et al.*, 2011a, 2011b; Barnes *et al.*, 2015; Baldissera *et al.*, 2016; Paciullo *et al.*, 2017; Gomes *et al.*, 2019).

The degree of shading effects will depend on several factors, including the type of the species, genetic factors, soil nutritional status, water availability, season, grazing intensity and frequency, percentage of the shaded area, quality of the light, duration of the exposure to shade, among other environmental and management factors.

As grass growth can be affected by shading, pruning or periodic harvestings are recommended, especially when the level of shading decreases pasture production and uniformity. However, several issues must be considered when pruning the trees to avoid shaded pastures, including the reduction of animal well-being and overall legume production or even N-fixation. Another possible factor is interspecies competition for water and nutrients. The grass growing near the trees possibly faced stronger competition compared to the grass located further from the tree's double rows. According to Isaac and Borden (2019), to increase the sustainability of agroforestry systems it is fundamental that trees and herbaceous crops interact harmonically regarding nutrient acquisition on nearby sites, which is very complex to achieve. Several factors including the type of species in the systems, type of the soil, characteristics of root length and distribution, type of nutrient and its availability in the soil,

water availability, soil microbes, climate and weather, among others factors can modify competition force (Gregory, 1996; Schroth, 1998; Isaac and Borden, 2019; Bayala and Prieto, 2020).

Herbage N content

The increase in N content in the forage near the legume trees in the silvopastoral systems was possibly associated with the N-transfer phenomena. N transfers can occur from the decomposition of the litter and dead roots of the legume trees (Sierra and Nygren, 2006; Thilakarathna *et al.*, 2016), and also from root exudates (Jalonen *et al.*, 2009), with subsequent N uptake by the grass closer to the trees or near N release sites. Also, it is worth mentioning that these plants near the tree rows possibly were less developed, as evidenced by the greater herbage mass at distant areas from the trees (Fig. 4). This possibly contributed to a reduction in C compounds in the plants near the trees and a relative increase in the N content of the forage. Paciullo *et al.* (2011a) evaluated nutrient characteristics of signalgrass under a silvopastoral system in the function of the distance from the trees (0, 3, 6, 9, 12 and 15 m). They observed that crude protein content in signalgrass varied with the distance from the trees. The minimum value was 65 g/kg, estimated at 13.5 m distance from the tree row, while under the tree canopy, the content was 97 g/kg.

Litter N content

There was litter accumulation within the legume rows as well as at the five transect evaluation points. Litter N in the *M. caesalpinifolia* system was greater than in the *G. sepium* system, with an average of 18.5 and 17.6 g N/kg, respectively. Silva *et al.* (2013) evaluated litter mass and soil fertility in a degraded pasture of *U. decumbens* after the implantation of tree legumes. They observed greater litter N in the treatment that was composed of *M. caesalpinifolia*. Greater litter N was possibly associated with the largest deposition of litter biomass from the legume trees, which probably resulted in more soil N within the areas that were covered by legumes.

Litter mass

Apolinário *et al.* (2016) observed annual litter deposition of 4540 kg OM/ha in *M. caesalpinifolia* under a silvopastoral system, and the greatest amounts of litter was reported to be found under the tree canopy. Litter represents an important source of nutrient input to the soil, besides providing soil cover (Dubeux *et al.*, 2017; Herrera *et al.*, 2020). It also improves the physical and chemical properties of the soil and its degradation will result in one of the main mechanisms of nutrient recycling in agroforestry systems. Unlike *M. caesalpinifolia*, *G. sepium* did not show differences in litter mass between the rainy and dry seasons, demonstrating that litter deposition of this species is less variable throughout the year than *M. caesalpinifolia*. Depending on the type of tree species, a different dynamic regarding litter mass and composition may be found in the different silvopastoral systems using legume trees.

Soil organic matter

The interaction between tree species and distance on the SOM emphasizes the importance of the type of legume tree used in the system, but overall, the SOM was greater far from the trees. Lima *et al.* (2018) evaluated soil attributes in a silvopastoral system composed of *U. decumbens*, *M. caesalpinifolia* and *G. sepium*

trees, and found that the light fraction of SOM was not affected by the grass; however, within the silvopastoral systems, it was greater in the *M. caesalpinifolia* than in the *G. sepium* (64 vs. 45 mg/kg, respectively). Evaluating six herbaceous legumes on pasture of signalgrass, Silva (2018) observed that for the fractionated SOM there was no difference between treatments regardless of harvesting time and soil depth. According to Dias *et al.* (2007), the influence of trees can reach projections beyond their crowns, reaching more distant sites from the tree component. Farias *et al.* (2009) evaluated soil chemical characteristics at different distances and depths from the eucalyptus trees and observed that the pH decreased significantly with the distance from the trees, but this effect varied with soil depth. Variations in soil physical-chemical and microbiological characteristics have been reported to be influenced by the type of vegetation (Prescott and Grayston, 2013; Sul *et al.*, 2013; Tian *et al.*, 2017).

Legume trees in silvopasture systems

It should be considered that the interaction between grass and legume trees is subjected to interspecific competition for water, light and nutrients, and there was an indication that grass growing near the trees was subjected to stronger competition than further from the double rows. The intensity of this competition, especially for water, could be worsened during the dry season. Silvopasture managers must consider these seasonal effects on the overall development of the pastures and trees under the integrated system. In silvopasture systems, pruning may help to reduce the negative impacts of the shades from legume trees on the nearby grass, however, should be considered several other factors such as the reduction of shading for animals, the provision of forage via legume regrowth, modification of legume architecture due to emissions of new branches, time to recover from harvesting and reduction of the N₂ fixation. Opting for grass less affected by shading is another approach for composing silvopastoral systems.

Conclusions

Tree legumes had a marked influence on most of the characteristics of the signalgrass herbage and litter. Signalgrass did not develop near the trees, which was evidenced by the absence of growth in the nearby areas between 0 and 1.8 m, also, greater herbage mass occurred in the pasture areas at further distances from the trees (7.5 m). N content in the forage was favoured by the proximity to the legume trees. Signalgrass was not present under the canopy of *M. caesalpinifolia*. There is a need for further research to better understand the mechanisms of competition among these species.

Litter mass was greater under the tree canopy, especially in the signalgrass-*M. caesalpinifolia* system. Litter N content was also greater near the trees. Future research should target companion species that have compatible growth and are capable of coexisting in the silvopastoral systems. This includes herbaceous legumes that could be competitive and persist with trees and grass components.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0021859624000200>.

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Author contribution. J. C. B. D. Jr., M. V. F. d. S.: supervision, conceptualization, funding acquisition, project administrator; A. M. G. d. S.: investigation,

sampling, data curation, laboratory analysis, formal writing; S. B. d. M. C., D. d. L. C., E. R. d. S. S., N. G. d. M. S., B. M. M. d. O.: sampling and laboratory analysis; V. X. d. O. A., J. J.: writing review.

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Ethical standards. Not applicable.

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