

## Sampling representativeness of soil carbon and physiological parameters of marandu palisadegrass in a tropical silvopastoral system

Jaqueline de Cássia de Oliveira<sup>1</sup>, Alcinei Místico Azevedo<sup>1</sup>, Juliana Martins Ribeiro<sup>1</sup>, Igor Costa Freitas<sup>1</sup>, Rafael Ferreira Dias<sup>1</sup>, Ana Clara Santos Duarte<sup>1</sup>, Paula Franciele Melo<sup>1</sup>, Álvaro Luís Veloso<sup>2</sup>, Leidivan Almeida Frazão<sup>1</sup>\*

<sup>1</sup>Universidade Federal de Minas Gerais/Instituto de Ciências Agrárias, Av. Universitária, 1000 – 39404-547 – Montes Claros, MG – Brasil.

<sup>2</sup>Faculdades Integradas do Norte de Minas, Av. Osmane Barbosa, 11111 – 39404-006 – Montes Claros, MG – Brasil.

\*Corresponding author <lafracao@ica.ufmg.br>

Edited by: Luiz Alexandre Peternelli

Received June 24, 2020

Accepted October 26, 2020

**ABSTRACT:** The sampling methods for soil and plant evaluation in integrated production systems need to be refined for their use in experiments. This study purposed a sampling representativeness model to evaluate soil carbon and physiological parameters of marandu palisadegrass intercropped with double rows of eucalyptus in a silvopastoral system in southeastern Brazil. Four transects were delimited within the silvopastoral system and the soil was sampled at 0-5, 5-10, and 10-20 cm depths to evaluate two parameters of soil organic matter: total organic carbon (TOC) and soil microbial carbon (Cmic). We also evaluated three physiological parameters of marandu palisadegrass: stomatal conductance, transpiration rate, and photosynthetic rate. The sampling strategy was evaluated by the Hatheway and Modified Maximum Curvature (MMC) methods. We found that cultivation of the alley with forage grass and double rows of eucalyptus must be considered to ensure sampling representativeness in a silvopastoral system, once TOC and Cmic and physiological parameters showed variation within the integrated production system. The MMC method could be useful to evaluate silvopastoral systems for experiments and characterization of agricultural areas, as 11 soil samples are representative to evaluate Cmic and TOC, and 13 samples are enough to evaluate the physiological parameters in marandu palisadegrass. The Hatheway method could be applied to experiments that need higher accuracy with a reduction in the minimum difference to be detected between the treatments and a consequent increase in the number of samples to be collected.

**Keywords:** Eucalyptus, MMC method, Hatheway method, forage grass, soil sampling

### Introduction

Integrated production systems are sustainable models that combine livestock, forestry, and crop activities in the same area over time (Rodrigues et al., 2014), allowing to retain carbon (C) in the soil and in the biomass (Conceição et al., 2017). In addition, the adoption of these production systems could reduce erosion and loss of soil fertility, mitigating GHG emissions, reducing the silting of watercourses, preventing pollution of soil and water, and improving soil quality (Moraes et al., 2014; Oliveira et al., 2016a).

Silvopastoral systems combine trees, forages, and animals in the same area (Jose and Dollinger, 2019). The space and the size between the rows, known as alley, is defined according to the area of interest for forage grass cultivation (Figueiredo et al., 2018; Ghezehei et al., 2015; Oliveira et al., 2016b; Paula et al., 2013; Santos et al., 2018). Soil parameters are affected by the deposition of animal excrement and trampling, especially near trees, since animals seek shade (Borges et al., 2018). In addition, heterogeneity within the systems affects the soil C contents (Moraes et al., 2014). Therefore, it is important to determine total organic carbon (TOC) and microbial carbon (Cmic), once they are used to quantify the potential of SOC accumulation over time and the role of microorganisms in nutrient cycling, respectively.

Several studies have pointed out different sampling strategies; however, patterns and numbers of soil and

plant samples are variable (Borges et al., 2018; Guillot et al., 2019; Oliveira et al., 2016a). Therefore, to ensure sampling representativeness to evaluate experiments is necessary to consider the arrangement and the plant species within a silvopastoral system (Albrecht and Kandji, 2003; Nair et al., 2009; Oliveira et al., 2016a). Different models have already used heterogeneity in the soil (Lorentz et al., 2010; Li, 2019; Pereira et al., 2017; Saste and Sananse, 2015; Silva et al., 2019). While the modified maximum curvature (MMC) method correlates the ideal number of samples with the coefficient of variation (Facco et al., 2018; Pereira et al., 2017; Silva et al., 2019), the Hatheway method estimates the minimum difference between the treatments determining the optimal number of repetitions per plot (Donato et al., 2018; Mello et al., 2004).

This study purposed a sampling representativeness model to evaluate soil TOC and Cmic as well as physiological parameters of marandu palisadegrass intercropped with double rows of eucalyptus in a silvopastoral system in Minas Gerais State, southeast Brazil.

### Materials and Methods

#### Location and characterization of the study site

The study was carried out in the municipality of Francisco Sá (16°38'44.02" S and 43°42'43.77" W,

altitude 590 m), Minas Gerais, Brazil. Mean values of annual rainfall and air temperature over the last ten years were 887 mm and 28 °C, respectively (INMET, 2019). According to the Köppen classification (Alvares et al., 2013), the climate in the region is Aw, tropical savanna, with dry mild winters and rainy summers and high temperatures.

The study site is located in a transitional site between the *Cerrado* (Brazilian tropical savanna) and the Semideciduous Seasonal Forest. Almeida et al. (2021) characterized the soil as Haplic Cambisol with loam texture and medium soil fertility. Before the experiment was set up, the soil had grown a pasture of *Brachiaria brizanta* cv. Marandu, which was in a degradation process. The area was extensively grazed for 13 years (until 2011) by dairy cows without soil management nor replacement of nutrients contents. Since 2011, the pasture stand has been decreasing and spontaneous vegetation has emerged in the site. In Oct 2012, glyphosate herbicide was applied to desiccate the pasture and remove the weeds.

The integrated production system was introduced in Dec 2012 in an homogeneous area of 1.6 ha, where hybrid *Eucalyptus urograndis* (*E. grandis* × *E. urophylla*) was planted in double rows (Dec 2012) intercropped with forage sorghum (*Sorghum bicolor* L.) between rows in the first two years of the experiment. The initial fertilization consisted of the application of 120 g per plant of a fertilizer formulated NPK (6:30:10) with micronutrients (B, Zn and Cu). In 2014, sorghum was replaced by marandu palisadegrass (*Urochloa brizanta* cv. Marandu) pasture composing the silvopastoral system. The spatial arrangement within the system was composed by double rows of eucalyptus (2 × 3 m) and alleys (14 m) between the double rows. The alley space was used for grazing by 50 mixed-breed calves from black and white Holstein dairy cows, at an age ranging from 2 to 12 months.

### Soil sampling

In Jan 2018, an area of 884 m<sup>2</sup> within the silvopastoral system was divided into four transects 6 m distant from each other to determine the sampling representativeness. The soil samples were collected in the area comprising two double rows and one alley totaling 20-m length, where the samples were collected with a distance of 1 m from each other starting in the first eucalyptus for 20 sampling points (Figure 1A). The soil samples were collected in pits of 40 × 40 × 30 cm at 0-5, 5-10, and 10-20 cm depths to evaluate the total organic carbon (TOC) and the microbial carbon (Cmic).

### Sampling of the forage component

For marandu palisadegrass evaluation, we estimated the physiological parameters only in the alley (forage grass cultivation) in the four transects previously delimited

for soil sampling. The stomatal conductance (Gs) (mol m<sup>-1</sup> s<sup>-1</sup>), transpiration rate (E) (mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>), and carbon dioxide (CO<sub>2</sub>) assimilation rate or photosynthetic rate (A) (μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) of marandu palisadegrass were evaluated using the infrared gas analyzer (IRGA), model LCpro-SD, with a distance of 1 m from each other starting in the first eucalyptus for 15 sampling points (Figure 1B). All the evaluations were performed between 08h00 and 11h00 a.m. and each measurement was made in the middle part of the second youngest fully expanded leaf of the plant.

### Preparation of soil samples and TOC and Cmic determination

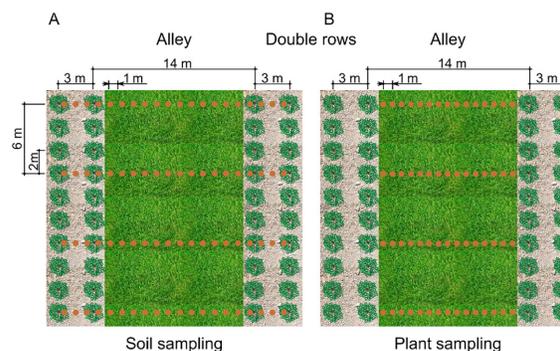
After soil sampling, the small plant fragments were picked manually from the all samples. After, the soil samples were air dried, homogenized, sieved through a 2-mm mesh, finely ground, and then sieved through 0.150 mm to determine TOC by wet oxidation (Yeomans and Bremner, 1988).

To evaluate Cmic, the initial moisture content of the samples was determined and water was added until reaching 60 % field capacity, the ideal moisture for proliferation of soil organisms. The fumigation-extraction method was used to determine the initial moisture content, according to Vance et al. (1987) and adapted by Silva et al. (2007). The Cmic was obtained by the C difference between fumigated and non-fumigated samples, applying the correction factor that represents extraction efficiency.

### Statistical analysis

All statistical analyses were performed using R statistical software (version 3.5) through functions of the "stats" package or by programming.

For TOC and Cmic at each soil depth, one soil sample represented one basic unit (BU), resulting in 12 BU within double rows (3 soil samples × 4 transects) and 56 BU in the alley (14 soil samples × 4 transects) (Figure 1A). The number of BU per plot in the alley (X = 1, 2,



**Figure 1** – Demonstrative design for sampling strategy of soil carbon (A) and physiological parameters of marandu palisadegrass (B) in a silvopastoral system in southeast Brazil.

4, 7, 8, 14, 28, and 56) was given by the 12 combinations of the four transects (1, 2, and 4) and number of samples per transect (1, 2, 7, and 14). Regarding the double row analyses, one was considered a continuity of the other, adding up to 8 transects, with three samples in each. Thus, the number of BU per plot (1, 2, 3, 4, 6, 8, 12, and 24) was given by all the 12 combinations of the number of transects (1, 2, 4, and 8) and of samples per transect (1, 2, and 3).

For the physiological parameters, each plant evaluated represented a BU, resulting in 60 BU in the alley (15 plant sampled  $\times$  4 transects) (Figure 1B). The number of BU per plot for these variables ( $X = 1, 2, 3, 4, 5, 6, 10, 12, 15, 20, 30,$  and  $60$ ) was obtained by the 12 combinations of the four transects (1, 2, and 4) and 15 samples (1, 3, 5, and 15) in each one of parameters.

We used the modified maximum curvature (MMC) method to determine the optimal number of samples to represent the double rows with eucalyptus and the alley with marandu palisadegrass for the response variables, fitting the following model:

$$CV_i = \left( \frac{A}{x_i^B} \right) + e_i \quad (1)$$

where:  $CV_i$  is the coefficient of variation estimated for the  $i$ -th sample size;  $x_i$  the number of BU associated with the  $i$ -th sample size;  $A$  and  $B$  coefficients of the regression model and  $e_i$  the experimental error associated to the  $i$ -th sample size.

The  $A$  value (intercept) represents the estimated coefficient of variation (CV) when plots composed of a single BU are accepted, in other words, plots composed of a single sample. Consequently, the value is very close to the CV considering the values of each sample individually:]

$$CV = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{X})^2}{n-1}}{\bar{X}} \quad (2)$$

where:  $x_i$  is the value of the attribute evaluated in the  $i$ -th sample;  $\bar{X}$  is the general mean and  $n$  is the total number of samples).

To facilitate estimation of the regression coefficients, the model was linearized by logarithmic transformation. Afterward, the coefficients were estimated by the least square method, with the assistance of the "lm" function. The maximum point of curvature (Meier and Lessman, 1971) was obtained by the following function:

$$X_{optimal} = \left( \frac{A^2 B^2 (2B + 1)}{(B + 2)} \right)^{\frac{1}{2B+2}} \quad (3)$$

where:  $X_{optimal}$  is the optimal size of the plot; and  $A$  and  $B$  are the regression coefficients of the fitted model.

The Hatheway (1961) method was applied to know the optimal number of replications according to the number of samples per plot for experiments in the randomized block design, which is an estimate of the true difference between the ideal numbers of samples with the percentage of the mean, estimated by the equation:

$$X = \sqrt{\frac{2(t_1 + t_2)^2 CV^2}{d^2 r}} \quad (4)$$

where:  $X$  = size of the plot;  $r$  = number of replications required to detect differences of  $d$  units;  $d$  = true difference between two means of treatments expressed in percentage of the mean;  $CV$  = estimate of the coefficient of variation in percentage for a basic unit;  $t_1$  = critical value of  $t$  for tests of significance (bilateral at 5 %) and  $t_2$  = critical value of  $t$  corresponding to  $2(1-P)$ , where  $P$  is equal to 0.80; and  $B$  = heterogeneity index of the soil. For better presentation of the optimal number of samples within the plot, contour plots were made for the size of the plot in accordance to the number of replications and treatments with the assistance of the Sigma Plot software (Systat Software, v. 11).

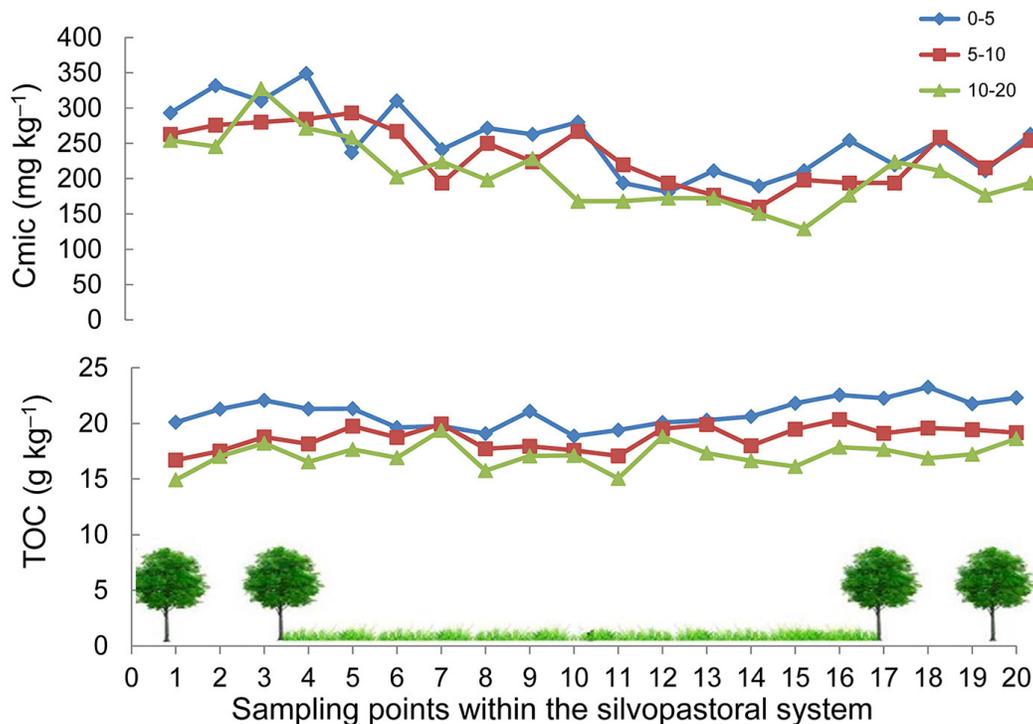
## Results

### Soil TOC and Cmic within the silvopastoral system

The soil TOC contents ranged from 14.92 (10-20 cm) to 23.26 g kg<sup>-1</sup> (0-5 cm) within the silvopastoral system (Figure 2). The soil Cmic values also varied with soil depths, ranging from 129.31 (10-20 cm) to 349.14 mg kg<sup>-1</sup> (0-5 cm). The evaluation of the transect showed that the soil Cmic and TOC contents had a different distribution pattern within the silvopastoral system, from the first eucalyptus tree to the alley cultivated with marandu palisadegrass. Thus, it can be inferred that to evaluate the changes in these two soil parameters in a silvopastoral system, a large number of soil samples are necessary to represent the study site. To ensure sampling representativeness, soil samples should be collected between the double rows of trees and in the alley cultivated with forage grass.

### Physiological parameters of marandu palisadegrass within the silvopastoral system

The physiological parameters  $A$ ,  $E$ , and  $G_s$  varied according to the sampling position in the alley of marandu palisadegrass (Figure 3). The assimilation of CO<sub>2</sub> ( $A$ ) ranged from 4.03  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  (nearer the double rows of Eucalyptus) to 14.4  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  (central points in the alley). The same pattern was observed for the transpiration rate ( $E$ ) and stomatal conductance ( $G_s$ ), with values ranging from 1.33 to 4.82 mols H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup> and from 0.03 mols m<sup>-1</sup> s<sup>-1</sup> to 0.16 mols m<sup>-1</sup> s<sup>-1</sup>, respectively, where the highest values were also



**Figure 2** – Total Organic Carbon = TOC ( $\text{g kg}^{-1}$ ) and microbial carbon = Cmic ( $\text{mg kg}^{-1}$ ) at three soil depths within 20 collected points per transect ( $n = 4$ ) in a silvopastoral system in southeast Brazil.

observed in the central points of the alley cultivated with marandu palisadegrass.

### Proposal of sampling representativeness within the silvopastoral system

#### Modified maximum curvature method

Applying the MMC method for the soil TOC contents, we found that at least seven sampling points are necessary to represent the silvopastoral system – three samples that should be taken within the alley (between two double rows of eucalyptus) and four samples within the double rows of eucalyptus (Table 1). For soil Cmic, we found that 11 sampling points within the silvopastoral system are needed: six samples within the double rows of eucalyptus and five samples within the alley (Table 1).

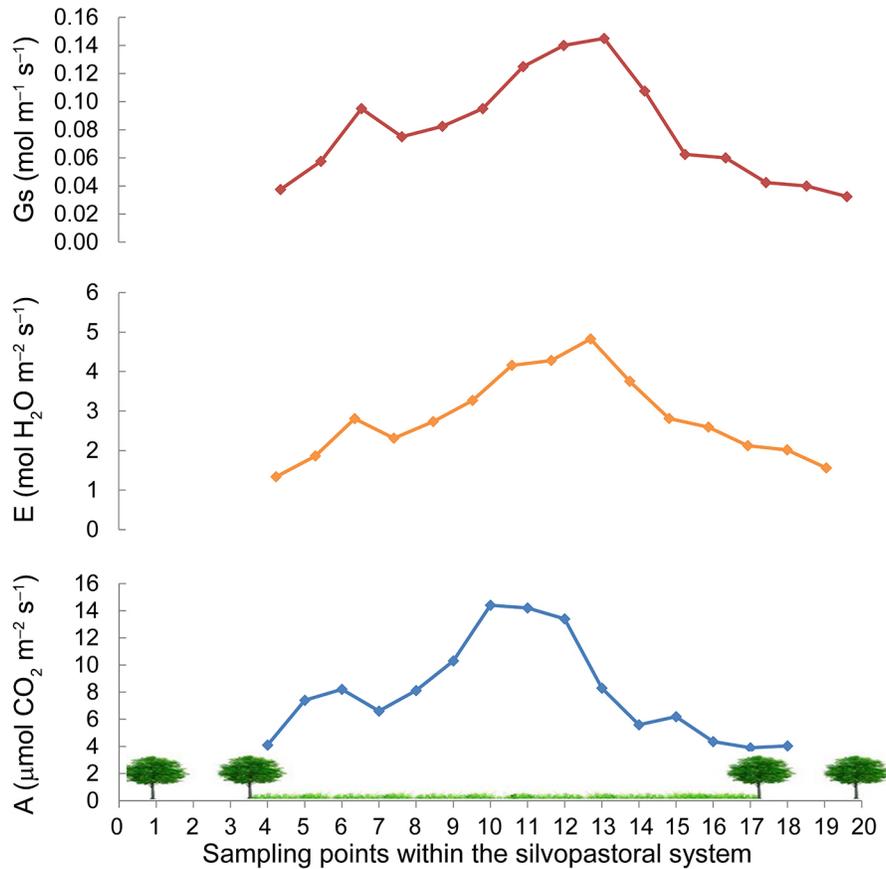
Considering the physiological parameters evaluated in marandu palisadegrass within the silvopastoral system, we found that the sampling method is representative when there are measurements for A, E, and Gs of at least 13, 11, and 12 points, respectively. Therefore, at least 13 points must be sampled since the physiological parameters are evaluated simultaneously by the using of IRGA model LCpro-SD (Table 1).

In the Figure 4, we are considering the values of A and B in Table 1. These results allow visualizing a stability of CV values with the recommended sampling size. Consequently, if the experiment had a larger

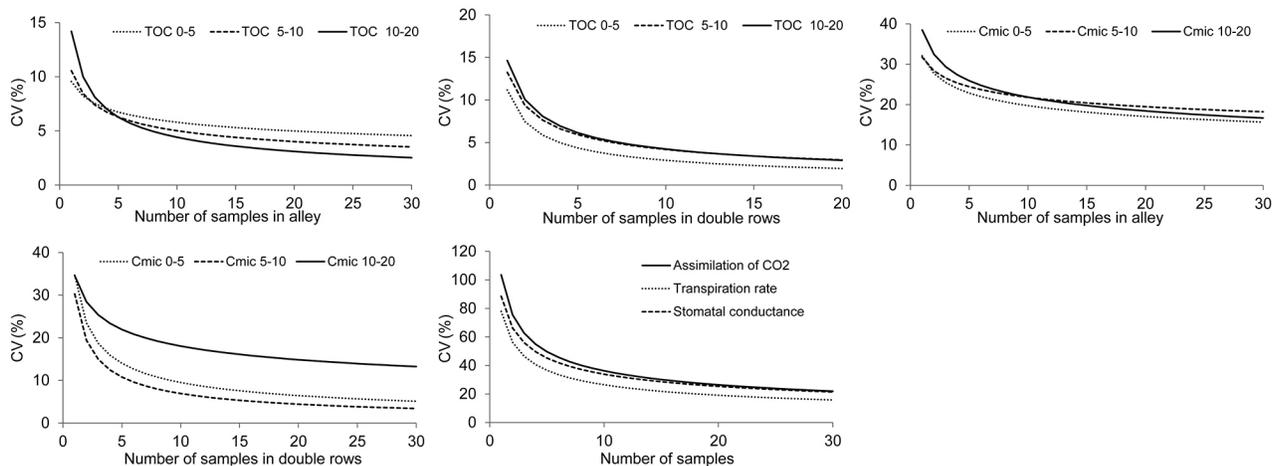
**Table 1** – Estimates of regression coefficients A and B of the potency model, coefficient of determination ( $R^2$ ), coefficient of variation (CV%), and maximum curvature point ( $X_c$ ) to estimate the optimal number of samples to represent a silvopastoral system evaluated in southeast Brazil.

Depth (cm)	A	B	$R^2$	CV%	$X_c$
TOCAlley (0-5)	9.549	-0.217	0.868	9.815	1.52
TOCAlley (5-10)	10.549	-0.323	0.913	12.128	2.219
TOCAlley (10-20)	14.193	-0.508	0.98	12.907	3.449
TOCRow (0-5)	11.166	-0.585	0.481	10.258	3.092
TOCRow (5-10)	13.245	-0.501	0.392	13.399	3.276
TOCRow (10-20)	14.627	-0.539	0.516	13.88	3.586
CmicAlley (0-5)	32.072	-0.211	0.402	36.427	4.038
CmicAlley (5-10)	31.739	-0.163	0.341	36.374	3.331
CmicAlley (10-20)	38.486	-0.246	0.638	40.044	5.161
CmicRow (0-5)	34.671	-0.562	0.216	31.056	6.304
CmicRow (5-10)	30.273	-0.641	0.413	34.172	5.828
CmicRow (10-20)	34.577	-0.282	0.229	38.401	5.101
A	103.543	-0.456	0.473	75.669	12.955
E	77.886	-0.469	0.439	53.902	10.664
Gs	88.51	-0.417	0.461	66.254	11.58

number of samples, the estimated optimal number does not have substantial changes because there is stability in the CV values. The MMC method showed higher CV values for soil Cmic and physiological parameters of marandu palisadegrass, due to their greater variability



**Figure 3** – Assimilation of CO<sub>2</sub> – A (μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>), Transpiration rate – E (mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>) and Stomatal conductance – Gs (mol m<sup>-1</sup> s<sup>-1</sup>) of marandu palisadegrass at 15 collected points per transect (n = 4) in a silvopastoral system in southeast Brazil.



**Figure 4** – Relationship between the coefficient of variation – CV (%) and plot size using the Modified Maximum Curvature Method to determine the number of samples for soil TOC and Cmic and physiological parameters of marandu palisadegrass in a silvopastoral system in southeast Brazil.

within the silvopastoral system. This model could also be applied to characterize agricultural areas where it is intended to evaluate only data from a single production site.

**Hatheway method**

The optimal number of samples was estimated by the Hatheway method considering a completely randomized

design (CRD) and a randomized block design (RBD). As the results were similar using the two experimental designs, the results include details of RBD.

Three soil depths (0-5, 5-10, and 10-20 cm) were considered to indicate the optimum number of samples to be collected within the silvopastoral system, choosing the number in accordance to the depth with greater data variability.

The soil TOC results showed that it is possible to differentiate close mean values of treatments (LSD of 20 %) with the use of small plot sizes (Figures 5A to 5F). The 5-10 cm soil depth, which showed the greatest data variation (Figures 5B and 5E), needs only six samples within the alley and 10 samples within the double rows if five treatments and four blocks are used, totaling 16 samples per plot. If great accuracy is not necessary to compare the treatments, a considerably smaller number of samples could be used.

For Cmic, which had a CV (%) approximately three-fold that found for TOC (Table 1), a very large number of samples per plot is necessary, if great accuracy is necessary to compare the treatments. Considering the soil layer with the greatest variability (10-20 cm) and an LSD of 40 % between the treatments, eight samples within the alley and six samplings within the double rows are necessary considering the use of five treatments and 10 blocks (Figures 5I and 5L). These results indicate the great difficulty to perform experiments with high accuracy to differentiate treatments with close mean values. If the aim is to set up an experiment with lower accuracy, the use of five treatments and four replications (5-10 cm depth) requires 27 samples within the alley and 13 within the double rows to achieve an LSD of 60 %, or four samples within the alley and two within the double rows to achieve an LSD of 70 %, totaling six samples per plot.

The estimation of the physiological parameters of marandu palisadegrass require a larger number of samples, due to their large CV (Table 1) and variability within the experimental site (Figure 3). The CV estimated for A, E, and Gs were approximately two-fold higher than those found for soil Cmic and six-fold higher than those found for soil TOC. Thus, the evaluation of marandu palisadegrass within a silvopastoral system considering an LSD of 70 % between the treatments (Figures 6A, 6B, and 6C), the best sampling arrangement obtained in accordance with the parameter of greatest variability (A) was 10 samples and seven blocks for an experiment with five treatments (Figure 6A). If five treatments and four blocks are used, considering an LSD of 70 %, 41, 9, and 31 samples are necessary for A, E, and Gs, respectively. On the other hand, considering an LSD of 80 %, the number of samples necessary decreases to 23, five, and 16 for A, E, and Gs, respectively.

Experimental accuracy for varied configurations of experiments could be seen for soil TOC within the alley (Figures 5A, 5B and 5C) and the double rows (Figures 5D, 5E and 5F), for soil Cmic within the alley (Figures 5G,

5H and 5I) and the double rows (Figures 5J, 5K and 5L), and for A (Figure 6A), E (Figure 6B), and Gs (Figure 6C) in the marandu palisadegrass, or estimated from the data in Table 1 and the Hatheway formula presented in the M&M section. In general, in experiment planning in RBD with five treatments and four replications, with 11 samples (mini pits) per plot (8 within the alley and 3 within the double rows), treatments with LSD lower than 23 % for TOC and 71 % for Cmic could be distinguished. For the physiological data, with five treatments, four replications, and 23 evaluations with IRGA within the plot, treatments with LSD lower than 80, 56, and 74 % of the mean for A, E, and Gs, respectively, could be distinguished.

## Discussion

### Soil TOC and Cmic within the silvopastoral system

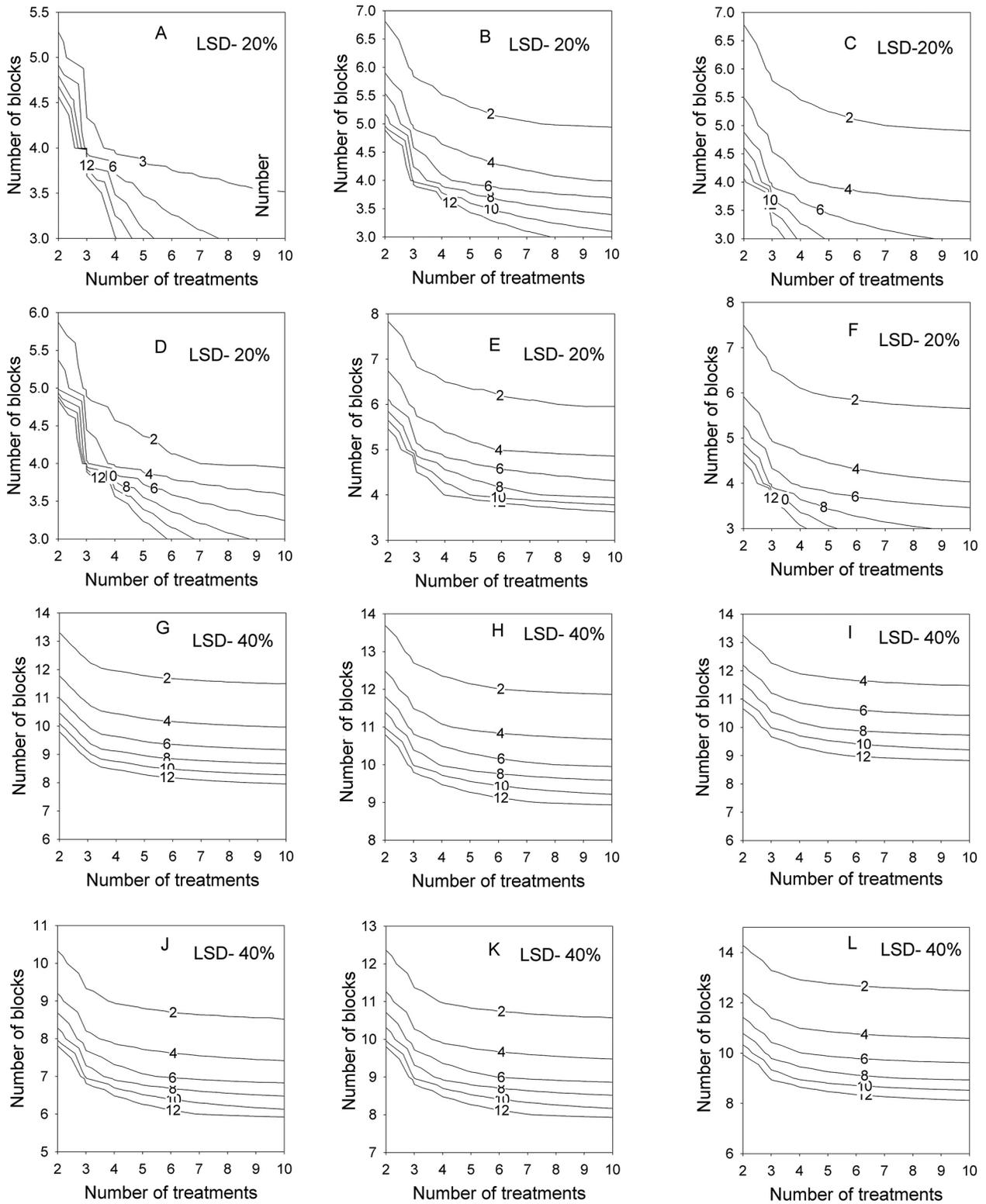
Our results showed that the accumulation of soil TOC can be favored by the cultivation of eucalyptus and marandu palisadegrass in the silvopastoral system (Figure 2), in agreement with other studies performed under agroforestry systems (Howlett et al., 2011; Lim et al., 2018; Tumwebaze et al., 2012).

Our findings for Cmic at 0-20 cm soil depth (Figure 2) is related to the greater amount of OM in the soil surface layer, an energy source for soil microorganisms. Microbial carbon is sensitive to changes in the soil environment (Acosta-Martínez et al., 2004; Oliveira et al., 2016a). The diversity of species within the silvopastoral system (e.g. forage plants, and eucalyptus) contribute to a greater amount of C in the surface layers because most root distribution occurs in the soil surface (Conceição et al., 2017).

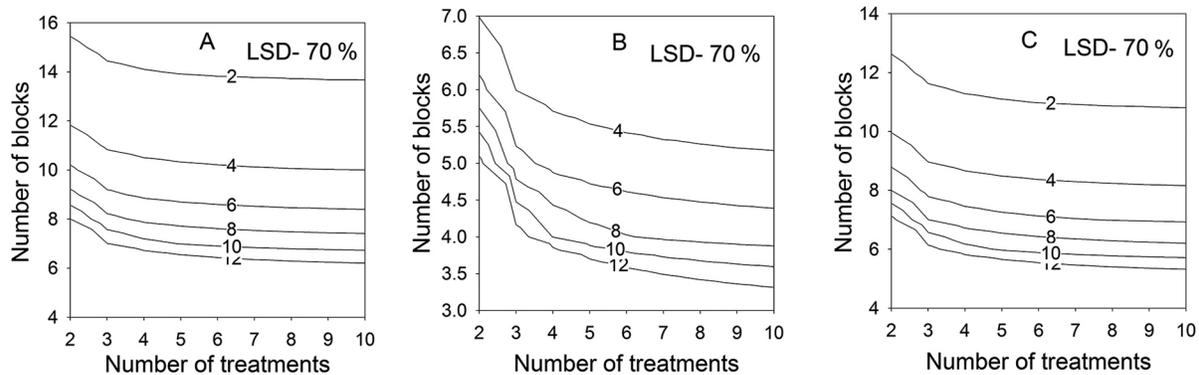
According to Moreira et al. (2018), the forest component within the system could affect the availability of nutrients for the other crops. This availability varies according to the distance from the tree, and significant changes in soil properties occur nearer the trees, due to greater litter deposition and lower exposure to solar radiation, factors that influence litter decomposition.

### Physiological parameters of marandu palisadegrass within the silvopastoral system

Our results showed that the sampling point near the double rows of eucalyptus contributed to variations in the physiological parameters of forage grass in relation to the alley (Figure 3). Santos et al. (2017) also found that shading contributed to the reduction in E and Gs for soybean plants within an agrosilvopastoral system, identifying that lower spacing from the eucalyptus affected the physiological parameters. The authors highlighted that reduction in light intensity and the high density of forage plants in addition to the greater proximity to eucalyptus trees could limit the photosynthetic rate, especially for C4 plants.



**Figure 5** – Optimal number of samples estimated by the Hatheway method considering the randomized block design (RBD) in accordance to the number of treatments and replications at 0-5 cm, 5-10 and 10-20 cm depths for soil total organic carbon (TOC) within the alley (A, B and C) and the double rows (D, E, and F), and for soil microbial carbon (Cmic) within the alley (G, H, and I) and the double rows (J, K, and L) in a silvopastoral system in southeast Brazil.



**Figure 6** – Optimal number of samples estimated by the Hatheway method considering the randomized block design (RBD) in accordance to the number of treatments and replications for Assimilation of  $\text{CO}_2$  – A (A), Transpiration rate – E (B), and Stomatal conductance – Gs (C) in the marandu palisadegrass cultivated within a silvopastoral system in southeast Brazil.

Rodrigues et al. (2014) also emphasized relevant differences in the quantity and quality of solar radiation in the understory of the silvopastoral system, due to the spatial arrangement of the trees and sampling locations, even when the forest component is planted in the east-west direction.

#### Proposal of sampling representativeness within the silvopastoral system

##### Modified maximum curvature method

The number of sampling points was defined according to the stability of the CV values (Table 1, Figure 4). Donato et al. (2018) have reported that CV values have more interference at the sampling points when data heterogeneity increases. Mello et al. (2004) also found smaller plot sizes with lower CV; however, when the plot size increased, the CV tended to stability. Similar to our findings, the authors pointed out that CV tends to increase under greater variability.

The proposed number of soil sampling for silvopastoral systems, according to MMC method (Table 1), was lower than that found by Feijó et al. (2006) evaluating soil chemical parameters before and after cultivation of cucurbits. The authors found that 11 samples for fallow and nine sample points after cropping were necessary to represent the variables analyzed.

Our results indicate the need for an increase in the number of soil samples to estimate soil Cmic according to the MMC method (Table 1 and Figure 4). The Cmic had higher variability within the system because the species, light intensity, animals, and their waste affect the quantity and distribution of soil microorganisms. Microbial biomass is sensitive to soil components and crop management operations (Aryal et al., 2019; Guillot et al., 2019).

To evaluate the physiological parameters of marandu palisadegrass applying the MMC method (Table 1), we found that heterogeneity of the silvopastoral

systems favors an increase in the number of samples to improve sampling representativeness (Figure 4). According to Gomes et al. (2019), the presence of trees in integrated systems influences the light incidence for forage grass, increasing with the development of the forest component.

For a decrease in CV (%) in the evaluation of soil Cmic and physiological parameters of marandu palisadegrass, the number of samples must be increased. However, the sampling size used in this research showed stability of CV (Figure 4). Therefore, if we consider a larger number of samples, the estimated number has no substantial changes because this increase has no significant effect on CV values (Mello et al., 2004).

##### Hatheway method

According to our findings, considering the heterogeneity of silvopastoral systems (composed of pasture and trees), the number of samples estimated by the Hatheway method is not feasible. An increase in the CV values indicates an increase in experimental error and, therefore, lower accuracy (Leite et al., 2006).

The highest CV values for Cmic can be explained by climate variations, in accordance to the quantity and quality of the plant residues deposited on the soil (Tracy and Zhang, 2008). As an example (Figures 5I and 5L), if five treatments and 10 blocks are considered, the method indicates the need to collect at least eight samples in the alley and six in the double rows, which implies opening 140 mini pits for soil sampling within the experimental site to be able to differentiate treatments with a mean LSD of 40 %.

The Hatheway method proposes an increase in stringency for experiment accuracy, with a reduction in the minimum difference to be detected between the treatments, which also cause significant increases in the number of samples. An increase in the number of replications and treatments is not desirable for sampling; therefore, it is easier to increase the number

of samples to obtain representativeness (Cargnelutti Filho et al., 2018). According to Mello et al. (2004), the Hatheway method allows to combine different plot sizes and forms to reconcile different numbers of repetitions. For Cargnelutti Filho et al. (2018), within the availability of the experimental area, the researcher must define the number of treatments to be evaluated and the accuracy to define which combination of sample numbers and repetitions is most appropriate.

Thus, treatments with LSD lower than 23 % for TOC and 71 % for Cmic can be distinguished to set up an experiment in RBD with five treatments, four replications, and eight samples in the alley and three in the double rows. For this same configuration of experiment, 23 evaluations in marandu palisadegrass between double rows of eucalyptus allows distinction of treatments with LSD lower than 80 % for the physiological parameters estimated.

## Conclusions

In order to perform soil sampling and determine the input of soil C in silvopastoral systems, the area cultivated with forage grass (alley) and eucalyptus (double rows) must be taken into consideration to ensure sampling representativeness.

The physiological parameters (A, E, and Gs) of marandu palisadegrass varied according to the sampling position in relation to the double rows of eucalyptus, requiring to increase the number of points collected to guarantee the sampling representativeness.

The MMC method could be useful to evaluate the silvopastoral systems for both experiments and characterization of agricultural areas, once 11 soil samples are representative to evaluate Cmic and TOC, and 13 samples are enough to evaluate physiological parameters in marandu palisadegrass.

The Hatheway method could be applied to experiments that need high accuracy, with a reduction in the minimum difference to be detected between the treatments and a consequent increase in the number of samples to be collected.

## Acknowledgments

This study was financed in part by the Coordination for the Improvement of Higher Level Personnel (CAPES) - Finance Code 001 and Grant N° 88881.068513/2014-01; Minas Gerais Research Foundation (Fapemig) - Grant N° PPM-00617-18; and Brazilian National Council for Scientific and Technological Development (CNPq) - Grant N° 430097/2016-6. We also thank the Barra Farm for logistical support offered during the study.

## Authors' Contributions

**Conceptualization:** Oliveira, J.C.; Frazão, L.A.; Azevedo, A.M. **Data acquisition:** Oliveira, J.C.;

Frazão, L.A.; Azevedo, A.M.; Freitas, I.C.; Melo, P.F.; Dias, R.F.; Duarte, A.C.S. **Data analysis:** Oliveira, J.C.; Frazão, L.A.; Azevedo, A.M. **Desing of methodology:** Oliveira, J.C.; Frazão, L.A.; Azevedo, A.M. **Software development:** Azevedo, A.M. **Writing and editing:** Oliveira, J.C.; Frazão, L.A.; Azevedo, A.M.; Ribeiro, J.M.; Freitas I.C.

## References

- Acosta-Martínez, V.; Zobeck, T.M.; Allen, V.G. 2004. Soil microbial, chemical and physical properties in continuous cotton and integrated crop-livestock systems. *Soil Science Society America Journal* 68: 1875-1884.
- Albrecht, A.; Kandji, S.T. 2003. Carbon sequestration in tropical agroforestry systems. *Agriculture Ecosystems Environment* 99: 15-27.
- Almeida, L.L.S.; Frazão, L.A.; Lessa, T.A.M.; Fernandes, L.A.; Veloso, A.L.C.; Lana, A.M.Q.; Souza, I.A.S.; Pegoraro, R.F.; Ferreira, E.A. 2021. Soil carbon and nitrogen stocks and the quality of soil organic matter under silvopastoral systems in the Brazilian Cerrado. *Soil and Tillage Research* 205: 104785. doi: 10.1016/j.still.2020.104785
- Alvares, C.A.; Stape, J.L.; Sentelhas, P.C.; Gonçalves, J.L.M.; Sparovek, G. 2013. Köppen's Climate Classification Map for Brazil. *Meteorologische Zeitschrift* 22: 711-28.
- Aryal, D.R.; Gomez-Gonzalez, R.R.; Hernandez-Nuriasmu, R.; Morales-Ruiz, D.E. 2019. Carbon stocks and tree diversity in scattered tree silvopastoral systems in Chiapas, Mexico. *Agroforestry Systems* 93: 213-227.
- Borges, W.L.B.; Calonego, J.C.; Rosolen, C.A. 2018. Impact of crop-livestock-forest integration on soil quality. *Agroforestry Systems* 93: 2111-2119.
- Cargnelutti Filho, A.; Neu, I.M.M.; Santos, G.O.; Facco, G.; Wartha, C.A.; Kleinpaul, J.A. 2018. Plot size related to numbers of treatments, repetitions, and the experimental precision in flax. *Comunicata Scientiae* 9: 629-636.
- Conceição, M.C.G.; Matos, E.S.; Bidone, E.D.; Rodrigues, R.A.R.; Cordeiro, R.C. 2018. Changes in soil carbon stocks under integrated crop-livestock-forest system in the Brazilian Amazon region. *Agricultural Sciences* 8: 904-913.
- Donato, S.L.R.; Silva, J.A.; Guimarães, B.V.C.; Silva, S.O. 2018. Experimental planning for the evaluation of phenotypic descriptors in banana. *Revista Brasileira Fruticultura* 40: e-962.
- Facco, G.; Cargnelutti Filho, A.; Lavezo, A.; Schabarum, D.E.; Chaves, G.G.; Silveira, D.L. 2018. Basic experimental unit and plot sizes for fresh matter of sunn hemp. *Ciência Rural* 48: e20170660. doi: 10.1590/0103-8478cr20170660
- Feijó, S.; Storck, L.; Lúcio, A.D.; Lopes, S.J. 2006. Heterogeneity of soil and sample size, before and after italian pumpkin cropping in a greenhouse. *Ciência Rural* 36: 1744-1748 (in Portuguese, with abstract in English).
- Figueiredo, C.C.; Oliveira, A.D.; Santos, I.L.; Ferreira, E.A.B.; Malaquias, J.V.; Sá, M.A.C.; Carvalho, A.M.; Santos Jr, J.D.G. 2018. Relationships between soil organic matter pools and nitrous oxide emissions of agroecosystems in the Brazilian Cerrado. *Science of the Total Environment* 618: 1572-1582.

- Ghezehei, S.B.; Everson, C.S.; Annandale, J.G. 2015. Can productivity and post-pruning growth of *Jatropha curcas* in silvopastoral systems be regulated by manipulating tree spacing/arrangement without changing tree density? *Biomass and Bioenergy* 74: 233-243.
- Gomes, F.J.; Pedreira, C.G.S.; Bosi, C.; Cavalli, J.; Holschuch, S.G.; Mourão, G.B.; Pereira, D.H.; Pedreira, B.C. 2019. Shading effects on marandu palisadegrass in a silvopastoral system: plant morphological and physiological responses. *Agronomy Journal* 111: 1-9.
- Guillot, E.; Hinsinger, P.; Dufour, L.; Roy, J.; Bertrand, I. 2019. With or without trees: resistance and resilience of soil microbial communities to drought and heat stress in a Mediterranean agroforestry system. *Soil Biology and Biochemistry* 129: 122-135.
- Hatheway, W.H. 1961. Convenient plot size. *Agronomy Journal* 53: 279-280.
- Howlett, D.S.; Mosquera-Losada, M.R.; Nair, P.K.R.; Nair, V.D.; Rigueiro-Rodríguez, A. 2011. Soil carbon storage in silvopastoral systems and a treeless pasture in northwestern Spain. *Journal of Environmental Quality* 40: 825-832.
- Instituto Nacional de Meteorologia [INMET]. 2019. Meteorological Databases for Teaching and Research = Banco de Dados Meteorológicos para Ensino e Pesquisa (BDMEP). INMET, Brasília, DF, Brazil (in Portuguese).
- Jose, S.; Dollinger, J. 2019. Silvopasture: a sustainable livestock production system. *Agroforestry Systems* 93: 1-9.
- Leite, M.S.O.; Peternelli, L.A.; Barbosa, M.H.P. 2006. Effects of plot size on the estimation of genetic parameters in sugarcane families. *Crop Breeding and Applied Biotechnology* 6: 40-46.
- Li, J. 2019. Sampling soils in a heterogeneous research plot. *Journal of Visualized Experiments* 143: 1-6.
- Lim, S.S.; Baah-Acheamfour, M.; Choi, W.J.; Arshad, M.A.; Fatemi, F.; Banerjee, S.; Carlyle, C.N.; Bork, E.W.; Park, H.; Chang, S.X. 2018. Soil organic carbon stocks in three Canadian agroforestry systems: from surface organic to deeper mineral soils. *Forest Ecology and Management* 417: 103-109.
- Lorentz, L.H.; Boligon, A.A.; Storck, L.; Lúcio, A.D. 2010. Plot size and experimental precision for sunflower production. *Scientia Agricola* 67: 408-413.
- Meier, V.D.; Lessman, K.J. 1971. Estimation of optimal field plot shape and size for testing yield in *Crambe abyssinica* Hochst. *Crop Science* 11: 648-650.
- Mello, R.M.; Lúcio, A.D.; Storck, L.; Lorentz, L.H.; Carpes, R.H.; Boligon, A.A. 2004. Size and form of plots for the culture of the Italian pumpkin in plastic greenhouse. *Scientia Agricola* 61: 457-461.
- Moraes, A.; Carvalho, P.C.F.; Lustosa, S.B.C.; Lang, C.R.; Deiss, L. 2014. Research on integrated crop-livestock systems in Brazil. *Revista Ciência Agronômica* 45: 1024-1031.
- Moreira, G.M.; Neves, J.C.N.; Magalhães, C.A.S.; Farias Neto, A.L.; Sauer, G.; Silva, J.F.V.; Fernandes, R.B.A. 2018. Soil chemical attributes in response to tree distance and sun-exposed faces after the implantation of an integrated crop-livestock-forestry system. *Revista Árvore* 42: 405-420.
- Nair, P.K.R.; Nair, V.D.; Kumar, B.M.; Haile, S.G. 2009. Soil carbon sequestration in tropical agroforestry systems: a feasibility appraisal. *Environmental Science Policy* 12: 1099-1111.
- Oliveira, W.R.D.; Ramos, M.L.G.; Carvalho, A.M.; Coser, T.R.; Silva, A.M.M.; Lacerda, M.M.; Souza, K.S.; Marchão, R.L.; Vilela, L.; Pulrolnik, K. 2016a. Dynamics of soil microbiological attributes under integrated production systems, continuous pasture, and native Cerrado. *Pesquisa Agropecuária Brasileira* 51: 1501-1510.
- Oliveira, C.H.R.; Reis, G.G.; Reis, M.G.F.; Leite, H.G.; Souza, F.C.; Faria, R.S.; Oliveira, F.B. 2016b. Dynamics of eucalypt clones canopy and *Brachiaria brizantha* production in silvopastoral systems with different spatial arrangements. *Agroforestry Systems* 90: 1077-1088.
- Paula, R.R.; Reis, G.G.; Reis, M.G.F.; Oliveira Neto, S.N.; Leite, H.G.; Melido, R.C.N.; Lopes, H.N.S.; Souza, F.C. 2013. Eucalypt growth in monoculture and silvopastoral systems with varied tree initial densities and spatial arrangements. *Agroforestry Systems* 87: 1295-1307.
- Pereira, A.S.; Silva, G.O.; Carvalho, A. 2017. Minimum plot size to evaluate potato tuber yield traits. *Horticultura Brasileira* 35: 604-608.
- Rodrigues, C.O.D.; Araújo, S.A.C.; Viana, M.C.M.; Rocha, N.S.; Braz, T.G.S.; Villela, S.D.J. 2014. Light relations and performance of signal grass in silvopastoral system. *Acta Scientiarum* 36: 129-136.
- Santos, M.V.; Ferreira, E.A.; Valadão, D.; Oliveira, F.L.R.; Machado, V.D.; Silveira, R.R.; Souza, M.F. 2017. *Brachiaria* physiological parameters in agroforestry systems. *Ciência Rural* 47: e20160150. doi: 10.1590/0103-8478cr20160150
- Santos, D.C.; Guimarães Júnior, R.; Vilela, L.; Maciel, G.A.; França, A.F.S. 2018. Implementation of silvopastoral systems in Brazil with *Eucalyptus urograndis* and *Brachiaria brizantha*: productivity of forage and an exploratory test of the animal response. *Agriculture, Ecosystems & Environment* 266: 174-180.
- Saste, S.V.; Sananse, S.L. 2015. Soil Heterogeneity to determine size and shape of plots: a review. *International Journal of Advanced Scientific and Technical Research* 5: 201-209.
- Silva, E.E.; Azevedo, P.H.S.; De-Polli, H. 2007. Carbon determination of soil microbial biomass (BMS-C) = Determinação do carbono da biomassa microbiana do solo (BMS-C). Embrapa Agrobiologia. Comunicado técnico 98 (in Portuguese).
- Silva, M.S.; Silva, S.O.; Donato, S.L.R.; Sampaio Filho, O.M.; Silva, G.M.A. 2019. Optimal size of experimental plots of papaya trees using a modified maximum curvature method. *Ciência Rural* 49: e20180930.
- Tracy, B.F.; Zhang, Y. 2008. Soil compaction, corn yield response, and soil nutrient pool dynamics within an integrated crop-livestock system in Illinois. *Crop Science* 48: 1211-1218.
- Tumwebaze, S.B.; Bevilacqua, E.; Briggs, R.; Volk, T. 2012. Soil organic carbon under a linear simultaneous agroforestry system in Uganda. *Agroforestry Systems* 84: 11-23.
- Vance, E.D.; Brookes, P.C.; Jenkinson, D.S. 1987. An extraction method for measuring soil microbial biomass C. *Soil Biology and Biochemistry* 19: 703-707.
- Yeomans, J.C.; Bremner, J.M. 1988. A rapid and precise method for routine determination of organic carbon in soil. *Communications in Soil Science and Plant Analysis* 19: 1467-1476.