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Nodulation and biological nitrogen fixation (BNF) in forage peanut (*Arachis pintoi*) cv. Belmonte subjected to grazing regimes

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ABSTRACT

Productivity of grassland ecosystems is highly dependent on the availability of nitrogen (N) from the soil and/or biological fixation (BNF) from the atmosphere. However, the high cost of N-fertilizers and the increasing awareness of the need for sustainable nutrient alternative sources have increased the strategic importance of the BNF. Defoliation interferes with plant growth and also with BNF in legumes, since it modifies the leaf area and the availability of energy to plants. In spite of the potential advantages of forage legumes to pastures, current knowledge regarding the effects of grazing management on BNF is scarce. In order to evaluate the effect of grazing management on aerial and root mass, nodulation and BNF in forage peanut (*Arachis pintoi* cv. Belmonte), two experimental protocols based on the continuous and intermittent (rotational grazing) stocking methods were used. In the continuous stocking protocol, treatments corresponded to four grazing intensities represented by four management heights - 5, 10, 15 and 20 cm. In the intermittent stocking protocol, treatments corresponded to combinations between two pre- (95% and maximum canopy light interception during regrowth – $LI_{95\%}$ and LI_{Max}, respectively) and two post-grazing (post-grazing heights equivalent to 40 and 60% of the pre-grazing height) conditions. It was hypothesized that hard grazing, represented by low management heights under continuous stocking or low post-grazing heights under intermittent stocking, causes reduction in root mass and nodulation interfering with the balance between root and aerial, with negative impacts on BNF. Under continuous stocking, greater number of nodules was associated with lower nodule mass and vice-versa throughout the year. Swards managed at 5 and 10 cm had greater root and nodule mass than those managed at 15 and 20 cm. However, greater BNF (%Ndfa and BNF) was recorded on swards managed at 15 and 20 cm relative to those managed at 5 and 10 cm. Under intermittent stocking, the combinations between frequencies and severities of grazing did not affect root mass or nodule number at both pre- and post-grazing conditions. Overall, swards managed with the LI_{Max} target showed greater BNF than those managed with the $LI_{95\%}$ target. For swards managed with the same LI target ($LI_{95\%}$ or LI_{Max}), hard grazing (40% of the pre-grazing height) resulted in lower %Ndfa and BNF during early and late spring and summer II. For both grazing methods, the range of management targets used did not result in negative impacts on root mass or nodulation. Regardless of grazing method, BNF was determined by aerial biomass, indicating that moderate intensities of grazing would be adequate for optimizing BNF.

1. Introduction

The necessary nitrogen (N) to sustain pasture growth may be provided through nitrogen fertilizers or from the use legumes capable of fixing atmospheric N (Thomas, 1992). Although N fertilization is considered essential to sustain grassland productivity in tropical regions, the increased use of N derived from mineral fertilizers has been strongly questioned not only for its cost (Euclides et al., 2007), but also for the energy cost, GHG and NH_3 emissions associated with industrial N fertilizer production and applications in agricultural systems (Qiao et al., 2014; Xu et al., 2019). Thus, the growing worldwide concern about issues of global warming, andenvironmental degradation and losses of

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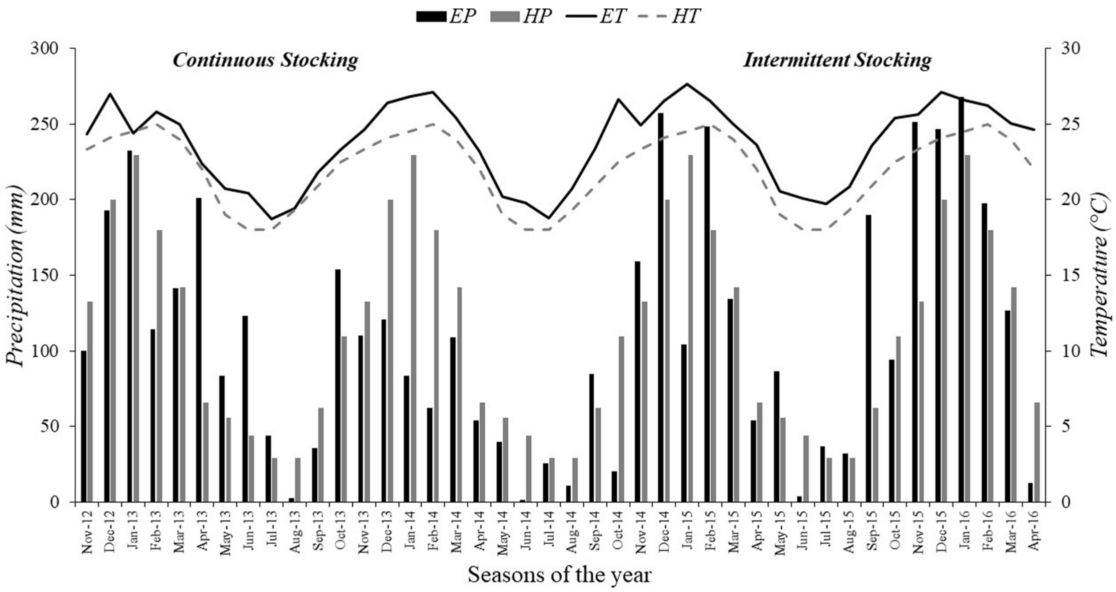

Fig. 1. Monthly precipitation (mm) and daily temperatures (°C) of the experimental period (EP and ET, respectively) and historical averages (HP and HT from 1917 to 2017, respectively) for the region of Piracicaba, SP, from November 2014 to April 2016.

natural resources has rekindled the interest in using pasture legumes as a source of biologically fixed N (Ledgard and Steele, 1992; Peoples et al., 1995).

The biological nitrogen fixation (BNF) probably is the most important natural biological process after photosynthesis (Unkovich, 2013), extremely relevant to sustainable agriculture (Udvardi and Poole, 2013; Oldroyd et al., 2011), since it represents the main form of N input for several terrestrial ecosystems. The understanding of mechanisms and feedbacks involved on the atmospheric N fixation has received growing attention in the literature (Menge and Hedin, 2009). However, information on regulation pathways and the impacts of defoliation on the symbiotic N_2 fixation in ecosystems other than crop production systems, in particular for tropical legumes subjected to grazing, is substantially weaker (Vitousek et al., 2002), particularly for tropical related to temperate conditions (Carlsson and Huss-Danell, 2003).

According to Hartwig (1998), the control of symbiotic N_2 fixation operates through a series of ecophysiological triggers which are also influenced by complex interactions between legume plants, soil (chemical, physical and biological properties) environmental conditions (temperature, water stress and CO_2), as well as other organisms in the ecosystem (Soussana and Tallec, 2010; Das et al., 2011). For forage legumes in pastures, the animal component also represents one kind of regulation of N_2 fixation. This is because the main parameter determining the amount of symbiotically fixed N_2 introduced into a terrestrial ecosystem is the legume biomass (Hartwig, 1998), which makes the defoliation regime a modulator of the performance of symbiotic N_2 fixation in each individual legume (Boucho et al., 2019; Carranca, 2013; Carranca et al., 2015; Lawson et al., 2000).

The defoliation regime corresponds to the frequency and severity of grazing that plants are subjected to. Its primary effect is related to the intensity of leaf area removal that, in turn, alters the balance between aerial and root biomass, interfering with the size and efficiency of the remaining photosynthetic apparatus, and affecting its potential of transforming the captured sunlight into chemical energy (carbohydrates) (Richards, 1993; Gordon et al., 1990). Bayne et al. (1984) and Hartwig et al. (1994) indicated that the metabolic activity of the nodules after defoliation is significantly reduced, with negative impacts on atmospheric N_2 fixation. The low photosynthetic activity of the remaining leaf area, consequence of long regrowth periods (Pedreira and Pedreira, 2007), and/or the small residual leaf area after severe grazing may result in restricted supply of assimilates immediately after defoliation and negatively affect nodules growth, development and activity (Lie, 1971; King and Purcell, 2001). In spite of the importance of forage legumes to pastures, information regarding the effects of grazing management on the BFN mechanisms is scarce (Carranca et al., 1999) and mainly derived from studies with temperate forage plants (e.g. alfalfa and white clover), highlighting the lack of information regarding tropical forage legumes.

Forage peanut (*Arachis pintoi* cv. Belmonte) is a perennial stoloniferous tropical legume, with a deep and dense root system (Fisher and Cruz, 1994), widely used under grazing conditions in the tropics, but with almost none information regarding the impacts of grazing on BNF. Thus, the objective of this study was to evaluate the effect of grazing on canopy leaf area index (LAI), root mass, nodulation and BNF using two experimental protocols based on continuous and intermittent stocking management. The hypothesis was that high intensities of grazing, represented by low management canopy heights under continuous stocking or low post-grazing heights under intermittent stocking, would alter the balance between aerial and root (reduction of assimilates to root relative to shoot), resulting in reduced root mass and nodulation, with negative impacts on BNF.

2. Material and methods

2.1. Experimental site, treatments and design

The experiments were carried out from November 2012 until April 2016 at "Luiz de Queiroz" College of Agriculture, University of São Paulo, Piracicaba, SP, Brazil (22°42′S, 47°37′W and 550 m a.s.l.). In order to avoid water soil deficits, a sprinkling irrigation system was installed and irrigation was conducted based on rainfall events, average air temperature and evapotranspiration from data collected in a Meteorological Station located at 500 m from the experimental area (Fig. 1).

The soil is a Eutric Kandiudalf, with high fertility. Average soil chemical characteristics for the 0–20 cm layer were: pH $CaCl_2$: 5.85; OM = 41 g/dm^3; P (ion-exchange resin extraction method) = 77 mg/dm^3; Ca = 87.5 mmolc/dm^3, Mg = 50 mmolc/dm^3; K = 16.3 mmolc/dm^3; H + Al = 26.5 mmolc/dm^3; sum of bases = 146.3 mmolc/dm^3; cation exchange capacity = 172.8 mmolc/dm^3; base saturation = 84%. These were considered adequate for the forage species used, with no need for additional fertilization. The vegetative material used for planting, the stolons, was not inoculated. Inoculation was based on native *Rhizobia* found in the soil.

The 0.4 ha experimental area was divided into 16 paddocks of approximately 210 m^2 each. Crossbred Holstein and Jersey dairy heifers weighing 230 ± 60 kg were used as grazers only. For both experimental protocols (continuous and intermittent stocking) treatments were assigned to experimental units according to a randomized complete block design, with four replications. The continuous stocking protocol was carried out from January 2013 to April 2014, after an adaptation period to the treatments of around 60 days (November and December 2012). The intermittent stocking protocol was carried out from February 2015 to April 2016, also after an adaptation period of 60 days (December 2014 and January 2015). Although the experiments were conducted continuously throughout each experimental period, evaluations were performed every season of the year.

For the continuous stocking protocol treatments corresponded to four grazing intensities represented by the management heights of 5, 10, 15 and 20 cm. The steady-state canopy heights were maintained by grazing that mimicked continuous stocking (Yasuoka et al., 2018). The intermittent stocking protocol treatments corresponded to combinations between two pre- (95% and maximum canopy light interception during regrowth – $LI_{95\%}$ and LI_{Max}, respectively) and two post-grazing (post-grazing heights equivalent to 40 and 60% of the pre-grazing height) conditions, the last being defined according to recommendations of Fonseca et al. (2012). Grazings were performed in the shortest possible time, preferably no longer than 12 h (day grazing), using the mob grazing technique (Gildersleeve et al., 1987).

2.2. Measurements

2.2.1. Canopy height

During the continuous stocking protocol canopy height was evaluated throughout the experimental period at 3 and 4 day-intervals (twice a week) adapted sward stick (Bathram (1985)). The paddocks were divided into nine sampling areas and three readings were taken from each one, totalling 27 readings per paddock. Grazings were performed once or twice a week with the objective of maintaining the targets of 5, 10, 15 and 20 cm considering a 10% maximum range of variation.

During the intermittent stocking protocol, the canopy height was measured at pre- and post-grazing adapted sward stick (Bathram, 1985), with a total of 50 random readings taken per paddock.

2.2.2. Canopy light interception

Monitoring of canopy light interception (LI) during the intermittent stocking protocol was carried out using a LAI 2000 canopy analyser (LI-COR, Lincoln, Nebraska, EUA) soon after each grazing and, afterwards, weekly during regrowth until the value of 90% was reached, and then every two days as a means of ensuring that the $LI_{95\%}$ and LI_{Max} targets were precisely achieved (average of 94.9 and 99.4%, respectively). LI_{Max} was defined as the value obtained from two consecutive equal readings. Measurements were carried out always at dusk, on eight points per paddock considered representative of the average sward condition (visual assessment of herbage mass and height) at the time of sampling. In each point one reference reading was taken above the canopy and five at ground level, totaling eight readings above the canopy and forty at ground level per paddock.

2.2.3. Aerial biomass

Two samples were harvested per paddock using 0.33 m^2 metallic frames (0.90 x 0.37 m). Frames were positioned at points representative of average sward conditions at the time of sampling (visual assessment of herbage mass and height) and all herbage within the sampling area was cut to ground level. Samples were divided in two subsamples, one for determining herbage mass and the other stored for BNF determination. Subsamples were oven dried at 65 °C until constant mass. The aerial biomass (ton ha^{-1}) was calculated using the sample dry weight and the area of the metallic frame. For the intermittent stocking protocol only the results from the pre-grazing measurements will be used and presented in this paper.

2.2.4. Sward leaf area index

The sward leaf area index (LAI) was determined using the same samples collected for aerial biomass determination. After manual separation of the morphological components, leaf laminae were passed through a LAI-3100 leaf area integrator (LI-COR, Lincoln, Nebraska, EUA) before oven drying, and the data used to calculate specific leaf area and leaf area index of samples. For the continuous stocking protocol average LAI values corresponded to values actually measured since paddocks were maintained at “constant” heights. For the intermittent stocking protocol average LAI values corresponded to the arithmetic mean between LAI measurements at pre- and post-grazing.

2.2.5. Root and nodule mass

Root samples were harvested using 15 cm-internal diameter cylinders introduced to a 20 cm-depth into the soil following the same sampling frequency and criteria used for BNF determinations. For the intermittent stocking protocol sampling was carried out at both pre- and post-grazing conditions. After sampling, samples were immediately sent to laboratory and standardized to 10 cm-depth removing the excess of material (dirt and roots). After washing and root separation, nodules (determinate nodules – Hirsch, 1992) were hand separated and the total number of nodules per sample (cylinder) was determined. Roots and nodules were oven dried at 65 °C until constant mass and weighed.

2.3. Total N, N derived from atmosphere and estimates of biological N fixation (BNF)

Herbage samples used for determinations of total N and BNF were subsamples of those harvested for aerial biomass determination. After drying, the material was ground in a Willey mill to pass a 0.85 mm sieve (Smith and Myng, 1990). The BNF was estimated using the ^{15}N isotope natural abundance technique (Shearer and Kohl, 1986). The technique is based on the fact that the available N on the majority of the soils is lightly enriched with the ^{15}N isotope relative to the atmosphere (Okito et al., 2004), indicating that a N-fixing plant will have a lower ^{15}N concentration than a non-fixing control plant, which is completely dependent of N from the soil. The samples were analysed for the $\delta^{15}N_{reference}$:$\delta^{15}N_{legume}$ and total N (reference and legume) at the aerial biomass using a mass spectrophotometer (Finigan Delta Plus). According to the methodology, the N derived from the atmosphere (% Ndfa) was calculated (exp 1) as follows:

$$\%Ndfa = \left(\frac{\delta^{15}N\ reference\ -\ \delta^{15}N\ legume}{\delta^{15}N\ reference\ -\ B}\right) x\ 100$$

Where:

- $\delta^{15}N$ reference = $\delta^{15}N$ value from the soil obtained from non-fixing plants growing together with the legume;
- $\delta^{15}N$ legume = $\delta^{15}N$ value for the N_2 fixing plant;
- B = a constant used to adjust the ^{15}N isotopic fractioning by legumes during the biological nitrogen fixation process.

Forage peanut was used as N-fixing plant and as non-fixing plants (control) four plants (their aerial biomass) growing nearby the experimental units were used: *Brachiaria decumbens* Stapf., *Cynodon dactylon* (L.) Pers., *Panicum maximum* (Jacq.) e *Paspalum notatum* (Flügge. Parodi). The adjustment of the isotopic fractioning was made according to the recommendation of Doughton et al. (1992), and a B value of -2.27‰, proposed by Okito et al. (2004) for non-inoculated *Arachis* *hypogeal* L. was used.

The amount of N biologically fixed in the aerial biomass of forage peanut (kg N ha^{-1}) was calculated (exp 2) as:

$$N_{bnf} = N_{total} \times \%N_{bnf}$$

Where:

- N_{bnf} = total amount of N in the aerial biomass of forage peanut derived from BNF (kg N ha^{-1});
- N_{total} = total amount of N in the aerial biomass of forage peanut as determined by dry combustion of samples (kg N ha^{-1});
- $\%N_{bnf}$ = %Nda in the aerial biomass of forage peanut.

2.4. Statistical analysis

Data were analysed separately for each experimental protocol (continuous and intermittent stocking), and grouped according to predetermined seasons of the year. For the continuous stocking protocol, the seasons of the year corresponded to summer I, autumn, winter, spring and summer II. For the intermittent stocking protocol, the seasons of the year corresponded to summer I, autumn/winter, early spring, late spring and summer II. The difference is because under intermittent stocking the duration of grazing cycles is completely variable and follows very closely the seasonality of herbage production. For the continuous stocking protocol, paddocks were maintained at steady-state and sampling could be performed exactly in each season of the year. All data set was subjected to ANOVA using the MIXED Procedure of SAS® (Statistical Analysis System). The choice of the variance/covariance matrix was made using the Schwarz's Bayesian Criterion (Littel et al., 2000). The matrix that was most indicated for each data set was selected for all variables, with the ARH(1) and TOEP type used for the continuous and intermittent stocking protocols, respectively. ANOVA was performed using the following variation causes: (i) continuous stocking protocol: canopy height, season of the year and the interaction between them (fixed effects) and blocks (random effect); (ii) intermittent stocking protocol: pre-grazing canopy light interception (LI), post-grazing height (% of the pre-grazing height), season of the year and their interactions (fixed effects) and blocks (random effect). For both protocols the season of the year was considered as repeated measure. Treatment means were estimated using the LSMEANS command and comparison, when necessary, made using Student *t*-test at 5% probability.

The data regarding canopy height for the continuous stocking protocol and pre-grazing canopy light interception and post-grazing height for the intermittent stocking protocol were presented using descriptive statistics only (mean and standard error of the mean for treatments within seasons of the year), since they were all control variables.

3. Results

3.1. Continuous stocking protocol

Root mass varied with canopy height ($p = 0.0352$) and season of the year ($p < 0.0001$). Greater values were recorded on swards managed at 5 and 10 cm, intermediate on swards managed at 20 cm and smaller values on swards managed at 15 cm. Throughout the year, greater values were recorded during winter, spring and summer II and smaller values during summer I and autumn (Table 1).

Total nodule mass varied with canopy height ($p = 0.0219$) and season of the year ($p < 0.0001$). Greater values were recorded on swards managed at 10 cm and smaller on swards managed at 15 and 20 cm. During the year, greater values were recorded in spring, intermediate in summer I, winter and summer II and smaller in autumn (Table 1). Nodule number was only influenced by season of the year ($p = 0.0046$), with greater values recorded during summer I and spring, intermediate during autumn and summer II and smaller values during winter (Table 1). Nodule size varied only with season of the year ($p < 0.0001$), with greater values recorded during spring and winter, intermediate during summer II and summer I and smaller values during autumn (Table 1).

Aerial biomass varied with canopy height ($p < 0.0001$), season of the year ($p = 0.0001$) and with the canopy height x season of the year interaction ($p = 0.0165$). Swards managed at 5 cm showed the smallest values, which increased gradually as canopy height increased. During the experimental period, greater values were recorded during the first pasture growing season (summer I and autumn) and smaller during the second pasture growing season (spring and summer II), with intermediate values recorded during winter (Table 1).

LAI varied with canopy height ($p = 0.0009$), season of the year ($p < 0.001$) and with the canopy height x season of the year interaction ($p = 0.0087$). Swards managed at 20 cm showed greater values than those managed at 5, 10 and 15 cm. Greatest values were recorded during summer I, but during autumn and winter swards managed at 10, 15 and 20 cm showed smaller LAI relative to the remaining seasons of the year, characterizing the canopy height x season of the year interaction (Table 2).

Total N percentage in the aerial biomass varied with canopy height ($p = 0.0062$) and season of the year ($p = 0.0006$). Swards managed at 15 and 20 cm showed lower total N percentage than those managed at 5 cm, with intermediate values on those managed at 10 cm. Throughout the year, highest values were recorded during spring, with no difference during the remaining seasons of the year (Table 1). %Ndfa varied with canopy height ($p = 0.005$) and season of the year ($p < 0.0001$). Higher values were recorded on swards managed at 15 and 20 cm relative to those managed at 5 and 10 cm. During the experimental period, higher values were recorded during winter, spring and summer II and lower during summer I and autumn (Table 1).

BNF varied with canopy height ($p < 0.0001$) and season of the year ($p = 0.0376$). BNF values increased with increased sward management height, with highest values recorded on swards managed at 20 cm. During the experimental period, higher values were recorded during spring, intermediate during winter and lower values during summer I, autumn and summer II (Table 1).

3.2. Intermittent stocking protocol

3.2.1. Pre-grazing condition

Total nodule mass varied with season of the year ($p < 0.0001$) and with the LI x season of the year interaction ($p = 0.0235$). Overall, the values were similar for the LI targets. For the LI_{Max} target, total nodule mass increased from summer I until late spring, when the greatest value was recorded, and then started to decrease until summer II. For the $LI_{95\%}$ target, total nodule mass increased continuously until reaching the greatest values during summer II (Table 3).

Nodule number varied only with season of the year ($p = 0.0072$), with greater values recorded during summer II, smaller during autumn/winter and early spring and intermediate during summer I and late spring (Table 3). Nodule size varied with season of the year ($p < 0.0001$) and with the LI x season of the year interaction ($p < 0.0001$). Differences between LI targets were detected only during early spring and summer II, with greater values recorded for $LI_{95\%}$ relative to LI_{Max}. For the $LI_{95\%}$ target, greater values were recorded during early and late spring and summer II and smaller during summer I and autumn/winter. For the LI_{Max} target, greater values were recorded during late spring relative to the remaining seasons of the year (Table 3).

Aerial biomass varied with LI ($p < 0.0001$) and with the LI x season of the year interaction ($p = 0.0253$). Overall, greater values were recorded for the LI_{Max} relative to the $LI_{95\%}$ target, except during early spring, when there was no difference between LI targets. For both LI targets aerial biomass decreased from the first (summer I, autumn/winter and early spring) to the second (late spring and summer II)

Table 1
Root mass, total nodule mass, nodule number, nodule size, aerial biomass, total nitrogen percentage in the aerial biomass (Total N), percentage of nitrogen derived from the atmosphere (Ndfa) and total nitrogen derived from biological nitrogen fixation (NBNF) (mean ± standard error of the mean) on *Arachis pintoi* cv. Belmonte swards managed with four grazing heights under continuous stocking from February 2013 to April 2014.

	Root mass (g cylinder^{-1})	Nodule mass (mg cylinder^{-1})	Nodule number (n^{o} cylinder^{-1})	Nodule size (mg nodule^{-1})	Aerial biomass (t DM ha^{-1})	Total-N (%)	Ndfa (%)	N_{BNF} (kg N ha^{-1})
Canopy height (cm)								
5	10.3 ± 0.76 A	231 ± 23.4 AB	678 ± 62.1	0.34 ± 0.02	4.9 ± 0.42 D	3.3 ± 0.09 A	53 ± 2.3 C	88 ± 7.4 D
10	10.6 ± 0.76 A	250 ± 23.4 A	626 ± 62.1	0.39 ± 0.02	7.5 ± 0.42 C	3.1 ± 0.09 AB	57 ± 2.3 BC	128 ± 7.4 C
15	7.8 ± 0.76 B	155 ± 23.4 C	458 ± 62.1	0.36 ± 0.02	10.3 ± 0.42 B	2.9 ± 0.09 B	62 ± 2.3 AB	177 ± 7.4 B
20	8.6 ± 0.76 AB	167 ± 23.4 BC	475 ± 62.1	0.36 ± 0.02	13.2 ± 0.42 A	2.9 ± 0.09 B	64 ± 2.3 A	244 ± 7.4 A
Season of the year								
Summer I	5.4 ± 0.85 B	208 ± 33.6 B	632 ± 73.4 A	0.32 ± 0.03 C	9.7 ± 0.45 AB	2.7 ± 0.11 B	51 ± 2.3 B	144 ± 6.2 B
Autumn	7.6 ± 0.85 B	92 ± 18.6 C	546 ± 66.9 AB	0.17 ± 0.03 D	10.1 ± 0.45 A	2.9 ± 0.10 B	49 ± 2.0 B	145 ± 5.4 B
Winter	10.4 ± 0.85 A	178 ± 22.1 B	414 ± 51.1 B	0.45 ± 0.03 AB	8.8 ± 0.45 BC	2.9 ± 0.11 B	66 ± 2.0 A	163 ± 8.2 AB
Spring	12.4 ± 0.85 A	325 ± 21.1 A	660 ± 34.2 A	0.50 ± 0.03 A	8.3 ± 0.45 CD	3.8 ± 0.15 A	65 ± 4.5 A	199 ± 16.1 A
Summer II	10.7 ± 0.85 A	200 ± 33.6 B	543 ± 56.9 AB	0.38 ± 0.03 BC	7.8 ± 0.45 D	3.0 ± 0.09 B	64 ± 2.2 A	146 ± 7.4 B

*Cylinder volume = 1800 cm^{3}.
Means followed by the same uppercase letters in columns are not statistically different each other ($p > 0.05$).

Table 2
Sward leaf area index (mean ± standard error of the mean) of *Arachis pintoi* cv. Belmonte swards managed with four grazing heights under continuous stocking from February 2013 to April 2014.

Season of the year/Canopy height (cm)	5	10	15	20
Summer I	4.03 ± 0.427 Ab	4.05 ± 0.427 Ab	5.05 ± 0.427 Ab	6.94 ± 0.427 Aa
Autumn	3.26 ± 0.365 Aa	3.56 ± 0.365 Aa	3.42 ± 0.365 Ba	3.63 ± 0.365 Ba
Winter	2.66 ± 0.525 Aa	2.00 ± 0.525 Ba	1.48 ± 0.525 Ca	2.52 ± 0.525 Ba
Spring	3.84 ± 0.712 Ab	4.48 ± 0.712 Ab	5.10 ± 0.712 Ab	7.85 ± 0.712 Aa
Summer II	3.08 ± 0.395 Aa	3.04 ± 0.395 ABa	3.29 ± 0.395 Ba	3.39 ± 0.395 Ba

Means followed by the same lowercase letter in rows and uppercase letters in columns are not statistically different each other ($p > 0.05$).

pasture growing season (Table 3).

LAI varied with LI targets ($p < 0.0001$) and with the LI x season of the year interaction ($p = 0.0178$). In general, greater values were recorded on swards managed with the LI_{Max} relative to the $LI_{95\%}$ target, except during summer II, when there was no difference between LI targets. Throughout experimental period, the $LI_{95\%}$ target showed greater values during early and late spring and summer II relative to the remaining seasons of the year. For the LI_{Max} target, greater values were recorded during autumn/winter and early spring relative to the remaining seasons of the year (Table 3).

Total nitrogen in the aerial biomass varied only with season of the year ($p = 0.0002$), with higher values recorded during early (3.48 ± 0.089) and later spring (3.50 ± 0.085) relative to summer I (2.80 ± 0.085) and summer II (3.13 ± 0.085), and intermediate values during autumn/winter (3.35 ± 0.085).

%Ndfa varied with post-grazing height ($p = 0.0303$), season of the year ($p < 0.0001$) and with the LI x post-grazing height x season of the year interaction ($p = 0.0372$). Values varied throughout the year, but highest values were recorded during summer I and early spring. Overall, there was no difference between LI targets for the same post-grazing height within season of the year. On the other hand, for the same LI target, higher values were recorded for the LI_{Max} target in early and late spring and $LI_{95\%}$ target in summer II when associated with the post grazing height target of 60% relative to 40% of the pre-grazing height (Table 5).

BNF varied with LI ($p < 0.0001$), post-grazing height ($p = 0.0113$), season of the year ($p = 0.0005$) and with the LI x post-grazing height x season of the year interaction ($p = 0.0474$). Values varied throughout the year, but highest values were recorded during autumn/winter and early spring. Within each season of the year, BNF varied according to LI and post-grazing height, with higher values recorded on swards managed with the LI_{Max} relative to the $LI_{95\%}$ target regardless of post-grazing height during summer I and autumn/winter. During early and late spring higher values were recorded for the LI_{Max} relative to the $LI_{95\%}$ target only for the post-grazing height target of 60% of the pre-grazing height, with no difference between LI targets for the post-grazing height of 40% of the pre-grazing height. During summer II the reverse happened, with higher values recorded for the LI_{Max} relative to the $LI_{95\%}$ target when associated with the post-grazing height of 40% of the pre-grazing height. No difference between LI targets were detected for the post-grazing height of 60% of the pre-grazing height (Table 5).

3.2.2. Post-grazing condition

Root mass varied with season of the year only ($p = 0.0274$), with greater values recorded during autumn/winter, smaller during summer I and intermediate during early spring and summer II (Table 4). Total nodule mass varied with season of the year ($p = 0.0016$) and with the LI x season of the year interaction ($p = 0.0247$). On swards managed with the LI_{Max} target values increased from summer I to autumn/winter, remaining stable until the end of the experiment in summer II. On swards managed with the $LI_{95\%}$ target greater values were recorded during summer II, smaller during summer I and autumn/winter and intermediate during early and late spring. Differences between LI targets were detected during autumn/winter and late spring, with greater values recorded for LI_{Max} relative to $LI_{95\%}$ (Table 4). Nodule number and nodule size (mass) were not affected by any of the variation causes (LI, post-grazing height, season of the year and the interaction between them).

4. Discussion

Biological nitrogen fixation (BNF) is an energy-intensive process, and to supply energy for N_2 fixation, carbohydrates are translocated from their sources to nodules to support their bacteroids respiration and growth (Osman et al., 1983). The hypothesis tested in this study for both grazing methods presumed that high intensity of defoliation would result in restrictions to root growth, reducing root mass that, in turn,

Table 3

Root mass, nodule number, total nodule mass, nodule size, aerial biomass and leaf area index at pre-grazing (mean ± standard error of the mean) of *Arachis pintoi* cv. Belmonte swards subjected to strategies of intermittent stocking management form February 2015 to April 2016. $LI_{95\%}$ and LI_{Max} correspond to pre-grazing targets when canopy light interception during regrowth reached 95% and maximum values, respectively.

Season of the year	Nodule number	Nodule mass		Nodule size		Aerial biomass		Leaf Area Index	
	(n° cylinder^{-1})	(mg cylinder^{-1})		(mg nodule^{-1})		(t DM ha^{-1})			
		$LI_{95\%}$	II_{Max}	$LI_{95\%}$	LI_{Max}	$LI_{95\%}$	LI_{Max}	$LI_{95\%}$	LI_{Max}
Summer I	777 ± 78.2 BC	247 ± 64.3 Ca	286 ± 64.3 Ba	0.33 ± 0.039 Ba	0.36 ± 0.039 Ba	9.1 ± 0.67 ABb	13.9 ± 0.67 ABa	3.85 ± 0.442 BCb	5.33 ± 0.442 Ba
Autumn/Winter	692 ± 78.2 C	231 ± 64.3 Ca	287 ± 64.3 Ba	0.37 ± 0.039 Ba	0.36 ± 0.039 Ba	9.5 ± 0.67 ABb	14.3 ± 0.67 Aa	3.56 ± 0.442 Cb	7.12 ± 0.442 Aa
Early Spring	655 ± 78.2 C	345 ± 64.3 BCa	344 ± 64.3 Ba	0.64 ± 0.041 Aa	0.40 ± 0.043 Bb	10.6 ± 0.67 Aa	12.1 ± 0.67 BCa	5.01 ± 0.442 Ab	6.39 ± 0.442 ABa
Late Spring	859 ± 78.2 AB	445 ± 64.3 ABa	594 ± 64.3 Aa	0.54 ± 0.041 Aa	0.61 ± 0.039 Aa	8.2 ± 0.67 Bb	11.7 ± 0.67 Ca	4.79 ± 0.442 ABb	6.01 ± 0.442 Ba
Summer II	954 ± 78.2 A	540 ± 64.3 Aa	388 ± 64.3 Ba	0.55 ± 0.039 Aa	0.40 ± 0.039 Bb	8.2 ± 0.67 Bb	10.8 ± 0.67 Ca	4.86 ± 0.442 ABa	5.64 ± 0.442 Ba

[*] Cylinder volume = 1800 cm^3.

Means followed by the same lowercase letter in rows and uppercase letters in columns are not statistically different each other ($p > 0.05$).

would affect nodule size and total nodule mass, reducing BNF. However, contrary to the hypothesis, higher intensities of grazing under the continuous stocking protocol or the different combinations between frequency and severity of grazing under the intermittent stocking protocol did not affect negatively root mass and nodulation. For both experimental protocols the percentage of N derived from atmosphere (% Ndfa) and the BNF (kg N ha^{-1}) were modulated by sward aerial biomass and not by root mass.

4.1. Continuous stocking protocol

Severe or moderate-severe grazing (management heights of 5 and 10 cm, respectively) resulted in greater root mass relative to moderate or lenient grazing (management heights of 15 and 20 cm, respectively) and could be associated with greater stolon segmentation induced by the more frequent defoliations normally associated with the lower management heights. Clonal segmentation is part of an ecological strategy developed during plant evolution to cope with environmental disturbances that may be induced as a response to certain stimuli like defoliation or grazing, or happen naturally following a seasonal programme of plant development (Thomas and Hay, 2004). In both cases, the benefits of such a strategy are associated with resource use optimization within the clone and persistency of the plant community.

The increase in root mass from autumn to winter indicates the possibility of a season pattern of plant segmentation, which was previously described by Fialho (2015) in a previous series of experiments in the same experimental area. According to the author, during autumn, winter and spring, plant traits associated with plant number (population) increase while plant traits associated with plant size decrease. In white clover (***Trifolium repens*** L.) the number of roots per plant increases throughout the winter, reaching a peak in early spring and then declining during summer and autumn (Brock et al., 1988), following a seasonal pattern similar to the observed for root mass in forage peanut in this study.

The clonal segmentation process seems to be directly related to the development of new ramifications (branches). Additionally, within the primary characteristics associated with clonal development, is the ability to form adventitious roots on nodes along the stolon axis, favouring the maintenance of greater root mass and leaf area. According to Thomas and Hay (2004), the evolutive mechanism related to the development of new branches in plagiotropic growing plants, like white clover and forage peanut, was based on a regulating mechanism associated with the presence of nodal roots.

The primordia responsible for nodal root formation are early formed within the buds and develop into roots under adequate conditions of soil humidity. Thus, the development of roots on one given node generates the stimuli that causes the activation and later development of axillary buds into new branches (Thomas and Hay, 2010). The successive axillary buds located at the distal positions along the stolon axis that is continuously elongating, although may receive such stimuli, normally produce branches in lesser number and with lower growth potential. According to Thomas and Hay (2007), this is because the intensity from which the signal derived from the root system is perceived decreases as the distance between the axillary buds and the rooting points increases. Since the growth potential of new branches along the stolon axis decreases, the fragmentation of long clones into small ramets consist in important strategy for maximizing plant persistence (Wang et al., 2013). Price and Marshall (1999) argued that fragmentation or natural segmentation (or programmed segmentation) consists in an adaptive strategy developed for many clonal plants in response to the spatial-temporal heterogeneity of the growth resources distribution.

Total nodule mass showed a variation pattern in agreement with the corresponding variations in root mass according to both effects: grazing intensity (management heights) and season of the year. Root systems with greater root mass result in larger volume of soil to be exploited,

Table 4

Root mass, nodule number, nodule size and total nodule mass at post-grazing (mean ± standard error of the mean) of *Arachis pintoi* cv. Belmonte swards subjected to strategies of intermittent stocking management from February 2015 to April 2016.

Season of the year	Root mass (g cylinder^{-1})	Nodule number (n° cylinder^{-1})	Nodule size (mg nodule^{-1})	Nodule mass (mg cylinder^{-1})	
				$LI_{95\%}$	LI_{Max}
Summer I	8.3 ± 0.11 C	615 ± 57	0.36 ± 0.047	186 ± 62.3 Ba	273 ± 62.3 Ba
Autumn/Winter	12.5 ± 0.11 A	617 ± 56	0.47 ± 0.047	181 ± 62.3 Bb	429 ± 62.3 Aa
Early Spring	11.0 ± 0.11 AB	685 ± 56	0.51 ± 0.047	295 ± 62.3 ABa	429 ± 62.3 Aa
Late Spring	9.7 ± 0.11 BC	710 ± 56	0.50 ± 0.047	266 ± 62.3 ABb	461 ± 62.3 Aa
Summer II	11.9 ± 0.11 AB	735 ± 57	0.49 ± 0.047	378 ± 62.3 Aa	393 ± 62.3 ABa

*Cylinder volume = 1800 cm^3.

Means followed by the same lowercase letter in rows and uppercase letters in columns are not statistically different each other ($p > 0.05$).

Table 5

Percentage of nitrogen derived from atmosphere (Ndfa) and biological nitrogen fixation (BNF, kg N ha^{-1}) (mean ± standard error of the mean) of *Arachis pintoi* cv. Belmonte swards subjected to strategies of intermittent stocking management characterized by the pre-grazing targets of $LI_{95\%}$ and LI_{Max} and the post-grazing targets of 40 and 60% of the pre-grazing height from February 2015 to April 2016.

Grazing severity	Ndfa (%)		N_{BNF} (kg N ha^{-1})	
	$LI_{95\%}$	LI_{Max}	$LI_{95\%}$	LI_{Max}
		Summer I		
R40%	60 ± 4.5 Aa	68 ± 4.5 Aa	151 ± 22.7 Ab	264 ± 22.7 Aa
R60%	66 ± 4.5 Aa	70 ± 4.5 Aa	193 ± 22.7 Ab	275 ± 22.7 Aa
		Autumn/winter		
R40%	58 ± 4.5 Aa	65 ± 4.5 Aa	210 ± 22.7 Ab	299 ± 22.7 Aa
R60%	63 ± 4.5 Aa	68 ± 4.5 Aa	183 ± 22.7 Ab	319 ± 22.7 Aa
		Early spring		
R40%	63 ± 4.5 Aa	58 ± 4.5 Ba	241 ± 22.7 Aa	224 ± 22.7 Ba
R60%	66 ± 4.5 Aa	74 ± 4.5 Aa	229 ± 22.7 Ab	309 ± 22.7 Aa
		Late spring		
R40%	48 ± 4.5 Aa	42 ± 4.5 Ba	132 ± 22.7 Aa	158 ± 22.7 Ba
R60%	57 ± 4.5 Aa	59 ± 4.5 Aa	182 ± 22.7 Ab	236 ± 22.7 Aa
		Summer II		
R40%	49 ± 4.5 Ba	58 ± 4.5 Aa	108 ± 22.7 Bb	194 ± 22.7 Aa
R60%	62 ± 4.5 Aa	62 ± 4.5 Aa	186 ± 22.7 Aa	201 ± 22.7 Aa

Means followed by the same lowercase letter in rows and uppercase letters in columns are not statistically different each other ($p > 0.05$).

which represents a larger area for infection and nodulation. However, the establishment of the symbiosis between the legume and the *Rhizobium* involves the infection of roots of the host plant and subsequent formation of nodules (Bauer, 1981), but old and/or mature roots are not susceptible to infection (Bhuvaneswari et al., 1981). Therefore, the establishment and development of roots in young plants (branches), originated from the programmed segmentation process during autumn and winter, would also explain the increasing values of total nodule mass from autumn until spring.

The nodule is the basic structure of the symbiotic relationship between host plant and *Rhizobium* and, for the equilibrium of this relationship, it is necessary the continuous supply of assimilates produced by the aerial biomass while the biological N fixation in the nodules provides N to plants. Greater total nodule mass, nodule number as well as greater nodule size were responses observed during spring, probably the result of the previous growth dynamics during autumn and winter. At that time of the year, the low availability of light and temperature induces reduction in growth rates of plants. As a result, the frequency of defoliation for a given height is lesser, allowing that the existing leaf area maintain certain stability in assimilate production. Since growth is happening slowly in tropical regions at lower temperatures relative to spring and summer, photoassimilates production may exceed plant demand for growth, allowing the assimilates to be allocated for root development and growth as well as for increasing nodule size.

From summer I to winter the number of nodules decreased while nodule size increased. According to Pate and Armstrong (1996), large nodules are stronger sinks for photoassimilates than small nodules, particularly in relation to the priority for assimilates use. In this study, high intensities of grazing (management heights of 5 and 10 cm) on forage peanut resulted in greater root mass and smaller number of smaller nodules relative to moderate and lenient intensities of grazing (management heights of 15 and 20 cm), indicating a compensation mechanism that may be triggered by plants when nutrient availability is not limiting (Quinn and Hall, 1996).

The percentage of N derived from BNF (%Ndfa) in above ground material was more closely related to sward aerial biomass than root mass, since BNF (kg N ha^{-1}) increased as management height increased (greater values recorded on swards managed at 15 and 20 cm). The amount of N derived from BNF is determined by two factors: (i) the aerial biomass (t DM ha^{-1}) that generates and provides energy (i.e. carbohydrates) for N fixation; and (ii) the efficiency of the BNF process or nodule activity, in this case expressed as % of N derived from BNF (Hartwig, 1998). As a result, despite the greater total nodule mass and nodule number recorded on swards managed at 5 and 10 cm relative to those managed at 15 and 20 cm, those variables alone did not express nodule activity or BNF efficiency, but in some legumes, nodule activity shows direct relationship with nodule size (King and Purcell, 2001). Tajima et al. (2007) demonstrated for *Arachis hypogaeaI* L. that nodules have very thin peripheric areas that are non-infected meanwhile the inner part are densely filled with infected cells, suggesting that bigger nodules have greater activity than smaller ones. In this study, forage peanut showed a positive relationship between average nodule size and percentage of N derived from BNF (%Ndfa) across seasons of the year (Fig. 2), but the same relationship was not verified across management heights.

Lenient grazing represented by the 20 cm management height resulted in both greater %Ndfa and BNF (kg N ha^{-1}) relative to more severe grazing (management heights of 5 and 10 cm). Differences in % Ndfa were more pronounced between swards managed at 5 and 10 cm relative to those managed at 20 cm. Lower intensities of grazing remove less aerial biomass at any single defoliation event (Silva et al., 2017), what probably resulted in higher %Ndfa. Possibly, under severe grazing (management heights of 5 and 10 cm), the energy required for the uptake of N from the soil is lesser relative to establishing a symbiotic relationship for acquiring the nutrient from the atmosphere, and this may be an efficient mechanism provided when the kinetics of N uptake from the soil is not limited (Polley and Detling, 1989). In general, the BNF process regulator for the continuous stocking protocol was aerial biomass since it was the responsible for capturing the incident light and transforming it into the chemical energy (assimilates) necessary for feeding the *Rhizobium* during the symbiotic association.

4.2. Intermittent stocking protocol

The different combinations between frequency and severity of defoliation evaluated in this study did not affect root mass or the number

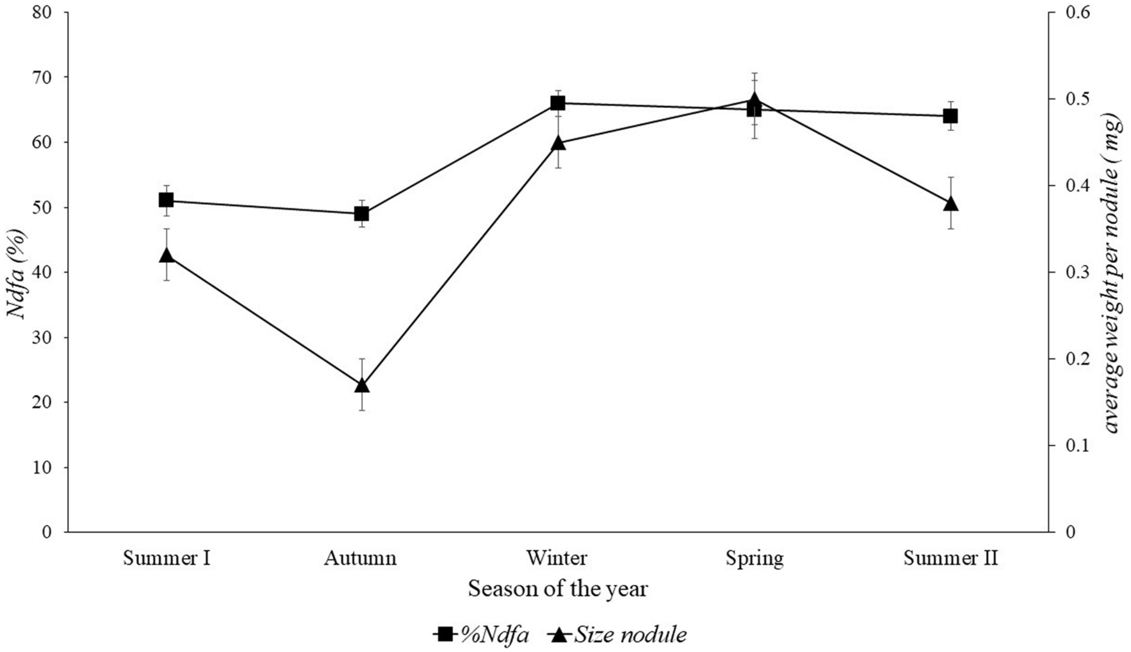

Fig. 2. Relationship between average nodule size and the percentage of nitrogen derived from the atmosphere (%Ndfa) in the aerial biomass of forage peanut subjected to intensities of continuous stocking management.

of nodules at both pre- and post-grazing conditions. Differently from the continuous stocking method, where defoliations with varying degrees of intensity depending on management height occur during the entire period during which animals remain on pastures, under intermittent stocking sward structure is abruptly modified every grazing cycle. In this grazing method (i.e. rotational grazing), the severity with which the leaf area is removed is modulated by the target of post-grazing height used. Photosynthesis is immediately reduced after defoliation. Consequently, defoliation regimes that cause the removal of large proportions of sward leaf area, herbage mass and/or pre-grazing height (Da Silva et al., 2014) may result in reduction or even cessation of root growth (Evans, 1971, 1973; Fownes and Anderson, 1991; Hodgkinson and Becking, 1978; Schuster, 1964) as a means of ensuring enough energy to restore leaf area. Root respiration and nutrients uptake from the soil are also reduced after defoliation but, according to Briske and Richards (1995), such reduction is smaller than the reduction in root growth. However, greater impact of defoliation intensity on root growth has been reported under defoliation regimes where the intensity of grazing exceeds 50% removal of the aerial biomass (Richards, 1993). In this context, the defoliation regimes used in this study possibly did not exceed the plants' tolerance threshold, creating growth conditions and microclimate that minimized the negative impacts on root growth.

Total nodule mass at post-grazing was affected by the grazing management targets used, and directly regulated through average nodule size. During regrowth, bigger nodules were detected when swards were managed with the LI_{Max} target and were associated with greater total nodule mass during autumn/winter and late spring. However, the effect of grazing frequency on total nodule mass disappeared when swards reached the pre-grazing LI target, condition at which the average nodule size was bigger for the $LI_{95\%}$ relative to the LI_{Max} target. Butler et al. (1959) reported that the restoration of root growth and nodulation in temperate climate legumes subjected to shading and defoliation is a species-dependent process widely related to plants growth form. Greater capacity of recovery in response to shading and defoliation was observed for white clover relative to red clover (*Trifolium pratense* L.) and bird's-foot trefoil (*Lotus uliginosus* L.). According to the authors, there was progressive death of old roots and nodules after defoliation followed by rapid reestablishment from the rooting of new stolons densely nodulated in white clover. For the other species, limitations to regrowth related to plants' growth form resulted in progressive decline in the ability to recover and nodulate after successive defoliations. The senescence of the nodular structure (Dupont et al., 2012) is related to the fact that the metabolic activity of nodules (nitrogenase activity) is significantly reduced few hours after defoliation (Gordon et al., 1990). Therefore, the restoration of the N fixation potential would depend on how fast the root system is renewed, since young roots have greater potential for nodulation than old roots (Carranca et al., 1999; Gage, 2004; Pommeresche and Hansen, 2017). However, BNF was related to grazing frequency regardless of the evaluated responses of root mass and nodulation at pre-grazing and, when the results are compared within the same LI target, the post-grazing target defined the %Ndfa and BNF. For both variation causes (LI pre-grazing and post-grazing height), the aerial biomass regulated the BNF process during early and late spring, since higher %Ndfa and greater BNF were recorded when swards were managed with the LI_{Max} target associated with the post-grazing height of 60% of the pre-grazing height relative to the remaining combinations between grazing frequency and severity.

In relation to the grazing frequency effects, Suzuki et al. (2011), studying the regulation process of nodulation in mutant plants of *Lotus japonicus* inoculated with *Mesorhizobium loti*, concluded that nodule formation is a photomorphogenetic response controlled by the shoot and modulated by the plant sensibility to the quality of the light environment around, decreasing as the red-to-far red ratio decreases. However, negative impacts of shading on nodulation and BNF, as expected when swards were managed with the LI_{Max} target, could be minimized if the plasticity in petiole elongation allowed leaflets to be positioned in the top strata with high light availability. Such mechanism of plastic adjustment, previously described for forage peanut (Fialho, 2015), could explain the greater BNF values recorded from autumn and winter on swards subjected to the $LI_{Max}/60$ treatment.

Sharp reduction in BNF and greater dependency on N from the soil from autumn/winter was observed on swards subjected to the $LI_{Max}/40$ treatment, indicating that this combination of pre- and post-grazing management targets is not adequate for BNF, even considering that there was no negative impact on root mass and nodulation. Severe grazings are well known for causing restrictions to carbon acquisition after defoliation and interfering negatively with nodule activity (Bayne

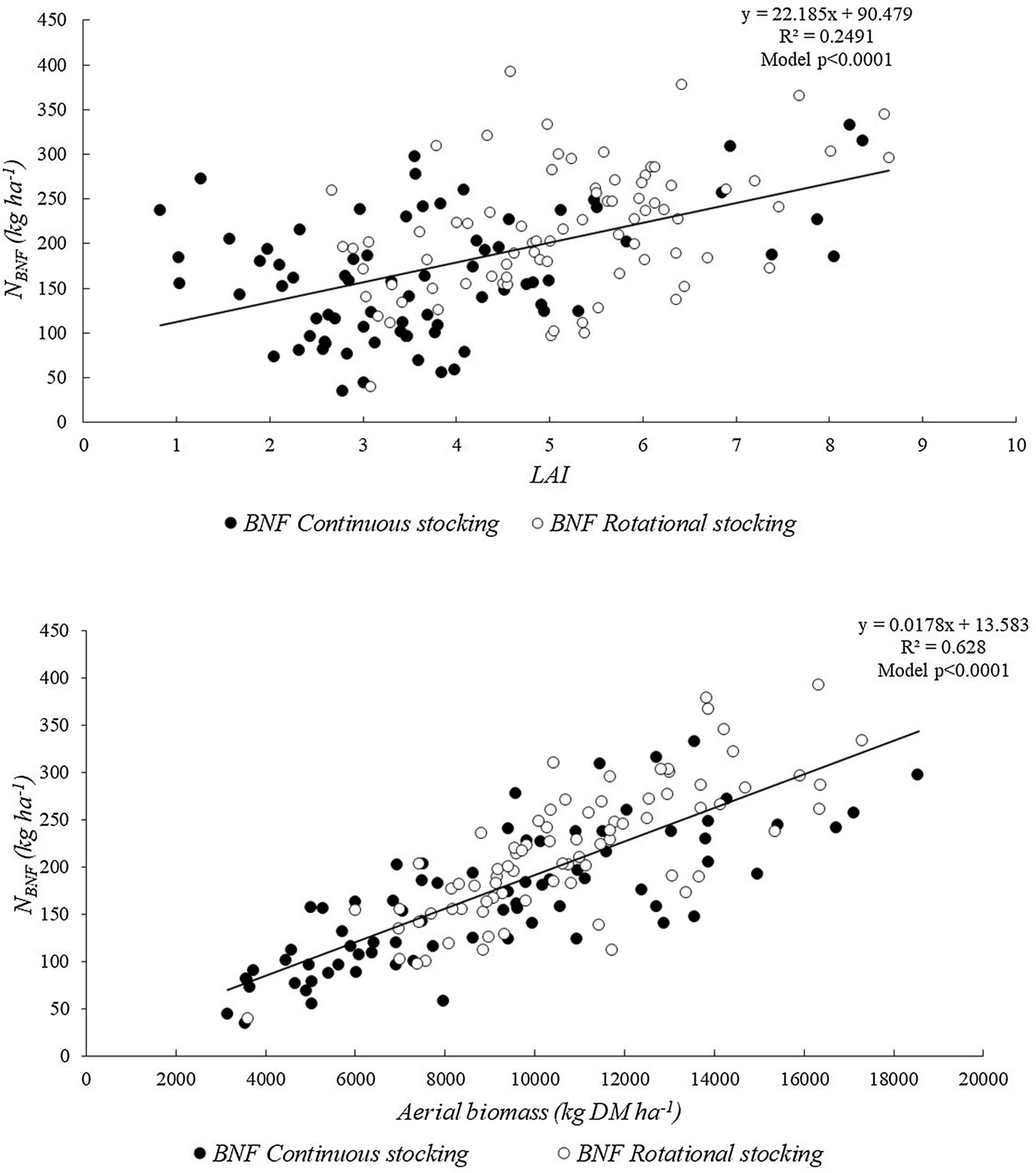

Fig. 3. Relationship between BNF and average sward leaf area index (LAI) (a) and aerial biomass (b) of forage peanut swards subjected to defoliation regimes under continuous and intermittent stocking management.

et al., 1984). The nitrogenase activity decreases rapidly when grazing severity exceeds 50% removal of sward leaf area (Hartwig et al., 1994), causing negative impact on the %Ndfa regardless of the grazing frequency adopted.

BNF represents a high energetic cost to plants (Gutschick, 1981; Houlton et al., 2008), since assimilate consumption to maintain nodule respiration only may correspond to up to 23% of the gross photosynthesis (Ryle et al., 1985). Therefore, under adverse conditions, such as those represented by the adoption of severe grazings (similar to the post-grazing height of 40% of the pre-grazing height in this study), plants seem to give priority to N uptake from the soil as a means of optimizing the use of the available energy supply. This energetic balance mechanism is known as BNF-facultative, according to which BNF is regulated positively or negatively depending on external factors such as the natural availability of N in the soil and, as observed in this study, the severity of defoliation (Menge and Hedin, 2009).

Inferences regarding the impacts of grazing management on the efficiency of the symbiosis based on BNF (kg N ha^{-1}) become interesting provided that they are related to the percentage of N derived from the atmosphere (%Ndfa). The reason is that swards with different aerial biomass may show similar values of BNF (kg N ha^{-1}), as observed by Querné et al. (2017) in a study of an integrated system between an arboreal no N fixating species (*Juglans nigra x regia* cv. NG23) and a N fixating legume species (*Medicago sativa* L.). The authors demonstrated that even though dry matter production was reduced in plants located close to tree ranks due to shading, the effect was compensated for by the higher %Ndfa, resulting in relatively stable values of BNF regardless of the distance from the trees. According to the authors, the arboreal component is the main sink for inorganic sources of N from the soil, and that the reduction in mineral N availability would act as a stimulus to BNF. Further, contrary to what was observed by Anglade et al. (2015), that found highly significant correlations between estimates of BNF and the total amount of N in the aerial biomass (%N_{total}) in temperate climate regions, the results of this study for both grazing methods (continuous and intermittent stocking protocol) indicate that %N_{total} should not be used to make inferences regarding the amount of N derived from the atmosphere. Nevertheless, additional studies are necessary to evaluate if this pattern of response happens in a similar manner in other regions of the country.

Throughout the year, both BFN and %Ndfa decreased from autumn/winter to late spring, when the lowest values were recorded, and the same trend was observed for the aerial biomass of the swards. This pattern is different of that observed for root mass, total nodule mass and average nodule size, whose greatest values were recorded in late spring. Therefore, similarly to what happened with the continuous stocking protocol, the postulated hypothesis for swards subjected to intermittent stocking management was partially rejected, since there was no direct relationship between root mass and BNF. Strategies of intermittent stocking management that remove high proportion of the aerial biomass or LAI through frequent grazings regardless of post-grazing height or long regrowth periods associated with low post-grazing height (severe grazing) result in decreased BNF.

Irrespective of grazing method, BNF showed a positive relationship with average sward LAI (Fig. 3a) and sward aerial biomass (Fig. 3b). Greater aerial biomass was associated with lesser number of large nodules, which are more efficient in the BNF process. However, the relationship between BNF and aerial biomass was more relevant than the relationship between BNF and LAI, indicating the importance of other morphological components like stolons and petioles e not only leaf area to N fixation. In general, regardless of the grazing method (continuous or intermittent stocking), the process of biological N fixation seems to be optimized when grazing intensity is moderate/lenient, a condition that allowed greater contribution of the N derived from BNF in the aerial biomass of the forage peanut swards. That is indicative of the deleterious effect of high intensities of grazing on the BNF process.

5. Conclusion

For both grazing methods, the range in grazing management targets used did not cause negative impacts on root mass and nodulation. Under continuous stocking, greater percentage of N derived from the atmosphere and greater amount of N from the BNF process were obtained when swards are subjected to moderate/lenient grazing (management heights of 15 and 20 cm). Under intermittent stocking, the percentage of N derived from the atmosphere remained relatively stable, regardless of grazing frequency. However, the amount of N from the BNF process was closely associated with sward aerial biomass, indicating that defoliation regimes characterized by high frequency ($LI_{95\%}$ target) and/or high intensity of aerial biomass or leaf area removal (LI_{Max}/40 treatment) negatively affect BNF. For both grazing methods, BNF was closely related to sward aerial biomass.

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Declarations of interest

None.

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References

Anglade, J., Billen, G., Garnier, J., 2015. Relationships for estimating N_2 fixation in legumes: incidence for N balance of legume-based cropping systems in Europe. Ecosphere 6, 1–24. https://doi.org/10.1890/ES14-00353.1.

Bathram, G.T., 1985. Experimental Techniques: The HFRO Sward Stick, Hill Farming Research Organization, Biennial report. Midlonthian. pp. 29–30.

Bauer, W.D., 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. 32, 407–449. https://doi.org/10.1146/annurev.pp.32.060181.002203.

Bayne, H.G., Brown, M.S., Bethlenfalvay, G.J., 1984. Defoliation effects on mycorrhizal colonization, nitrogen fixation and photosynthesis in the *Glycine-Glomus-Rhizobium* symbiosis. Physiol. Plant. 62, 576–580. https://doi.org/10.1111/j.1399-3054.1984.tb02802.x.

Bhuvaneswari, T.V., Bhagwat, A.A., Bauer, W.D., 1981. Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. Plant Physiol. 68, 1144–1149. https://doi.org/10.1104/pp.68.5.1144.

Boucho, A.C., Carranca, C., Redondo, R., Calouro, F., Madeira, M., 2019. Biomass, nodulation and N_2 fixing response by subclover and pink serradela to phosphorus fertilization. Arch. Agron. Soil Sci. https://doi.org/10.1080/03650340.2019.1566712. (just-accepted).

Briske, D.D., Richards, J.H., 1995. Plant Responses to Defoliation: A Physiological, Morphological and Demographic Evaluation. Wildland Plants: Physiological Ecology and Developmental Morphology. Society for Range Management, Denver.

Brock, J., Hay, M., Thomas, V., Sedcole, J., 1988. Morphology of white clover (*Trifolium repens* L.) plants in pastures under intensive sheep grazing. J. Agric. Sci. 111, 273–283. https://doi.org/10.1017/S0021859600083210.

Butler, G.W., Greenwood, R.M., Soper, K., 1959. Effects of shading and defoliation on the turnover of root and nodule tissue of plants of *Trifolium repens*, *Trifolium pratense*, and *Lotus uliginosus*. N. Z. J. Agric. 2, 415–426. https://doi.org/10.1080/00288233.1959.10418027.

Carlsson, G., Huss-Danell, K., 2003. Nitrogen fixation in perennial forage legumes in the field. Plant Soil 253, 353–372. https://doi.org/10.1023/A:1024847017371.

Carranca, C., 2013. Legumes: properties and symbiosis. In: Camisão, A.H., Pedroso, C.C. (Eds.), Symbiosis: Evolution, Biology and Ecological Effects. Animal Science, Issues and Professions. Nova Science Publishers, New York, pp. 67–94.

Carranca, C., Varennes, A., Rolston, D.E., 1999. Biological nitrogen fixation estimated by ^{15}N dilution, natural ^{15}N abundance, and N difference techniques in a subterranean clover–grass sward under Mediterranean conditions. Eur. J. Agron. 10, 81–89. https://doi.org/10.1016/S1161-0301(98)00056-2.

Carranca, C., Castro, I.V., Figueiredo, N., Redondo, R., Rodrigues, A.R.F., Saraiva, I., Maricato, R., Madeira, M.A.V., 2015. Influence of tree canopy on N_2 fixation by pasture legumes and soil rhizobial abundance in Mediterranean oak woodlands. Sci. Total Environ. 506, 86–94. https://doi.org/10.1016/j.scitotenv.2014.10.111.

Da Silva, S.C., Pereira, L.E.T., Sbrissia, A.F., Hernandez-Garay, A., 2014. Carbon and nitrogen reserves in marandu palisade grass subjected to intensities of continuous stocking management. J. Agric. Sci. 153, 1449–1463. https://doi.org/10.1017/S0021859614001130.

Das, S., Bhattacharyya, P., Adhya, T.K., 2011. Impact of elevated CO_2, flooding, and temperature interaction on heterotrophic nitrogen fixation in tropical rice soils. Biol. Fertil. Soils 47, 25–30. https://doi.org/10.1007/s00374-010-0496-2.

Doughton, J.A., Vallis, I., Saffigna, P.G., 1992. An indirect method for estimating ^{15}N isotope fractionation during nitrogen fixation by a legume under field conditions. Plant Soil 144, 23–29. https://doi.org/10.1007/BF00018841.

Dupont, L., Alloing, G., Pierre, O., El Msehli, S., Hopkins, J., Hérouart, D., Frendo, P., 2012. The Legume Root Nodule: From Symbiotic Nitrogen Fixation to Senescence. Senescence. InTechhttps://doi.org/10.5772/34438.

Euclides, V.P.B., Costa, F.P., Macedo, M.C.M., Flores, R., Oliveira, M.P., 2007. Eficiência biológica e econômica de pasto de capim-tanzânia adubado com nitrogênio no final do verão. Pesqui. Agropecu. Bras. 42, 1345–1355. https://doi.org/10.1590/S0100-204X2007000900017.

Evans, P.S., 1971. Root growth of *Lolium perenne* L. II. Effects of defoliation and shading. N. Z. J. Agric. 14, 552–562 doi: https://dx.doi.org/10.1093%2Faob%2Fmcr273.

Evans, P.S., 1973. The effect of repeated defoliation to three different levels on root growth of five pasture species. N. Z. J. Agric. 16, 31–34. https://doi.org/10.1080/00288233.1973.10421155.

Fialho, C.A. (2015). Características morfogênicas e estruturais de amendoim forrageiro (*Arachis pintoi* Krapovickas & Gregory cv. Belmonte) submetido a intensidades de pastejo sob lotação contínua. Piracicaba, pp. 121. Thesis (Doctor in Science) – "Luiz de Queiroz" College of Agriculture doi: https://doi.org/10.11606/T.11.2015.tde-04052015-110506.

Fisher, M.J., Cruz, P., 1994. Some ecophysiological building blocks in the domestication of an outstanding tropical pasture legume ideotype. In: Kerridge, P.C., Hardy, W. (Eds.), Biology and Agronomy of Forage *Arachis*. CIAT, Cali, Colombia, pp. 53–70 (In press).

Fonseca, L., Mezzalira, J.C., Bremm, C., Filho, R.S.A., Gonda, H.L., Carvalho, P.C.F., 2012. Management targets for maximising the short-term herbage intake rate of cattle grazing in *Sorghum bicolor*. Livest. Sci. 145, 205–211. https://doi.org/10.1016/j.

livsci.2012.02.003.

Fownes, J.H., Anderson, D.G., 1991. Changes in nodule and root biomass of *Sesbania sesban* and *Leucaena leucocephala* following coppicing. Plant Soil 138, 9–16. https://doi.org/10.1007/BF00011802.

Gage, D.J., 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. 68, 280–300. https://doi.org/10.1007/BF00011802.

Gildersleeve, R.R., Ocumpaugh, W.R., Quesenberry, K.H., Moore, J.E., 1987. Mob-grazing of morphologically different *Aeschynomene* species. Trop. Grassl. 21, 123–132.

Gordon, A.J., Kessler, W., Minchin, F.R., 1990. Defoliation-induced stress in nodules of white clover: I. Changes in physiological parameters and protein synthesis. J. Exp. Bot. 41, 1245–1253. https://doi.org/10.1093/jxb/41.10.1245.

Gutschick, V.P., 1981. Evolved strategies in nitrogen acquisition by plants. Am. Nat. 118, 607–637. https://doi.org/10.1086/283858.

Hartwig, U.A., 1998. The regulation of symbiotic N_2 fixation: a conceptual model of N feedback from the ecosystem to the gene expression level. Perspect. Plant. Ecol. 1, 92–120. https://doi.org/10.1078/1433-8319-00054.

Hartwig, U.A., Heim, I., Lüscher, A., Nösberger, J., 1994. The nitrogen-sink is involved in the regulation of nitrogenase activity in white clover after defoliation. Physiol. Plant. 92, 97–105. https://doi.org/10.1111/j.1399-3054.1994.tb08824.x.

Hirsch, A.M., 1992. Developmental biology of legume nodulation. New Phytol. 122, 211–237. https://doi.org/10.1111/j.1469-8137.1992.tb04227.x.

Hodgkinson, K.C., Becking, H.B., 1978. Effect of defoliation on root growth of some arid zone perennial plants. Aust. J. Agric. Res. 29, 31–42. https://doi.org/10.1071/AR9780031.

Houlton, B.Z., Wang, Y.P., Vitousek, P.M., Field, C.B., 2008. A unifying framework for dinitrogen fixation in the terrestrial biosphere. Nature 454, 327–330. https://doi.org/10.1038/nature07028.

King, C.A., Purcell, C., 2001. Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Sci. 41, 1099–1107. https://doi.org/10.2135/cropsci2001.4141099x.

Lawson, A.R., Kelly, K.B., Sale, P.W.G., 2000. Defoliation frequency and cultivar effects on the storage and utilisation of stolon and root reserves in white clover. Aust. J. Agric. Res. 51, 1039–1046. https://doi.org/10.1071/AR00039.

Ledgard, S.F., Steele, K.W., 1992. Biological nitrogen fixation in mixed legume/grass pastures. Plant Soil 141, 137–153. https://doi.org/10.1007/BF00011314.

Lie, T.A., 1971. Symbiotic nitrogen fixation under stress conditions. Plant Soil 35, 127–137. https://doi.org/10.1007/BF02661845.

Littel, R.C., Pendergast, J., Natarajan, R., 2000. Modelling covariance structure in the analysis of repeated measures data. Stat. Med. 19, 1793–1819. https://doi.org/10.1002/1097-0258(20000715)19:13%3C1793::AID-SIM482%3E3.0.CO;2-Q.

Menge, D.N.L., Hedin, L.O., 2009. Nitrogen fixation in different biogeochemical niches along a 120 000-year chronosequence in New Zealand. Ecology 90, 2190–2201. https://doi.org/10.1890/08-0877.1.

Okito, A., Alves, B.R.J., Urquiaga, S., Boddey, R.M., 2004. Isotopic fractionation during N_2 fixation by four tropical legumes. Soil Biol. Biochem. 36, 1179–1190. https://doi.org/10.1016/j.soilbio.2004.03.004.

Oldroyd, G.E., Murray, J.D., Poole, P.S., Downie, J.A., 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Gene 45, 119–144. https://doi.org/10.1146/annurev-genet-110410-132549.

Osman, A.K., Wynne, J.C., Elkan, G.H., Schneeweis, T.J., 1983. Effect of leaf removal on symbiotic nitrogen fixation in peanut. Peanut Sci. 10, 107–110. https://doi.org/10.3146/i0095-3679-10-2-17.

Pate, J.S., Armstrong, E.L., 1996. Photoassimilate partitioning and consumption in nitrogen-fixing crop legumes. In: Zamski, E., Schaffer, A.A. (Eds.), Photoassimilate Distribution in Plants and Crops Source-Sink Relationships, pp. 625–642.

Pedreira, B.C., Pedreira, C.G.S., 2007. Fotossíntese foliar do capim-xaraés *Brachiaria brizantha* (A. Rich.) Stapf. cv. Xaraés e modelagem da assimilação potencial de dosséis sob estratégias de pastejo rotativo. R. Bras. Zootec. 36, 773–779. https://doi.org/10.1590/S1516-35982007000400004.

Peoples, M.B., Herridge, D.F., Ladha, J.K., 1995. Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? Plant Soil 174, 3–28. https://doi.org/10.1007/978-94-011-0053-3_1.

Polley, H.W., Detling, J.K., 1989. Defoliation, Nitrofen, and competition: effects on plant growth and nitrogen nutrition. Ecology 70, 721–727. https://doi.org/10.2307/1940222.

Pommeresche, R., Hansen, S., 2017. Examining Root Nodule Activity on Legumes. FertilCrop Technical Note.

Price, E.A., Marshall, C., 1999. Clonal plants and environmental heterogeneity–An introduction to the proceedings. Plant Ecol. 141, 3–7. https://doi.org/10.1023/A:1009838300691.

Qiao, Y., Miao, S., Han, X., You, M., Zhu, X., Horwath, W.R., 2014. The effect of fertilizer practices on N balance and global warming potential of maize–soybean–wheat rotations in Northeastern China. Field Crops Res. 161, 98–106. https://doi.org/10.1016/j.fcr.2014.03.005.

Querné, A., Battie-laclau, P., Dufour, L., Wery, J., Dupraz, C., 2017. Effects of walnut trees on biological nitrogen fixation and yield of intercropped alfalfa in a Mediterranean agroforestry system. Eur. J. Agron. 84, 35–46. https://doi.org/10.1016/j.eja.2016.12.001.

Quinn, M.A., Hall, M.H., 1996. Compensatory growth response of the legume, *Medicago sativa*, to defoliation and denodulation. Entomol. Exp. Appl. 78, 243–252. https://doi.org/10.1111/j.1570-7458.1996.tb00788.x.

Richards, J.H., 1993. Physiology of plant recovery from defoliation. In: Baker, M.J. (Ed.), Grassland for Our World. SIR Publishing, Wellington, pp. 46–54.

Ryle, G.J.A., Powell, C.E., Gordon, A.J., 1985. Defoliation in white clover: regrowth, photosynthesis and N_2 fixation. Ann. Bot. 56, 9–18. https://doi.org/10.1093/oxfordjournals.aob.a086998.

Schuster, J.L., 1964. Root development of native plants under three grazing intensities. Ecology 45, 63–70. https://doi.org/10.2307/1937107.

Shearer, G., Kohl, D.H., 1986. N_2 fixation in field settings - estimations based on natural ^{15}N abundance. Aust. J. Plant Physiol. 13, 699–756. https://doi.org/10.1071/PP9860699.

Silva, G.P., Fialho, C.A., Carvalho, L.R., Fonseca, L., Carvalho, P.C.F., Bremm, C., Da Silva, S.C., 2017. Sward structure and short-term herbage intake in *Arachis pintoi* cv. Belmonte subjected to varying intensities of grazing. J. Agric. Sci. 1, 1–8. https://doi.org/10.1017/S0021859617000855.

Smith, J.L., Myung, H.U., 1990. Rapid procedures for preparing soil and KCl extracts for ^{15}N analysis. Commun. Soil Sci. Plant Anal. 21, 2173–2179. https://doi.org/10.1080/00103629009368368.

Soussana, J.F., Tallec, T., 2010. Can we understand and predict the regulation of biological N_2 fixation in grassland ecosystems? Nutr. Cycl. Agroecosys. 88, 197–213. https://doi.org/10.1007/s10705-009-9335-y.

Suzuki, A., Suriyagoda, L., Shigeyama, T., Tominaga, A., Sasaki, M., Hiratsuka, Y., Yoshinaga, A., Arima, S., Agarie, S., Sakai, T., Inada, S., 2011. *Lotus japonicus* nodulation is photomorphogenetically controlled by sensing the red/far red (R/FR) ratio through jasmonic acid (JA) signaling. Proc. Natl. Acad. Sci. U. S. A. 108, 16837–16842. https://doi.org/10.1073/pnas.1105892108.

Tajima, R., Lee, O.N., Abe, J., Lux, A., Morita, S., 2007. Nitrogen-fixing activity of root nodules in relation to their size in peanut (*Arachis hypogaea* L.). Plant Prod. Sci. 10, 423–429. https://doi.org/10.1626/pps.10.423.

Thomas, R.J., 1992. The role of the legume in the nitrogen cycle of productive and sustainable pastures. Grass Forage Sci. 47, 133–142. https://doi.org/10.1111/j.1365-2494.1992.tb02256.x.

Thomas, R.G., Hay, M.J.M., 2004. Evidence suggests plagiotropic clonal species have evolved a branching physiology emphasizing regulation by nodal roots. Evol. Ecol. Res. 18, 409–427. https://doi.org/10.1007/s10682-004-5137-5.

Thomas, R.G., Hay, M.J.M., 2007. Cumulative activation of axillary buds by nodal roots in *Trifolium repens* L. J. Exp. Bot. 58, 2069–2078. https://doi.org/10.1093/jxb/erm063.

Thomas, R.G., Hay, M.J.M., 2010. The role of nodal roots in prostrate clonal herbs: ‘phalanx’ versus ‘guerrilla. Evol. Ecol. 24, 1489–1504. https://doi.org/10.1007/s10682-010-9400-7.

Udvardi, M., Poole, P.S., 2013. Transport and metabolism in legume-rhizobia symbioses. Annu. Rev. Plant Biol. 64, 781–805. https://doi.org/10.1146/annurev-arplant-050312-120235.

Unkovich, M., 2013. Isotope discrimination provides new insight into biological nitrogen fixation. New Phytol. 198, 643–646. https://doi.org/10.1111/nph.12227.

Vitousek, P.M., Cassman, K.E.N., Cleveland, C., Crews, T., Field, C.B., Grimm, N.B., Howarth, R.W., Marino, R., Martinelli, L., Rastetter, E.B., Sprent, J.I., 2002. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57, 1–45. https://doi.org/10.1023/A:1015798428743.

Wang, Z., Van Kleunen, M., During, H.J., Werger, M.J.A., 2013. Root foraging increases performance of the Clonal Plant *Potentilla reptans* in heterogeneous nutrient environments. PLoS One 8. https://doi.org/10.1371/journal.pone.0058602.

Xu, R., Tian, H., Pan, S., Prior, S.A., Feng, Y., Batchelor, W.D., Chen, J., Yang, J., 2019. Global ammonia emissions from synthetic nitrogen fertilizer applications in agricultural systems: empirical and process-based estimates and uncertainty. Glob. Change Biol. Glob. 25, 314–326. https://doi.org/10.1111/gcb.14499.

Yasuoka, J.I., Pedreira, C.G.S., Da Silva, V.J., Alonso, M.P., Da Silva, L.S., Gomes, F.J., 2018. Canopy height and N affect herbage accumulation and the relative contribution of leaf categories to photosynthesis of grazed *Brachiaria* grass pastures. Grass Forage Sci. 73, 183–192. https://doi.org/10.1111/gfs.12302.