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Clitoria ternatea L. as a Potential High Quality Forage Legume

Matheus Lima Corrêa Abreu[1], Ricardo Augusto Mendonça Vieira*, Norberto Silva Rocha[1], Raphael Pavesi Araujo[1], Leonardo Siqueira Glória[1], Alberto Magno Fernandes, Paulo Drude de Lacerda[2], and Antonio Gesualdi Júnior

Laboratório de Zootecnia e Nutrição Animal (LZNA), Centro de Ciências e Tecnologias Agropecuárias (CCTA), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Av. Alberto Lamego 2000, Campos dos Goytacazes, RJ, Brazil, CEP 28013-602

ABSTRACT: Samples of *Clitoria ternatea* L. (Cunhã) were harvested at 35, 50, 70, and 90 d after a uniformity harvest in a field study designed as a completely randomized design with a total of 18 experimental plots. The dry matter yield of the whole plant was separated quantitatively into leaves, stems, and pods at each harvesting age. Chemical analyses and *in vitro* gas production kinetics were performed to assess the quality of the plant parts. Yields, chemical composition, and estimates of gas production parameters were analyzed by fitting a mixed statistical model with two types of covariance structures as follows: variance components and an unrestricted structure with heterogeneous variances. Fast and slow gas yielding pools were detected for both leaves and stems, but only a single pool was detected for pods. The homoscedasticity assumption was more likely for all variables, except for some parameters of the gas production kinetics of leaves and stems. There was no presence of typical pods at 35 and 50 d. In the leaves, the fibrous fractions were affected, whereas the non-fibrous fractions were unaffected by the harvesting age. The harvesting age affected the majority of the chemical constituents and gas kinetic parameters related to the stems. The leaves of this legume were the least affected part by the aging process. (**Key Words:** *Clitoria ternatea* L., Forage Yield, Chemical Composition, Gas Production Kinetics)

INTRODUCTION

The legume scientifically known as *Clitoria ternatea* L. and by many common names, such as Cunhã, Clitoria, Butterfly pea, Blue pea, Conchitas, and Kordofan pea, belongs to the *Fabaceae* family, *Faboidae* subfamily, *Phaseoleae* tribe, and *Clitoriinae* subtribe. The origin of Cunhã is obscure (Hall, 1985; Gomez and Kalamani, 2003; Cook et al., 2005). Some authors attribute the origin of this legume to tropical America (Upadhyaya and Pachauri, 1983), but it is more likely that its origin is the Ternate Island in the Molluca archipelago, Indonesia (Gupta et al., 2010). In Brazil, the beginning of Cunhã cultivation is also obscure, but this legume may be considered a well adapted perennial, vigorous, twining, climbing, and summer growing tropical plant with pinnate leaves, bearing 5 to 7 elliptical and 3 to 5 cm long leaflets. The flowers are solitary or paired and the fruits are linear flat and sparsely pubescent pods containing 8 to 10 dark seeds at its maturity (Staples, 1992; Gomez and Kalamani, 2003; Cook et al., 2005). Cunhã has been cultivated as pure stands to be grazed for short periods (protein bank), fed as a fresh fodder, and harvested for haymaking. It can also be sown with other forage plants forming both cultivated and natural permanent pastures (Staples, 1992; Araújo Filho et al., 1996; Avalos et al., 2004).

Besides its good palatability and nutritive value, Cunhã is a good forage source for the hot and semi-arid (BSh climate, Köppen standards) northeastern Brazil due to its

* Corresponding Author: Ricardo Augusto Mendonça Vieira. Tel: +55-22-2748-6397, Fax: +55-22-2739-7194, E-mail: ramvieira@uenf.br
[1] Graduate Program in Animal Science, CCTA/UENF, Brazil.
[2] Empresa de Assistência Técnica e Extensão Rural do Estado do Rio de Janeiro (EMATER - RIO), Escritório local de Saquarema, Av. Saquarema 5123, Centro, Saquarema, RJ, Brazil, CEP 28993-000.
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adaptability and resilience under drought conditions, and to its potential for regrowth soon after the onset of the rainy season (Barros et al., 1991; Araújo Filho et al., 1994). However, the widespread presence of Cunhã in humid and low altitude areas in the tropics, its response to irrigation (Gomez and Kalamani, 2003; Avalos et al., 2004; Juma et al., 2006), and its use as a forage source by smallholders of the Rio de Janeiro state, has motivated us to investigate the potential nutritive value of this legume. Although some information exists regarding animal performance, forage yield, and chemical composition at different stages of development of Cunhã (Barro and Ribeiro, 1983; Araújo Filho et al., 1994; Barros et al., 2004), a detailed chemical analysis and an investigation on the gas production kinetics as an auxiliary measure of the potential nutritive value of this tropical legume at different harvesting ages may be useful as basic quantitative indices and constituted the goal of the present study.

MATERIAL AND METHODS

Cunhã (*C. ternatea* L.) was sown in early spring (October) of 2008 in a 900 m^2 area containing Xanthic and Eutrophic Oxisols with 10% declivity located in Northern Rio de Janeiro State (21°42′33″ S and 41°20′23″ W; 12 m of altitude), Brazil. The area is located in a region where an Aw climate (Köppen standards) predominates with an annual rainfall of 800 mm. The seeds were manually scarified over a concrete-made sidewalk for approximately one minute prior to being inoculated with Rhizobium of the cowpea group, and 4 to 6 seeds were sown manually at every 0.2 m along 0.8±0.3 m spaced lines.

A completely randomized design was set up by demarcation of 18 plots formed by 5 lines (3 m in length) on August 22nd of 2010, i.e., 22 months after planting. Uniformity of the plots was achieved by harvesting all herbage mass greater than 0.2 m in height. All plots were manually weeded and kept without weeds during the course of the experiment. Plots received single doses of limestone, K_2O, and P_2O_5 equivalent to 1,500, 100, and 50 kg/(ha·yr), respectively, in addition to a single dose of goat manure (approx. 20 ton/(ha·yr)). A uniformity harvest was performed one month before the end of the winter (June 21st to September 23rd) of 2010 during which the minimum temperature and minimum relative humidity had risen (Figure 1a and 1b). The harvesting ages (treatments) were defined according to a pilot trial conducted to gather an idea of the plant developmental stages. Therefore, four treatments were randomly assigned to the experimental units after the day of the uniformity harvest as follows: five plots were harvested after 35 d (early vegetative growth), four plots were harvested after 50 d (flowering), four plots were harvested after 70 d (early seed pod), and five plots were harvested after 90 d (ripe seed pod). The area was irrigated according to the rainfall occurrence. Since the uniformity harvest, the rainfall accumulated 3 mm for 30 d, 43 mm for 60 d, and 269 mm for the entire 90 d period of the field trial. Therefore, the irrigation was performed once to twice a week in the first 60 d; however, the irrigation was stopped during the last 30 d of the experimental period because of the rise in the rainfall and in the relative humidity (Figure 1).

Plots were harvested at the same time of the day for all treatments, which was after dew drying (approx. 08:00 to 09:00 h). The herbage mass of the plots harvested at a cutting height of 0.2 m above the ground was collected, and the fresh weight was recorded to the nearest 0.005 kg. The fresh forage was dried at 55°C for 72 h in a forced air oven. The total air dried mass of each plot was then quantitatively separated into leaves, stems, and pods (whenever present), and the respective weights were recorded to the nearest 0.005 kg. The separated masses of leaves, stems, and pods

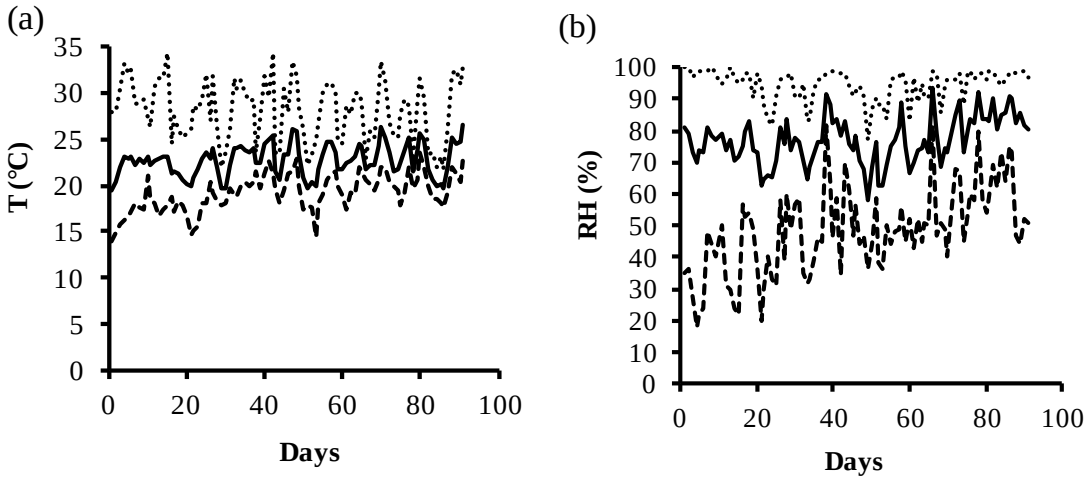

Figure 1. Maximum (dotted lines), means (solid lines), and minimum (dashed lines) of the daily temperature of the air (T, °C; panel a), and daily relative humidity (RH, %; panel b) during the 91 d experimental period.

were ground through a 5 mm screen in a Wiley-type mill, and samples of approx. 0.1 kg were then ground through a 1 mm screen for chemical analyses and *in vitro* gas production.

Forage samples were analyzed for dry matter (DM, method 967.03; AOAC, 1998), crude fat (CF, method 2,003.06; Thiex et al., 2003), and ash (method 942.05; AOAC, 1998). The crude protein (CP) content was obtained by digesting samples (0.25 g) with 5 mL of H_2SO_4 and approximately 1.5 g of a 56:1 mixture of Na_2SO_4 and $Cu_2SO_4.5H_2O$ in 100 mL tubes using aluminum digestion blocks according to the guidelines outlined in method 984.13 and 2,001.11, including N recovery assays with certified $NH_4H_2PO_4$, and lysine-HCl (AOAC, 1998; Thiex et al., 2002). The insoluble fiber content (aNDFom) was assayed with sodium sulfite and two additions of a standardized solution of heat-stable amylase, and with ash excluded according to method 2002.04 (Mertens, 2002). The non-fibrous carbohydrates were estimated with the following equation: NFC = DM–CP–CF–Ash–aNDFom. The lignin content was assessed by two methods. The first method assessed lignin (sa) by the sulfuric acid method (973.18; AOAC, 1998) after a sequential neutral detergent and acid detergent extraction (ND $\rightarrow$ AD; Van Soest et al., 1991). The second method assessed lignin (pm) as the weight loss after permanganate oxidation (Goering and Van Soest, 1970) in the following sequence: ND $\rightarrow$ AD $\rightarrow$ 72% H_2SO_4 $\rightarrow$ $KMnO_4$ $\rightarrow$ ash (Van Soest, 1994, p. 147).

The *in vitro* incubations were performed in a 39°C water bath using 100 mL serum amber bottles sealed with butyl rubber stoppers and aluminum crimp seals. Individual samples of air-dried leaves, stems, and pods (approx. 0.5 g; nearest to 0.1 mg) were transferred into the flasks. The forage samples were incubated with 40 mL of a reduced culture medium with 10 mL of rumen inoculum as previously described by Goering and Van Soest (1970). The culture medium, reducing solution, and inoculum were prepared as a single batch (Hall and Mertens, 2008). The inoculum was obtained from a six-year-old healthy Holstein-Zebu steer that weighed 550 kg. The steer was maintained in a pasture of palisade grass (*Urochloa brizantha* (Hochst. ex A. Rich) R. Webster) during the winter (dry period), and the steer was supplemented *ad libitum* with chopped 1.5-year-old sugar cane (*Saccharum* spp.) and 1 kg/d of a concentrate containing 290 g/kg of soybean meal, 680 g/kg of ground corn, and 30 g/kg of a commercial mineral salt. The liquid and fibrous mat of the rumen contents were abundant and presented characteristic appearance, color, and odor. The rumen fluid and fibrous mat were collected separately to completely fill respective thermal bottles (2,000 mL each). Approximately 250 g of the fibrous mat was then blended for 60 s with 500 mL of the rumen fluid under continuous CO_2 gassing, and the mixture was then filtered through four layers of cheesecloth. The filtered inoculum was added to the reduced culture medium in a 4:1 ratio, and the mixture was maintained at 39°C with CO_2 gassing until the mixture was transferred to the flasks.

Time profiles of cumulative gas production were obtained for leaves, stems, and pods using a non-automated device similar to the one used by Malafaia et al. (1999), slightly modified as follows. A 0 to 8 psi manometer (0.05 increments) was attached to a three-way plastic valve. One of the ways of the valve was connected to a silicone tube (i.d. 5 mm; 1.5 m in length) with a 20 gauge needle attached to the loose extremity of the tube. The second way was attached to the manometer by a small piece of the silicone tube (i.d. 5 mm; 0.3 m in length) and plastic clamps. The third way was connected by another silicone tube (i.d. 5 mm; 1.3 m in length) to the top of a graduated 25 mL pipette (0.1 mL increments), which had its conical end connected to the stem of a separating funnel (1,000 mL) by the same type of silicone tube (i.d. 5 mm; 0.4 m in length). The funnel and pipette were attached to a metal support stand in a vertical and static position. The connecting system was filled with resazurin solution (0.1 g/L) to the zero mark of the pipette, i.e., allowed for atmospheric pressure equilibration. The system was filled with caution to avoid the formation of air bubbles.

The gas pressure hold in the airspace of the fermentation flask was read in the manometer by inserting the 20 gauge needle of the loose extremity through the butyl rubber stopper of the crimp sealed flask, and the gas volume produced was read after changing the position of the three-way valve to allow the top-down displacement of the liquid inside the pipette. The objective of the loose extremity was to read the pressure and volume without removing the bottle from the water bath. However, the bottles were shortly removed every day of the incubation period to be slightly shaken in the early morning and early evening to mix contents. Pressure and volume readings were performed at 1, 2, 3, 6, 8, 10, 12, 16, 20, 24, 30, 36, 48, 72, and 96 h of incubation. Volume displacement in the pipette was allowed only after sensible pressure readings. Volume readings were expressed as mL/0.1 g of DM. Because blank incubations introduces bias in the cumulative gas profiles due to the absence of substrates to be fermented and to the direct relationship among fermentation of substrates, VFA production, and the amount of gas produced and released by the culture medium (Beuvink and Spoelstra, 1992; Hall et al., 1998; Rymer et al., 2005), we considered that blank corrections loose generality and were not performed.

The time profiles generated exhibited four different shapes according to the forage samples. Therefore, the following four different models proposed by Zwietering et al. (1990) and revisited by Schofield et al. (1994) were

fitted accordingly:

$$V_t = V_f[1-exp(-kt)]+\varepsilon \quad \text{(Eq. 1)}$$

$$V_t = V_f\, exp\{-exp[1+ke(L-t)]\}+\varepsilon \quad \text{(Eq. 2)}$$

$$V_t = V_{f1}\,[1-exp(-k_1t)] + V_{f2}\, exp\{-exp[1+k_2e(L-t)]\}+\varepsilon \quad \text{(Eq. 3)}$$

$$V_t = V_{f1}\, exp\{-exp[1+k_1e(L-t)]\} + V_{f2}\, exp\{-exp[1+k_2e(L-t)]\}+\varepsilon \quad \text{(Eq. 4)}$$

V_t (Eqs. (1) to (4)) is the cumulative gas production over time (t, h) ; V_f (Eqs. (1) to (2)) is the asymptotic gas volume reached for a single pool substrate; k (h^{-1}) is the fractional rate constant of cumulative gas production inferable as the digestion rate of a single pool substrate (Eqs. (1) to (2)); and L (Eq. (2)) is the discrete lag time (h). Eq. (3) is a dual-pool model designed to estimate the asymptotic gas production of fast (V_{f1}) and slow (V_{f2}) digesting substrates (pools) with their respective k_1 and k_2 degradation rates, which are both expressed as h^{-1}. In Eq. (3), the fast digesting pool is fermented as a first-order process without lag, and the second pool follows a logistic pattern with a lag time (L; h). Eq. (4) was designed to fit sigmoid-shaped patterns in which fast and slow digesting pools yield asymptotic gas volumes (V_{f1} and V_{f2}) at k_1 and k_2 rates (h^{-1}) after a common lag time (L; h) for both pools. The term e is the base of the natural logarithms (Eqs. (2) to (4)), and ε is the random error term (Eqs. (1) to (4)). The mean digestion time (MDT, h) for each equation was calculated as follows: $MDT = 1/k$ for Eq. (1); $MDT = 1/k+L$ for Eq. (2); and $MDT = 1/k_1+1/k_2+L$ for Eq. (3) and for Eq. (4).

Only one gas production profile per experimental unit was obtained, i.e., only one fermentation vessel was used per individual forage sample of the leaves, stems, and pods taken at the experimental unit (plot). Therefore, the error variance includes the variations among plots (assumed to be the experimental error) and among replicates of the incubation vessels (error within each plot, which was not estimated).

The linearity between the observed volume and pressure readings of the gas production technique (Theodorou et al., 1994) was checked by means of the robust regression method of SAS and the quality of fit of the linear model was compared to the higher order polynomials by computing Akaike information criteria. The parameters of the different nonlinear models (Eqs. (1) to (4)) were estimated with the NLIN procedure of SAS. The likelihoods of Eqs. (1) to (4) in mimicking the gas production profiles were also assessed by computing Akaike criteria (Akaike, 1974; Burnham and Anderson, 2004; Vieira et al., 2012).

The variables related to the dry matter yield of the whole plant (DMY; kg/ha); separated DMY (kg/ha) for leaves, stems, and pods; proportion of leaves (dimensionless); chemical components; and gas production parameters at the different harvesting ages (treatments) were analyzed by the following linear mixed model:

$$Y_{ij} = \mu+\alpha_i+e_{ij} \quad \text{(Eq. 5)}$$

Y_{ij} is the observation measured in the j-th plot at the i-th harvesting age (α_i) after the uniformity harvest. The fixed effects in Eq. (5) are the mean (μ) and α_i, and the random effect is the usual error term (e_{ij}) that estimates the error among experimental plots. Eq. (5) was fitted using the PROC MIXED procedure of SAS (version 9) with maximum likelihood as the estimation method. The repeated command was used with plots as subjects. The variance-covariance matrix was modeled as variance components, and as the unrestricted variance-covariance structure with treatment grouping to account for heterogeneous variances (Littell et al., 2006). The likelihood of the different variance-covariance structures was assessed by computing Akaike information criteria as suggested by Vieira et al. (2012). Null hypotheses regarding treatments, and their linear, quadratic, and cubic effects were rejected when $p<0.05$. Tendencies regarding treatment effects were considered when $0.05<p<0.10$.

For significant regressions and trends, the estimated 95% confidence intervals (95% CI) were presented as follows: $\hat{y}_x \pm (U_r - L_r)/2$; where $\hat{y}_x$ is the predicted dependent variable for a given harvesting age, x; and U_r and L_r are the upper and lower limits, respectively, of the 95% CI. For absent treatment effects, the 95% CI for the least squares means of the dependent variable at each harvesting age ($\bar{y}_x$) was provided as follows: $\bar{y}_x \pm (U_r - L_r)/2$. The approximate 99% confidence interval (99% CI) was estimated for x_m as the abscissa coordinate of the maximum or minimum of the fitted quadratic polynomial according to Neter and Wasserman (1974).

RESULTS

The homoscedasticity assumption, as verified by the Akaike criteria, was more likely for all variables, except for the following variables: k_1 of leaves; and the k_1, k_2, and MDT of stems. The alternative model used in these exceptions was the unstructured variance-covariance matrix with treatment grouping to account for heterogeneous variances.

The DMY (kg/ha) of the whole plant resembled a crescent sigmoid-shaped pattern after a visual appraisal; an evidence for that was the cubic trend observed in Table 1. However, the DMY of leaves presented a significant

Table 1. p-Values regarding the linear (L), quadratic (Q), and cubic (C) effects for the dry matter yield (DMY), the leaf:plant dry mass ratio (LR), the chemical components[a], and gas production parameters for the whole butterfly pea, its leaves, and stems, and p-values for the *F*-ratio test for pods

Variable	Whole plant			Leaves			Stems			Pods
	L	Q	C	L	Q	C	L	Q	C	*F*-ratio
DMY (kg/ha)	<0.001	0.088	0.056	<0.001	0.004	0.455	<0.001	0.486	0.498	0.049
LR	<0.001	0.303	0.003	-	-	-	-	-	-	-
DM[b]	0.161	0.063	0.372	-	-	-	-	-	-	-
Ash[c]	<0.001	0.683	0.448	0.810	0.634	0.890	0.002	0.011	0.439	0.917
CF[c]	<0.001	0.710	0.001	0.131	0.914	0.037	<0.001	0.001	0.314	0.129
CP[c]	0.006	0.146	0.129	0.728	0.363	0.493	0.057	0.087	0.548	0.417
NFC[c]	<0.001	0.587	0.125	0.113	0.938	0.486	<0.001	0.333	0.050	0.089
aNDFom[c]	<0.001	0.188	0.514	0.015	0.284	0.921	<0.001	0.002	0.056	0.301
Lignin (sa)[c]	<0.001	0.818	0.237	0.004	0.777	0.878	<0.001	0.020	0.410	0.010
Lignin (pm)[c]	<0.001	0.583	0.437	0.079	0.730	0.101	<0.001	0.070	0.094	0.008
Lag (h)	-	-	-	0.001	0.956	0.152	0.007	0.777	0.690	-
V_{f1} [d]	-	-	-	0.395	0.111	0.139	<0.001	0.002	0.742	-
V_{f2} [d]	-	-	-	0.695	0.049	0.070	0.022	0.019	0.502	-
V_f [d]	-	-	-	-	-	-	-	-	-	<0.001
k_1 (h^{-1}) [e]	-	-	-	0.286	0.284	0.295	0.016	0.047	0.237	-
k_2 (h^{-1}) [e]	-	-	-	0.933	0.776	0.081	0.109	0.139	0.631	-
k (h^{-1}) [e]	-	-	-	-	-	-	-	-	-	0.611
MDT (h) [e]	-	-	-	0.956	0.010	0.145	0.027	0.050	0.867	0.616

[a] DM = Dry matter; CF = Crude fat; CP = Crude protein; NFC = Non-fibrous carbohydrates; aNDFom = Neutral detergent fiber assayed with amylase and sulphite and expressed exclusive of residual ash; Lignin (sa) = Sulfuric acid lignin; Lignin (pm) = Permanganate lignin.
[b] g/kg of the fresh harvested mass. [c] g/kg of DM.
[d] V_{f1}, V_{f2}, and V_f are fast, slow, and single pools gas productions, expressed as mL/0.1 g of DM.
[e] k_1, k_2, and k are fractional rates of the fast, slow, and single pools, respectively, and *MDT* is mean digestion time.

quadratic fit, and the DMY of stems increased linearly. There was no presence of typical pods at the first and second harvesting ages. The flowers had become protruded at 35 and 50 d after the uniformity harvest, but no characteristic pods were noticeable at these ages. Therefore, the mass of such flowers was considered merely as the mass of leaves, and the mass of typical pods was detectable only at 70 and 90 d after the uniformity harvest. Therefore, only a conclusive *F*-ratio test was applied to demonstrate the mass accumulation of pods between the last two harvesting ages with respective p-values as shown in Table 1. The leaf:whole plant DM mass ratio (LR) presented a sigmoid decreasing pattern after visual inspection. This dimensionless proportion was transformed as $2\arcsin\sqrt{LR}$ prior to analysis, and a significant cubic effect was detected (Table 1), which agreed with the observed decreasing sigmoid behavior. The DMY of leaves, stems, pods, and of the whole plant presented uncorrelated errors and homoscedasticity as evidenced by the greater likelihood of the simplest variance components structure. With regard to the related quadratic effect (Table 1), the leaf DMY peaked at x_m = 73±3 d after the uniformity harvest, and this maximum DMY for leaves was 1,541±238 kg/ha. Confidence intervals for the variables and parameters studied are displayed in Table 2 and 3.

There was evidence of a quadratic trend for the DM content of the whole plant (Table 1). The value reached a minimum DM content of 170.7±14.2 g/kg of fresh forage at 57±4 d. A negative linear effect for the ash content of the whole plant (approximately –0.3 g/kg/d) and a decreasing quadratic effect for the stems were observed. The 95% CI of the minimum ash content was 45.6±4.9 g/kg DM at x_m = 72±3 d. Ash contents in the leaves and pods were unaffected by the harvesting age (Table 1).

Although a cubic trend was detected for the CF content of the whole plant, its behavior was difficult to justify biologically. Therefore, a linear decrease was considered more likely with aging (Table 1). A cubic effect was also evident for the CF content of the leaves, but only the general mean was reported. The CF content in stems was quadratic and reached a minimum of 10.2±0.8 g/kg DM within the 82±4 d range. The CF contents of pods at 70 and 90 d did not differ (Table 1).

The CP content of the whole plant decreased linearly, approx. –0.3 g/(kg·d), with the harvesting age (Table 1). A quadratic trend for the CP contents of stems was observed, and the minimum content predicted was 103.3±9.5 g/kg DM within the approximated 72±2 d range. The CP and NFC contents of leaves and pods were unaffected by the harvesting age (Table 1). The NFC content decreased linearly in the whole plant at an approx. rate of –1.4 g/(kg·d). The cubic effect for the NFC content in stems was

Table 2. Confidence intervals (95% CI) for the predicted dry matter yield (DMY, kg/ha), the chemical components[a], and gas production parameters for the leaves and stems at each harvesting age (d), and for 70 d and 90 d pods of Cunhã

Variables	95%CI for plant parts									
	Leaves				Stems				70 d Pods	90 d Pods
	35	50	70	90	35	50	70	90		
DMY	436±274	1,134±221	1,533±250	1,326±280	1,401±128	366±92	666±87	967±135	850±440	1,443±393
Ash[c]	96.7±3.9	95.7±47.4	96.2±4.4	97.2±3.9	63.8±5.5	52.1±4.5	45.7±5.1	49.7±5.7	55.6±5.1	56.0±5.7
CF[c]	50.6±4.8	55.0±5.3	45.6±5.3	48.1±4.8	17.1±1.0	13.5±0.8	10.7±0.9	10.4±1.0	22.5±7.5	30.7±8.4
CP[c]	320.2±17.3	320.0±19.4	311.5±19.4	326.3±17.3	123.9±10.6	110.3±8.5	103.3±9.7	108.9±10.8	237.4±21.2	249.1±23.8
NFC[c]	313.3±29.0	293.5±32.4	296.6±32.4	278.3±29.0	251.4±15.2	235.3±10.8	213.9±10.3	192.4±15.9	281.1±34.4	238.1±38.5
aNDFom[c]	223.5±17.4	232.0±12.6	243.2±13.3	254.5±21.0	540.0±16.4	592.6±13.3	631.6±15.0	635.1±16.8	403.4±32.3	426.2±36.1
Lignin (sa)[c]	38.7±4.6	41.5±3.3	45.2±3.5	48.9±5.6	94.5±6.7	108.4±5.4	117.1±6.1	114.5±6.9	58.3±10.1	80.4±11.3
Lignin (pm)[c]	19.7±3.2	22.5±2.3	26.2±2.2	29.9±3.4	81.4±6.7	94.5±5.1	105.2±6.2	108.0±6.9	55.6±7.4	73.0±8.3
Lag (h)	3.2±0.4	2.8±0.3	2.3±0.3	1.8±0.4	2.17±0.4	1.9±0.3	1.5±0.3	1.1±0.4	-	-
V_{f1} [d]	11.3±2.9	10.9±3.2	6.8±3.2	10.6±2.9	9.9±1.0	7.0±0.8	5.5±1.0	6.6±1.1	-	-
V_{f2} [d]	7.2±1.9	8.9±1.5	9.0±1.7	6.8±2.0	8.1±0.5	8.5±0.4	8.3±0.5	7.2±0.5	-	-
V_f [d]	-	-	-	-	-	-	-	-	19.1±1.2	14.7±1.1
k_1 [(h-1) e]	0.087±0.004	0.090±0.004	0.267±0.347	0.089±0.018	0.095±0.007	0.110±0.007	0.119±0.010	0.112±0.013	-	-
k_2 [(h-1) e]	0.027±0.013	0.023±0.014	0.041±0.014	0.023±0.013	0.018±0.001	0.018±0.001	0.022±0.012	0.015±0.001	-	-
k [(h-1) e]	-	-	-	-	-	-	-	-	0.077±0.006	0.079±0.003
MDT (h) [e]	59.7±16.5	59.0±18.5	38.6±18.5	63.0±16.5	69.9±2.0	61.3±2.8	62.4±3.7	78.0±5.0	23.7±2.1	24.9±2.4

[a] CF = Crude fat; CP = Crude protein; NFC = Non-fibrous carbohydrates; aNDFom = Neutral detergent fiber assayed with amylase and expressed exclusive of residual ash; Lignin (sa) = Sulfuric acid lignin; Lignin (pm) = Permanganate lignin.
[b] g/kg of the harvested mass. [c] g/kg of DM.
[d] V_{f1}, V_{f2}, and V_f are fast, slow, and single pools gas productions, expressed as mL/0,1 g DM.
[e] k_1, k_2, and k are fractional rates of the fast, slow, and single pools, respectively, and *MDT* is mean digestion time.

difficult to explain, so a linear decrease (approx. –1.1 g/(kg·d)) was considered more appropriate.

We can observe in Table 1 that the fibrous fractions (aNDFom, lignin (sa), and lignin (pm), g/kg DM) of the whole plant increased linearly (approx. 2.4, 0.5, and 0.6 g/(kg·d), respectively) as the harvesting age increased. The aNDFom, lignin (sa), and lignin (pm) increased linearly in the leaves (approx. 0.6, 0.2, and 0.2 g/(kg·d), respectively), and they were considered to increase in a quadratic fashion for the stems with quadratic trends visually resembling an asymptotic profile in the 90 d interval. The cubic trends for aNDFom and lignin (pm) of stems shown in Table 1 were disregarded because no apparent inflection points characteristic of sigmoid-shaped patterns in those trends were visually identified. The aNDFom contents of pods at 70 and 90 d did not differ, but significant differences were observed for the other fibrous fractions at these ages. For stems, the aNDFom, lignin (sa), and lignin (pm) contents peaked at 82±4, 75±4, and 87±10 d, respectively, after the uniformity harvest with peak contents of 638.0±12.7, 117.5±5.6, and 108.1±5.9 g/kg DM, respectively.

The linearity assumption between pressure and volume readings of the gas production technique held true. The Eq. (4) was adopted for describing the gas production kinetics of leaves and stems because of its greater likelihood. For pods, all profiles were better described by Eq. (1).

The estimated lag time of the cumulative gas production profiles decreased linearly for leaves and stems (Table 1).

Table 3. Confidence intervals (95% CI) for the predicted dry matter yield (DMY), the leaf:plant dry mass ratio (LR), and the chemical components[a] for the whole Cunhã plant at each harvesting age

Variables	95% CI			
	35 d	50 d	70 d	90 d
DMY (kg/ha)	592±383	1,401±428	3,192±428	3,684±383
LR (dmls)	0.786±0. 046	0.755±0.051	0.492±0.051	0.356±0.046
DM[b]	182.9±15.4	172.0±12.4	174.8±14.1	197.3±15.7
Ash[c]	78.14±30.5	73.3±21.8	67.0±20.8	60.7±32.0
CF[c]	44.3±3.5	40.5±2.5	35.3±2.4	30.3±3.6
CP[c]	273.3±12.8	265.5±9.2	255.1±8.7	244.7±13.4
NFC[c]	315.0±16.6	294.8±11.9	267.9±11.3	241.0±17.4
aNDFom[c]	289.3±8.3	325.9±6.0	374.7±5.7	423.4V8.8
Lignin (sa)[c]	50.6±3.1	57.4±2.2	66.4±2.1	75.5±3.3
Lignin (pm)[c]	32.1±2.5	41.2±1.8	53.3±1.7	65.4±2.6

[a] DM = Dry matter; CF = Crude fat; CP = Crude protein; NFC = Non-fibrous carbohydrates; aNDFom = Neutral detergent fiber assayed with amylase and expressed exclusive of residual ash; Lignin (sa) = Sulfuric acid lignin; Lignin (pm) = Permanganate lignin.
[b] g/kg of the fresh harvested mass. [c] g/kg of DM.

There was no detectable lag for pods at 70 and 90 d. The gas volume produced from the slow digesting fraction of the leaves peaked at 9.3±1.9 mL/0.1 g DM close to 61±3 d. The gas volume produced from the fast digesting pool of the stems reached a minimum of 5.5±1.0 mL/0.1 g DM at almost 61±3 d, and the slow digesting pool of the stems peaked at 8.5±0.5 mL/0.1 g DM when x_m was approx. 55±3 d. The k_1 and k_2 of the leaves and k of the pods were unaffected by the harvesting age (Table 1). For stems, the k_1 peaked at 0.119±0.010/h close to 68±3 d, and the *MDT* reached a minimum at 60±3.6 h close to 59±2 d.

DISCUSSION

Barro and Ribeiro (1983) and Araújo Filho et al. (1994) observed quadratic responses in forage DMY with a growth profile resembling an asymptotic first-order behavior with no apparent inflection point during the 42 (growth), 56 (beginning of flowering), 70 (pod formation), and 84 d (seed) of regrowth under irrigation in the semiarid region of Northeastern Brazil. In this region, specifically at Vale do Curu (CE), after an approximately 820 d field trial, Araújo Filho et al. (1994) recommended 56 d as the best harvesting age with a cutting height of 0.1 m; they argued that the practiced cutting heights did not affect plant DMY, and forage production was maximized with 6.5 cuts/yr, namely 21,850 kg/(ha·yr).

The calculated leaf to stem mass ratios, based on the predicted DMY values shown in Table 2 at 35, 50, 70, and 90 d, were 3.11, 3.10, 2.30, and 1.37, respectively. Barro and Ribeiro (1983) reported that the same ratio after haymaking has values of 1.26, 0.87, 0.73, and 0.97 at 42, 50, 70, and 84 d, respectively. This disparity may have been partly due to the foliage loss during haymaking. Because hay is more difficult to produce in areas with high relative humidity and heavy rain, the herbage mass of Cunhã may be better used as fresh fodder to ruminants. The county of Campos dos Goytacazes (Northern Rio de Janeiro State) offers good haymaking conditions due to lower rainfall (600 to 800 mm) and prevailing dry northeastern winds during the spring-summer period. For other regions of the Rio de Janeiro State (e.g., the northwestern and highland regions with rainy spring-summer seasons), however, the production of good quality hay is more difficult to achieve.

Foliage, what includes senescent leaves, is the preferable part of the forage legume or browse mass eaten by ruminants (Pfister and Malechek, 1986; Van Soest, 1996). In the present study, the leaf DMY peaked near 70 d after the uniformity harvest, i.e., later than the recommend harvesting age proposed by Araújo Filho et al. (1994). At the practiced cutting height (0.2 m), there were negligible amounts of senescent leaves in the harvested herbage mass; nonetheless, we observed considerable amounts of senescent leaves on the ground at 70 d. This might indicate that the ideal harvesting age may be performed sooner than the age at which maximum leaf DMY have occurred. However, there are variables other than the climate related ones, such as soil type, fertility, cultivar, spacing, seed density and stand density, which may influence DMY (Hall, 1985; Avalos et al., 2004), and therefore, the ideal harvesting age. There is no additional evidence observed in the present study against the 56 d as the ideal harvesting age recommended by Araújo Filho et al. (1994). Nevertheless, these projections need to be strengthened or revisited by long-term multi-location trials.

The chemical composition of legume leaves varies much less when compared to grass counterparts as the plant matures because there is no decisive structural support role for the leaves of legumes or other forbs (Van Soest, 1996). Lignification with maturity affects mostly plant cell walls, but leaves the digestibility of the cell contents relatively unaffected (Van Soest, 1967; 1996; Huhtanen et al., 2006). The ash, CF, CP, and neutral detergent solubles (NDS) are nutritional uniform entities (Lucas, 1964; Huhtanen et al., 2006). According to the Lucas theory (Lucas, 1964), given that NFC are contained in the NDS fraction, it can be assumed that NFC also behaves uniformly. In this sense, the leaves were the Cunhã plant parts with nutritional entities less affected by lignification and cell wall thickening (Tables 1, 2, and 3). Therefore, the leaf DMY should be regarded for increasing the amount of edible parts of the forage mass and taken into account for management purposes.

There are differences in the composition among plant parts (Van Soest, 1996), and these differences were confirmed for the legume Cunhã in the present study. Compared to all studied plant parts, the stems were the more lignified and fibrous part of the plant (Tables 2 and 3). As a result of cell wall thickening with maturity (Van Soest, 1994), the amounts of the nutritional entities, including ash, CF, CP, and NFC of the stems were reduced (Tables 1 and 2). Barro and Ribeiro (1983) reported that the insoluble fiber content of the whole Cunhã plant resembles a quadratic behavior with the neutral detergent residue of the plant accessions peaking at 511 g/kg DM near 70 d. They also reported a possible linear increase for permanganate lignin, and at 70 d the permanganate lignin reported was 152 g/kg DM. Juma et al. (2006) reported an insoluble fiber content for the whole plant of 605 g/kg DM at a flowering state of 60% (near 60 d from the uniformity harvest in the present study). Barros et al. (1991) found at the stage of pod formation, 497 and 83 g/kg DM of insoluble fiber (as a neutral detergent residue) and permanganate lignin, respectively. This disparity in the literature for reported fibrous contents for the whole plant compared to the aNDFom and lignin contents in Table 2 and 3 may be

because, in the present study, the entire fresh material of the plot was quantitatively collected with no loss of leaves, which was the plant part richest in NDS. The cutting height may be another interfering factor on quality, which means more or less stem mass in the harvested herbage. Other differences with respect to literature values may be due to differences among methods for forage fiber and lignin analyses (Mertens, 2002). Therefore, a sequential analysis for lignin was adopted in the present study to avoid interferences due to DM solubility differences in the neutral and acid detergents (Van Soest et al., 1991).

The kinetic aspects of the *in vitro* gas production may reveal some intrinsic properties of the feedstuff studied. The lag time, fractional rates and asymptotic gas production are affected by inhibitory substances, such as lignin, tannins, and other phenolic and plant defensive compounds that naturally occur in the substrate. Tropical legumes are an example of feedstuffs rich in these inhibitory substances (Van Soest, 1994; Longland et al., 1995; Mertens, 2005). In the present study, these chemicals were not quantified in the Cunhã parts, but Juma et al. (2006) reported an average tannin content of 17.1 g/kg DM.

Schofield and Pell (1995) identified two gas yielding pools for the whole plant DM of both clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) with asymptotic gas productions of 21.0 and 20.2 mL/0.1 g of DM, respectively. Moreover, they also reported values of 0.64 and 0.54 as the $V_{f1}/(V_{f1}+V_{f2})$ ratio for clover and alfalfa, respectively, and they reported that the fractional rates of the fast (non-fibrous) and slow (fibrous) digesting carbohydrate pools are 0.153 and 0.028/h, respectively, for clover, and 0.110 and 0.030/h, respectively, for alfalfa. The calculated *MDT* from the kinetic values reported by Schofield and Pell (1995) were 42.8 and 43.5 h for clover and alfalfa, respectively. In the present study, the fast digesting pool at 70 d presented $V_{f1}/(V_{f1}+V_{f2})$ ratios of 0.53 and 0.40 for leaves and stems, respectively, and the gas production from pods was more likely to have originated from a uniform digesting pool. At 50 d, i.e., close to the ideal harvesting age of 56 d (Araújo Filho et al., 1994), the same ratios were 0.55 and 0.45 for leaves and stems, respectively. The basic difference between the *MDT* calculated from Schofield and Pell (1995) and the *MDT* calculated in the present study is based on the magnitude of the faster lag times presented by Schofield and Pell (1995).

Consistent with the lack of the harvesting age effect observed over the nutritive entities was the absent treatment effect over the fractional rate and asymptotic gas produced from the fast digesting pool of the leaves. This fact kinetically confirm the uniformity of the soluble components of the DM as an ideal nutritional entity according to H. L. Lucas terms (Lucas, 1964), and also agrees to the absence of treatment effect over the soluble material (Van Soest, 1967; 1996; Huhtanen et al., 2006). The gas produced from the fast digesting pool is associated to the digestion and subsequent fermentation of soluble carbohydrates of the cell contents (Hall et al., 1998). However, despite the fact that the lignin content had increased in the plant DM with maturity, this aspect is of little or no influence on the rate and extent of digestion of cell contents (Van Soest, 1967). Therefore, whatever is the harvesting age chosen within 35 to 90 d, our findings indicate that the contents of ideal nutritional entities of the leaves (CP, CF, and NFC) remained constant. Given that the harvesting age affected V_{f2} of both leaves and stems, we inferred that the extent of digestion of fibrous carbohydrates might had also been affected. The extent of digestion of the fibrous carbohydrates probably peaked between 50 to 60 d, i.e., at the same age the maximum V_{f2} for leaves and stems had occurred. The lignin content affects the extent of digestion of fibrous carbohydrates (Smith et al., 1972). Lignification completely prevents digestion of xylem and tracheary tissues of legumes. Nonetheless, other factors prevent microbial digestion too. The accessibility to inner surfaces of the cell walls, the release of phenolic-carbohydrate complexes in the vicinity of the sites where adherent microorganisms are digesting the forage particles, and the release of other inhibitory secondary compounds of the forage plant that may alter the fractional rate of fiber digestion (Wilson and Mertens, 1995; Mertens, 2005). Nonetheless, in our study k_2 was unaffected by the harvesting age on both leaves and stems of Cunhã within the 35 to 90 d range.

Ruminant nutrition models have been built to provide means of quantifying the nutritional value of diets and the expected animal performance. These models are based on the intrinsic substrate fractionation and its digestion kinetics in the rumen to estimate the availability of substrates (carbohydrates and protein) for microbial growth. The escape of dietary substrates and microbial mass from the rumen that become available to the host digestive process in the intestines, and the absorption of VFA are computed to estimate metabolizable energy and protein values (Fox et al., 2004; Tedeschi et al., 2008; Tylutki et al., 2008; Tedeschi et al., 2010). In this regard, inferences from gas production parameters are useful for estimating the kinetic aspects of feed fractions in ruminant nutrition models (Schofield et al., 1994; Schofield and Pell, 1995; Hall et al., 1998). Because amino acids are not the energy-yielding substrates preferred by rumen microorganisms in the presence of non-fibrous and fibrous carbohydrates (Russell, 2002), gas production profiles provide important information about potentially digestible soluble and insoluble carbohydrates and its respective fractional rates of digestion (Hall et al., 1998). Therefore, our findings may provide relevant information for allowing the computation of the nutritive value of

Cunhã, so that this important forage could figure in as an additional resource in feed libraries of the ruminant nutrition models.

The 95% CI values reported in Table 2 and 3 allow a high nutritive value to be inferred for Cunhã comparable to clover and alfalfa, at least in terms of the chemical composition and characteristics of the fermentation kinetics. Information on animal performance is scarce, but Avalos et al. (2004) reported similar performances for Brown-Swiss cows eating equivalent amounts of Cunhã and alfalfa, which may support an expected high nutritional value for Cunhã. Despite the favorable features of Cunhã, such as the high protein content of leaves (Table 2), perennial nature, vigorous regrowth capacity (Gomez and Kalamani, 2003), and estimates of gas production kinetics comparable to alfalfa and clover at 70 d (i.e., closest to the maximum leaf DMY), this harvesting age may not be close enough to the optimum harvesting age of Cunhã (Araújo Filho et al., 1994). Therefore, a compromise between a sustainable forage production, which means resilience of the forage crop in terms of number of cuts per year that could be achieved, and the quantity and quality of the herbage mass yield, particularly foliage yield, has to be sought for an efficient utilization of Cunhã as a forage crop.

IMPLICATIONS

The forage legume, Cunhã (*C. ternatea* L.), has a good potential to be cultivated under irrigation because it yields good quality fodder, especially if this legume is offered fresh and with a high content of leaves. The leaves of this legume are the least affected part by the maturation process. Cunhã yields forage with a potential nutritive value comparable to the traditionally cultivated forage legume crops (e.g., alfalfa or clover) despite the possible positive linear effect of maturity on aNDFom and lignin (sa) contents in its leaves. Here we provided evidences for defining an ideal management strategy for this forage crop: an absent harvesting age effect over the contents of nutritional entities, over the fractional rate, and over the asymptotic gas production of the fast digesting fraction of the leaves. There is also supporting evidence that the gas production from the slow digesting fraction of leaves peaks within the 50 to 70 d range for the harvesting age of Cunhã.

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