# INFLUENCE OF NITROGEN LEVELS ON LEAF ANATOMY AND NUTRITIVE VALUE OF MILLENNIUM GRASS

## *INFLUÊNCIA DE NÍVEIS DE NITROGÊNIO NA ANATOMIA FOLIAR E VALOR NUTRITIVO DE CAPIM-MILÊNIO*

**Kelen Cristina BASSO[1]; Ulysses CECATO[2]; Leandro Martins BARBERO[3]; Beatriz LEMPP[4]; José Augusto Nogueira GOMES[5]; Simony Marta Bernardo LUGÃO[6]**

1. Pós-doutoranda, Universidade Federal de Uberlândia, UFU, Uberlândia, MG, Brasil. Kelen.basso@gmail.com; 2. Professor, Doutor, Departamento de Zootecnia, Universidade Estadual de Maringá - UEM, Maringá, PR, Brasil; 3. Professor, Doutor, Departamento de Medicina Veterinária - UFU; 4. Professora, Doutora, Departamento de Zootecnia, Universidade Federal da Grande Dourados - UFGD, Dourados, MS, Brasil; 5. Mestre em Zootecnia; 6. Pesquisadora do Instituto Agronômico do Paraná - IAPAR, Paranavaí, PR, Brasil

**ABSTRACT:** The objective of this study was to evaluate the influence of nitrogen levels (control = without N, 150, 300 and 450 kg N/ha), on proportions of tissues of leaf blades and the nutritional value of Millennium grass, on grazing system with intermittent stocking during the summer of 2007 in a completely randomized design. The last and penultimate expanded leaves of vegetative tillers were collected for this study. The proportion of abaxial epidermis (EPIaba) and vascular tissue (VT) decreased linearly with increasing levels of nitrogen. The percentage of sclerenchyma (SC) was 40,8% e 36,4% lower in pasture fertilized with 150, 300 kg N/ha and 25% lower for those with 450 kg in comparison to pasture without fertilization. The percentage of mesophyll (MES) increased linearly with nitrogen levels, with up to 20,3% higher for pasture with 450kg of N in relation to control level. The morphological characteristics, leaf area (LA), specific leaf area (SLA) and length, increased linearly with increasing nitrogen levels. The percentage of crude protein (CP ) and in vitro digestibility of dry matter (IVDDM ) were higher for treatments with higher fertilization (300 and 450 kg). The EPIaba and the levels of acid detergent fiber (ADF) and neutral detergent fiber (NDF) had positive correlation. The same occurred between IVDDM and parenchyma bundle sheath (PBS). The nitrogen applied to pasture has influence on improving the nutritive value of leaf blades of millennium grass and on the proportion of tissues considered of high digestibility.

**KEYWORDS:** Anatomical characteristics. Intermittent grazing. Morphological characteristics. *Panicum maximum*. Pasture fertilization.

## INTRODUCTION

Studies on participation of different tissues of leaves blades are relevant because they complement information on factors that affect forage grass quality, once chemical analysis digestibility do not always explain all variations of forage grass consumption (LEMPP, 2007).

Mineral nutrition contributes to the structural organization of the leaf blade, being nitrogen the nutrient that has direct influence on the morphologic and anatomic aspects, constituting many components of the vegetal cell, including aminoacids and nucleic acids (TAIZ; ZEIGER, 2010). The supply of this nutrient through fertilization becomes, in many cases, essential to express the genetic potential of forage mass production and even to maintain the grass survival in pasture, whenever other factors that lead to pasture degradation are not limited.

The association between tissue proportion, measured by transversal sections of leaves blades of forage grass, has been studied since 1972 (WILKINS, 1972). Akin e Amos (1975) demonstrated that cell of MES and of the phloem, with thick cell wall, are rapidly digested, while those of the epidermis and of PBS are digested slowly and partly. The sclerenchyma (SCL) and the xylem, which present thick and lignified cell wall, are indigestible (AKIN, 1989). They are the main morphologic component of forage $C_4$ associated to animal performance. However, few studies have been carried out to obtain information about the effect of nutrients, such as the nitrogen, on the tissue proportion of leaves blades.

Due to correlations between nutritional value and the percentage of leaf tissues, the anatomy may benefit the selection of superior genotypes in breeding forage programs. Gomes et al. (2011) determined the relative importance of anatomical, morphological and physiological characteristics to discriminate young nutritive value of promising genotypes evaluated 23 genotypes of *Panicum maximum* and found that the anatomical characteristics showed relative importance of 70% in the formation of genotypes groups, with much

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greater relative importance than the morphological characteristics.

The objective of this study was to evaluate the effect of levels of nitrogen fertilization in anatomic tissues proportion on transversal sections of leaves blades and on the nutritional value of *Panicum maximum* Jacq. cv. IPR-86 Millennium.

## MATERIAL AND METHODS

The experiment was carried out at the Experimental Station of IAPAR, in Paranavaí-PR - Brazil, located at 23º 05' S of latitude 52º 26' W of longitude at an average altitude of 480m. The predominant climate of the region is cfa – mesothermal humid subtropical weather according to Köeppen. It is characterized by the predominance of hot summers, with low frequency of frost and a tendency to concentrate rains in spring and summer. The annual average temperature is 22ºC and annual rainfall is around 1.200mm.

The Millennium grass (*Panicum maximum* Jacq cv. IPR-86 Milênio was established in October 1995 and its fertilizations were started in November 1997. The data in this study refer to the period of summer 2007. The soil of the experimental area was characterized as Red Dystrophic Podzolic of sandy texture, the topography was basically flat with desirable drainage, whose values of physical features showed: 89% of sand; 10% of clay and 1% of silt (Embrapa, 2006). The soil analysis (0-20 cm), carried out at the beginning of the installation of the experiment, showed pH ($CaCl_2$), 5.67; C (g/dm$^3$), 10.03; P (mg/dm$^3$), 11.00; K (cmol$_c$/dm$^3$), 0.23; Ca (cmol$_c$/dm$^3$), 2.05; Mg (cmol$_c$/dm$^3$), 1.44; H+Al (cmol$_c$/dm$^3$), 2.55; SB (cmol$_c$/dm$^3$), 4.8; T (cmol$_c$/dm$^3$), 6.22; V (%), 59.95.

With the first nitrogen fertilization, whose source used was the ammonium nitrate, the phosphate fertilization was carried out, having the simple super-phosphate as source, aiming to elevate the phosphorus level to 15 mg/dm$^3$. The potash fertilization was shared with the nitrogen, using chlorate of potassium as source, aiming to increase saturation of K to 4% of the cation exchangeable capacity (CEC). The fertilization was carried out at throw right after the removal of the animals from the paddock.

The treatments utilized were: without fertilized (considered the control); 150; 300 and 450 kg de N/ha year$^{-1}$. The design was completely randomized, being that, each treatment was comprised of eight experimental units (paddocks) totaling an area of 2.02 ha. The pasture cycles were determined by the height of pre-grazing (90 cm) and post-grazing (40 cm). The Millennium grass is very similar in mass production to Mombaça grass (*P. maximum*), so the grazing management recommendations followed were the same used in Mombaça.

To lower the pastures was used crossbreed steers (Zebu x European) with initial weight of 170 kg. The period of occupation of animals and pasture regrowth was variable according to the growth rate of each paddock.

When the pastures reached nearly the pre-grazing height (90 cm), twenty-three blades of each repetition were collected, being them collected from vegetative tillers. It was selected the last and penultimate expanded leaves of vegetative tillers being cut at the ligule region and conditioned to plastic bags. Out of these twenty-three leaf blades, twenty were immediately conditioned in plastic bags and kept in a freezer at -10°C until morphologic evaluations be started. Monteiro et al., (2005) reviewing several studies that consistently show that the leaf blades of the two newly expanded leaves reflect the nutritional status of grasses as *Brachiara Brizantha* cv. Marandu e *Panicum maximum* cv. Mombaça e Tanzânia.

For morphologic evaluations, twenty leaf blades were measured with a ruler, measuring the width at the central portion of the blade and the length was taken from the apex to the base of the ligule insertion. The leaves area was measured, utilizing the equipment Licor Modelo 3100, obtaining values through the average of the readings. The blades were weighed and placed in an stove at 60°C for 72 hours, afterward, they were weighed again and the specific leaf area (SLA) was estimated dividing the area (cm) by the dry weight (g) obtained after drying (RADFORD, 1967).

The three remaining leaf blades, right after collected, were cut at their median region, obtaining fragments of approximately 1 cm, which were conditioned in glasses with capacity for 10mL and covered with FAA$_{50}$ stored until the beginning of the histological preparation (JOHANSEN, 1940).

The fragments of each leaves blade were submitted to series of alcohol concentration with tertiary butyric acid for about 40 hours to remove gradual water preventing the cellular plasmolysis from inflicting progressive dehydration (DAYKIN; HUSSEY, 1985). After the series of alcohol concentration, the paraplast inclusion was used. It was sectioned transversally at 10 µm, with a rotator microtome, carrying out the triarca quadruple coloring of tissues (HAGQUIST, 1974) and the assembling of permanent blades for anatomical quantification.

The proportions of different leaves tissues were determined with the use of binocular optical microscope coupled to the Software of Image Analysis, model Axion Vision version 3.1. It was measured the total area of the transversal section of the blade projected on the video, afterward, it was measured the adaxial and abaxial epidermis (EPIada and EPIaba), of the sclerenchyma (SCL), of the parenchyma bundle sheath (PBS) and of the vascular tissues (VT). The mesophyll (MES) was calculated by the difference between the total area of the transversal section and the areas of the other tissues.

During the summer were cuts carried out in the pasture to estimate the o valor nutritive of leaves existents in forage mass using a square frame of 1 $m^2$, allocated in 5 representative points of the paddock and the forage contained inside the frame was cut at 40 cm height. The cuts number were variable and according the grazing cicles and the results shown are for the medium of the sample collected in the summer. After weighing, it was collected a sub-sample which was separated into leaves blades, stem+sheath and dead matter. The leaves blades were weighed and dried in an stove with forced air circulation at 60ºC up until the constant weight. After that, they were ground in a 1-mm sieve and submitted to analysis on crude protein estimates (CP) AOAC (1990); neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin in sulfuric acid (LIG) (VAN SOEST et al., 1994); in vitro digestibility of dry matter (IVDDM) (TILLEY; TERRY, 1963).

The means were submitted to the regression analysis and partial correlation analysis of *Pearson* among the morph-physiological characteristics; nutritional value and anatomic proportion of tissues. It was applied the ANOVA of data and for that, it was used the MIXED procedure of the SAS statistical package (SAS INSTITUTE, 2003), in which the RANDOM and TEST commands were utilized. The comparison of the means was carried out by the Tukey test, adopting 5% of significance level.

## RESULTS AND DISCUSSION

The results obtained for the proportion EPIada and PBS in the transversal sections of the leaves blades of millennium grass showed no differences between the control and those pastures that received increasing levels of nitrogen fertilization (Table 1).

**Table 1.** Proportion of adaxial epidermis (EPIada) and parenchyma bundle sheath (PBS) (%) in the transversal section of leaves blades of millennium grass submitted to levels of nitrogen fertilization.

| Treatments | Variables | |
|---|---|---|
| | EPIada | PBS |
| 0 | 16.62 | 31.31 |
| 150 | 15.13 | 32.71 |
| 300 | 15.19 | 33.51 |
| 450 | 14.38 | 33.94 |
| Means | 15.33 | 32.87 |
| SEM | 0.88 | 0.64 |

SEM: Standard error of the mean. Tukey P<0.05.

The outer walls of the epidermis cells became thick, lignificated and covered with a layer of cuticle and wax, as they developed, being more expressive in EPIaba than in EPIada (WILSON, 1993). The same author reported that PBS cells of tropical grasses presented two-wall cells, constituting tissues partly degraded showing in their inner parts high level of protein and starch, and they generally account for 20 to 35% of the transversal section (LEMPP, 2007), and these cells are usually associated to biomass deposition, because as they grow there is also an increase of the width and of the LA of leaves.

The EPIaba and VT proportions responded in a decreasing linear way to the increase of nitrogen levels applied (Figure 1). This tissues that are considered of slow degradation and indigestible, respectively (AKIN, 1989), thus lowering the proportion of these tissues may affect the plant in improving the digestibility.

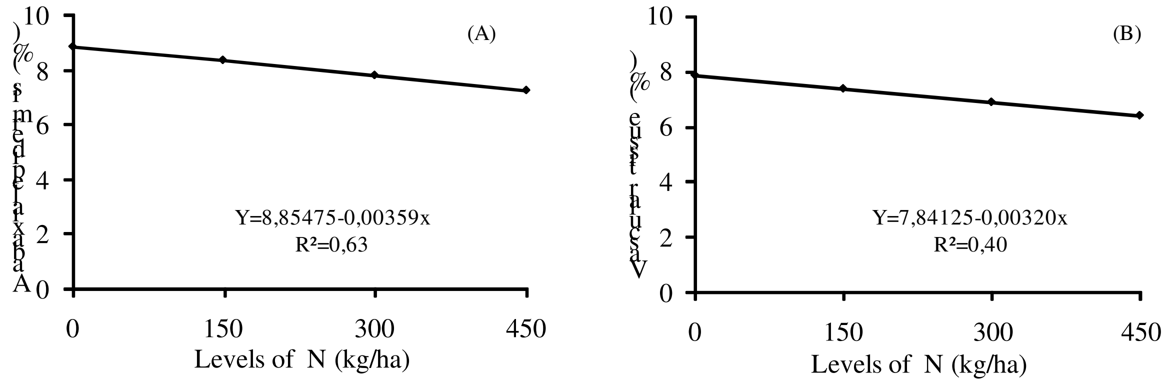


**Figure 1.** (A) Abaxial epidermis (EPIaba) and (B) Vascular tissue (VT) expressed in % in the transversal section of leaves blades of millennium grass, submitted to levels of nitrogen.

The leaves blades from pastures that did not receive nitrogen had on average 12% more of the EPIaba and VT proportions than the blades that received the treatment with fertilization. In a parallel experiment, it was reported elevation of stem deposition resulting from increases of Nitrogen levels (BASSO et al., 2010), which can explain the reduction of VT percentage in the blades of millennium grass, once the leaves of this cultivar, with the lengthening of stems, presented to be more curved, which can diminish the number of VT used in sustainability.

The proportion of MES in the leaves blades of millennium grass increased linearly with the increment of application of nitrogen levels (Figure 2A).The SCL proportion responded in a quadratic way to the nitrogen does, being the 276 kg de N/ha the point of lowest value found (Figure 2B).

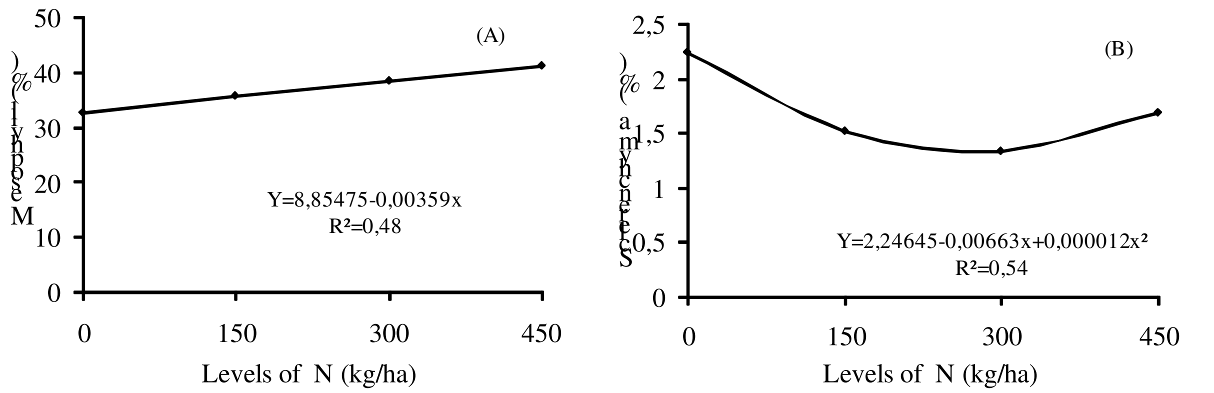


**Figure 2.** (A) Mesophyll and (B) Relative proportion of sclerenchyma (SCL), expressed in % in the transversal section of leaves blades of millennium grass, submitted to levels of nitrogen.

The MES proportion was on average 14,2% higher for pastures submitted to nitrogen fertilization (Figure 2A, B, C and D). High proportions of MES are important from the qualitative perspective of forage grass, being along with the phloem, the tissue that presents the highest digestibility.

The millennium grass presented higher proportion of SCL in the transversal section of the leaves blade (1.7%) this grass shows leaves with more erect tillers and therefore the blades can have a more structural role, which is mainly attributed to SCL, support tissue, or to SCL and VT associated.

Van Soest (1994) cited that one the differences between blades of grass $C_3$ and $C_4$, is that in the $C_4$ they also play a structural role, which can explain the increase of SCL proportion with the increment of nitrogen levels.

The SCL can affect direct or indirectly the degradability of the leaves blades. Directly as a result of how it represents along the transversal section due to its indigestible nature (AKIN, 1989). Indirectly because of its location on the leaves blade, once it can form the structure girder I or girder T. The girder I structure is denominated when the SCL is located between the EPI, adaxial and abaxial, and the PBS cells, and girder T is denominated when it located between EPI, adaxial and abaxial, and the PBS. Wilson et al., (1983) attributed a lower degradability observed on blades of *P. maximum* cv. Petrie in relation to *Lolium perenne,* due to the higher presence of girder structure in a cv. Petrie.

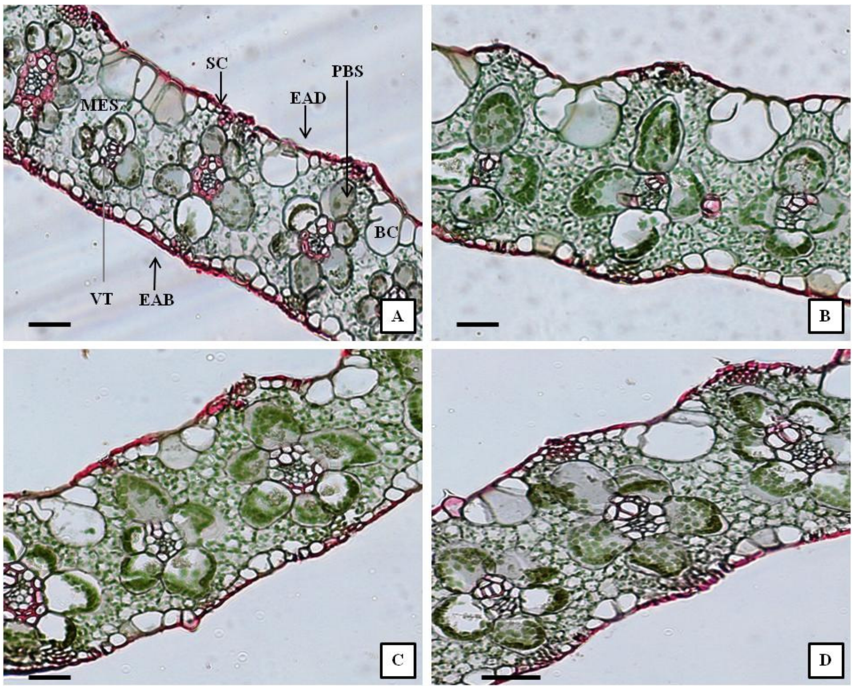


**Figure 2.** Cross section of leaves of millennium grass (A, B, C and D) subjected to levels of nitrogen (0, 150, 300 and 450 kg N, respectively). EDA-Adaxial epidermis; EAB-abaxial epidermis; VT-vascular tissue; PBS-parenchyma bundle sheath; SC-sclerenchyma; MES-mesophyll.

Regarding the morph-physiological characteristics, only the width was not different between the treatments, remaining on average around 3.01 cm. The length increased linearly with the nitrogen levels, the pastures non-fertilized with nitrogen presented average values of 8,8%; 20,7% and 11,5% lower than those submitted to nitrogen fertilization, respectively to length, LA and SLA (Figure 3A, B and C).

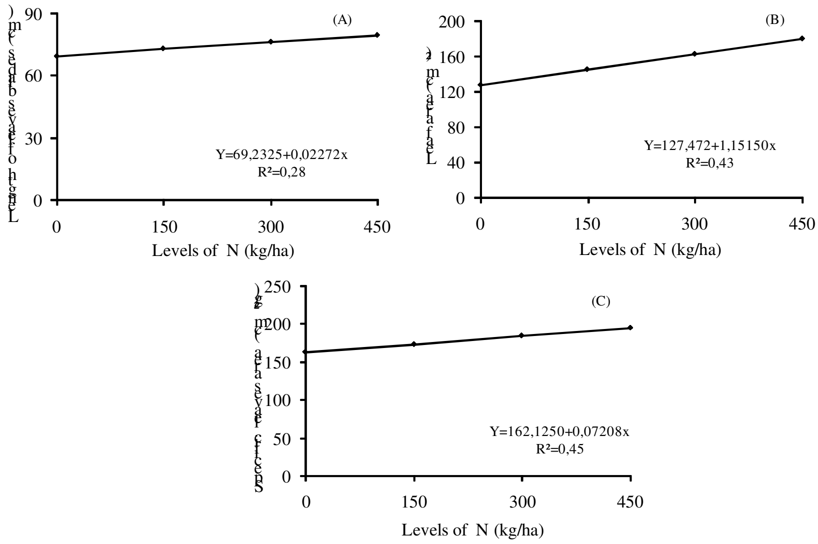


**Figure 3.** (A) Length of leaves blades (cm), (B) Leaves area (LA, cm²) and (C) Specific leaves area (SLA, cm²/g) of millennium grass, submitted to levels of nitrogen fertilization in the summer season.

The expansion rate and the final length of leaves increase with the application of nitrogen, resulting in a more increase of the leaf surface (BÉLANGER, 1998). Thus there is an increase in leaf area index (FAGUNDES et al., 2012) and leaf size (CABRAL et al. 2012). Therefore, there is greater interception of incident light that result in higher rates of pasture growth (BÉLANGER et al., 1993) and increase in forage mass (JOHNSON et al., 2001).

The CP level responded in a positive and linear way to the increment of nitrogen levels, (Figure 4A). Generally, the nitrogen applied to forage plants increases the concentration of protein in the dry matter (EUCLIDES et al. 2007).

The IVDDM responded in quadratic way to the increase of nitrogen levels, with highest point reached at 299 kg of N (Figure 4B). The levels of NDF and ADF decreased linearly with the increment of nitrogen levels. Regarding NDF levels, there was a decrease of even 23% for paddocks that received 450kg of N in relation to the non-fertilized and as for ADF, of around 31%, comparing these same treatments (Figure 4C, D).

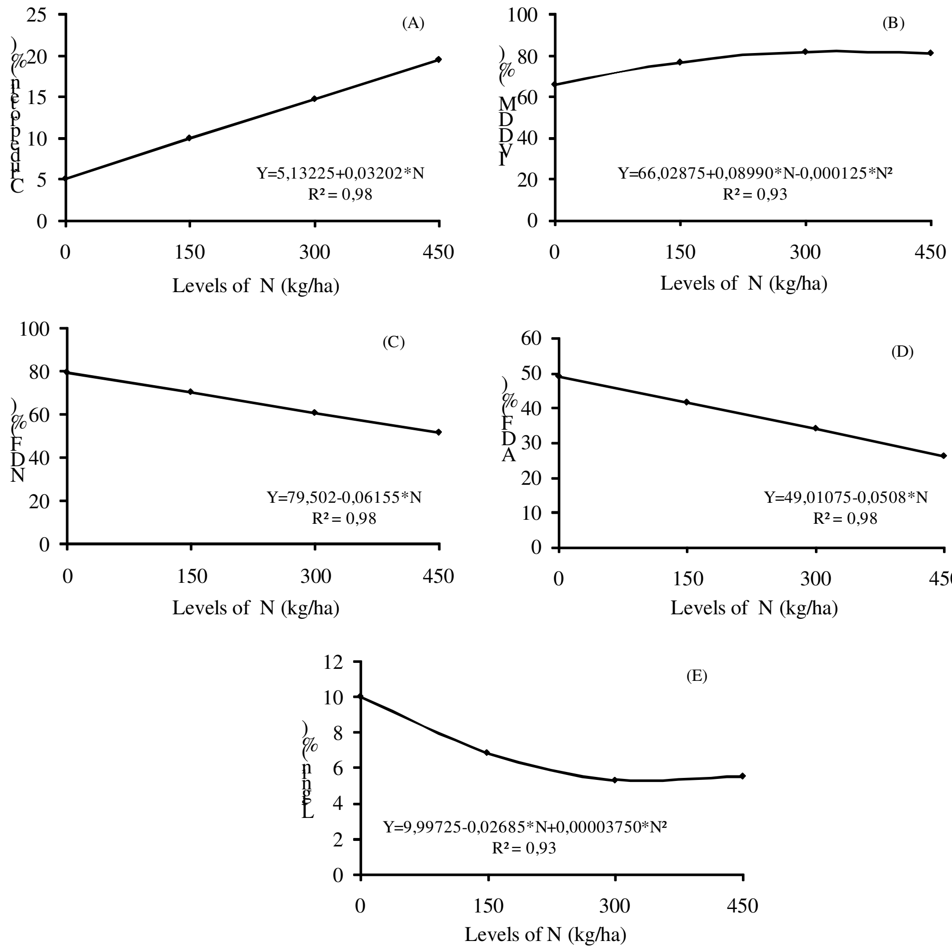


**Figure 4.** (A) Crude Protein (CP), (B) Digestibility *in vitro* of dry matter (IVDDM), (C) Neutral detergent fiber (NDF), (D) Acid detergent fiber (ADF) and (E) Lignin percentage on leaves blades of millennium grass, submitted to levels of nitrogen fertilization in the summer season.

The quadratic response of levels of lignin (Figure 4E) indicate that if there is increase of mass production, can be have increment of the percentage of structures that provide support to tropical grasses, which can implicate on forage quality, once lignin is linked to indigestibility of cell wall components (VAN SOEST, 1994). This could happen if the grazing management strategies of the plant were not respected, by adjusting in the stocking rate.

There was significant correlation with most anatomic and morph-physiological characteristics evaluated (Table 2).

**Table 2.** Coefficients of linear correlation between tissues proportion and morph-physiological characteristics of leaves blades of millennium grass.

| Tissues | Features | | | |
|---|---|---|---|---|
| | Length (cm) | Width (cm) | LA[7] (cm²) | SLA[8] (cm²/g) |
| EPIada[1] | -0.45*** | -0.48** | -0.60** | -0.58** |
| EPIaba[2] | -0.43*** | -0.51** | -0.41** | -0.40** |
| VT[3] | 0.07 | -0.10 | -0.21 | -0.20 |
| PBS[4] | 0.40** | 0.30* | 0.61*** | 0.61*** |
| SCL[5] | 0.00 | -0.23 | -0.23* | -0.20* |
| MES[6] | 0.13 | 0.53** | 0.42* | 0.42* |

[1]Adaxial epidermis; [2]abaxial epidermis; [3]vascular tissue; [4]parenchyma bundle sheath; [5]sclerenchyma; [6]mesophyll; [7] leaf area; [8]specific leaf area. ***P<0.001, **P<0.01, *P<0.05.

There was negative correlation between the length and EPIada and EPIaba, which indicates that with the length increase of the blades, which occurred in pastures with higher fertilization, there was reduction in the structures that compose the epidermis. There was also negative correlation between the length and the epidermis.

The PBS cells and the length correlated positively, and the same behavior occurred between PBS and the length, PBS and LA and PBS and SLA, that is, the PBS cells elevate as a result of the increase of morphologic characteristics. The PBS cells presented a higher volume than the SCL and VT together (WILSON, 1997), which can explain its high correlation with LA and SLA, therefore we can consider that with the LA increase there is an increment in proportion of cells rich in protein as in the case of PBS.

In leaf blades, there is a strong relationship between leaf anatomy and morph-physiological characteristics, as width, leaf area and specific leaf area (GOMES et al., 2011). These relationships can assist in selection of genotypes with higher nutritional value. According MacAdam & Mayland (2003) can be associated some morphological characteristics with animal preference. These authors reported that the width of leaf blades in *Festuca arundinacea* is associated with animal preference, which allows this characteristics be used to select genotypes.

Regarding the MES proportion, there was positive correlation with the length and LA indicating that the leaves have larger active area for photosynthesis can present higher number of MES cells.

Wilson et al., (1989) verified that genotypes of *Cenchrus ciliaris* with heavier leaves and with high SLA (g de MS/cm²) were associated to higher proportions of thick wall tissues (PBS+VT+SCL) in the transversal section of the leaf blade.

The SCL that develops the secondary thick wall and the leaves blades of grass is located above and below the vascular beams (WILSON, 1993), which presented negative correlation with LA and SLA.

There was positive correlation between EPIaba and NDF, ADF and negative between EPIaba and IVDDM. With the increase of tissue percentage of EPIaba there was reduction of IVDDM, and with the increase of nitrogen levels, there was increase in the digestibility and reduction of EPIaba (%) (Table 3).

**Table 3.** Coefficients of linear correlation between the tissues and the nutritional value of leaves blades of millennium grass.

| Tissues | Features | | | | |
|---|---|---|---|---|---|
| | CP[7] | NDF[8] | ADF[9] | IVDDM[10] | LIG[11] |
| EPIada[1] | 0.06 | -0.05 | 0.21 | -0.25 | 0.22 |
| EPIaba[2] | -0.50 | 0.65*** | 0.62** | -0.49* | 0.41 |
| PBS[3] | 0.35 | -0.13 | -0.16 | 0.24 | -0.33 |
| MES[4] | 0.19 | -0.25 | -0.64*** | 0.56** | -0.44 |
| VT[5] | 0.18 | 0.07 | 0.32 | -0.33 | 0.38 |
| SCL[6] | -0.30 | 0.59** | 0.71*** | -0.64** | 0.58** |

[1]Adaxial epidermis; [2]abaxial epidermis; [3]vascular tissue; [4]parenchyma bundle sheath; [5]sclerenchyma; [6]mesophyll; [7]crude protein; [8]fiber in neutral detergent; [9] fiber in acid detergent; [10] digestibility *in vitro* of dry matter; [11] lignin. ***P<0.001, **P<0.01, *P<0.05.

The PBS cells constitute a significant source of the cell content readily digestible, however this content may not be available to microorganisms of the rumen due to the thickening of cell walls and to the formation of girder I or T formation (WILSON, 1997).

There was positive correlation between MES and IVDDM and negative between MES and ADF. Akin et al., (1983) found 50% of MES disappearance in the first six hours of *in vitro* incubation. Therefore, the MES cells constitute important subtract for all bacteria in the rumen, once they are digested by extra cellular enzymes. Akin (1989) cited that MES, as well as the phloem, present quick degradability and do not form physical barriers to digestion, in $C_3$ grasses also in $C_4$. One of the greatest differences between the nutritional values of the grasses $C_3$ and $C_4$ is attributed to the higher MES proportion in grasses from temperate climate.

There was no correlation between the VT and PBS percentage in any of the characteristics of nutritional value studied. With the increase of SCL cells there was increase in NDF and ADF levels, reduction of IVDDM and increase in the lignin level. The lignin retards the hydrolysis of the epidermis cells and SCL, and also prevents ingestion of vascular tissues (HARBERS et al., 1980), therefore the lignin found in organs and tissues as structural component can reduce the negative effect of fiber digestibility.

Paciullo et al. (2001) found that the thickness of cell walls of sclerenchyma and metaxylem were negatively correlated with digestibility, and concluded that the estimated proportions of mesophyll, xylem and sclerenchyma, with the cell wall thickness, can be combined with the chemical composition to improve the estimation of the nutritive value of forage.

## CONCLUSION

Associated with de correct grazing management the use of nitrogen fertilization in millennium grass reduces the proportion of slow-digestible tissues and increases crude protein and digestibility. The increase of the nutritional value can be correlated with the proportion of tissues on leaves blades of millennium grass.

**RESUMO:** Este trabalho foi realizado com objetivo de avaliar a influência do uso de doses de nitrogênio (controle=sem N; 150; 300 e 450 kg de N/ha), nas porcentagens de tecidos de lâminas foliares e no valor nutritivo de capim-milênio, em sistema de pastejo com lotação intermitente no período do verão de 2007, em delineamento inteiramente casualizado. Foram coletadas a última e penúltima folha expandida de perfilhos vegetativos. A porcentagem de esclerênquima (ESC) foi 40,8% e 36,4% menor para os pastos adubados com 150 e 300 kg de N/ha e 25% menor para aqueles com adubação de 450 kg em relação aos não adubados. A porcentagem de mesofilo (MES) aumentou linearmente com as doses de nitrogênio, sendo até 20,3% maior para os pastos com 450 kg de N em relação aos não adubados. Area foliar (AF), área foliar especifica (AFE) e comprimento aumentaram linearmente com o aumento das doses de nitrogênio. Houve correlação positiva entre AF e bainha parenquimática dos feixes (BPF), AFE e BPF, mesofilo (MES) e largura das folhas. A porcentagem de proteína bruta (PB) e digestibilidade *in vitro* da matéria seca (DIVMS) foram maiores para os pastos mais adubados (300 e 450 kg de N/ha). A epiderme abaxial (EPIaba) e os teores de fibra em detergente ácido e neutro (FDA e FDN) tiveram correlação positiva, o mesmo ocorreu entre a DIVMS e BPF. O nitrogênio aplicado influencia na melhoria do valor nutritivo de lâminas foliares do capim-milênio e na proporção de tecidos considerados de alta digestibilidade.

**PALAVRAS-CHAVE:** Adubação de pastagens. Características anatômicas. Características morfológicas. *Panicum maximum*. Pastejo intermitente.

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