Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v40i1.39369

BOTANY

Regrowth age modifies the leaf anatomy of *Brachiaria* genotypes

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ABSTRACT. Changes in leaf anatomy were evaluated in genotypes of *Brachiaria brizantha*, *Brachiaria decumbens*, *Brachiaria ruziziensis* and three *Brachiaria ruziziensis* clones through tissue proportion in internerval and midrib regions at three regrowth ages. Plants were grown and cutting was performed after 60 days. Further, leaves were sampled at 8, 15 and 29 regrowth days and processed with usual plant microtechnique. The internerval region showed higher parenchyma percentage at 15 days for Clone 95 and similar values at 15 and 29 days for Clone 1. The proportion of vascular bundles was lower after 15 days in Clones 1 and 95 and 29 days in *B. brizantha*. In the midrib, the parenchyma proportion was higher at 29 days in *B. brizantha* and lower at 15 days in *B. ruziziensis*. The proportion of vascular bundles was higher at 8 days in *B. decumbens*, *B. brizantha* and Clone 1, and lower at 29 days for Clones 97 and 95 and at 8 days in *B. ruziziensis*. Therefore, the regrowth age modifies the percentage of leaf tissues in *Brachiaria* genotypes, in which the fibers and vascular bundles increase at 29 days and 8-day-old leaves are not fully developed.

Keywords: digestibility; lignina; *Urochloa*; *Brachiaria brizantha*; *Brachiaria decumbens*; *Brachiaria ruziziensis.*

A idade de regeneração modifica a anatomia foliar dos genótipos de *Brachiaria*

RESUMO. A anatomia foliar foi avaliada em genótipos de *Brachiaria brizantha*, *Brachiaria decumbens*, *Brachiaria ruziziensis* e três clones de *Brachiaria ruziziensis;* quanto à proporção de tecidos foliares, em três idades de rebrota. As plantas foram cultivadas e cortadas após 60 dias. As folhas foram amostradas aos 8, 15, 29 dias de rebrota e analisadas pela metodologia de microtécnica vegetal. Na região internervural, a proporção de parênquima foi maior aos 15 dias para o Clone 95, e aos 15 e 29 dias para o Clone 1. Proporção de feixes vasculares foi menor aos 15 dias para os Clones 1 e 95 e aos 29 dias para genótipo *B. brizantha*. Na nervura central, a proporção de parênquima foi maior aos 29 dias para *B. brizantha* e menor, aos 15 dias, para *B. ruziziensis*. Proporção dos feixes vasculares foi maior aos 8 dias para genótipo *B. decumbens*, *B. brizantha* e para o Clone 1, e menor aos 29 dias para os Clones 97 e 95; aos 8 dias, para genótipo *B. ruziziensis*. Portanto, a idade de rebrota modifica a porcentagem de tecidos foliares em genótipos de *Brachiaria;* contudo, fibras e feixes vasculares aumentam aos 29 dias; aos 8 dias, as folhas não estão totalmente desenvolvidas.

Palavras-chave: digestibilidade; lignina; *Urochloa*; *Brachiaria brizantha*; *Brachiaria decumbens*; *Brachiaria ruziziensis*.

Introduction

The quality of a forage plant is determined by the degree of tissue digestibility (Jerba, Medeiros, & Fernandes, 2004). The microorganisms which inhabit the rumen of ruminants possess the capacity for digesting cellulose, but not lignin (Clipes et al., 2010; Vanholme, Demedts, Morreel, Ralph, & Boerjan, 2010; Marković et al., 2012). Therefore, lignin deposition in the cell wall is a factor that reduces tissue digestibility. Such deposition of lignin occurs simultaneously to plant physiological maturity (Paciullo, 2002). In addition to leaf lignification, older leaves show increased proportion of lignified tissues (Brito, Rodella, Deschamps, & Alquini, 1999). The lignified and non-lignified tissues are either digested by ruminants according to their chemical composition, structure and cell wall thickness (Brito et al., 1999; Paciullo, 2002; Jung, 2012). Generally, chemical analyses of stems and leaves are conducted to identify and quantify the compounds affecting forage digestibility (Jerba et al., 2004).

However, the quantitative plant anatomy has become a significant tool for evaluation of forage quality. This method uses the unique digestibility of each tissue to predict leaf digestibility (Brito et al., 1999; Paciullo, 2002). Likewise, the study of the different tissues in forage leaves is essential to

provide new actions in breeding programs (Basso et al., 2014). There are many works using that method as a complementary contribution to evaluate the digestibility potential of leaf tissues. Brito, Rodella, and Deschamps (2004) evaluated the anatomy of leaves at different insertion levels in plants of *Brachiaria brizantha* and *B. humidicola* and found larger area of lignified tissues related to the lower digestibility of *B. brizantha*. Bauer, Gomide, Silva, Regazzi, and Chichorro (2008) evaluated the influence of anatomical traits upon the nutritive value of molasses grass (*Melinis minutiflora* Pal. De Beauv), brachiaria grass (*Brachiaria decumbens* Staph.), sape grass (*Imperata brasiliensis* Trin.) and jaragua grass (*Hyparrhenia rufa* (Nees) Staph.) and concluded that lower digestibility coefficients were related to high proportion of xylem and sclerenchyma. In addition, plant anatomy is useful for the comparison of different species, cultivars and the forage maturity (Brito et al., 2004). Furthermore, the decline of nutritive value upon maturity is due to increased lignification (Brito et al., 1999; Paciullo, Gomide, Silva, Queiroz, & Gomide, 2002; Paciullo, 2002; Carvalho & Pires, 2008; Pariz et al., 2010; Medeiros, Pinto, Castro, Rezende, & Lima, 2011; Mauri et al., 2015).

Therefore, the leaf quantitative anatomy evaluation is a good method on the selection of leaves with higher digestibility potential and is a low-cost method which presents results on a short term. Thus, this study aimed to verify how the proportion of leaf tissues of *Brachiaria* genotypes changes along the regrowth age.

Material and methods

Plant material

Genotypes of brachiaria grass were selected for the experiment as follows: *Brachiaria brizantha* (Hochst.) Stapf. (cv. Marandu), *Brachiaria decumbens* Stapf. Prain. (cv. Basilisk), *Brachiaria ruziziensis* R. Germ & Evrad (cv. Kennedy) and three clones (1,95 and 97) of *Brachiaria ruziziensis,* which are under development in the breeding program of *Embrapa Gado de Leite, Juiz de Fora* – State of Minas Gerais, Brazil. Those genotypes were grown in the *Embrapa Gado de Leite* experimental field, localized in the municipality of Valença (State of Rio de Janeiro, Brazil). The soil used for propagation was classified as Haplic Gleysol (*Empresa Brasileira de Pesquisa Agropecuária* [Embrapa], 2013) and fertilization was made based on previous soil analysis according to Mauri et al., (2015). Soil correction was conducted with limestone and planting fertilization consisted of the application of 300 kg ha^{-1} of the 08-28-16 NPK + Zn. Topdressing fertilization was carried out later by applying 50 kg N ha^{-1}. Irrigation was performed when soil moisture reached 50% of the field capacity. The applied water volume was enough to restore 100% of the field capacity according to previous analyses in the experimental area. The cutting to obtain uniformity of plots was done 60 days after propagation. Experimental leaves were collected at different regrowth ages in the periods of 8, 15 and 29 days after cutting. The experimental design was completely randomized with three treatments (leaf ages) and nine replications.

Anatomical analysis

Leaves were collected at the second node from the culm apex and fixed in $F.A.A_{70\%}$ (formaldehyde: acetic acid: ethanol 70% at the proportion of 0.5:0.5:9 v v^{-1}) for 72 hours (Johansen, 1940). Further, leaves were stored in 70% ethanol, at room temperature until analysis. Cross sections of the leaves were accomplished free-hand, using steel blades on the lower third of the leaf, soon after the end of the sheath. The midrib and internerval regions were evaluated. Analyses were made on the lower third of the blade because the vascular system of leaves shows acropetal development, therefore the leaf base has faster development of the sclerenchyma and vascular bundles (Beck, 2010).

Sections were submitted to 50% sodium hypochlorite (v v^{-1} of the commercial solution), washed twice in distilled water for 10 min. The sections were stained with a solution of safranin and Astra blue [0.01% safranin (m v^{-1}) and 0.99% Astra blue (m v^{-1})] according to Kraus and Arduim (1997) and mounted on semi-permanent slides with 50% glycerol (v v^{-1}). Images of the slides were captured under Leica DMLS microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) coupled to the digital camera Nikon SIGHT DS- SI1.

Nine slides were mounted per treatment and four fields were analyzed per slide. Images were analyzed with the ImageJ software obtaining the area of the tissues: adaxial epidermis; abaxial epidermis; chlorenchyma; phloem area in a single vascular bundle; area of the parenchyma layer of the vascular bundle sheath; area of the vascular bundles; area of the xylem in a single vascular bundle; area of the fibers in a single vascular bundle and total area of the leaf in the image. For obtaining the proportions, the

following equation was used: (area of the tissue/area of the leaf)*100.

Statistical analysis

Data were tested by analysis of variance and the means compared by the Scott-Knott test for $p \leq 0.05$. The statistical analysis was accomplished using the Sisvar 5.0 statistical software (Ferreira, 2011).

Results and discussion

The proportion of the adaxial epidermis showed no influence of the regrowth age for the *B. ruziziensis*, Clone 97 and Clone 95. However, it was higher at 15 days in *B. brizantha* and showed similar values at 8 and 15 days for the Clone 1 and at 15 and 29 days in *B. decumbens* (Figure 1A). The proportion of the abaxial epidermis showed no differences for *B. decumbens*, *B. ruziziensis* and Clone 1. Nevertheless, it was higher at 15 days for *B. brizantha* and Clone 97, whereas equivalent values were found at 15 and 29 days for Clone 95 (Figure 1B). The proportion of chlorenchyma showed no differences due to the regrowth age for *B. decumbens*, *B. ruziziensis* and Clone 97. However, it was higher at 15 days for the Clone 95 and similar means were found at 15 and 29 days for the Clone 1. On the other hand, *B. brizantha* showed higher values at 8 and 29 days (Figure 1C).

The proportion of vascular bundles showed no significant effect of the regrowth age for *B. decumbens*, *B. ruziziensis* and Clone 97. However, lower means were found at 15 days for the Clone 1 and Clone 95, as well as at 29 days for *B. brizantha* (Figure 2A).

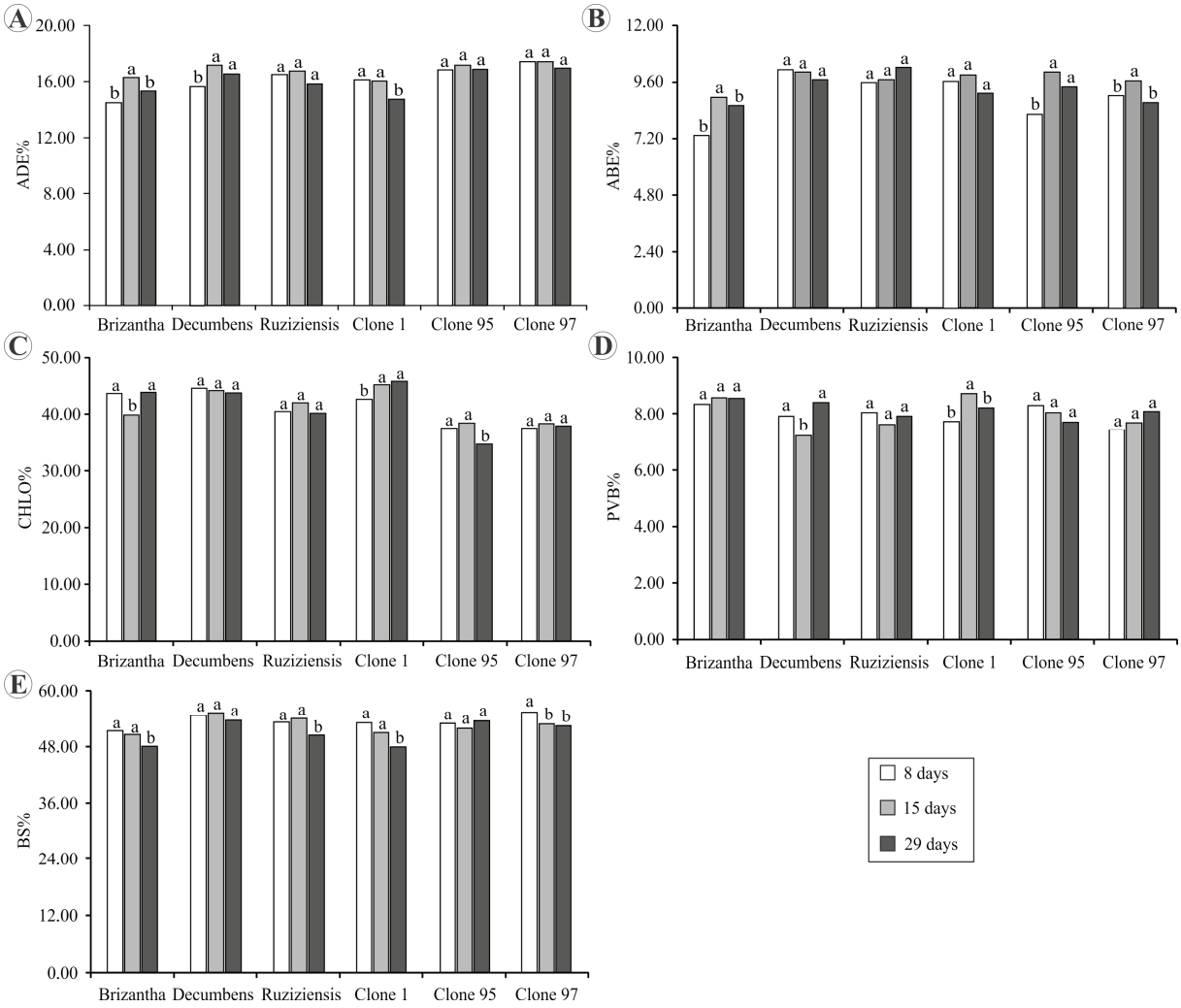

Figure 1. Modifications in the tissues of the internerval region of the leaf blade from the *B. brizantha*, *B. decumbens, B. ruziziensis,* Clone 1, Clone 95 and Clone 97 at different regrowth ages. Means followed by the same letter within genotypes are not significantly different by the Scott-Knott test at 5% significance. A) proportion of adaxial epidermis (ADE%); B) proportion of abaxial epidermis (ABE%); C) proportion of chlorenchyma (CHLO%); D) proportion of phloem in the vascular bundle (PVB%); E) proportion of bundle sheath (BS%).

The proportion of xylem in the vascular bundle showed no significant differences between regrowth date for *B. decumbens* and Clone 1. However, it was lower at 8 days in *B. brizantha*. In addition, higher means were found at 15 days for Clone 97, whereas *B. ruziziensis* and Clone 95 presented lower means (Figure 2B). Regarding the proportion of fibers in the vascular bundle, no significant effect was detected between regrowth age for Clone 95. However, it was higher at 29 days in *B. brizantha*, *B. ruziziensis*, Clone 1 and Clone 97 and lower at 8 days in *B. decumbens* (Figure 2C). The proportion of the adaxial epidermis in the midrib showed no significant effects due to regrowth age for the *B. brizantha*. However, it was higher at 15 days for *B. decumbens*, Clone 1, Clone 95 and Clone 97 whereas lower means were found at 8 days for *B. ruziziensis* (Figure 3A).

The proportion of abaxial epidermis showed no significant differences between regrowth date for *B. decumbens*, *B. ruziziensis* and Clone 95. Nevertheless, it was higher at 15 days for *B. brizantha* and lower at 29 days for Clone 1, but Clone 97 showed higher means in that regrowth age (Figure 3B).

The proportion of ground parenchyma in the midrib showed no significant modifications between regrowth age for Clone 1 and Clone 97. However, it was higher at 29 days in *B. brizantha* and lower at 15 days in *B. ruziziensis* (Figure 3C). The proportion of phloem in the vascular bundle showed no significant influence of the regrowth age for *B. brizantha*, *B. ruziziensis* and Clone 97. This variable was higher at 15 days for the Clone 1 but lower in *B. decumbens* and Clone 95 (Figure 3D).

The proportion of bundle sheath cells showed no significant effect of the regrowth date for Clone 95. Nevertheless, it was higher at 15 days for *B. ruziziensis* and at 8 days for *B. decumbens*. However, Clone 1 and Clone 97 showed higher means at 8 days (Figure 3E). The proportion of vascular bundles was higher at 8 days in *B. decumbens*, *B. brizantha* and Clone 1. Lower means were found at 29 days for Clone 97 and Clone 95 and at 8 days in *B. ruziziensis* (Figure 4A).

The proportion of xylem in the vascular bundles was not significantly modified due to the regrowth age in *B. brizantha*, Clone 97 and Clone 95. However, it was lower at 8 days for the *B. decumbens* but higher for *B. ruziziensis* and at 15 days in Clone 1 (Figure 4B). The proportion of fibers in the vascular bundle showed no differences due to the regrowth age for *B. decumbens* and Clone 95. However, lower means were found at 8 days for Clone 97 and at 15 days for *B. ruziziensis*; however higher means were found at 29 days for Clone 1 (Figure 4C).

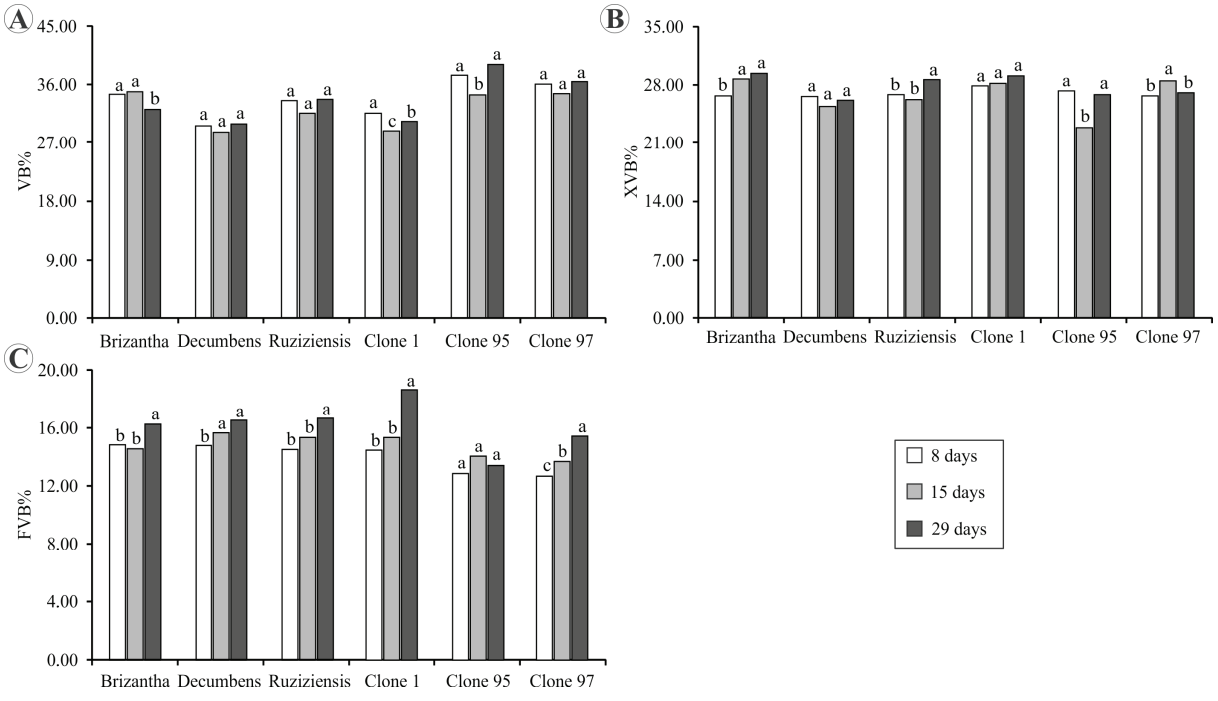

Figure 2. Structure of the vascular bundles in the internerval region of *B. brizantha*, *B. decumbens, B. ruziziensis,* Clone 1, Clone 95 and Clone 97 at the different regrowth ages. Means followed by the same letter within genotypes are not significantly different by the Scott-Knott test at 5% significance. A) proportion of vascular bundle (VB%); B) proportion of xylem in the vascular bundle (XVB%); C) proportion of fibers in the vascular bundle (FVB%).

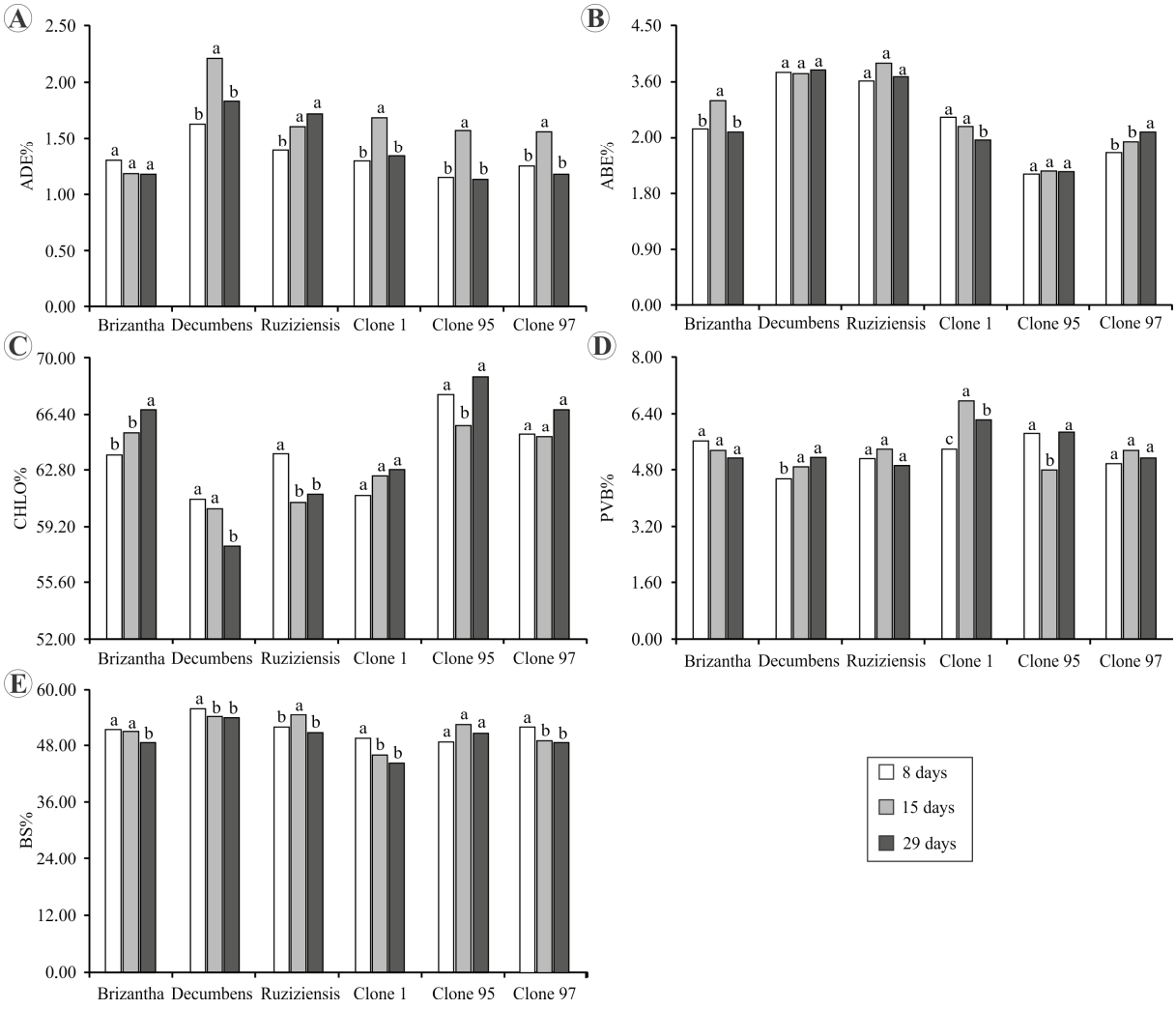

Figure 3. Proportion of midrib tissues in *B. brizantha*, *B. decumbens, B. ruziziensis,* Clone 1, Clone 95 and Clone 97, in the different regrowth ages. Means followed by the same letter within genotypes are not significantly different by the Scott-Knott test at 5% significance. A) proportion of adaxial epidermis (ADE%); B) proportion of abaxial epidermis (ABE%); C) proportion of chlorenchyma (CHLO%); D) proportion of phloem in the vascular bundle (PVB%); E) proportion of bundle sheath (BS%).

The six *Brachiaria* genotypes showed anatomical differences in the leaf development due to regrowth ages. At all leaf ages sampled, the internerval region showed uniseriate epidermis on both the abaxial and adaxial faces in all ages studied (Figure 5). The 15-day-old leaves showed higher development of the chlorenchyma and bundle sheath cells with the Kranz structure well established (Figure 5B, E, H, K, N and Q). Leaf thickness increased from 8 to 15-day-old leaves, increasing the size of the internerval region. The vascular bundles are collateral and were larger at 29 days (Figure 5). The mestomatic layer of the vascular bundle sheath showed expansions comprised of sclerenchyma fibers and, after 29 regrowth days, more developed fibers were found (Figure 5).

The midrib region showed uniseriate epidermis both on the adaxial and abaxial surfaces and little modifications in this tissue was observed due to the regrowth age, whereas the cuticle was more developed in older leaves (Figure 6). On the adaxial side, the midrib showed fiber strands that increased with regrowth age (Figure 6). Ground parenchyma was found in a large area of the central region of the midrib; this tissue was already developed at 8 days of regrowth; however, cell expansion was verified in older leaves (Figure 6). Larger vascular bundles on the abaxial surface of the midrib showed sheaths comprised of sclerenchyma fibers, which are more developed after 29 days of regrowth (Figure 6).

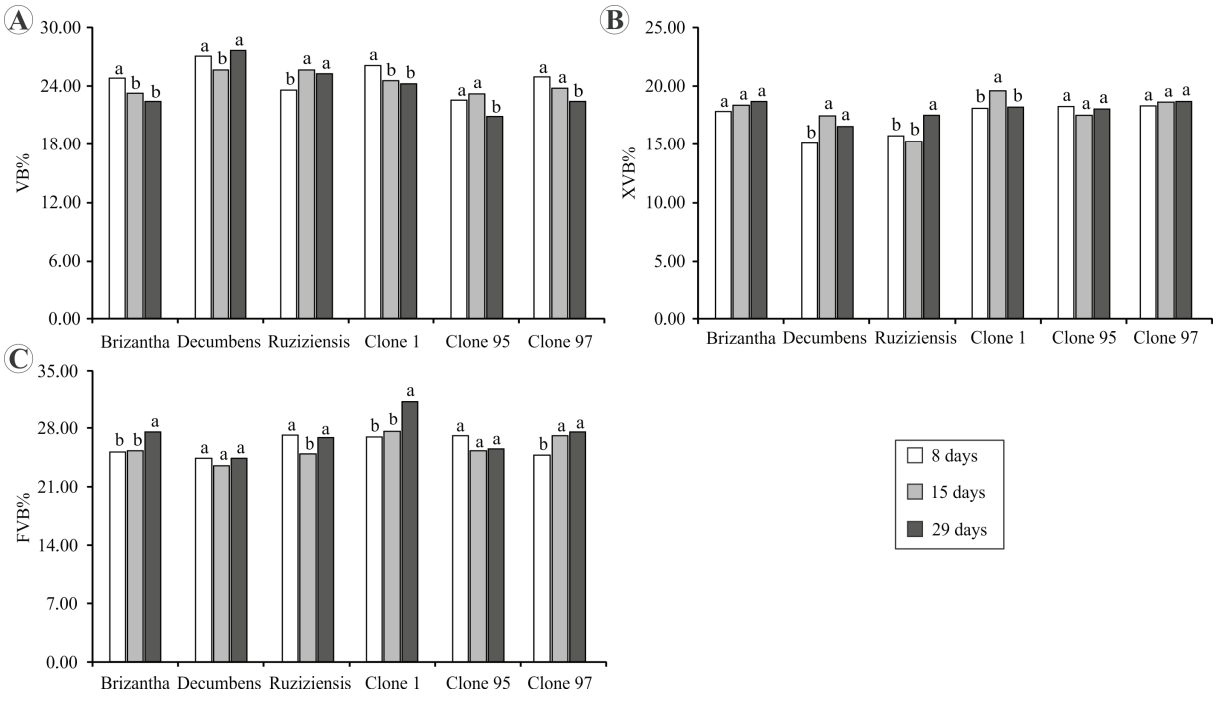

Figure 4. Structure of the vascular bundles in the midrib of *B. brizantha*, *B. decumbens, B. ruziziensis,* Clone 1, Clone 95 and Clone 97, at the different regrowth ages. Means followed by the same letter within genotypes are not significantly different by the Scott-Knott test at 5% significance. A) proportion of the vascular bundle (VB%); B) proportion of xylem in the vascular bundle (XVB%); C) proportion of fibers in the vascular bundle (FVB%).

The increase of the regrowth age promotes the expansion of the sclerenchyma and xylem in the *Brachiaria* leaves. It is well known that the cell expansion in leaf tissues is followed by cell differentiation at maturity (Sinha, 1999). During leaf development, cell division and differentiation occur at different regions and meristems (Beck, 2010). In this study, leaf tissues of the *Brachiaria* genotypes were already differentiated at 8 days of regrowth, therefore, they were no longer meristematic and no cell division was observed. However, *Brachiaria* leaves increased in size due to the cell expansion, and further, the cell wall deposition was found in the sclerenchyma and xylem cells with increased regrowth age.

The 8-day-old leaves were not fully expanded. Likewise, at 15 days, the chlorenchyma was better developed in the internerval region, which may promote higher photosynthetic potential to these leaves when compared to 8-day-old ones. Fully expanded leaves show photosynthesis at the maximum capacity and the photoassimilates may be used for plant growth (Silva, Nascimento Júnior, & Euclides, 2008). In addition, 15-day-old leaves showed larger vascular bundles, which reduce the forage quality due to lower digestibility, once it is proved that increased lignin content in the fibers is found in older plants (Lev-Yadun, 2010; King, Mceniry, Richardson, & O'Kiely, 2013).

It is well known that forage growth is accompanied by thickening of the cell wall and increasing lignin content, reducing forage quality (Brito et al., 1999; Paciullo et al., 2002; Jerba et al., 2004; Bauer et al., 2008). Therefore, the knowledge of the ideal regrowth age is critical to avoid the unnecessary content of lignified tissues. As shown in this work, *Brachiaria* leaf tissues are still expanding and differentiating at longer regrowth ages, improving biomass content. As the most appropriate age for use of the forage is that provided by the best combination of forage yield and quality (Moura, Bona, Rodrigues, Oliveira, & Lopes, 2014), the 15-day-old leaves of *Brachiaria* genotypes evaluated may be more efficient to use as forage.

There are studies on *Brachiaria* forages seeking for the ideal regrowth age, however, the anatomical analysis was not performed in these works. Costa et al. (2007) found that the recommended date to forage regrowth was between 28 and 42 days for *Brachiaria brizantha* cultivar Marandu and between 28 and 35 days for BRA-004308 and BRA-003395 genotypes. However, Deminicis, Abreu, Vieira, and Araújo (2010) reported that 56 regrowth days resulted in higher yield for *Brachiaria humidicola*. However, those works disregarded the structural evaluations of tissues, which may show declines in digestibility with increasing age.

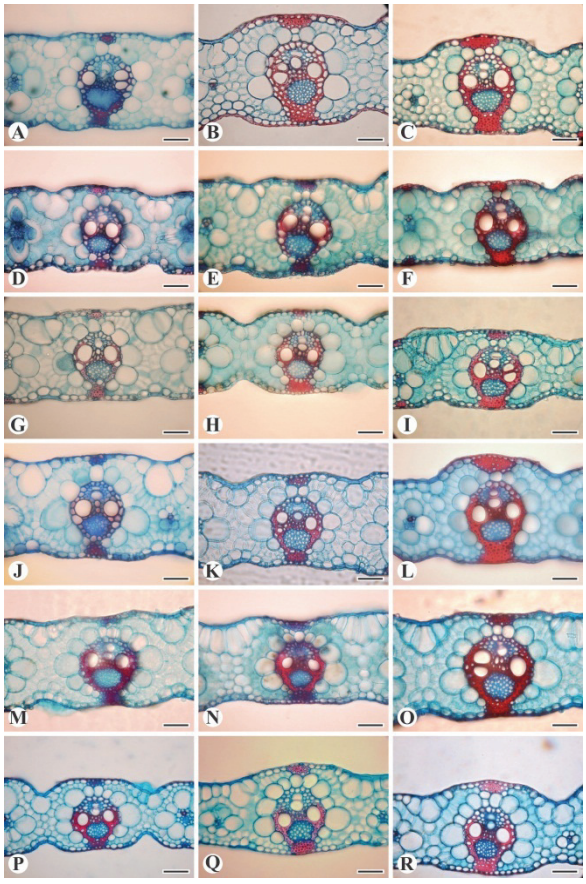

Figure 5. Anatomical structure of the internerval region, indicating the regrowth age in the columns (left 8-day-old leaves, middle 15-day-old leaves and right 29-day-old leaves) and in the rows of the *Brachiaria* genotypes: *B. brizantha* (A, B and C), *B. decumbens* (D, E and F), *B. ruziziensis* (G, H and I), Clone 1 (J, K and L), Clone 95 (M, N and O) and Clone 97 (P, Q and R). Bar = 50 μm.

Each leaf tissue presents particular digestion inside the ruminant's digestive system. It is well known that tissues with lignified cell walls show poor digestibility. This lower digestibility is because microorganisms that aid in the digestion have no ability to digest lignin (Clipes et al., 2010). According to Brito et al. (1999), the digestion potential of forage tissues has the following decreasing order in the ruminant's organism: parenchyma > phloem > epidermis > bundle sheath > xylem and sclerenchyma. Following those considerations, the current study presented results of higher proportion of tissues with better digestibility in the 15-day-old leaves. The 8-day-old leaves are still expanding and increasing the total biomass in further dates. In that sense, on the basis of the set of anatomical characteristics evaluated, the date of 15 days may be the most adequate in relation to the 8 days.

The 29-day-old *Brachiaria* leaves showed high proportion of poorly digestible tissues and this may reduce the forage quality. It is worthwhile remembering that the presence of high proportions of both xylem and sclerenchyma is linked to lower digestibility coefficients of forages (Bauer et al., 2008). In this sense, 15 days of regrowth also stood out in relation to the 29 days for presenting lower rates of lignified tissues. Considering the leaf regrowth age of *Brachiaria* in the literature, along with the results of the present study, it would be interesting to reduce the regrowth age closer to 20 days.

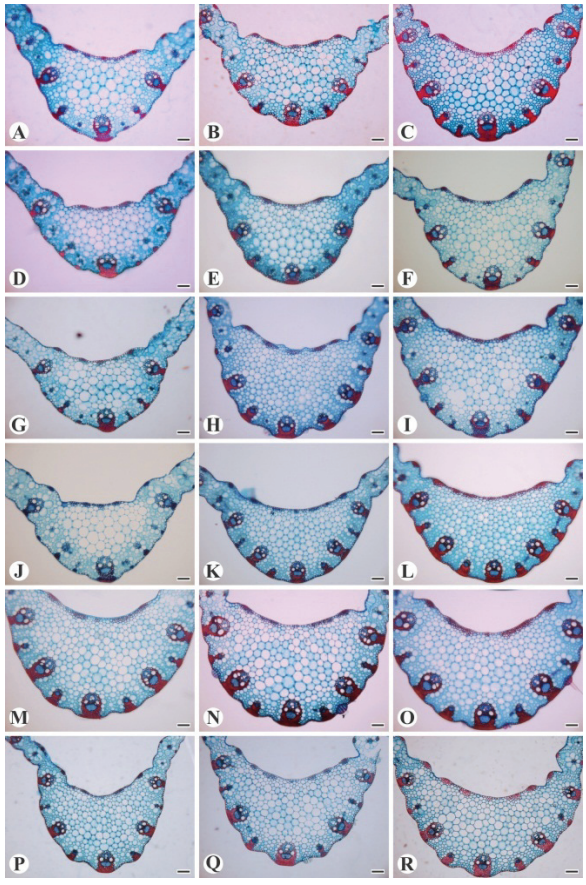

Figure 6. Anatomical structure in cross section of the midrib region, indicating the regrowth age in the columns (left 8-day-old leaves, middle 15-day-old leaves and right 29-day-old leaves) and in the rows the *Brachiaria* genotypes: *B. brizantha* (A, B and C), *B. decumbens* (D, E and F), *B. ruziziensis* (G, H and I), Clone 1 (J, K and L), Clone 95 (M, N and O) and Clone 97 (P, Q and R). Bar = 100 μm.

Conclusion

The leaf development of *Brachiaria* forages is not complete at 8 days of regrowth. The regrowth age modifies the percentage of all tissues in *Brachiaria* leaves. Likewise, fibers, xylem and vascular bundles increase in proportion in older leaves and show significantly higher proportion at 29 regrowth days.

Acknowledgements

The authors thank CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico*), Capes (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*) for funding and research grants awarded to complete the present study and *Embrapa Gado de Leite.*

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Received on September 7, 2017.
Accepted on February 21, 2018.

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